Development of a split-ubiquitin based, cytosolic yeast two-hybrid system and its application to the structure-function analysis of scUril1p

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Zusammenfassung


Im Weiteren haben wir uns auf die molekulare Funktion von Ura1p konzentriert, da eine wichtige biologische Rolle für dieses weitgehend uncharakterisierte Protein postuliert wurde. Ura1p ist ein atypisches Prefoldin Chaperon, welches mit der Nährstoff Signalübertragung in Verbindung gebracht wurde (Gstaiger et al., 2003). Die Mehrheit der in der CytoY2H Selektion identifizierten Ura1p-Interaktoren verstärkte den Verdacht, dass Ura1p eine Funktion in mRNA-Translation und im Proteinfaltungsprozess einnimmt. Mittels CytoY2H Technik wurde eine Interaktionskarte zwischen Ura1p und dessen Bindungspartner erstellt, welche eine enge Verbindung zwischen Translation und Proteinfaltung via Elongationsfaktor 1A enthüllte. Struktur-Funktionsanalysen zeigten, dass die mittlere saure Region der Ura1p Proteinsequenz dessen funktionelle Einheit ist und als Bindungsstätte für die meisten Ura1p Interaktoren dient. Mit Hilfe verschiedener Algorithmen wurde berechnet, dass
Uri1p ein intrinsisch unstrukturiertes Protein ist. Dies erklärt, weshalb es Uri1p möglich ist nicht nur über die mittlere saure Region, sondern über einen ausgedehnten Teil seiner Proteinsequenz Kontakte mit verschiedenen Bindungspartnern einzugehen. Biochemische und genetische Studien wiesen auf eine molekulare Rolle von Uri1p in der Initiation der Translation hin, durch die Rekrutierung des eIF2-GTP-tRNA<sub>Met</sub> Dreifachkomplex zum 43S ribosomalen Preinitiations-Komplex (A. Deplazes, Manuskript in Vorbereitung). Die Evaluation der urilΔ Phänotypen im Zusammenhang mit Resultaten zusätzlicher genetischer Studien und der mRNA Profile in URI1-deletierten Hefezellen unter verschiedenen zellulären Stressbedingungen (Gasch et al., 2000) lassen vermuten, dass Uri1p in die allgemeine zelluläre Stressantwort involviert ist.
Summary

Since interactions between proteins provide a close insight into the function of proteins, they represent a major topic in life science and numerous technologies have evolved to tackle protein interactions.

The yeast two-hybrid system (Fields and Song, 1989) provides a well-established tool for the analysis of protein interactions in vivo. However, since not all protein classes are amenable to this method, a novel genetic screening assay, the cytosolic yeast two-hybrid system (cytoY2H) has been developed in this work, which is based on the split-ubiquitin technique (Johnsson and Varshavsky, 1994) and which detects protein-protein interactions in the cytoplasm. The assay was used to probe defined protein interactions between a wide range of transcriptionally active proteins, including the tumor suppressor p53 and members of the NF-κB complex. This kind of proteins can hardly be applied as bait proteins in the classical yeast two-hybrid system. Moreover, we utilized the cytoY2H system to cDNA library screening of different proteins and identified a well characterized interactor of the Simian Virus 40 large T antigen and new interaction partners of the S. cerevisiae protein Uri1p. The cytoY2H system is a very versatile technology which extends existing proteomic methods by providing a convenient solution for the identification and characterization of protein interactions of a wide range of transcriptionally active proteins.

We then focused on the molecular function of Uri1p, since an important biological role for this rather uncharacterized protein has been proposed. Uri1p is an atypical prefoldin chaperone and may be implicated in nutrient signaling (Gstaiger et al., 2003). The majority of the Uri1p interactors identified in the cytoY2H screen support a role for Uri1p in mRNA translation and related Uri1p to the protein folding process. Using the cytoY2H system, a protein interaction map between Uri1p and its binding partners was established, revealing the tight interconnection of translation and protein folding through translation elongation factor 1A. Structure-function analyses identified the large central acidic region as the functional portion of Uri1p and as a binding site for most Uri1p interactors. Applying different algorithms, the structure of Uri1p was predicted to be intrinsically disordered, which explains why Uri1p is able to contact multiple binding partners not only by the acidic region, but over a large part of its entire sequence. Biochemical and genetic studies indicate a molecular role for Uri1p in translation initiation by recruitment of the eIF2-GTP-tRNA^Met ternary complex to the 43S ribosomal pre-initiation complex (A. Deplazes, manuscript in preparation). Integrating uri1Δ phenotypes, additional results from genetic studies and uri1Δ mRNA profiles under environmental stress (Gasch et al., 2000), we hypothesize that Uri1p might be involved in the general cellular stress response.
Opening Remark

The work I have done during my PhD thesis was split into two interdependent projects. The first project aimed at developing of an in vivo method, termed cytoY2H system, suitable for the detection of interactions between transcriptionally active proteins. Therefore, a general introduction of existing experimental technologies for the detection of protein-protein interactions is given in Chapter 1, whereas the development of the cytoY2H system is described in Chapter 2. Pros and cons of the novel method, compared to alternative systems are discussed in Chapter 5.

The cytoY2H method was tested and applied to several transactivating proteins, including Uri1p, a yeast protein with unknown molecular function. A cytoY2H cDNA library screen with a Uri1p bait yielded many interesting putative interaction partners, which were validated and studied in detail by structure-function analyses. The complete work on Uri1p function, including the introduction of Uri1p is covered in Chapter 4.
1. Chapter - General introduction

The importance of detecting protein-protein interactions
A huge challenge of postgenomic biology is to understand how genetic information results in the concerted action of gene products in time and space to generate function. Proteins play a key role in all biological processes by mediating numerous regulatory, structural and metabolic cellular events. The performance of proteins is highly dependent on interactions with other proteins or protein complexes. Therefore, the identification of protein interactions and protein networks provides one important clue to their molecular function. In this context, an immense variety of techniques has emerged, including high-throughput assays, to identify protein-protein interactions and to cope with the huge amount of data generated by sequencing multiple genomes.

The adaptation from a small to a large scale application in protein chemistry is highly challenging since the physiochemical properties of proteins differ and the activity of a given proteome is regulated with respect to time in an organism, subcellular setting, expressed tissue and external conditions. The diversity of proteins indicates the need of different experimental strategies to uncover the entity of protein interaction networks and the detailed characterization of individual protein interactions.

Two key technologies for the detection of protein interactions
To date, most of the interactions that have been detected experimentally have relied on one of two technologies, the yeast two-hybrid system (Fields and Song, 1989) and mass spectrometry of co-affinity purified protein complexes (co-AP/MS) (Pandey and Mann, 2000). Co-AP/MS detects interactions mainly between protein complexes without revealing direct binary interactions. Contrarily, the yeast two-hybrid (y2h) system senses interactions between two individual proteins, revealing the path of energy or information flow through a complex. Surprisingly, the datasets of the two techniques have only a very small overlap. This difference may be due to the difficulties of each method for distinct types of interactions, false positives generated by these methods or may be caused by incomplete saturation (von Mering et al., 2002). Since the two methods are complementary, they are particularly powerful when used in combination, as shown for yeast (Hazbun et al., 2003).
Limitations of the yeast two-hybrid system
Although the y2h system is the most robust technology for the identification of protein interactions in vivo, it is biased against certain protein classes, like membrane proteins or proteins involved in transcriptional regulation. To overcome these limitations the original y2h method has evolved into a variety of experimental niches (Fashena et al., 2000). However, certain protein classes are either still inaccessible or the existing tools are insufficient for thorough analysis, as it is the case for transcriptional activators. Thus, we are challenged today to develop new and optimized assays to tackle the remaining niches.

Idea of the cytoY2H system – filling a gap
As one important focus of current research concerns transcriptional activators, the availability of a suitable in vivo system would be of broad interest. In this work, a novel genetic screening assay is proposed, the split-ubiquitin based cytosolic yeast two-hybrid (cytoY2H) system, which is particularly useful at screening both for protein interactions involving transcriptional activators and cytosolic proteins.

The scope of this introduction is therefore to survey the most important techniques for the identification of novel protein-protein interactions. Furthermore, I will focus on two-hybrid and split-ubiquitin related methods which can be used to study transactivating proteins.
1. General introduction

Identification of protein-protein interactions *in vitro*

Mass spectrometry based identification of co-immunopurified protein interactions
The classical biochemical techniques for detecting protein interactions *in vitro* are immunoprecipitation and pull-down assays, both of which are based on affinity purification of a bait protein associated with its binding partners. These techniques were further developed for proteomic applications. Of the various high-throughput experimental methods used so far to identify protein-protein interactions, tandem affinity purification (TAP), (Rigaut et al., 1999) of affinity-tagged proteins followed by MS has provided the best coverage and accuracy (von Mering et al., 2002). TAP-tagged baits expressed in a target cell are allowed to form complexes with untagged physiologically expressed prey proteins. Protein complexes are subsequently purified using the TAP-tag in two consecutive steps of affinity purification under mild conditions (Fig. 1.1). Protein complexes are isolated from electrophoresis gels, before subjecting them to MS.

Applying TAP/MS, the most comprehensive attempts made so far to identify protein complexes in eukaryotic cells was made in recent studies by Gavin et al., 2006 and Krogan et al., 2006. About 76 percent of yeast proteins were identified and half of the proteome was reported to be in complexes. Substantial protein sharing between complexes was noticed (Gavin et al., 2006), which suggests that proteins combined in multiple ways to diversify the functional repertoire within cells.

In TAP/MS, yeast bait proteins are endogenously tagged, which is achieved rapidly since genetic manipulation of yeast is easy, in contrast to higher eukaryotes. Endogenous expression of bait and preys is one important factor for the high accuracy reached by TAP/MS, but has restricted the method for high-throughput applications to yeast. As a consequence, co-AP/MS proteome-wide screens in higher eukaryotes are in an early stage yet (Marsischky and LaBaer, 2004; Uetz and Finley, 2005).
Protein microarrays

DNA-based array technology has fueled the transition of protein microarrays by providing platforms and software tools (Espina et al., 2004) and today, the protein microarray technology is essential for various applications including high-throughput analysis of entire proteomes.

Different proteins are immobilized onto solid supports in a spatially resolved manner and the binding of an interaction partner – which can be a protein, DNA, RNA, a lipid, peptides or a small molecule – is detected in parallel for each protein. One of the most challenging aspects of this technology is the functional immobilization of a large number of different proteins with different physicochemical properties. Capturing is performed by antibodies or directly using metal chelators or streptavidin. Typically, the readout of protein arrays is indirect and uses fluorescence or chemoluminescent probes. However, direct detection by mass spectrometry and label-free solid-phase detection such as surface plasmon resonance are promising readout strategies for protein arrays (Smith and Corn, 2003). At present, protein arrays still require complex and expensive equipment for sample handling and detection, but this technology is developing rapidly, and has the potential to integrate the identification of interaction partners and detailed characterization of the interaction (Ptacek et al., 2005).

Phage display

In contrast to protein microarrays, the phage display method is relatively restricted for the identification of antibodies and peptides binding to a specific target. However, phage display is an important tool in the discovery of therapeutic and diagnostic antibodies and in protein engineering.
A prey protein is displayed on the surface of a phage as a fusion with one of the viral coat proteins. The prey encoding DNA is housed within the virion. By cloning large numbers of DNA sequences into the phages, display libraries are produced with a repertoire of many billions of unique displayed proteins which makes the method very powerful for screening antibodies. Interaction occurs by incubating a plate coated with the target protein, with a phage library. In a process called biopanning, the binding phage is enriched and subsequently rescued. Phage selection is not limited to solid support biopanning, but has also been used \emph{in vivo} and against intact cells for selection of tissue and cell targeting proteins (Paschke, 2006).

\textbf{Identification of protein-protein interactions \emph{in vivo}}

\textbf{The principle of the yeast two-hybrid system}

The screening and identification of protein-protein interactions within the cell requires suitable biological or spectroscopic reporter systems. A breakthrough in the screening of protein interaction \emph{in vivo} was made 18 years ago by inventing the yeast two-hybrid (y2h) method (Fields and Song, 1989). This technique was inspired by the modular nature of transcription factors containing a DNA-binding domain (DBD) and a transcription activation domain (AD). Splitting the DBD and the AD inactivates the yeast transcription factor (for example the yeast transcription factor Gal4p used by Fields and Song in their initial experiments), but its function can be restored by fusing the DBD and the AD to two interacting proteins, thereby providing an easy method to identify protein-protein interactions. When a DBD-fused protein X (bait) and an AD-fused protein Y (prey) are expressed in yeast, a quasi native functional transcription factor is generated upon interaction of bait and prey (Fig. 1.2B). Consequently, this two-hybrid transcription factor activates common prototrophic reporter genes such as \textit{HIS3}, \textit{LEU2}, \textit{URA3}, \textit{ADE2} or a \textit{lacZ} marker, such that yeast cells grow on selective medium or turn blue in a \textit{\beta}-galactosidase assay.

Since the y2h method is rapid and easy in use, it has become the most popular \emph{in vivo} method for the identification of proteins and was shown to be very suitable for large scale applications in several organisms including yeast (Fromont-Racine et al., 2000; Uetz et al., 2000; Drees et al., 2001; Ito et al., 2001), worm (Li et al., 2004), fly (Stannyon et al., 2004; Formstecher et al., 2005) and human (Rual et al., 2005; Stelzl et al., 2005).
Figure 1.2. The yeast two-hybrid system - principle and large-scale applications. (A) Co-expression of a DBD-fused bait and an non-interacting AD-fused prey does not lead to a functional hybrid transcription factor. Therefore, transcription of reporter genes is not activated in the y2h-assay. (B) The DBD-fused bait is interacting with an AD-fused prey and thereby activating the reporter gene leading to positive growth selection or a colorimetric readout. (C) Y2H matrix or array approach, suitable for high-throughput applications. Please see text for details. (D) Y2H-cDNA library screening. A cDNA library, expressed in haploid yeast cells are conjugated with a bait construct, expressed in haploid yeast cells of the opposite mating type. Diploids, expressing a bait and a prey, are selected on several large plates for interactions.
Yeast two-hybrid: matrix approach or cDNA library screening

High-throughput two-hybrid approaches have used two complementary methods for screening large sets of proteins, the matrix approach and the cDNA library screening.

A matrix of full-length prey clones is created by dispensing one yeast clone (mating type a) expressing a given AD-Y fusion protein into each well of a multiwell plate. Using a robot, the bait clone (mating type α), expressing a DBD-X fusion is then transferred into the individual wells of the plate containing the preys. Since bait and prey are expressed in cells of different mating types, they will conjugate. Then, diploids are spotted by the robot on selective medium for bait and prey plasmids and on medium selecting for a protein-protein interaction. Alternatively, a colorimetric assay can be used to measure lacZ activity. Since the position of a given prey and a bait is defined, their identity can be figured out easily.

In the library screening approach, a DBD-fused bait is screened against an entire library of clones expressing full-length open reading frames or fragments of genes fused to the AD. The bait expressed from one mating type is mated with preys expressed in the opposite mating type. Diploids are plated on medium selecting for interaction by prototrophic growth. Prey plasmids of positive clones have to be isolated and sequenced to identify their identity.

An advantage of the matrix approach is the rapid identification of interactions, the high accuracy of the robot assisted procedures and a low rate of false positives, since the interactions are defined. However, the required equipment is expensive and only full-length preys can be found as binding partner for a given prey. This can be a disadvantage, because certain proteins bind with distinct regions to a given bait, but not as a full-length protein. In contrast, a cDNA library contains both full-length and random protein fragments. A drawback of cDNA library screening is the exhaustive labor and the risk of false positives and negatives, which might be higher than in the matrix approach, since cDNA libraries are generally not normalized.

Both approaches were applied to identify protein interactions of various organisms including higher eukaryotes (Formstecher et al., 2005; Rual et al., 2005).
Adaptions of the yeast two-hybrid method

Some protein classes, including membrane proteins and transcription factors cannot be studied in the y2h-system. To outwit these constraints and to broaden the scope of experimental feasible questions, the two-hybrid method has evolved into numerous directions by means ranging from relatively simple tricks up to sophisticated yeast two-hybrid inspired methods (Fashena et al., 2000; Auerbach et al., 2002; Piehler, 2005; Suter et al., 2006).

The repressed transactivator system (RTA)

Hirst and coworkers used the general transcription repressor Tup1p to develop a method for probing proteins with intrinsic transcriptional activity such as transcription factors (Hirst et al., 2001). The conventional yeast two-hybrid assay is not suitable for this class of proteins, since a DBD fused bait is capable to activate reporter genes in absence of an interacting prey protein.

The yeast repressor Tup1p is recruited by Mig1p to upstream repressing sequences of glucose repressed genes. Gene repression by Tup1p is dependent on the corepressor Ssn6p and might involve modulation of the RNA polymerase II holoenzyme function and organizing nucleosomes (Hirst et al., 2001).

To detect protein interactions with transactivating baits by the RTA method, the bait is fused to the DBD of the Gal4p yeast transcription factor (Fig. 1.3). The repressing domain (RD) of Tup1p is attached to the prey. Without an interaction, the Gal4-DBD-bait is directed to the upstream activating sequences of GAL4 (UASGal4) and induces transcription of the reporter gene URA3. Expression of Ura3p selects for prototrophy and 5′-FOA (fluoroorotic acid) sensitivity, since Ura3p converts the compound 5-FOA into a toxic metabolite. If the DBD-bait is coexpressed with an interacting prey-Tup1-RD fusion, URA3 is repressed and the readout of the interaction follows by 5-FOA resistant yeast cells.
Figure 1.3. The repressed transactivator system (RTA). (A) A transactivating bait is fused to the Gal4p-DBD. The bait is recruited to the upstream activating sequences of \textit{GAL4} (UAS\textsubscript{GAL4}) by the Gal4p-DBD. Due to the intrinsic transcriptional activity of the bait, it is capable of recruiting the RNA polymerase II complex and activate transcription of \textit{URA3}. The expression of Ura3p leads to a \textit{URA3}\textsuperscript{+}-phenotype and 5-FOA resistance in the reporter strain. (B) Upon interaction between the DBD-fused transactivator bait with a prey fused to the Tup1p-repressor domain (Tup1-RD) the \textit{URA3} reporter gene is repressed. Thus, interaction can be selected by 5-FOA resistance.

The RNA polymerase III based two-hybrid (Pol III) system

The Pol III system was designed to screen and study protein interactions of transcription factors as well. The method exploits the fact that polymerase II and III based transcription is mediated by different transcription factors and is based on the activation of a modified \textit{SNR6} reporter gene transcribed by the polymerase III (Marsolier et al., 1997). U6, the transcript of the \textit{SNR6} gene, is an essential small nuclear RNA involved in splicing. Transcription of \textit{SNR6} is stimulated by binding of the transcription factor τ138p, a subunit of TFIIIC, to a specific DNA sequence located downstream of the \textit{SNR6} gene, the so-called B-block. Deletion of this B-block abolishes binding of τ138p and inactivates transcription of \textit{SNR6}.

The reporter construct of the Pol III system bears five Gal4p binding sequences (UAS\textsubscript{G}) in place of the original B-block (UAS\textsubscript{G}-\textit{SNR}). This otherwise inactive reporter gene can be activated by expression of a fusion protein between τ138p and the Gal4p-DBD (Fig. 1.4A). Activation of UAS\textsubscript{G}-\textit{SNR} can also be achieved by fusing τ138p to a protein that interacts with a known partner fused to the Gal4p-DBD, analogous to the conventional y2h system.
1. General introduction

For screening procedures a SNR6 knock out strain and a temperature sensitive episomally expressed SNR6 mutant is used (survival construct), which allows growth at 30°C, but not at 37°C (Petrascheck et al., 2001). Plasmids encoding the survival construct, the τ138p- and Gal4p- fused potential interactors are inserted into the SNR6 deleted reporter strain by triple transformation. Yeast harboring an interaction then is selected at the non-permissive temperature of 37°C.

Equal to the conventional y2h system and the RTA system, the RNA pol III system detects an interaction between bait and prey in the nucleus. In contrast, the following methods presented will sense an interaction and trigger the read out of an interaction outside of the nucleus in the cytoplasm or at the plasma membrane.

The SOS recruitment system
The SOS recruitment system is based on the essential Ras signal transduction pathway and was developed to study transcriptionally active proteins or proteins which are associated with the membrane (Aronheim et al., 1994; Young et al., 1998). Ras guanyl exchange factor Cdc25p stimulates guanyl nucleotide exchange on Ras and promotes downstream signaling events that ultimately lead to cell growth. A Cdc25-2 temperature sensitive yeast strain can be complemented by the human homolog SOS, which is active but unable to localize with the membrane. A soluble protein X (bait) is fused to SOS, whereas a protein y (prey) is associated by myristylation with the membrane (Fig. 1.4B). Since the Ras pathway is absolutely dependent on the membrane recruited human SOS, cells expressing an interaction between bait and prey grow on the non-permissive temperature of 36°C.
1. General introduction

RNA Polymerase III system

A

Nucleus

t138 X

SOS reporter gene UASGal

RNA pol III

Y

ts U6 snRNA

tn survival construct

Growth at 37°C

SOS recruitment system

B

Cytoplasm

X SOS

No growth at 36°C

Y

ts Cdc25

Downstream signaling and growth at 36°C

Ras recruitment system (RRS)

C

Cytoplasm

yRas GDP

No growth at 36°C

X mRas GTP

Downstream signaling and growth at 36°C

Reverse Ras recruitment system (reverse RRS)

D

Cytoplasm

yRas GDP

No growth at 36°C

X mRas GTP

Downstream signaling and growth at 36°C

Figure 1.4. Y2H-based techniques for the identification of protein interactions. (A) The RNA polymerase III system. Since the temperature sensitive (ts) survival construct is not functional at 37°C, only those cells are selected at 37°C which harbor an interacting t138-X and Y-Gal4-AD. The hybrid transcription factor binds to the operator sites, recruits the RNA pol III, which leads to the expression of the essential U6 snRNA. (B) The SOS recruitment system. Top: No selective growth at 36°C, due to a lacking binding partner Y. The guanine exchange factor SOS remains in the cytoplasm below the membrane anchored prey Y, recruits X-SOS to the membrane, since X and Y do interact. Nucleotide exchange by SOS renders yeast Ras active and downstream signaling occurs. (C) The RRS and the reverse RRS use the same ts Cdc25-2 strain as the SOS system. Activated X-mRas is directed to the membrane, upon interaction between the bait X and a membrane associated prey Y. (D) The reverse RRS uses an integral membrane protein as bait (X). Contrarily to the RRS, here the prey Y is fused to activated mRas. The readout follows analogous to the RRS. Please see also text for details.
Ras recruitment systems
The Ras pathway was also exploited for the Ras Recruitment System (RRS) (Broder et al., 1998) and the reverse RRS (Hubsman et al., 2001). Equal to the SOS system, a Cdc25-2 temperature sensitive yeast strain is used in both the RRS and the reverse RRS. In the RRS, a soluble protein under investigation X is expressed as a fusion with activated mammalian Ras (mRas), localized in the cytoplasm (Fig. 1.4C). Since the mRas is constitutively active, no Cdc25p is required for the guanyl nucleotide exchange. The only requirement for activating the Ras pathway is the recruitment of mRas to the plasma membrane. Co-expression of X-mRas with an interacting protein Y that was targeted to the plasma membrane by a myristylation signal recruits X-mRas to the plasma membrane. As the endogenous Cdc25-2 mutant is active at 30°C, but inactive at 36°C, only those cells are selected for growth at 36°C which harbor an interaction between the membrane anchored prey Y and X-mRas. The RRS method can be applied on cytosolic and nuclear bait proteins, but not for membrane associated or integral membrane proteins.

To circumvent this problem the reverse RRS system was created, for which an integral membrane protein serves as bait protein. The bait is coexpressed unmodified with a binding partner Y fused to activated mRas. This leads to the recruitment of mRas-Y to the membrane protein X upon interaction and positive growth selection at 36°C (Fig. 1.4D). However, the reverse RAS is very limited for screening procedures since membrane proteins expressed as bait (RRS) or from a cDNA library (reverse RRS) will lead to unspecific results.

G-protein fusion technology
In a similar approach, the yeast mating pathway was used to study protein-protein interactions involving integral membrane proteins as baits (Ehrhard et al., 2000), but this system has not been adapted and used for yeast cDNA library screenings.
The split-ubiquitin system

The 76 amino acids yeast ubiquitin is highly conserved in eukaryotes (Ozkaynak et al., 1984). Many regulatory proteins are subject to rapid proteolysis following conjugation to an ubiquitin chain. The poly-ubiquitinylated protein is recruited to the 26 proteasome, where the ubiquitin moieties are recognized by ubiquitin binding proteases (UBPs) and cleaved prior to protein degradation (Hershko and Ciechanover, 1992).

The split-ubiquitin system takes advantage of the highly specific cleavage by UBPs (Johnsson and Varshavsky, 1994). The expression of a fusion protein consisting of ubiquitin and a C-terminally attached reporter in yeast results in fast and complete cleavage by UBPs. Cleavage of ubiquitin in an N-terminal (Nub) and a C-terminal half (Cub) leads to the spontaneous reassociation of the so called “split-ubiquitin”, which is functional since it is recognized by UBPs. Mutations were engineered into Nub by replacing the isoleucine residues at position 3 and 13 into glycine, alanine or valine to reduce its affinity for Cub and thereby suppress the spontaneous reassembly of Cub and mutated Nub. In these cases efficient reassociation is only seen if the two halves are located in close proximity to each other by the fusion to two interacting proteins X and Y. Subsequently, a Cub-attached reporter is cleaved off from split-ubiquitin.

To date two applications of the split-ubiquitin system have been described: the rUra3p based split-ubiquitin system and the transactivator based split-ubiquitin system.

The rUra3p based split-ubiquitin system

According to the N-end rule of protein degradation, proteins that carry a destabilizing amino acid at the N-terminus are rapidly tagged with ubiquitin and then degraded by the 26S proteasome (Bachmair et al., 1986; Hershko et al., 1986). This property can be conferred onto any protein by simply exchanging its N-terminal residue for a destabilizing one (Varshavsky, 1997). In the rUra3 based split-ubiquitin system (Wittke et al., 1999), a destabilized Ura3p protein (rUra3p) is fused to Cub, which is attached to a protein X (Fig. 1.5A).

Since Ura3p converts the compound 5-FOA into a toxic metabolite, cells that express the X-Cub-rUra3p fusion protein are unable to grow on plates supplemented with 5-FOA. In a second step, an interacting protein Y is expressed as a fusion to NubG. Like described above, the reporter protein rUra3p is cleaved off upon an interaction between X and Y.
The now liberated N-terminal arginine on rUra3p is destabilized and thus, the entire protein is quickly degraded. This degradation step is very efficient, so that cells containing interacting X-Cub-rUra3p and Y-NubG fusions will soon have no rUra3p in the cytoplasm left and will therefore grow on selective medium containing 5-FOA. Since the rUra3 based split-ubiquitin system does not rely on a transcriptional readout, it is very versatile and can be applied on nuclear, cytoplasmic and integral membrane proteins (Laser et al., 2000; Kerkman and Lehming, 2001; Raquet et al., 2001; Eckert and Johnsson, 2003).

The transactivator split-ubiquitin system

Same as the rUra3p system, the transactivator split-ubiquitin system relies on the reconstitution of split-ubiquitin due to two interacting proteins fused to Cub and Nub and the following cleavage of a reporter by UBPs. However, in contrast to the rUra3 system, an artificial transcription factor serves as reporter in the transactivator system, composed of the *Escherichia coli* LexA-DBD fused to the strong transactivating domain VP16 of *Herpes simplex* (Stagljar et al., 1998). The cleaved LexA-VP16 reporter protein is directed into the nucleus, where it binds LexA operator DNA sequences located in the promoter region of reporter genes (Fig. 1.5B). DNA-binding of LexA-VP16 recruits the RNA polymerase II complex to the promoter and results in transcription of the reporter genes, whose activity is then assayed by appropriated means. For instance, prototrophic and colorimetric reporter genes such as *HIS3* and *lacZ* respectively, present in the genome of the host yeast strain allow selection on plates with minimal medium lacking the amino acid histidine or yields blue colonies in a β-galactosidase assay.

A number of reports have shown the capacity of the MbYTH system to detect interactions involving all types of integral proteins and membrane-associated proteins (Cervantes et al., 2001; Obrodlik et al., 2004; Pandey and Assmann, 2004; Yan et al., 2005; Ihara-Ohori et al., 2007). In addition the MbYTH system has been used successfully to find novel interactors by cDNA library screening (Thaminy et al., 2003; Wang et al., 2004; Matsuda et al., 2005) and by a large scale matrix approach (Miller et al., 2005). The latter has identified 1985 putative interactions involving 536 integral membrane proteins of *S. cerevisiae*. 131 interactions were assigned with a very high confidence level by bioinformatics means. Since previous studies have focused on soluble proteins, the majority
of the interactions were not previously described. Thus, this study is a valuable extension of the yeast protein interaction map in which interactions of integral membrane proteins are largely underrepresented.

Figure 1.5. The rUra3p and the transactivator split-ubiquitin system. (A) Top: A membrane or a soluble bait protein serve as bait X in the rUra3 system. Without an interacting prey Y, cells do not grow on 5′-FOA, but on minimal medium lacking uracil. Below: Upon interaction with prey, split-ubiquitin is restored, recognized and cleaved by the ubiquitin binding proteases (UBPs). The freed RUr3p reporter is quickly degraded due to the destabilizing N-terminus. Cells grow on 5′-FOA, but not on medium lacking uracil. (B) Top: An integral membrane protein serves as bait, fused to Cub and the transcription factor LexA-VP16. Since LexA-VP16 is immobilized at the membrane, no transcription of reporter genes occurs. Below: Upon interaction between NubG-fused Y and X, the same process leads to cleavage of the reporter as in (A). LexA-VP16 is directed into the nucleus, where it activates reporter genes which will lead to prototrophic growth and β-galactosidase activity.
Split-protein sensors

Ideally, the interaction-induced reassociation of split-protein sensors provides the cell with a growth advantage, thus allowing a selection for interacting proteins. A common feature of the presented protein fragment complementation techniques so far, is the more or less indirect and time-shifted response, as they rely on the transcription of a reporter gene, Ras- or G-protein downstream signaling events or the degradation of the rUra3p protein. Since the introduction of split-ubiquitin, a variety of other split-protein sensors have been developed with the aim to provide a more direct read-out, including pairs of fragments of dihydrofolate reductase (Remy and Michnick, 1999), β-galactosidase (Rossi et al., 1997), β-lactamase (Galarneau et al., 2002), green fluorescent protein (Beauchemin et al., 2007) and the split-Trp (Tafelmeyer et al., 2004).

Among these systems, only split-ubiquitin was successfully applied to screen for binding partners. The other sensors were used to study the interactions between selected pairs of proteins to date rather than to find new partners by a random library approach.

Resonance energy transfer

A promising technique which is suitable for the detection and in vivo monitoring of protein interactions combines resonance energy transfer with flow cytometry and has the potential for large-scale applications. An interaction is traced by fusing the protein of interest X to the “energy donor” and the putative binding partner Y to the “energy acceptor”. Upon interaction of the X- and Y- fusions, fluorescence is analyzed by microscopy or flow cytometry. The fluorescence resonance energy transfer (FRET) technique commonly uses variants of fluorescent proteins for energy donors and acceptors (Shibasaki et al., 2006), which creates a relatively high background of cellular autofluorescence due to the requirement of external illumination. This limitation is avoided in bioluminescence resonance electron transfer (BRET) by using a bioluminescent energy donor, such as Renilla luciferase (Xu et al., 1999; Boute et al., 2002). BRET has been successfully applied for demonstrating the oligomerization of G-protein coupled receptors (Angers et al., 2000; Pfleger and Eidne, 2003) and was used for high-throughput screening for antagonists of the chemokine receptor CCR5 (Hamdan et al., 2005).

However, these methods are technically and experimentally rather demanding. Furthermore, the previously mentioned two-hybrid reporter systems are readily implemented and have a more robust read-out for identifying and screening proteins.


\textit{Saccharomyces cerevisiae} as model organism

The majority of \textit{in vivo} methods utilize yeast \textit{S. cerevisiae} for finding novel protein interactions. Yeast is genetically easy to manipulate and many genes are conserved between yeast and human. In addition, the yeast research community is exceptionally well organized, offering publicly available genomic and proteomic yeast data and providing multiple useful tools such as GFP- or TAP-tagged yeast collections and yeast deletion strains.

However, some proteins of higher eukaryotes participating in signal transduction pathways undergo post-translational modifications or need cofactors, which are absent in yeast, to function properly. Co-expression of heterologous modifying enzymes or cofactors with putative interacting protein pairs can solve this constraint in yeast.

Detection of protein interactions in mammalian cells

Some experimental attempts were performed to identify defined protein interactions or to screen for protein binding partners in mammalian cells, despite the fact that mammalian cells are difficult to manipulate genetically. The mammalian methods include a split-ubiquitin based system using the modified guanine phosphoryltransferase reporter protein Rgp2 (Rojo-Niersbach et al., 2000) and the complex two-hybrid inspired MAPPIT screening method (Tavernier et al., 2002). The Tavernier group has used the MAPPIT method frequently for the characterization of protein interactions (Ulrichts, 2007; Montoye, 2006) as well as for a small compound screening (Caligiuri et al., 2006). Although MAPPIT can be used for the matrix approach, this procedure has not yet been adapted for proteome-scale screens.

Recently, the LUMIER (for luminescence-based mammalian interactome mapping) technology was developed (Barrios-Rodiles et al., 2005; Ozdamar et al., 2005), an automated high-throughput technology to map protein-protein interactions systematically in mammalian cells. This strategy uses \textit{Renilla} luciferase enzyme fused to proteins of interest, which are then coexpressed with Flag-tagged partners in mammalian cells. Interactions are determined by performing an RL enzymatic assay on immunoprecipitates using an antibody against Flag. Applying the technique to study interactions of the transforming growth factor-\(\beta\) superfamily, 11914 interactions were detected between 947 proteins.
Confirmation of *in vivo* identified interactions by independent methods

The identification of protein interactions *in vivo* requires validation *in vitro*, as false positive results can be generated. Coimmunoprecipitation, GST-pull down and plasmon surface resonance are the most frequently used methods for this purpose. Furthermore, interacting partners need to be characterized with respect to their function in biological processes to finally validate them.

*In silico* prediction and validation of protein-protein interactions

As genome sequences continue to be completed and the amount of interaction data increases, a growing number of computational methods for predicting protein interactions are emerging. These methods compare the presence or absence of genes within genomes, the spatial relationships among genes, gene fusion events, and co-evolution among protein pairs to assess the possibility of protein interactions. Moreover, when the molecular structure of two proteins is known, the prediction of protein interactions can be analyzed.

To confirm data resulting from large-scale studies, bioinformatics methods are often used (Miller et al., 2005; Gavin et al., 2006; Krogan et al., 2006). By the application of different algorithms, results are integrated with available data on expression, localization, function, evolutionary conservation, protein structure and binary interactions.

The next challenge is to integrate all data produced by functional genomics and proteomics and their dynamics into a more comprehensive picture of cellular machines and their function.

Concluding remarks

The development of large-scale applications to cope with the tremendous amount of data provided for proteomic research should, however, not mask the fact that proteins are assigned with unique physiochemical features and protein interactions are regulated with regard to different dimensions such as time, space and environment. Research at small scale remains important to take to account this singularity and all the dimensions of a given protein interaction. Moreover, small scale applications are required to detect and characterize protein partners, which are not amenable to high-throughput procedures, but significant for the life of an organism. Appropriate tools for this aim are a prerequisite.
2. Chapter - Development of the split-ubiquitin based cytosolic yeast two-hybrid system

Introduction
Inducible transcription factors regulate immediate and long-lived cellular responses necessary for organismal adaptation to their environment. Such responses are mediated to a large degree through changes in gene expression. Malfunctioning of transcription factors and transcriptional regulation often interfere with the development of multicellular organisms and can lead to severe disorders like inflammatory diseases or cancer. Understanding how the transcriptional potential, activity and selectivity of transcription factors are regulated is therefore a topic of intense investigation in numerous laboratories.

Transactivating proteins such as transcription factors cannot be studied in the original y2h system. Alternative systems such as the repressed transactivator assay (Hirst et al., 2001), the RNA pol III method (Petrascheck et al., 2001) or the rUra3 split-ubiquitin system (Laser et al., 2000) outwit drawbacks of the y2h system, but bear their own particular limitations or have not seen frequently in use.

In order to create a broadly applicable and flexible screening system for transactivating proteins, we have modified the well-established split-ubiquitin system (Johnsson, 1994). By combining the split-ubiquitin system with a stringent transcriptional output using multiple independent reporter genes, a screening system was established which can be used to detect interactions between transcriptionally active proteins and for the identification of novel protein interaction partners through cDNA library screening.

Self-activating proteins
Bait proteins are fused to the DBD of the reporter transcription factor in the y2h system. If expression of the DBD fused bait in the host strain leads to activation of y2h-reporter system, the bait protein is termed self-activating due to its intrinsic transactivating properties. These self-activating proteins trigger transcriptional activation due to their ability to recruit components of the RNA polymerase II complex to the promoter of the reporter genes (Fig. 2.1B).
It is in the nature of the y2h system (since it is based on the action of transcription factors), that many transcriptional regulators and especially transcription factors constitute a major class of self-activating proteins. Also large proteins, proteins with acidic clusters or amphipathic helices tend to self-activate when used as bait in the y2h assay.

![Diagram](image)

Figure 2.1. Self-activating proteins in the yeast two-hybrid system. (A) The DBD-fused bait is interacting with an AD-fused prey and thereby activating the reporter gene. (B) Certain DBD-fused baits, like transcription factors, can autonomously activate transcription of the reporter genes in absence of an AD-fused prey. DBD, DNA-binding domain. AD, activation domain.

**Principle of the cytosolic yeast two-hybrid system**

The cytoY2H system uses the mechanism of the well-established split-ubiquitin system (Johnsson and Varshavsky, 1994; Stagljar et al., 1998). A soluble protein of interest (the bait) is fused to the C-terminus of the integral membrane protein Ost4p (Kim et al., 2003) and the N-terminus of a reporter cassette composed of the C-terminal half of ubiquitin (Cub) and an artificial transcription factor LexA-VP16 (Fig. 2.2). A prey protein is fused to the N-terminal half of the mutated ubiquitin (NubG). Interacting baits and preys bring NubG and Cub close enough together to reconstitute ubiquitin, which is then recognized by ubiquitin-binding proteases (UBPs). Upon cleavage of UBPs, the transcription factor LexA-VP16 is released from the membrane and directed into the nucleus, where it activates reporter genes present in the yeast genome of the reporter strain. The transcriptional readout is analogous to the MbY2H system.

Thus, the cytoY2H system translates an interaction taking place outside of the nucleus into a defined transcriptional response. In contrast to other genetic interaction assays, the bait remains in the cytosol since it is fused to the membrane anchor and never moves to the nucleus, thereby avoiding transcriptional activation of the reporter genes in the absence of a bait-prey interaction.
2. Development of the split-ubiquitin based cytosolic yeast two-hybrid system

![Diagram of the cytoY2H system]

Figure 2.2. (A) Principle of the cytoY2H system. A protein of interest (the bait) is inserted between the membrane anchor Ost4p and the C-terminal half of ubiquitin (Cub) followed by the artificial transcription factor LexA-VP16. A second protein (the prey) is fused to the mutated N-terminal half of ubiquitin (NubG). Bait and prey are co-expressed in a reporter yeast strain. (B) If bait and prey interact, Cub and NubG complement to form functional ubiquitin, which is recognized and cleaved by ubiquitin binding proteases (UBPs). Cleavage leads to translocation of LexA-VP16 to the nucleus, where it activates LexA-responsive reporter genes. Yeast cells harboring an interacting protein pair are either selected for prototrophy or by measuring β-galactosidase activity. Cub, C-terminal half of ubiquitin; Nub, mutated N-terminal half of ubiquitin (113G).

**The membrane anchor Ost4p**

For the association of the bait-reporter cassette at the membrane a small type I membrane protein is required, which can efficiently anchor the bait construct at the membrane and which has no or only few interaction partners of its own. Ost4p fulfills these criteria, since it is an extraordinarily small (3.7 kDa) type I membrane protein of the *S. cerevisiae* oligosaccharyl complex, localized in the membrane of the endoplasmic reticulum. Topology studies have demonstrated that Ost4p was able to anchor a protein of 128 kDa fused to its C-terminus at the membrane of the endoplasmic reticulum (Kim et al., 2003). Therefore, we selected Ost4p as a membrane anchor for the cytoY2H system.
Development of the split-ubiquitin based cytosolic yeast two-hybrid system

Cytoplasmic split-ubiquitin based y2h system

Membrane based split-ubiquitin y2h system

Prey

Figuic 2.3. Comparison of the cytoY2H system with its progenitor, the membrane based split-ubiquitin system (MbYTH). Note, the bait in the cytoY2H is a soluble protein, whereas the bait in the MbYTH is composed of any transmembrane protein with a cytoplasmic tail. In contrast, in the cytoY2H system a type I transmembrane protein is merely used to anchor the bait construct at the membrane to prevent its entry into the nucleus.

Difference between the membrane based split-ubiquitin and the cytoY2H system

In the MbY2H system (Stagljar et al., 1998) the protein of interest is a transmembrane protein, whose cytosolic C-terminus is attached to Cub-LexA-VP16 (Fig. 2.3). The main purpose of the MbY2H system is to screen for interacting membrane proteins or to analyze defined interactions between two membrane proteins. In contrast to the membrane based split-ubiquitin system, transmembrane proteins cannot be used as bait proteins in the cytoY2H assay. Instead, the novel assay is designed for soluble proteins which can be either full-length proteins containing acidic regions or transactivation domains and is suitable for cytosolic and nuclear proteins. The cytoY2H system is very flexible and can be applied to essentially any soluble protein, protein subfragment or domain. Thus, the application areas of the two assays are complementary, although their experimental setup is similar (Fig. 2.3).

False positives in two-hybrid assays

False positives are defined as artifactual interaction partners (preys) which are selected in a y2h screen although they do not interact specifically or in a physiologically plausible way with the bait. The number of false positives in y2h library screenings varies considerably (Serebriiskii et al., 2000). There are two main categories of false positives: the first category
encompasses proteins that bind unspecifically to the bait protein or to the promoter region upstream of the reporter gene, thereby activating the reporter gene in the absence of a true protein interaction. Often, proteins of this class are sticky and their biological function involves binding non-specifically to a broad range of different proteins or DNA sequences, especially when overexpressed. The second category of false positives is independent of the bait under investigation or the promoter region and seems to mediate their effects rather through indirect metabolic effects. Some of these false positives have the capability to alter the growth rate of yeast and to alter the yeast metabolism in order to overcome selection. A list of frequent false positives of the classical y2h system is provided by E. Golemis (www.fccc.edu/research/labs/golemis/).

Bait-dependency test

To avoid extra work, false positives in two-hybrid screening should be identified and removed as early in the screen as possible. The bait-dependency test is one effective tool to identify these false positive clones, which however follows at a relatively late step in screening procedures (Fig. 2.14). Prey plasmids are rescued from the original clones and re-transformed together with the original bait or unrelated bait constructs into the yeast reporter strain. Preys which can be reproducibly selected for prototrophic growth and β-galactosidase activity with the original bait are considered to be bait-dependent. Preys which are not positive in interaction assays with the original bait or are positive with the unrelated baits as well, can be classified as false positive interactors. For use as bait controls, proteins are chosen that are not functionally or physically linked to the original bait, but preferentially share similarities like subcellular localization or stem from the same organism.

A quality control which is applied at an earlier step of the screening procedures comprises PCR-amplification of prey plasmid inserts of the selected clones, DNA sequencing and the evaluation of data by using bioinformatics means. Taking into account all possible biological and technical data available, different confidence levels are ascribed to the novel interactors.
Methods & Materials

Bait and prey construction

To construct the bait vector pSATO, the cDNA encoding the entire open reading frame of the yeast endoplasmatic reticulum OST4 was amplified by PCR from a genomic S. cerevisiae library using a forward primer with an Xba I site and a reverse primer with the restriction sites Sfi I - Stu I - Neo I. To shorten and optimize the multiple cloning site (MCS), Cub was amplified from the bait vector pTMBV4 using a forward primer with an Sfi I - Neo I site and reverse primer with a Not I site. The vector backbone, restricted by Xba I and Not I, was joined by a triple ligation with the amplified OST4 and Cub cassette. To generate pASO and pCO, the ADHI and CYC1 promoter were excised by the restriction sites Swa I and Xba I from pAMBV4 and pCMBV4, respectively. To exchange the mutagenized nuclear localization signal to the original wild type LexA, a 2.8kDa fragment, containing wild type LexA was excised by Afl II and Not I from pCAS and introduced by the same sites into pCO to create pCOWT. If not mentioned otherwise, vectors and coding DNA libraries serving for the construction of the cytoY2H- bait or -prey vectors, were provided by Dualsystems Biotech.

Maps and sequences of most vectors can be viewed on www.dualsystems.com/support.

Table 2.1. CDS inserted in the cytoY2H bait vectors pCO, pASO, pSATO or pCOWT

<table>
<thead>
<tr>
<th>CDS of bait proteins</th>
<th>Abbreviation</th>
<th>Organism</th>
<th>Amino acids</th>
<th>Bait vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large T Antigen</td>
<td>SV40 LT</td>
<td>Simian Virus</td>
<td>84-708</td>
<td>pASO</td>
</tr>
<tr>
<td>Tumor suppressor p53</td>
<td>p53</td>
<td>Human</td>
<td>full-length</td>
<td>pASO</td>
</tr>
<tr>
<td>Uri1p/Bud27p (YFL023w)</td>
<td>Uri1p</td>
<td>S. cerevisiae</td>
<td>full-length</td>
<td>pASO</td>
</tr>
<tr>
<td>Transcription factor Gal4p</td>
<td>fl-Gal4p</td>
<td>S. cerevisiae</td>
<td>full-length</td>
<td>pCOWT</td>
</tr>
<tr>
<td>Activation domain of Gal4p</td>
<td>Gal4p-AD</td>
<td>S. cerevisiae</td>
<td>aa 768-881</td>
<td>pCOWT</td>
</tr>
<tr>
<td>N-terminal region of Gal4p</td>
<td>Gal4-NT</td>
<td>S. cerevisiae</td>
<td>aa 2-841</td>
<td>pCOWT</td>
</tr>
<tr>
<td>Gal80p</td>
<td>Gal80p</td>
<td>S. cerevisiae</td>
<td>full-length</td>
<td>pCOWT</td>
</tr>
<tr>
<td>I kappa-B-alpha</td>
<td>IxB-α</td>
<td>Human</td>
<td>full-length</td>
<td>pASO</td>
</tr>
<tr>
<td>I kappa-B kinase alpha</td>
<td>IKKα</td>
<td>Human</td>
<td>full-length</td>
<td>pASO</td>
</tr>
<tr>
<td>I kappa-B kinase beta</td>
<td>IKKβ</td>
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<td>pASO</td>
</tr>
<tr>
<td>I kappa-B kinase gamma</td>
<td>IKKγ</td>
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<td>pASO</td>
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</tbody>
</table>
The coding DNA sequence (CDS) of the proteins studied in the cytoY2H system were cloned via Sfi I sites downstream of the OST4 sequence into the bait vectors or downstream of the NubG cDNA into the prey vectors pDSL-Nx or pADSL-Nx. All constructs were verified by sequencing and the expression of all baits and preys was verified by Western blotting analysis (data not shown). An overview of the cloned baits and preys is given in Table 2.1 and 2.2.

Table 2.2. CDS inserted in the split-ubiquitin prey vectors pDSL-Nx and pADSL-Nx

<table>
<thead>
<tr>
<th>Prey proteins</th>
<th>Abbreviation</th>
<th>Organism</th>
<th>full length/truncation residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large T Antigen</td>
<td>SV40 LT</td>
<td>Simian Virus</td>
<td>84-708</td>
</tr>
<tr>
<td>Tumor suppressor p53</td>
<td>fl-p53</td>
<td>Human</td>
<td>full-length</td>
</tr>
<tr>
<td>N-terminally truncated p53</td>
<td>Δp53</td>
<td>Human</td>
<td>full-length</td>
</tr>
<tr>
<td>Ubiquitin-protein ligase E3 Mdm2</td>
<td>Mdm2</td>
<td>Human</td>
<td>full-length</td>
</tr>
<tr>
<td>Transcription factor Gal4p</td>
<td>fl-Gal4p</td>
<td>S. cerevisiae</td>
<td>full-length</td>
</tr>
<tr>
<td>Activation domain of Gal4p</td>
<td>Gal4p-AD</td>
<td>S. cerevisiae</td>
<td>768-881</td>
</tr>
<tr>
<td>N-terminal region of Gal4p</td>
<td>Gal4-NT</td>
<td>S. cerevisiae</td>
<td>2-841</td>
</tr>
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<td>Transcriptional regulator Gal80p</td>
<td>Gal80p</td>
<td>S. cerevisiae</td>
<td>full-length</td>
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<td>RNA polymerase subunit Rpb5</td>
<td>Rpb5p</td>
<td>S. cerevisiae</td>
<td>full-length</td>
</tr>
<tr>
<td>Prefoldin 6 subunit = Gim1</td>
<td>Pfd6p</td>
<td>S. cerevisiae</td>
<td>full-length</td>
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<td>NF-kappa B subunit p65</td>
<td>fl-p65</td>
<td>Human</td>
<td>full-length</td>
</tr>
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<td>p65 linker-transactivation domain</td>
<td>p65-LTA</td>
<td>Human</td>
<td>303-551</td>
</tr>
<tr>
<td>NF-kappa B subunit p65, ΔN-terminus</td>
<td>Δp65</td>
<td>Human</td>
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</tr>
<tr>
<td>I kappa-B kinase alpha</td>
<td>IKKα</td>
<td>Human</td>
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<tr>
<td>I kappa-B kinase beta</td>
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<td>Human</td>
<td>full-length</td>
</tr>
<tr>
<td>I kappa-B kinase gamma = NEMO</td>
<td>IKKγ</td>
<td>Human</td>
<td>full-length</td>
</tr>
<tr>
<td>Lamin C</td>
<td>lamC</td>
<td>Human</td>
<td>66-230</td>
</tr>
<tr>
<td>Neuregulin receptor degradation</td>
<td>Nrdp1</td>
<td>Human</td>
<td>full-length</td>
</tr>
</tbody>
</table>

For functional control assays the prey vectors pNubG-Alg5, pNub1-Alg5, pNubG-Ost1 and pNubI-Ost1, pNubG-Tom20, pNubI-Tom20, were employed. NubI corresponds to the unmutated N-terminal half of ubiquitin, thus wild type Nub.
Membrane extracts
The cell pellet of 850 ml yeast NMY32 culture, optical density (OD$_{546}$) 1.0, expressing a cytoY2H bait construct, was frozen in liquid nitrogen and resuspended in 4ml TMM (50 mM Tris-HCl pH 7.4, 1 mM MgCl$_2$, 1 mM MnCl$_2$) supplemented by protease inhibitor cocktail (Roche). Cell lysis followed by using glass beads and vortexing at 4°C. Cell debris was removed by centrifugation and the extract was spun down at 20'000 g for 3h at 4°C. The supernatant was removed and the pellet dissolved in TMM supplemented with protease inhibitor, 1 % Triton X-100 and 35% glycerol to dissolve membrane proteins.

Construction of NMY32
The novel yeast reporter strain NMY32, bearing the additional reporter gene $ADE2$, was constructed mainly according to (James et al., 1996). An $ADE2$-reporter cassette consisting of 1044bp of the $ADE2$ 5' untranslated region, followed by 8 LexA-operator half-sites, the 774bp GAL2 promoter and 709 bp of the C-terminal $ADE2$ coding sequence was constructed into the vector p417-CYC for introduction into the $ADE2$ locus of L40 (MATa trp1-901 leu2-3,112 his3A200 ade2 LYS2::{lexAop}$^4$-HIS3 URA3::{lexAop}$^8$-lacZ), Invitrogen) by homologous recombination. To select for adenine 2 complementation, the $ADE2$ reporter cassette was transformed together with the interacting protein pair pLexA-Ap53 (bait) and pAct-largeT (prey) into L40 and plated on the minimal medium SD-Ade-Trp-Leu, selecting for adenine complementation and for the bait and prey plasmids (minimal medium containing 2% glucose (Sigma, Buchs, Switzerland), 0.67% yeast nitrogen base (BD Biosciences), complete amino acid mixture lacking adenine, leucine and tryptophan (Qbiogene), and 2% bacto agar (BD Biosciences). Loss of the plasmids pLexA-Ap53 and pAct-largeT was achieved by several passages of NMY32 cultivation in the complete medium YPAD (1% yeast extract, 2% peptone, 2% glucose, 2% bacto agar (all BD Bioscience), 40mg adenine hemisulphate (Applichem)).

Yeast transformation, relative growth and spotting
The S. cerevisiae reporter strain L40A or NMY32 (MATa trp1-901 leu2-3,112 his3A200 LYS2:: {lexAop}$^4$-HIS3 ade2:: {lexAop}$^8$-ADE2 URA3:: {lexAop}$^8$-lacZ) were co-transformed with bait and prey plasmids using the lithium acetate method (Gietz and Woods, 2005). Half of the co-transformants were plated on SD-Trp-Leu medium. The second half, selecting for interaction, was plated on SD-Trp-Leu-His or SD-Trp-Leu-His-Ade medium.
(minimal medium as described above, but lacking tryptophan, leucine, histidine and adenine). After 3 days of incubation at 30°C, relative growth was estimated from the number of colonies on selection plate for interaction over the colony number on selection plate for plasmids.

For spotting assays several colonies from double transformants grown on SD-Trp-Leu were transferred to liquid SD-Trp-Leu medium and grown overnight to an OD$_{546}$ of 1.0. 5 microliters of different dilutions (undiluted, 1:10, 1:100 and 1:1000) were spotted onto SD-Trp-Leu and SD-Trp-Leu-His-Ade medium and grown for 2 days at 30°C.

Analysis of β-Galactosidase activity by pellet X-gal assay
For each interaction pair, several colonies were picked from the selection plates and inoculated in 2ml deep-well 96 plates, containing 1ml of SD-Leu-Trp. Cultures were grown from an OD$_{546}$ < 0.1 to an OD$_{546}$ 1.0. One OD unit of yeast was pelleted by centrifugation at 2000 g for 5 min. The supernatant was discarded and cell lysis was performed by two freeze-thaw cycles (3 min in liquid nitrogen, 3 min in a 37°C water bath). Pellets were subsequently resuspended in 20 μl water, transferred to a transparent flat bottom 96-well microtiter plate, mixed with 100 μl PBS buffer pH 7.4, containing 500 μg/ml X-gal (Applichem), and 0.05% (v/v) β-mercaptoethanol and incubated at room temperature. The β-gal enzymatic activity was monitored by recording the color development at different time points using a flatbed scanner (Mockli and Auerbach, 2004).

Self-activation test
7 μg of yeast cDNA library were transformed by a high-efficiency transformation into the yeast reporter strain NMY32 expressing the bait to be titrated, using the lithium acetate protocol (Gietz and Woods, 2005). After transformation, the cells were allowed to recover for 90 minutes in 2xYPAD complete medium. Cells were resuspended in 3.6 ml NaCl 0.9%. 0.3 ml of the cell suspension was plated on the following round 150mm diameter plates: SD-Ade-Leu-Trp and SD-Ade-His-Leu-Trp supplemented with 0, 2.5, 5, 7.5, 10 and 15 mM 3-AT. Plates were incubated at 30°C for 3-4 days and growing colonies observed and counted. Transformation efficiency had to exceed 10$^5$ colony forming units/μg prey plasmid to cover the cDNA library complexity.
Artificial screen for rescuing known interactors

This assay probed to rescue an interactor of a certain bait in an artificial cytoY2H- pilot screen. A prey plasmid encoding the interactor fused to NubG was mixed in low ratio (1:500) with a cDNA library. Screening procedures were performed as in the self-activation test. Co-transformants in NMY32, expressing a bait construct- library vector mix, were selected for growth under the conditions defined in the self-activation test.

CytoY2H screen

28 µg yeast cDNA library transformed into the yeast reporter strain L40 or NMY32 harboring the bait plasmid, aiming at least one million total colonies screened. A high-efficiency transformation protocol, using the lithium acetate protocol described above was employed for this purpose. Transformed cells were resuspended in 4.8 ml NaCl 0.9%. 0.3ml was plated on each of 16 round 150mm diameter plates, containing the medium that was defined in the self-activation test. Transformants were grown for 3-4 days at 30°C, classified according size and restreaked on the same selective media. cDNA libraries were constructed by Dualsystems or according their standard procedures.

Yeast plasmid rescue, restriction analysis and sequencing

Restreaked yeast was grown over night in 5ml SD-Leu-Trp. Yeast DNA was extracted using the NucleoSpin® Plasmid extraction kit (Macherey-Nagel) according standard procedures with the following changes. 0.1 ml lyticase solution (1.2M Sorbitol, 0.1M NaPO4 buffer, pH 7.4, 5mg/ml Lyticase) was added after cells were resuspended in 0.2ml buffer A1. The spin columns were washed with both guanidine hydrochloride (AW) and ethanol (A4) before yeast DNA was eluted from the column. 5 µl of the eluate were transformed into E. coli to amplify the rescued prey plasmid. DNA was extracted from two different E. coli colonies per clone. 6 µl were digested with the restriction endonuclease Sfi I (NEB) for insert size of the prey DNA. The identity of cDNA inserts was determined by sequencing (Microsynth) and performing BLAST searches (Altschul et al., 1990) against GenBank.
2. Development of the split-ubiquitin based cytosolic yeast two-hybrid system

**Yeast colony PCR**

A very tiny amount of yeast was resuspended in 25 μl lyticase solution (1mg lyticase/ml in 10mM Tris, pH7.5), incubated for 30min at 37°C and mixed with 25 μl 2x lysis mix (0.1% SDS, 28 mM β-mercaptoethanol). 0.5 μl of the solution was used to amplify DNA per PCR (20 μl total volume, Phusion polymerase from Finnzymes).

**Co-Immunoprecipitation**

300ml yeast cultures were grown until mid-log phase, centrifuged and washed in 50ml H₂O. The cell pellet was resuspended in 2ml IP-buffer (20mM Tris pH7.5, 100mM NaCl, 5mM MgCl₂, 2% NP40, 10% glycerol, 20mM β-glycerophosphate, 1mM NaF, 0.5mM DTT, protease inhibitor cocktail (Roche Diagnostics)) and disrupted using a one shot cell disrupter (Constant Systems), followed by centrifugation at 14’000g for 10 min at 4°C. For co-immunoprecipitations 10μg mouse monoclonal anti-HA antibody (Covance) were added to 2mg of cell-lysate and gently mixed for 2h at 4°C. 50μl of pre-washed protein G sepharose beads (Amersham) were added and mixed for another hour. The beads were washed 5 times for 5min in IP-buffer containing 300mM to 600mM NaCl, eluted in 30μl 3x Laemmli-buffer and subjected to immunoblot analyzes. Unspecific binding of preys to Protein G sepharose beads was excluded by performing co-immunoprecipitation with or without anti-HA antibody.
Results

Development of the cytoY2H-system

Our intention was to create an *in vivo* method, useful to study interactions of proteins which are self-activating in the conventional yeast two-hybrid assay. A novel method, the cytosolic yeast two-hybrid (cytoY2H) system, was designed by employing the split-ubiquitin technique (Johnsson and Varshavsky, 1994; Stagljar et al., 1998). Here, I present the development of two applications for the cytoY2H system: the detection of defined protein-protein interactions using pairwise interaction assays and a screening assay to find novel protein interaction partners. Both applications were developed in parallel and results from one application influenced the experimental strategy of the other and *vice versa*. Data are presented chronologically and follow the development of the cytoY2H system. The crosstalk of the two applications is summarized in Fig. 2.10. The strategic approach is illustrated in Fig. 2.4.

Starting bait vectors for the cytoY2H system

We started the development of the cytoY2H system by using the membrane protein Ost4p to anchor the bait construct at the membrane of the endoplasmic reticulum (ER). The tumor suppressor protein and transcription factor p53 served as a test bait. Full-length p53 cannot be studied in the conventional yeast two-hybrid assay, since its transactivating domain is highly self-activating. Furthermore, p53 is very well characterized and its interactions with a large number of proteins have been studied in-depth due to its significant role in cancer (Somasundaram, 2000). Initially, the empty bait vector expressing an Ost4p-MCS-Cub-LexA-VP16 fusion protein and the p53 bait vector were expressed under the control of the strong TEF1 promoter, resulting in the vectors pSATO and pSATO-p53 respectively (Fig. 2.4).
2. Development of the split-ubiquitin based cytosolic yeast two-hybrid system

Yeast reporter strain L40:
MATa trp1 leu2 his3 ade2 LYS2:lexAop1-3 P16/23 HIS3 URA1:lexAop1-3 P16/23 LacZ

Initial experimental set up

Bait: pSATO
Prey: pNubG-HA

Self-activating Why?
1. Bait expression level too high? yes
   2. Prey expression level too high? yes
   3. Bait anchored at the membrane? yes
   4. Reporter strain stringent enough? no
   5. Is p53 suitable for screening? no

Bait expression levels lowered:
Pcycl, ADH1

Prey expression level lowered:
Pcycl

Ost4 retained as membrane anchor
additional reporter gene: ADE2
different baits tested

nuclear transport increased for pCO (pCOWT, wt Lex)

CytoY2H Baits:
pCOWT
pASO
pSATO

CytoY2H-Preys:
pDSL-Nx
pADSL-Nx

NMY32:
MATa trp1 leu2 his3 ade2
LYS2:lexAop1-3 P16/23 HIS3
ade2::lexAop1-3 P16/23 ADE2
URA1:lexAop1-3 P16/23 LacZ

Figure 2.4. Strategic approach for the development of the cytoY2H system. Since the bait vector pSATO and pSATO-p53 co-expressed with the empty prey vector pNubG-HA turned out to be self-activating in the host strain L40 (pink: initial cytoY2H set up), we tried to find the reason for self-activation by analysis of several parameters (expression levels, bait localization) and to improve the system also by trial and error. Green: modifications of the cytoY2H system. Lower panel: the improved cytoY2H system with new components was subsequently used to reconstitute known interactions and to perform cDNA library screenings.
Weakened NLS in the DNA-binding domain of LexA in cytoY2H-bait vectors
The artificial transcription factor in the cytoY2H assay consists of the *E. coli* LexA-DBD and the *Herpes simplex* virus transactivator domain. The LexA-DBD is bearing a strong nuclear localization signal (NLS), directing LexA-VP16 into the nucleus after cleavage from Cub. For a proper transcriptional readout in the cytoY2H assay, it is important that the bait construct is anchored immediately after translation at the membrane and is not guided into the nucleus. Since Ost4p is expressed endogenously in our host strains, the Ost4p-bait-reporter cassette constructs have to be inserted in addition to endogenous Ost4p into the ER-membrane. We supposed that extra amounts of Ost4p could increase the time for membrane incorporation. To prevent that the bait constructs are directed into the nucleus, before anchoring them at the membrane, the transcription factor was weakened by a mutation in the NLS (R157G) of the LexA-DBD (Rhee et al., 2000) in the initial cytoY2H-bait constructs. The transport of the artificial transcription factor into the nucleus was therefore impaired in the bait vectors pSATO and its derivatives pASO and pCO (Table 2.3).

Self-activation test for the initial bait constructs pSATO and pSATO-p53
Since the cytoY2H assay aims for proteins which are self-activating in the conventional y2h system, it had to be tested if bait proteins are completely and steadily anchored at the membrane. Incomplete immobilization at the membrane or cleavage of the bait by proteases can lead to the liberation of the attached transcription factor and to a positive transcriptional read-out.

Thus, the empty bait vector bearing the reporter cassette Ost4p-Cub-LexA-VP16 expressed with the NubG-moiety (empty prey vector), should not interact with any subfragment of the prey vector and therefore not lead to a transcriptional readout in the cytoY2H system. However, cotransformants were capable of activating the HIS3 reporter gene and were therefore considered self-activating.

To suppress subluminal self-activation, the selection medium was supplemented with 3-aminotriazole (3-AT). 3-AT is a competitive inhibitor of the HIS3 gene product which lowers cellular histidine concentrations. Therefore selection stringency is increased with increasing concentrations of 3-AT in the growth medium. If yeast cells expressing a certain bait and empty prey vector still grow under strong selective pressure, this bait cannot be
studied by library screening, since it activates the reporter genes autonomously in the absence of an interacting prey.

Since supplementation with high 3-AT concentrations can lead to false negatives, maximal 15mM 3-AT was generally added. A bait was termed self-activating when still growing on selective medium supplemented with 15mM 3-AT. According to these procedures the initial vector pSATO, expressing Ost4p-MCS-Cub-LexA-VP16, was found to be weakly self-activating and the bait construct pSATO-p53 was found to be strongly self-activating, making it unsuitable for screening.

From Western blottings it was concluded that the expression levels of pSATO and pSATO-p53 might be too high, leading to self-activation (Fig. 2.5C). The next step was therefore to lower the bait expression levels by exchanging the TEF1 promoter for the moderate ADH1 and the weak CYC1 promoter, resulting in the bait vectors pASO and pCO, respectively.

### Table 2.3. CytoY2H bait and prey vectors

<table>
<thead>
<tr>
<th>Bait vectors</th>
<th>Promoter</th>
<th>Expression</th>
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<tr>
<td>pCO</td>
<td>CYC1</td>
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</tr>
<tr>
<td>pASO</td>
<td>ADH1</td>
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<tr>
<td>pSATO</td>
<td>TEF1</td>
<td>strong</td>
</tr>
<tr>
<td>pCOWT¹</td>
<td>CYC1</td>
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</table>

<table>
<thead>
<tr>
<th>Prey vectors</th>
<th>Promoter</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDSL-Nx</td>
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<td>weak</td>
</tr>
<tr>
<td>pADSL-Nx</td>
<td>ADH1</td>
<td>moderate</td>
</tr>
<tr>
<td>pNubG-HA</td>
<td>0.7kb</td>
<td>moderate-strong</td>
</tr>
</tbody>
</table>

**Defined protein-protein interactions in the cytoY2H assay**

To test whether the cytoY2H assay is capable of detecting protein interactions outside of the nucleus and in the cytosol, the p53 oligomerization and the known interaction between p53 and *Simian virus 40* large T antigen (SV40LT) were chosen. Full-length p53 (fl-p53) served as bait and SV40LT and N-terminally truncated p53 (Δp53) as prey proteins. Since pSATO (-p53) co-expressed with pNubG-HA showed to be self-activating, fl-p53 and the preys were also expressed under the control of weaker promoters such as P<sub>ADH1</sub> and P<sub>CYC1</sub>.
In the membrane based split-ubiquitin system we generally observed fewer colonies growing on plates selecting for interaction than for the plasmids only. A bait-prey interaction is generally indicated if this so called relative growth exceeds 20 percent, but is rarely higher than 80 percent.

p53 oligomerization was clearly detectable in the cytoY2H assay when bait and preys were driven by the stronger promoters \textit{P}_{ADH1} or \textit{P}_{TEF1}, but not if they were expressed under the control of \textit{P}_{CYC1} (Fig. 2.5A and B). However, highly expressed baits (pASO and pSATO) and preys (pADSL-Nx) led to background growth between 15 and 20 percent with the unrelated prey protein lamin C (Fig. 2.5B).

![Graph showing relative growth of colonies](image)

**Figure 2.5.** Reconstitution of known protein interactions and the influence of bait and prey expression levels. (A and B) The yeast reporter strain L40 was co-transformed with the according bait and prey plasmids and plated on medium selecting for the plasmids only (SD-Leu-Trp) and for a bait-prey interaction (SD-His-Leu-Trp). Colonies were counted and the growth ratio indicated as percentage in the graphs. (A) Preys were expressed under the control of \textit{P}_{CYC1} (B) Preys were expressed under the control of \textit{P}_{ADH1}. The pASO-p53 bait – pADSL-SV40LT prey interaction was not detected reproducibly. (C and D) Expression levels of empty bait vectors expressing Ost4-Cub-LexA-VP16 only or the bait p53 vectors under the control of different promoters. (D) Expression levels of prey proteins under the control of \textit{P}_{CYC1} (pDSL-Nx) and \textit{P}_{ADH1} (pADSL-Nx). (C and D) Equal loading was verified by Ponceau staining.
Western blottings revealed that the expression levels of baits and preys largely correlated with the relative growth. Thus, elevated protein levels favored the detection of defined protein interactions but also resulted in background growth with control proteins, whereas very low protein levels prevented the detection of the p53-oligomerization. The interaction between fl-p53 bait and SV40LT prey was not detected reproducibly, probably due to low SV40LT protein levels (Fig. 2.5C). In addition, it was shown that the expression levels of different proteins in the same prey vector and under the control of the same promoter varied enormously (Fig. 2.5D). CytoY2H plasmids are shown in Table 2.3, details of the vectors are shown in Fig. 2.6 and 2.7.
2. Development of the split-ubiquitin based cytosolic yeast two-hybrid system

Figure 2.6. (A) Maps of the mostly used cyto2H-bait vectors pCOWT and pASO. Downstream from OST4 follows the multiple cloning site (MCS) which is in frame with the OST4 CDS and the Cub-LexA-VP16 reporter cassette. The centromeric constructs carry a LEU gene for selection in yeast and bear a Kanamycin resistance gene for propagation in E. coli. The vectors differ in their promoter CYCA or ADH1, respectively to regulate the expression level of individual bait proteins. In contrast to pCOWT, LexA in pASO carries a mutation in the NLS R157G, which reduces the transport of LexA to the nucleus. The only difference between pCOWT and pCO (not depicted) is the LexA-NLS, which is mutated in pCO contrarily to pCOWT. The only difference between pSATO and pASO concerns the promoter. Whereas the bait in pASO is driven by an ADH1 promoter, the bait in pSATO is under control of the TEF1 promoter. (B) Multiple cloning site of the cyto2H bait vectors pCOWT, pCO, pASO, pSATO. cDNA encoding the bait protein is easily inserted in one direction by Sfi I restriction sites.
Multiple cloning site pDSL-Nx and pADSL-Nx

NubG

BamHI (598)

Sfi I (598)

Sfi I (627)

EcoRI (599)

Sal I (646)

Amp

pDSL-Nx

6336bp

CYC1p

HA tag

pBS ori

Cytoplasmic Yeast Two-Hybrid System

2micron

Self-activation test for the bait constructs pCO (-p53) and pASO (-p53)

Next, we tested the lower expression bait vectors pCO(-p53) and pASO(-p53) for self-activation in the reporter strain L40 co-expressed with the prey vector pADSL-Nx. pADSL-Nx carries the entire 1.5kb ADH1 promoter, which is considered to be weaker than the 0.7kb ADH1 promoter used in the first self-activation assay. pCO, pCO-p53 and pASO were not self-activating as no colonies grew on SD-His-Leu-Trp supplemented with 3-AT in the concentration range from 7.5mM (pCO, pASO) to 15mM 3-AT (pCO-p53). In contrast, pASO-p53 was strongly self-activating at 15mM 3-AT (Table 2.4A and B).
Subcellular localization of bait vectors

We were wondering why the higher expressed p53 bait constructs were self-activating in the cytoY2H system and considered that the baits were not associated completely at the membrane, possibly due to insufficient anchoring by Ost4p or due to protein degradation of the bait protein. Membrane and cytosolic extracts from yeast cells expressing pASO, pASO-p53, pSATO and pSATO-p53 were isolated and the bait constructs detected by an antibody directed against the C-terminal LexA domain of the bait. The p53- and empty bait constructs were detected only in the membrane fractions indicating that the p53 baits, as well as the empty baits, were completely attached at the membrane of the ER (Fig. 2.8). If the bait construct was degraded and LexA-VP16 liberated, we would have found a signal at 30kD in the cytosolic fraction, which was not the case.

New experimental approach

Taken together, these results show that it is possible to detect an interaction between proteins in the cytoY2H system if bait and preys are expressed moderately or strongly, but that at high expression levels of bait and preys, firstly, a background is generated with an unrelated prey protein such as lamin C and secondly, the p53 bait constructs are self-activating. Yeast extract fractionation suggested a complete attachment of the cytoY2H bait at the membrane and showed no sign of bait degradation responsible for self-activation. Therefore no alternative protein was used to anchor the bait construct at the membrane and Ost4p was maintained as membrane anchor (Fig. 2.10).
1. Increase of the stringency in the yeast reporter strain

By increasing the stringency of the host strain L40, we tried to lower the background in defined interaction assays and to avoid self-activation of the pASO and pSATO constructs in cDNA library screenings. We introduced the additional reporter gene \( ADE2 \) into L40, since \( ADE2 \) is known to be a very stringent marker (James et al., 1996). The newly generated reporter strain NMY32 bears three different reporter genes (\( ADE2, HIS3, lacZ \)), each under LexA responsive elements and the control of distinct promoters to prevent those false positives which bind unspecifically to a certain promoter region.

NMY32 was compared to L40 for protein interaction and background growth in defined protein interaction assays employing p53 oligomerization and unrelated preys. p53 oligomerization was detected with the bait vector pASO-p53 in L40 as well as in NMY32 even when the \( \Delta p53 \) prey was expressed at a lower protein level (Fig. 2.9A). The membrane protein Alg5p, fused to NubG, served as negative control, since it is not related to p53. pAlg5-NubI was utilized as positive control. NubI corresponds to the unmutated, thus wild type N-terminal half of ubiquitin. NubI and Cub spontaneously reassociate into functional ubiquitin, which is recognized by UBPs. The expression of a cytoY2H-prey fused to NubI serves therefore as an excellent positive control in the split-ubiquitin system and is termed functional test, since it only leads to a positive readout if the bait construct is well expressed and correctly localized.

Whereas the functional test, using pAlg5-NubI was positive, the negative control preys pADSL-Nrdp1 and pAlg5-NubG showed no background growth when expressed with pASO-p53 in NMY32, in contrast to L40. Thus, by employing the more stringent reporter strain NMY32, background growth for pASO-p53 was eliminated in a defined protein interaction assay, while still detecting the p53-p53 protein interaction.

2. Enhanced nuclear transport of the cleaved LexA-VP16 in pCOWT

Since p53 oligomerization could not be detected using pCO-p53, the nuclear transport of the cleaved transcription factor was improved by switching the mutated NLS of LexA back to wild type NLS creating pCOWT and pCOWT-p53. Comparison of pCOWT-p53 with pASO-p53 showed that the same defined protein interactions were detected (p53 dimerization and the functional test using pAlg5-NubI) as with pASO-p53, without producing background growth with the unrelated preys Nrdp1 and Alg5p (Fig. 2.9B). In contrast to pASO-p53, the interaction between pCOWT-p53 and pDSL-\( \Delta p53 \) was detected only weakly.
Figure 2.9. Improvements of the initial experimental set up: the novel host strain NMY32 and an improved low expression bait vector pCOWT. (A) Comparison of the p53 oligomerization in the reporter strain NMY32 and L40. Both strains were co-transformed with either empty vector or the bait construct pASO-p53 and the indicated preys. Selection for interaction was performed on either SD-His-Leu-Trp for L40, SD-Ade-Leu-Trp for NMY32 or by lacZ assay. Results from growth selection are indicated by percental relative growth. β-galactosidase activity is indicated by increasing numbers of crosses. Unspecific growth generated in L40, was abolished in NMY32. (B) Comparison of the novel bait vector pCOWT with pASO by reconstitution of the p53-oligomerization in NMY32. Interaction is indicated as growth ratio and by blue levels in the lacZ assay. p53 oligomerization was detected in both bait vectors, which was not the case in the precursor pCO. No unspecific growth was detected for pCOWT, pASO-p53 or the p53 preys.

Self-activation test for pCOWT and pASO with various baits in NMY32

Defined protein interactions were detected for p53 using two different bait vectors and growth with unrelated preys could be eliminated. Since transformation efficiency is about hundredfold increased in self-activation assays compared to defined protein interaction assays, the question was if NMY32 would be stringent enough to avoid self-activation of the novel bait constructs. Indeed, self-activation could be eliminated for pASO and pASO-p53 when co-expressed with low expression prey vectors (Table 2.4A and B). Self-activation for pCOWT-p53 decreased when lowering the expression level of the prey, resulting in no self-activation with pDSL-Nx on SD-Ade-His-Trp-Leu (Table 2.4B, Fig. 2.10D). This shows that expression levels of both bait and preys are critical. Additional proteins were tested by using different bait and library vectors for self-activation in the host strain NMY32: the unconventional yeast prefoldin Uri1p/Bud27, SV40 LT, the yeast transcription factor Gal4p and IκB-α, and an inhibitor of NF-κB (Table 2.4C). Conditions of zero growth were detected for all baits studied in the novel reporter strain. Thus, it was demonstrated that self-activation could be prevented for all baits by adjustment of bait and prey levels and by use of the more stringent reporter strain NMY32.
### Basic prerequisites for the cytoY2H method accomplished

p53 oligomerization was detected in the cytoY2H system without generating unspecific growth and screening conditions were defined under which the same bait constructs did not activate the reporter genes in absence of an interaction partner. Therefore we concluded that the requirements for the identification of defined protein interactions and for cDNA library screening using the cytoY2H system were accomplished.

Table 2.4. Self-activation assays performed in the cytoY2H system. SD-HWL, SD-His-Trp-Leu; SD-AHWL, SD-Ade-His-Trp-Leu.

(A) Self-activation assay for empty bait vectors, expressing Ost4p-MCS-Cub-LexA-VP16 only

<table>
<thead>
<tr>
<th>Empty bait vectors</th>
<th>Prey vectors</th>
<th>Reporter strain</th>
<th>Selection</th>
<th>3-AT concentration/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSATO</td>
<td>TEF1 mutated</td>
<td>pNubG-HA</td>
<td>0.7 kBADH1</td>
<td>L40</td>
</tr>
<tr>
<td>pSATO</td>
<td>TEF1 mutated</td>
<td>pADSL-Nx</td>
<td>ADH1 fli</td>
<td>L40</td>
</tr>
<tr>
<td>pCOWT</td>
<td>CYC1 mutated</td>
<td>pADSL-Nx</td>
<td>ADH1 fli</td>
<td>L40</td>
</tr>
<tr>
<td>pCOWT</td>
<td>ADH1 mutated</td>
<td>pADSL-Nx</td>
<td>ADH1 fli</td>
<td>L40</td>
</tr>
<tr>
<td>pCOWT</td>
<td>CYC1</td>
<td>pADSL-Nx</td>
<td>ADH1 fli</td>
<td>L40</td>
</tr>
</tbody>
</table>

(B) Self-activation assay for p53 bait vectors.

<table>
<thead>
<tr>
<th>p53 bait vectors</th>
<th>Prey vectors</th>
<th>Reporter strain</th>
<th>Selection</th>
<th>3-AT concentration/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSATO-p53</td>
<td>TEF1 mutated</td>
<td>pNubG-HA</td>
<td>0.7 kBADH1</td>
<td>L40</td>
</tr>
<tr>
<td>pCOWT-p53</td>
<td>CYC1 mutated</td>
<td>pADSL-Nx</td>
<td>ADH1 fli</td>
<td>L40</td>
</tr>
<tr>
<td>pCOWT-p53</td>
<td>CYC1 mutated</td>
<td>pADSL-Nx</td>
<td>ADH1 fli</td>
<td>L40</td>
</tr>
<tr>
<td>pASO-p53</td>
<td>ADH1 mutated</td>
<td>pADSL-Nx</td>
<td>ADH1 fli</td>
<td>L40</td>
</tr>
<tr>
<td>pCOWT-p53</td>
<td>CYC1</td>
<td>pADSL-Nx</td>
<td>ADH1 fli</td>
<td>L40</td>
</tr>
<tr>
<td>pCOWT-p53</td>
<td>CYC1</td>
<td>pNubG-HA</td>
<td>0.7 kBADH1</td>
<td>NMY32</td>
</tr>
<tr>
<td>pCOWT-p53</td>
<td>CYC1</td>
<td>pADSL-Nx</td>
<td>ADH1 fli</td>
<td>NMY32</td>
</tr>
</tbody>
</table>

(C) Self-activation assay for further bait constructs.

<table>
<thead>
<tr>
<th>Bait vectors</th>
<th>Prey vectors</th>
<th>Reporter strain</th>
<th>Selection</th>
<th>3-AT concentration/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>pASO-Uri1p</td>
<td>ADH1 mutated</td>
<td>pNubG-HA</td>
<td>0.7 kBADH1</td>
<td>NMY32</td>
</tr>
<tr>
<td>pCOWT-Uri1p</td>
<td>CYC1</td>
<td>pADSL-Nx</td>
<td>CYC1</td>
<td>NMY32</td>
</tr>
<tr>
<td>pASO-Uri1p</td>
<td>ADH1 mutated</td>
<td>pADSL-Nx</td>
<td>CYC1</td>
<td>NMY32</td>
</tr>
<tr>
<td>pASO-SV40LT</td>
<td>ADH1 mutated</td>
<td>pADSL-Nx</td>
<td>CYC1</td>
<td>NMY32</td>
</tr>
<tr>
<td>pSATO-SV40LT</td>
<td>TEF1 mutated</td>
<td>pADSL-Nx</td>
<td>CYC1</td>
<td>NMY32</td>
</tr>
<tr>
<td>pCOWT-Gal4p</td>
<td>CYC1</td>
<td>pNubG-HA</td>
<td>0.7 kBADH1</td>
<td>NMY32</td>
</tr>
<tr>
<td>pASO-αB-øy1p</td>
<td>ADH1 mutated</td>
<td>pADSL-Nx</td>
<td>CYC1</td>
<td>NMY32</td>
</tr>
</tbody>
</table>
2. Development of the split-ubiquitin based cytosolic yeast two-hybrid system

Host strain: L40
Reporter genes (HIS3, lacZ)

pSATO/ pSATO-p53 → Self-activating

Lower bait and prey expression levels

Generate: pCO(-p53) and pASO(-p53)/ [pSATO (-p53)]

Self-activating? p53-oligomerization

Interaction detected? background?

No Yes Yes

A

Bait constructs attached at the membrane?

Membrane and cytosolic fractions

Yes Ost4p retained as membrane anchor

B

New approaches:

1. Increase stringency of the yeast reporter strain

Generate NMY32: two reporter genes ADE2 and HIS3

pCOWT (-p53) and pASO (-p53)

Self-activating? p53-oligomerization

Interaction detected? background?

No Yes No

C

2. Enhance transport of LexA-VP16 in pCO

Generate bait vector pCOWT: wild type LexA, P_{Cyc1}

pCOWT (-p53) and pASO (-p53)

Self-activating? p53-oligomerization

Interaction detected? background?

Yes No

D

Figure 2.10. Experimental approach for the cytoY2H system. The aim at first was to find a bait construct which was not self-activating and for which a known protein interaction could be detected. (A) Since pSATO(-p53) was self-activating, the lower expressed bait vectors pCO(-p53) and pASO(-p53) were tested for self-activation and protein interaction. (B) The bait construct was localized at the membrane and seemed not to be degraded. (C) Since the same cytoY2H baits were either self-activating or did grow with unrelated preys, we tried to improve the system by the indicated approaches. (D) By using a host strain with increased stringency, a modified low expression vector (pCOWT), and by adjusting expression levels also for prey vectors, we found pCOWT-p53 and pASO-p53 to fulfil the requirements needed for the cytoY2H system.
Reconstitution of various protein interactions in the cytoY2H system

We wanted to verify that the cytoY2H system could be used to detect protein interactions of different nature and probed several known protein pairs for interaction by using the bait vectors pCOWT, pASO or pSATO, the prey vectors pDSL-Nx or pADSL-Nx and the reporter strain NMY32 (Fig. 2.11).

Protein interactions of the transcription factor p53

Since the interaction between fl-p53 bait and a SV40LT prey was not detected reproducibly, an SV40LT bait was co-expressed with a Δp53 prey. The protein pair arranged in this way yielded robust growth under selection and strong blue coloration in a β-galactosidase assay (Fig. 2.11A). Furthermore, fl-p53 was found to interact not only with Δp53 prey, but also with a fl-p53 prey (Fig. 2.11B). The protein interaction between p53 and the E3 ligase Mdm2, an inhibitor of p53, was not detected reproducibly.

Despite low SV40LT prey levels, the oligomerization of SV40LT was detectable in the cytoY2H assay (Fig. 2.11C). This finding demonstrates the ability of the cytoY2H system to detect interactions between membrane-anchored proteins and proteins which are predominantly localized in the nucleus, such as SV40LT (Fanning and Knippers, 1992). The interactions involving the SV40LT and fl-p53 baits were specific since they interacted neither with NubG expressed from the empty prey vector, nor with several unrelated preys, including Gal80p, Rpb5p or the p65 subunit of the NF-κB complex (Fig. 2.11A and B). An overview of the reconstituted protein interactions is given in Table 2.5.

Interactions of the NF-κB/ IkB-α complex

To investigate whether the cytoY2H system can also be applied to other transcription factors, we chose to assay interactions involving proteins of the IkB-kinase/NF-κB complex. We selected the NF-κB subunit p65 and its interaction to IkB- α since this interaction is well characterized and p65 is notoriously difficult to handle in y2h assays (Jacobs and Harrison, 1998). Upon co-expression, the specific association of IkB-α bait with p65 prey was clearly detectable (Fig. 2.11D).
2. Development of the split-ubiquitin based cytosolic yeast two-hybrid system

Figure 2.11. Defined protein-protein interactions in the cytoY2H system. Using the according growth medium, it was selected for yeast, bearing the bait or prey plasmids only (SD-Leu-Trp) or for yeast harbouring a bait-prey interaction (SD-Adc-His-Leu-Trp). Interaction was also indicated by β-galactosidase activity using the lacZ reporter gene. The bait proteins associated with known interactors or protein subfragments, whereas they did not interact with unrelated control preys. Note that most baits and preys were crosswise tested as negative controls. In absence of a bait-prey interaction, the ADE2 reporter gene is not transcribed in NMY32 and a visible, red-colored intermediate accumulates in the adenine biosynthetic pathway. Contrarily, the ADE2 marker gene is induced when NMY32 is bearing a bait-prey interaction and the color of yeast cells is white to faint pink. Thus, also when selecting for bait and prey only, the white color can be a hint, but is not enough to reveal interaction, as shown for the fl-p53/Rpb5 interaction (B).
Next, we probed the inhibitor kinase complex (IKK) of IκB-α for homo- and heteromerization between the cytosolic subunits, IKKα, IKKβ and IKKγ. Several of these interactions were detected in the cytoY2H, such as the homodimerization of all three subunits and the association of the kinase subunits IKKα and IKKβ with IKKγ, showing that the cytoY2H is suitable for the detection between cytosolic proteins (Table 2.5).

**Yeast Gal4p-Gal80p interaction**

Since Gal4p, a classical yeast transcription factor, cannot be used as bait in a conventional y2h system due to strong stimulation of transcription in the absence of a protein interaction (Lohr et al., 1995), we wondered whether Gal4p can be studied in the cytoY2H system. In fact, the classical y2h system is based on the use of the DNA-binding and activation domains of Gal4p to detect a protein interaction (Fields and Song, 1989). We tested both full-length Gal4p and its activation domain alone for interaction with its inhibitor Gal80p. Full-length Gal4p and the Gal4p activation domain alone interacted specifically with the Gal80p prey, but not with NubG alone or unrelated preys (Fig. 2.11E and F). We deleted the binding site for Gal80p in the transcription factor Gal4p (Gal4p residues 841-881), (Melcher and Xu, 2001) for use as further specific negative control and found that the N-terminally truncated Gal4p (Gal4-NT) interaction with Gal80p was impaired or nearly abolished. The Gal4p-Gal80p interaction was detected when Gal4p and Gal80p were used for both bait and prey (Table 2.5). This shows the robustness of the cytoY2H method in detecting protein interactions, since often an interaction between two proteins can be detected only in one arrangement in the conventional y2h system.

**Interactions between Uri1p and Pfd6p or Rpb5p**

A surprisingly high number of cytosolic proteins also autonomously activate transcription when used as baits in the y2h system, most likely due to the presence of clusters of acidic residues in their primary sequence. To test whether the cytoY2H system can be used to investigate interactions involving such proteins, we chose Uri1p (Gstaiger et al., 2003) as bait. We have been unable to use Uri1p in a conventional y2h system, presumably due its strongly acidic region. As shown in Figure 2.11G, full-length Uri1p bait interacted strongly with two of its known binding partners, Pfd6p and Rpb5p, when tested in the cytoY2H assay, whereas it did not interact with seven control preys (not all shown in Fig. 2.11G). Uri1p, Pfd6p and Rpb5p are all found in the cytosol (Huh et al., 2003; Delgermaa et al., 2004), Thus, the
The cytoY2H system can also be used to detect interactions of self-activating cytosolic proteins that are unsuitable for use with the conventional y2h system.

Table 2.5. Reconstitution of known interaction in the cytoY2H

<table>
<thead>
<tr>
<th>Bait vector</th>
<th>Prey vector</th>
<th>Growth assay</th>
<th>lacZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV40 LT pASO fl-p53 pADSL-Nx</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>SV40 LT pASO Δp53 pADSL-Nx</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>SV40 LT pASO SV40 LT pADSL-Nx</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>fl-p53 pASO fl-p53 pDSL-Nx</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>fl-p53 pASO Δp53 pDSL-Nx</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>fl-p53 pASO Mdm2 pADSL-Nx</td>
<td>+/-*</td>
<td>+/-*</td>
<td></td>
</tr>
<tr>
<td>fl-p53 pASO SV40 LT pADSL-Nx</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Uri1p pASO Rpib5p pDSL-Nx</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Uri1p pASO Pfd6p pDSL-Nx</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>fl-Gal4p pCOWT Gal80p pDSL-Nx</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Gal4p-AD pCOWT Gal80p pDSL-Nx</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Gal4-NT pCOWT Gal80p pDSL-Nx</td>
<td>+/-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Gal80p pCOWT fl-Gal4p pDSL-Nx</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Gal80p pCOWT Gal4p-AD pDSL-Nx</td>
<td>+++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Gal80p pCOWT Gal4-NT pDSL-Nx</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>IkB-alpha pASO p65 pADSL-Nx</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IKKα pASO IKKα pDSL-Nx</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IKKβ pASO IKKβ pDSL-Nx</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>IKKα pASO IKKγ pDSL-Nx</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IKKβ pASO IKKγ pDSL-Nx</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>IKKγ pASO IKKγ pDSL-Nx</td>
<td>+++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IKKβ pASO p50 pDSL-Nx</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* The fl-p53 - Mdm2 interaction was not detected reproducibly. For the proteins in bold, different expression levels were tested and the indicated one was found to be most suitably (strongest interaction without the appearance of background growth.)
The cytoY2H method as a genetic screening system

The most widely used application of genetic screening systems in yeast is the identification of novel interaction partners through cDNA library screening. In this subchapter I first show preparative analyses for screening. Subsequently, the results of cDNA library screenings (Table 2.6) and the comparison of bait-prey verification for different baits are presented. Finally an overview for the individual steps of cytoY2H screening procedures is given (Fig. 2.14).

Rescue of a known interactor in artificial cytoY2H-pilot screens

After the screening conditions for p53 have been developed and determined, we wanted to verify whether it was possible to rescue a p53-interactor in an artificial cytoY2H- pilot screen. For this purpose, pDSL~Ap53 prey vector was added in a low molecular ratio (1:500) to a corresponding cDNA library. The p53 - library mixture was co-expressed in NMY32 and selected for prototrophic growth. Preys of four of the five colonies grown on selective medium encoded for Ap53. The artificial pilot screen was repeated for SV40 LT-bait and Ap53 prey, where 36% of the 33 total colonies growing corresponded to Ap53, and for the interaction pair lKB-α/p65, where 60% of the total 34 colonies corresponded to p65. The results demonstrate that it is possible to rescue a known interactor in a simulated cytoY2H screen.

fl-p53 cDNA library screenings

A human colon cDNA library was constructed for use in the p53 screen. Since we wanted to be sure that potential p53 interactors were represented in the library, the presence of the MDM2 and the P53 gene in the human colon cDNA library was verified by PCR. A p53 screen with COWT-p53 was performed under the conditions defined in previous self-activation assays (Table 2.4) using the human colon cDNA library. Surprisingly, the primary growth selection resulted in approximately 2500 colonies per large plate, which suggested that the p53 bait was strongly self-activating. Thus, pCOWT-p53 was titrated on SD-Ade-His-Leu-Trp supplemented with up to 15mM 3-AT against the human colon cDNA library, resulting in reduced numbers of colonies growing on medium with 10 and 15mM 3-AT (Fig. 2.12).
Prey plasmids from 21 yeast clones of the pilot screen were rescued, analyzed for insert sizes of preys, and sequenced. In parallel, the prey plasmids were co-transformed with pCOWT-p53 and re-tested for interaction. None of the co-transformants grew on selection plates or was positive in a β-galactosidase assay. Sequence analysis of the tested preys revealed common false positives, such as cytochrome c oxidase or ribosomal RNA. Thus, the false positives which were selected by this titration could be eliminated by the subsequent bait-prey interaction test. As the cDNA library plays an important role for the outcome of a screen, a complete screen with pCOWT-p53 using a Jurkat T cell cDNA library was performed. Over 100 colonies were selected and 48 clones processed as described before. Screening procedures were stopped at this point, as none of the candidates were positive in a second bait-prey interaction test (Table 2.6).

**Ur1p cDNA library screen**

Next, we used a Ur1p bait to screen a NubG-fused yeast *S. cerevisiae* cDNA library. Seven million clones were screened, yielding 63 primary interacting clones. These clones were analyzed in detail, showing that many encoded the same protein. Overall, 21 different putative interactors of Ur1p were found in the screen. 15 clones interacted reproducibly with Ur1p in a bait-dependency test and did not interact with the unrelated preys Gal4p and IκB-α (Fig. 2.13B). The other clones did either not interact reproducibly with Ur1p or failed to produce results in restriction analysis (Fig. 2.13A) or sequencing. Since many bait-dependent preys were significant in connection with Ur1p, they were analyzed and studied thoroughly. Results of this work are presented in chapter 4.
Figure 2.13. CytoY2H Uri1p-screen. (A) Analysis for insert sizes. For plasmid rescue, two individual *E. coli* colonies were picked for each yeast clone. *E. coli* plasmids were digested by Sfl I restriction digest to determine insert sizes of preys. (B) Bait-dependency test of Uri1p preys. Numbers correspond to original Uri1p - clones. Preys were re-transformed into NMY32 with the original bait vector, pCOWT-Uri1 or the unrelated control baits fl-Gal4p or IkB-α. Bait-dependent preys grow only with Uri 1 p on plates selective for interaction and are positive in the lacZ assay. As the lacZ marker is very sensitive, β-galactosidase activity corresponding to background is visible for some clones with Gal4p. Growth selection for interaction revealed four non reproducible interaction (6; 12; 13; 44) and two clone interacting also weakly with controls (47; 54). These clones were considered not bait-dependent.

**Gal4p-pilot screen - false positives in the cytoY2H**

Since a cDNA library has a critical impact on the outcome of a screen, we wanted to exclude that the bait-dependent Uri1p-candidates were unspecific binders of this yeast cDNA library, interacting with many different bait proteins. A pilot screen was performed using the yeast transcription factor Gal4p with the same *S. cerevisiae* cDNA library as used for the Uri1p-screen. 7 of the 15 candidates detected corresponded to Egd2p, a protein that has a dual role as being a subunit of the nascent polypeptide associated complex and enhancing the binding of Gal4p to the DNA and its transcriptional activity. Egd2p thus makes sense as an interactor of Gal4p. However, the appearance of Egd2p in the Gal4p screen is contradictory, since Egd2p was also found in the Uri1p-screen and Egd2p was not bait-dependent (Fig. 2.13).
Other preys which were found in both screens include Ssslp and Tsc13p. Both proteins were shown to interact with Ost4p in the split-ubiquitin system previously. Whereas the Ssslp-Ost4p interaction was described as a specific interaction (Chavan et al., 2005), Miller and coworkers ascribed a low confidence value to the Ost4p-Tsc13p association (Miller et al., 2005). Interestingly, both binding partners interacted with the unrelated IκB-α bait and were therefore considered as false positive interactors in the cytoY2H system (Fig. 2.13).

Although four interactors were found in both the Uri1p and the Gal4p pilot screen, the majority of the Uri1p candidates which could be grouped in two different but related biological processes did not appear in the Gal4p pilot screen (Chapter 4, Fig. 4.4).

cDNA library screening with IκB-α and SV40 LT

We further screened a Jurkat T cell line cDNA library with IκB-α and SV40 LT. The screen with IκB-α resulted in zero clones, whereas the SV40LT screen yielded 19 clones, including importin alpha 2, a well characterized interactor of SV40LT (Catimel et al., 2001).

Table 2.6. cDNA library screenings performed in the cytoY2H system.

<table>
<thead>
<tr>
<th>Bait construct</th>
<th>Library vector</th>
<th>Selection</th>
<th>3-AT concentration/mM</th>
<th># clones detected</th>
<th>Result/ comment</th>
<th>bait-dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCOWT-FL-p53</td>
<td>human colon</td>
<td>SD-AWL</td>
<td>0</td>
<td>&gt; 100</td>
<td>negative</td>
<td>nd</td>
</tr>
<tr>
<td>pCOWT-FL-p53</td>
<td>Jurkat T</td>
<td>SD-AHVL</td>
<td>15</td>
<td>0</td>
<td>self-activating</td>
<td>0</td>
</tr>
<tr>
<td>pCOWT-Uri1</td>
<td>yeast</td>
<td>SD-AHVL</td>
<td>15</td>
<td>&gt; 100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pCOWT-SV40LT</td>
<td>Jurkat T</td>
<td>SD-AHVL</td>
<td>1, 5</td>
<td>19</td>
<td>importin alpha 2</td>
<td>1</td>
</tr>
<tr>
<td>pASO-IκB-alpha</td>
<td>yeast</td>
<td>SD-AHVL</td>
<td>1</td>
<td>64</td>
<td>potential interactors</td>
<td>36</td>
</tr>
<tr>
<td>pCOWT-Gal4</td>
<td>yeast</td>
<td>SD-AHVL</td>
<td>0</td>
<td>15</td>
<td>Egd2p</td>
<td>nd</td>
</tr>
</tbody>
</table>

Bait-dependency test

The outcome of the bait-dependency test for p53, Uri1p and SV40LT antigen revealed different results. None of the p53-candidates were confirmed in a bait-prey interaction test. In contrast, 70% of the original Uri1p-candidates were bait-dependent. The SV40LT bait-dependency test was performed with nine candidates against SV40LT, IκB-α, pASO and p53. Importin alpha 2, a known T antigen interactor, was bait-dependent as it showed β-galactosidase activity only with SV40LT. However, yeast co-expressing SV40LT-importin alpha 2 grew only very weakly on selective media despite moderate expression in Western blotting of this bait. The other eight preys from the SV40LT screen were either negative with all baits or positive also with the control baits. As control protein the empty bait vector pASO
was initially used. However, it turned out that pASO leads to higher background growth than several control baits including transcription factors like Gal4p. Possibly the MCS of empty vector, which is three times longer than in bait constructs, led to unspecific cleavage at the MCS, liberation of LexA-VP16 and consequently activation of reporter genes.

CytoY2H system: experimental procedures

1. Bait construction
   - Bait cloning and sequencing
   - Western blotting
   - Functional control assay
   - Defined bait-prey interaction

2. Self-activation test
   - Defining screening stringency

3. cDNA library screen

   Analysis of clones I
   - Plasmid recovery from clones
   - Restriction analysis for insert size

4. Confirmation of preys I
   - Bait-prey interaction test

5. Analysis of clones II
   - Normalized Western blottings of preys

6. Confirmation of preys II
   - Bait-dependency test

7. Identification of preys
   - Sequencing and BLAST analysis
   - In silico research

8. Validation of biological function

   1. Bait construction
   2. Self-activation test
   3. cDNA library screen
   4. Confirmation of preys I
   5. Analysis of clones II
   6. Confirmation of preys II
   7. Identification of preys
   8. Validation of biological function

Figure 2.14. Experimental procedures of the cytoY2H system. 1) The bait coding sequence is introduced in the bait vector pASO or pCOWT. Adequate expression is tested by Western blotting and a functional control assay using wild type Nub, and adapted if necessary by use of a different promoter. The bait is tested for interaction with a known protein interactor (if available) in a cytoY2H assay. 2) In a pilot screen, the bait is co-expressed with empty library vector in the reporter strain NMY32 and by probing media of increasing stringency, selective pressure for the subsequent cDNA library screen is defined and self-activation tested. 3) NMY32 expressing the bait construct is transformed with an appropriate cDNA library expressing potential interactors. Interactors are selected under the conditions defined in 2). 4) Prey plasmids are isolated from the selected clones and analyzed for insert size. 5) In a first in vivo confirmation assay, the rescued preys are re-tested for protein interaction with the original bait by co-expressing bait and prey in NMY32 and selecting for interaction under the conditions used in the cDNA library screen. 6) Normalized Western blottings help to estimate the expression level of the individual preys and check for overexpression. 7) Expressed preys are re-tested in the cytoY2H assay for unspecific interaction using the original and unrelated bait. 8) Preys, confirmed in 7) are sequenced and analyzed by BLAST and literature research. 9) Promising candidates are confirmed by an unrelated, usually in vitro method. 10) By studying the biological function in relation to the bait, the novel interactor is validated as functional interactor.
Discussion

It was shown in this work that the cytoY2H system can evade limitations of the conventional y2h system, as this assay is able to detect interactions between transcription factors or strongly acidic proteins which often pose major problems in the conventional y2h system. Numerous defined and previously characterized interactions were detected in the cytoY2H assay between nuclear, cytosolic, full-length proteins, or only protein subfragments of phylogenetically distant organisms such as viral, human, and yeast. Moreover, using the cytoY2H system, we demonstrated that novel protein interactions can be identified by screening a cDNA library against Uri1p, a yeast protein which is self-activating in the conventional y2h system.

In the following section I focus on the achievements of the novel method, my concerns in context with the genetic screening system and discuss new approaches to optimize the cytoY2H system.

Defined protein-protein interactions in the cytoY2H

The cytoY2H system proved to be very robust in identifying defined protein interactions. However, the p53-Mdm2 interaction which has been identified in the conventional yeast two-hybrid assay could not be reconstituted by the novel method. The p53 bait in the cytoY2H system is fused both N-terminally to the Ost4p anchor and C-terminally to the Cub-LexA-VP16 reporter protein, in contrast to only one N-terminal fusion (the DBD) in the conventional y2h-assay. Since Mdm2 binds to the N-terminus of p53, the Ost4p fusion protein and the closeness to the membrane may have inhibited the protein interaction.

We were wondering if a prey that is predominantly directed to a subcellular localization, distant from the ER membrane anchored bait construct, could ever interact with a certain prey in the cytoY2H assay. However, a majority of proteins are localized at different subcellular settings during their life cycle and transcription factors (TFs) which represent a major focus in cytoY2H studies, are shuttled between the cytoplasm and the nucleus to switch from the activated into the inactive state (Lee and Hannink, 2003; Kim and Tucker, 2006).
Indeed, we performed successful functional cytoY2H assays with nuclear proteins and wild type Nub-fused membrane proteins of different subcellular locations such as the mitochondrial extracellular membrane (data not shown). Furthermore SV40LT and the transcription factors p65 and Gal4p, utilized as preys, confirmed that interactions between membrane anchored bait constructs and mainly nuclear proteins can be detected in the cytoY2H system.

A critical point in the detection of defined protein interactions is to distinguish between a weak interaction, such as the SV40LT-importin alpha 2 interaction and unspecific interactions. This problem was solved by testing baits and preys against different proteins in a bait-dependency test as shown in the bait-dependency test for Uri1p (Fig. 2.13).

cDNA library screenings in the cytoY2H system
CytoY2H cDNA library screenings were performed for five different bait proteins of completely different nature. A well-known interactor, importin alpha 2 (Catimel et al., 2001), was identified in the SV40LT-screen, novel interactors found for Uri1p and a promising candidate, Egd2p, identified in the Gal4p-screen. Some of the Uri1p interactors were confirmed in vitro, whereas we did not attempt to verify the Gal4p-Egd2p interaction by other means. The low number of screenings performed does not allow a statistical evaluation of the results. However, since we have chosen bait proteins with several known interactors, it was surprising to find not more than one known interactor in five screenings and to detect a high percentage of false positives in the p53 and Gal4p screen.

It was expected that the outcome of the cytoY2H library screenings correlated with the degree of “self-activation” for a certain bait, since it was suggested that slightly self-activating proteins which produces unspecific growth on selective medium without 3-AT produce more false positives than transcriptionally inactive proteins (Serebriiskii et al., 2000). However, we found no such correlation from the results of five cytoY2H screenings.
Possible reasons for false negatives
The occurrence of false negatives depends on many parameters which can influence a screen, such as protein levels of preys, the lack of interactors in cDNA libraries, or the need for posttranslational modifications and/or stabilizing interactions through third binding partners (Legrain et al., 2001). Furthermore, every system, including proteomics systems like co-affinity purification in combination with mass spectrometry (co-AP/MS), is suitable for certain protein interactions, whereas others cannot be detected in the particular system (see also general discussion). As initially mentioned this might be one reason for the small overlap of data between different methods (Uetz and Finley, 2005).

In case of the p53 screen, the presence of two interactors, p53 and Mdm2, was verified in the used cDNA library beforehand. However, the precise percentage of clones encoding p53 present in the cDNA library cannot be estimated by standard PCR, nor is it possible to analyze if the p53 cDNA are in a continuous reading frame with the NubG fusion. The p53 bait was expressed at a low level in order to avoid self-activation in the cDNA library screen. Thus, since we rescued around hundred times fewer p53 preys in the artificial library screen than calculated, a low p53 bait and prey concentration under screening conditions might have been a reason to not detect the high affinity p53 oligomerization.

In contrast to p53, Uri1p has been shown to be associated with the ribosome and a molecular function for Uri1p in translation and protein folding has been suggested (Chapter 3, Deplazes et al., in preparation). I assume that the rather “physiological localization” at the ER membrane might have favored the identification of potential interactors. On the other hand, the forced localization at the ER membrane may have been detrimental for the p53 bait, since p53 is involved in various cellular processes distant from the ER, and nuclear localization may be necessary in order to detect certain interactions.

Screening a T-cell line cDNA library with an IkB-α-bait resulted in zero clones, despite the use of the least stringent conditions possible. Binding partners for IkB-α were previously identified by TAP/MS (Bouwmcester et al., 2004), indicating that false negatives occurred not due to the lack of stabilizing interactions or certain post-translational modifications, missing in yeast. However, surprisingly no reports were found of any successful y2h cDNA library screenings, where the well-characterized IkB-α was used as bait protein. Maybe the identification of interaction partners in two-hybrid based systems is not possible due to technical constraints.
Possible reasons for false positives

The rate of false positives in cytoY2H screenings varied considerably from low (Urilp screen) to very high (p53 screen). The different types of false positives do not provide a clear cause for their occurrence.

Mildly self-activating proteins can favor the selection of false positives in cDNA library screenings. Thus, this could have been one reason to identify multiple obvious false positives for p53, since strong selective pressure had to be applied in order to suppress unspecific growth. In contrast to the p53 screen, where a high number of preys encoding ribosomal RNA or ubiquitin binding proteins were identified, no such clones were found in the other screens. Again, this supports the assumption that the p53 bait may have been unsuitable for screening in such a genetic system.

Since the abundance of proteins localized at the membrane of the ER is higher than at the plasma membrane or in the cytosol, anchoring the bait protein at the ER may also favor the occurrence of false positives for some baits like p53. Nevertheless, it is important to mention that false positives can be eliminated by a bait-dependency test with unrelated baits in cyctoY2H screenings. Additionally, experience with the cytoY2H system will reveal typical false positives of the system and for each cDNA library. Sss1p and Tsc13p might be cytoY2H specific false positives.

Thus, sequencing of the selected clones by cDNA library screenings and computational analysis of the clones could provide an alternative for the bait-dependency test to identify false positives in the future. This strategy is frequently used to determine the reliability of data resulting from large-scale screening (Formstecher et al., 2005; Gavin et al., 2006; Krogan et al., 2006).

Importance of expression levels of bait and preys in the cytoY2H system

Multiple self-activation assays for a variety of baits and the careful analysis of the novel Url1p interactors revealed that bait and prey expression levels are one of the most critical factors in the cytoY2H system. Overexpressing of the bait can lead to activation of the reporter genes in absence of an interacting prey (Table 2.4). Moreover, extremely strong expression of preys led to an unspecific interaction with the bait as seen for the association of
Uri1p with the elongation factor 1Bγ (Tef4p) (data not shown). Contrarily, the oligomerization of p53 was hardly detected when the p53 prey was driven by a low promoter. The most promising attempt to exclude false positives and negatives is to express baits and preys under their endogenous promoter. This can be achieved rather easily for yeast proteins, but not for heterologous proteins in yeast. Hence, it is important to carefully examine protein levels of heterologously expressed interaction partners during validation of an interaction.

Expression levels of proteins might not only be important in two-hybrid based technologies, but likely concern also in vitro systems such as Co-AP/MS. Identification of protein complexes by high-throughput mass-spectrometry was originally performed by inducible overexpression of the bait protein (Ho et al., 2002). The technique was improved later on by means of the TAP/MS system, in which proteins are endogenously expressed.

Finally, it has to be taken into account that gene expression is generally regulated and endogenous protein levels vary under different conditions. Consequently and ideally, a protein-protein interaction has to be studied under diverse or expected physiological circumstances.

**Optimization of the cytoY2H-screening system**

The causes for the appearance of false positives and false negatives in the cytoY2H screening system seem to be diverse and are not exactly known. Therefore different approaches are imaginable.

**Matrix approach**

Since the detection of defined protein interactions in the cytoY2H system works well, the identification of novel interactors could be tackled by a different promising technique, the matrix approach. This experimental procedure is frequently used for high-throughput applications and is therefore robot assisted. The matrix approach is suitable for sequenced organisms and limited defined protein subgroups of higher eukaryotes. A disadvantage of the matrix approach is the requirement of expensive laboratory equipment. In addition, the construction of the prey array is time-consuming when compared to the construction of cDNA libraries. However, the matrix approach seems to be the system of choice for present and future global analysis of higher eukaryotic proteomes (Rual et al., 2005). To date many full-length clone collections are commercially available and research is progressing rapidly for the efficient transfer of open reading frames in suitable acceptor vectors (Temple et al., 2006).
Different membrane anchor

Empty bait and the p53-bait constructs are anchored at the membrane and not degraded according cell fractionation experiments (Fig. 2.8). However, the genetic cytoY2H assay proved to be much more sensitive than Western blotting, so that even if no signal was detectable in the cytosolic fraction, it is possible that a very small portion of the bait construct or the cleaved transcription factor was localized in the nucleus, which may have been sufficient to activate the cytoY2H reporter system, leading to self-activation.

Use of a different membrane anchor might prevent background growth seen for the p53 and the Ure1p bait on selective plates without 3-AT supplementation. One approach could be to anchor the bait at the plasma membrane where the cellular protein concentration is expected to be lower than at the ER membrane. This may lead to fewer random collisions between the bait and unspecific preys and consequently, fewer false positives. Additionally, we speculate that “real” interactors would not be insulated from the bait due to lower protein concentration at the membrane, which could lead to fewer false negatives.

γ-secretase activity was reconstituted in yeast using a β-amyloid precursor protein (APP)-based type I transmembrane reporter protein of human APP fused to the 100kD Gal4p transcription factor (Edbauer et al., 2003). The Aβ-Gal4p fusion was effectually anchored at the membrane without activating the lacZ reporter gene in absence of the γ-secretase components. This indicates that the Aβ-domain could provide a valuable alternative for the Ost4p anchor. The heterologously expressed Aβ-domain does not compete with endogenous proteins in yeast. Degradation due to overexpressed endogenous protein levels – which is imaginable in case of Ost4p – would be avoided by use of the Aβ-anchor. There are no endogenous binding partners in yeast, thus the chance to find false positives interacting with the anchor, and not with the bait, could be prevented.

Further increase of selective pressure

Increasing selective pressure in cytoY2H cDNA library screenings by the simultaneous use of three prototrophic reporter genes such as HIS3, ADE2 and URA3 is believed to be a powerful approach to reduce false positives in the novel system. This selection system was applied previously (Ito et al., 2001) in a large scale protein interaction study.
CEN/ARS based prey vectors in cytoY2H screenings

Preys are encoded on 2 micron plasmids which facilitates plasmid recovery from yeast after screening. However, using a 2 micron cDNA library might not be advantageous for screenings. The varying copy number of prey plasmids per yeast cell (roughly 60-100) can lead to overexpression of prey candidates during library screening which might favor false positives. To confirm at first the individual prey clones as real bait interactors, the original bait and the rescued prey are co-transformed and probed again in NMY32 for interaction. The interaction cannot always be reproduced in the two-hybrid system, as seen for 30% of the initially processed Uri1p- and 90% of the SV40LT- candidates in the cytoY2H screens. Thus, the varying copy number - which can be higher in cDNA library screenings than in double-transformations - could be one cause for the observed non-reproducibility. Centromeric cDNA library plasmids might therefore lower false positives and enhance reproducibility of subsequent bait-prey interaction confirmation assays.
3. Chapter - The cytoY2H system

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Natalie Möckli, Igor Stagljar, Michael O. Hottiger and Daniel Auerbach conceived the screening system and suggested experiments.
Natalie Möckli established the cytoY2H system, cloned bait and with the exception of pDSL-Pex4 and pADSL-p65 (-LTA), tested defined protein interactions and performed the screen with Uri1p.
Paul O. Hassa cloned bait and prey constructs of the NF-kB complex.
Anna Deplazes and Matthias Peter analyzed results from the cytoY2H screen and designed methods to confirm interactions and to establish the relevance of the interactions.
Anna Deplazes carried out co-immunoprecipitations to confirm the interactors.
Zhaolei Zhang performed the bioinformatics analysis on the Uri1p interactors with assistance of Natalie Möckli.
Natalie Möckli and Daniel Auerbach wrote the manuscript.
Yeast split-ubiquitin based cytosolic screening system to detect interactions between transcriptionally active proteins

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Abstract

Interactions between proteins are central to most biological processes, and consequently, understanding the latter requires identification of all possible protein interactions within a cell. To extend the range of existing assays for the detection of protein interactions, we present a novel genetic screening assay, the cytosolic yeast two-hybrid system (cytoY2H), which is based on the split-ubiquitin technique and detects protein-protein interactions in the cytoplasm. We show that the assay can be applied to a wide range of proteins that are difficult to study in the classical yeast two-hybrid system, including transcription factors like p53 and members of the NF-κB complex. Furthermore, we applied the cytoY2H system to cDNA library screening and identified several new interaction partners of Uri1p, an uncharacterized yeast protein. The cytoY2H system extends existing methods for the detection of protein interactions by providing a convenient solution for screening a wide range of transcriptionally active proteins.
Introduction

In recent years, the discovery and characterization of protein interactions on a large scale has helped to elucidate many novel biological pathways (1). Due to their relative speed and ease of use, genetic screening systems such as the yeast two-hybrid (y2h) system (2) have been especially successful in finding novel protein interactions. However, the y2h system is biased against certain classes of proteins, such as transcription factors (3). When fused to a DNA-binding domain, these proteins autonomously activate transcription and therefore cannot be used in a y2h assay. Alternative methods have emerged, such as the repressed transactivator (RTA) system (4) or the RUr3-based split-ubiquitin assay (5). Although capable of circumventing the limitations of the y2h system with regard to self-activating proteins, these assays have their own particular drawbacks and have not seen frequent use to date.

In order to create a broadly applicable and flexible screening system for transactivating proteins we have modified the well-established split-ubiquitin system (6). The split-ubiquitin system is one of the most widely used protein complementation systems and has been applied successfully to the identification of protein interactions involving integral membrane proteins, membrane-associated proteins and soluble proteins (7). By combining the split-ubiquitin assay with a stringent transcriptional output using multiple independent reporter genes we have established a screening system which can be used to detect interactions between transcriptionally active proteins and to identify novel interactors of a protein of interest using cDNA library screening.
Materials and Methods

Bait and prey constructs
To construct the bait vector pCOWT the cDNA encoding the entire open reading frame of the yeast endoplasmic reticulum OST4 was amplified by PCR from a genomic *S. cerevisiae* library (Dualsystems Biotech, Zurich, Switzerland) using a forward primer with an *Xba I* site and a reverse primer with an *Sfi I* restriction site. The OST4 cDNA was inserted upstream of the multiple cloning site into the vector pCCW (Dualsystems Biotech, Zurich, Switzerland). The following bait proteins were cloned via *Sfi I* sites downstream of the OST4 sequence into pCOWT: N-terminally truncated Simian virus 40 large T antigen (amino acids 84-708), full-length human p53, full-length *S. cerevisiae* Uri1p (Systematic gene name YFL023w) and full-length human IkB-α. The following prey proteins were cloned downstream of the NubG cDNA into the prey vector pDSL-Nx (Dualsystems Biotech, Zurich, Switzerland): truncated human p53 (amino acids 72-390), full-length human p53, full-length human NF-κB subunit p65 and truncated p65 (amino acids 303-551) and, full-length *S. cerevisiae* Pex4p, Pfd2p, Pfd6p, and Rpb5p. All constructs were verified by sequencing and the expression of all baits and preys was verified by Western blotting analysis (data not shown). Sequences of all constructs and details of the construction process are available upon request.

Yeast transformation and spotting
The *S. cerevisiae* strain NMY32 [MATa trp1 leu2 his3 ade2::lexA-HIS3 ade2::lexA-ADE2 URA3::lexA-lacZ] (Dualsystems Biotech, Zurich, Switzerland) was co-transformed with bait and prey plasmids using the lithium acetate method (8). Transformants were selected for the presence of bait and prey plasmids during 3 days of growth at 30°C on SD-trp-leu medium (minimal medium containing 2% glucose (Sigma, Buchs, Switzerland), 0.67% yeast nitrogen base (BD Biosciences, Basel, Switzerland), complete amino acid mixture lacking leucine and tryptophan (Qbiogene, Basel, Switzerland), and 2% bacto agar (BD Biosciences). Several colonies were transferred to liquid SD-trp-leu medium and grown overnight to an optical density (OD$_{540}$) of 1.0. 5 microliters of different dilutions (1:10, 1:100 and 1:1000) were spotted onto SD-trp-leu and SD-trp-leu-his-ade medium (minimal medium as described above, but lacking tryptophan, leucine, histidine, and adenine) and grown for 2 days at 30°C.
β-Galactosidase assay

β-Galactosidase activity was analyzed by the pellet X-gal assay as described previously (9).

CytoY2H screen

A yeast cDNA library (Saccharomyces cerevisiae, strain Jel1) in the prey vector pNubGx (Dualsystems Biotech) was transformed into the yeast reporter strain NMY32 harboring pCOWT-Url1p using the lithium acetate protocol (8). Transformants were selected on SD-trp-leu-his-ade medium supplemented with 15mM 3-aminotriazole (Applichem, Darmstadt, Germany) for bait–prey interaction. Library plasmids were isolated from 63 positive clones, amplified in E. coli and analyzed by restriction analysis for insert sizes. The plasmids that contained an insert and were shown to be expressed in Western blots were further processed by a bait-prey interaction test. For this purpose the individual prey plasmids were co-transformed with the Url1p-bait into NMY32 and spotted as described above on medium selective for the plasmids or for protein interaction. Positive clones were sequenced and retested for bait dependency by carrying out a bait-prey interaction test with the Url1p-bait and unrelated baits like Gal4p or IxB-α. The identity of cDNA inserts was determined by performing BLAST searches (10) against GenBank.

Co-Immunoprecipitation

300ml yeast cultures were grown until mid-log phase, centrifuged and washed in 50ml H2O. The cell pellet was resuspended in 2ml IP-buffer (20mM Tris pH7.5, 100mM NaCl, 5mM MgCl2, 2% NP40, 10% glycerol, 20mM β-glycerophosphate, 1mM NaF, 0.5mM DTT, protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland)) and disrupted using a one shot cell disrupter (Constant Systems, Northants, UK), followed by centrifugation at 14'000g for 10 min at 4°C. For co-immunoprecipitations 10μg mouse monoclonal anti-HA antibody (Covance, Denver, PA, USA) were added to 2mg of cell-lysate and gently mixed for 2h at 4°C. 50μl of pre-washed protein G sepharose beads (Amersham, Buckinghamshire, UK) were added and mixed for another hour. The beads were washed 5 times for 5min in IP-buffer containing 300m to 600mM NaCl, eluted in 30μl 3x Laemmli-buffer and subjected to immunoblot analyzes. Unspecific binding of preys to Protein G sepharose beads was excluded by performing co-immunoprecipitation with or without anti-HA antibody.
Results and Discussion

The cytoY2H system takes advantage of the well-established split-ubiquitin system (6,11). A protein of interest (the bait) is anchored to the membrane via a fusion to the small integral membrane protein Ost4p (12) and fused to a reporter cassette composed of the C-terminal half of ubiquitin (Cub) and the artificial transcription factor LexA-VP16 (Fig. 1A). Anchoring of baits to the membrane allows screening of transcriptionally active proteins by preventing their transition to the nucleus. A second protein is fused to a mutated version of the N-terminal half of ubiquitin (NubG), which lacks intrinsic affinity for Cub. If the two proteins interact, Cub and NubG are forced into close proximity and re-associate to form split-ubiquitin, which is then recognized by cellular ubiquitin-specific proteases (UBPs). The UBPs cleave the polypeptide chain C-terminal to Cub, releasing the LexA-VP16 transcription factor from the membrane. LexA-VP16 then moves to the nucleus, where it activates the LexA-responsive reporter genes HIS3, ADE2 and lacZ integrated in the genome of the yeast reporter strain. Detection of the protein interaction is achieved by measuring the output of the activated reporter genes. Thus, the cytoY2H system converts an interaction taking place outside of the nucleus into a defined transcriptional readout, resulting in growth of yeast colonies under selection or blue coloration in a standard ß-galactosidase assay.

As an initial interaction pair, we chose the Simian virus 40 large T antigen and the N-terminally truncated tumor suppressor protein p53 (13). Due to its intrinsically self-activating transactivation domain at the N-terminus, full-length p53 cannot be used in a classical y2h assay (14). Instead, the N-terminally truncated variant (Δp53) is normally used. To reconstruct the interaction between p53 and the large T antigen, the latter was fused to Ost4p and Cub-LexA-VP16 as a bait and Δp53 was fused to NubG for use as a prey. Co-expression of the large T antigen bait and the Δp53 prey yielded robust growth under selection and strong blue coloration in a ß-galactosidase assay (Fig. 1B). No interaction of the large T antigen bait was observed with either NubG expressed from the empty prey vector, or with several unrelated preys (Fig. 1B).

As the advantage of the cytoY2H system over the conventional y2h system is its ability to screen transcriptionally active proteins, we sought to investigate whether full-length p53 could be used in the system. We first assayed the dimerization of full-length p53 (15).
3. The cytoY2H system

We found that a full-length p53 bait interacted strongly with both a full-length p53 prey and a Δp53 prey, whereas it showed no interaction with unrelated preys or NubG expressed from the empty prey vector (Fig. 1C). The fact that dimerization of full-length p53 is detectable in the cytoY2H system shows that interactions involving transcriptionally active proteins can be assayed without any of the background usually associated with investigating transactivating proteins in a classical yeast two-hybrid assay.

Next, we chose to assay interactions involving proteins of the IκB/NF-κB complex since the NF-κB subunit p65 and its inhibitor IκB-α are well characterized and p65 is notoriously difficult to handle in y2h assays (16). Upon co-expression, an IκB-α bait interacted with the full-length p65 prey. The interaction was shown to be specific since the IκB-oc bait no longer interacted with a truncated Δp65 prey lacking the N-terminal binding site for IκB-α (Fig. 1D).

We then investigated whether the cytoY2H system could also be applied to those cytosolic proteins which are self-activating in a conventional y2h system. For this purpose, we chose the yeast

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**Figure 1.** (A) Principle of the cytoY2H system. A protein of interest (the bait) is inserted between the membrane protein Ost4p and the C-terminal half of ubiquitin (Cub) followed by the artificial transcription factor LexA-VP16. A second protein (the prey) is fused to the mutated N-terminal half of ubiquitin (NubG). NubG has no intrinsic affinity for Cub and the two halves do not interact when co-expressed within the cell. However, if bait and prey interact, Cub and NubG complement to form split-ubiquitin, followed by cleavage and translocation of LexA-VP16 to the nucleus and transcriptional activation of endogenous reporter genes. (B-E) Detection of defined protein interactions using the cytoY2H system. Yeast co-expressing the indicated baits and preys were plated either on medium selecting for the presence of both bait and prey (left column) or on medium selecting for a protein interaction (right column). β-galactosidase activity of yeast transformants was measured by the pellet X-gal assay (9).
protein Uri1p/Bud27 (17). Uri1p is a cytoplasmic unconventional prefoldin, whose molecular function has not been characterized so far. Previously, we have been unable to use Uri1p in a conventional y2h system, most likely due to its large acidic region. As shown in Fig. 1E, full-length Uri1p bait interacted strongly with two of its known binding partners, Pfd6p and Rpb5p, demonstrating that interactions between cytosolic proteins can also be detected using the cytoY2H system.

To exclude any non-specific interactions, each prey was also assayed against at least one non-cognate bait. For example, the Δp53 and Pfd6p preys were assayed against the 1kB-α bait since neither p53 nor Pfd6p interact directly with IkB-α (Fig. 1D).

In order to be useful as a research tool, any yeast based protein interaction assay must be amenable to cDNA library screening, with the aim of uncovering novel protein interactions. To investigate whether the cytoY2H system can be applied for this purpose, we used the Uri1p bait to screen a NubG-fused yeast S. cerevisiae cDNA library. The cDNA library had a complexity of 4x10^6 independent clones. 7x10^6 clones were screened, yielding 63 primary interacting clones. These clones were analyzed in detail, showing that many encoded the same protein. Overall, 21 different putative interactors of Uri1p were found in the screen. 15 clones interacted reproducibly with Uri1p in a bait-dependency test and did not interact with the unrelated preys Gal4p and IkB-α (Fig. 2A). The other clones either did not interact reproducibly with Uri1p or failed to produce results in restriction analysis or sequencing. The 15 bait-dependent Uri1p interactors were classified according to their molecular function (Table 1). Several interactors were found to be involved in protein translation, supporting a previously suggested role for Uri1p in this process (17; Deplazes et al., manuscript in preparation). Another group of interactors were found to be chaperones or proteins involved in protein folding. As Uri1p contains an α-class prefoldin domain at its N-terminus and is known to interact with the chaperone prefoldin 6, the identification of additional chaperones as interaction partners of Uri1p fits well.

We assembled a local protein interaction network centered on Uri1p, which consists of the 15 new interactors discovered in the cytoY2H screen. We then searched SGD (18) and BioGrid (19) for previously known physical and genetic interactions among these proteins and assembled this information into a local interaction network of Uri1p (Fig. 2B). To further confirm the observed cytoY2H-interactions in a different system, we used co-immunoprecipitation to assay binding of Uri1p to its putative interactors Tef1p, Tef4p, Ssb1p, and Fpr1p (Fig. 2C).
Table 1. Interactors of Url1p identified in the cytoY2H screen. Confirmation of interactors was carried out by coimmunoprecipitation. Occurrence: number of independent clones identified in the screen that encoded the same protein. ND, not done.

<table>
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<tr>
<th>Class</th>
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<th>Occurrence</th>
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<th>Confirmed</th>
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<td>yes</td>
</tr>
<tr>
<td></td>
<td>SIS1</td>
<td>1</td>
<td>Hsp40 family, dnaJ homolog</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>CPR1</td>
<td>1</td>
<td>Cyclophilin, prolyl-isomerase</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>FPR1</td>
<td>1</td>
<td>Rapamycin binding, prolyl-isomerase</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>EGD2</td>
<td>5</td>
<td>NAC subunit</td>
<td>ND</td>
</tr>
<tr>
<td>3-PGK</td>
<td>5</td>
<td>3-Phosphoglycerate kinase</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>DEF1</td>
<td>1</td>
<td>RNA pol II degradation factor</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>VMA4</td>
<td>1</td>
<td>Vacuolar ATPase</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>PEP1</td>
<td>1</td>
<td>Vacuolar sorting protein</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ERG1</td>
<td>1</td>
<td>Squalene monooxygenase</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Taken together, these results show that Url1p interacts with several members of the translational and protein folding machinery and confirm the proposed role of Url1p in protein translation and folding.

We note that we did not find the previously known Url1p interactors Pfd6p and Rpb5p, although pairwise interaction assays did detect their interaction with the Url1p bait (Fig. 1E). An explanation for this false negative result may be that the two interactors may not have been present in the cDNA library or that variations in the copy number level of the library plasmids may have prevented expression of sufficient levels of the preys to enable detection.
To present a useful tool for functional proteomics studies, any new screening system must tackle those classes of proteins for which no suitable systems currently exist. In this report, we show that the cytoY2H system fulfills precisely such a role, as it is able to detect interactions between a wide range of proteins, including transcription factors, strongly acidic proteins and cytosolic proteins. Moreover, we demonstrate that novel protein interactions can be identified by screening cDNA libraries against a protein of interest. Compared to other alternative genetic screening methods, such as the repressed transactivator (RTA) system (4), the RUra3-based split-ubiquitin system (5) and the RNA polymerase III based (Pol III) system (20), the cytoY2H assay offers several advantages. For example, the RTA system and the RUra3-based split-ubiquitin system use resistance to 5-fluoro-orotic acid (5-FOA) as the selection mechanism. 5-FOA selection

Figure 2. (A) Bait-dependency with Urrlp-candidates. An Urrlp-candidate is considered bait-dependent if it interacts with Urrlp, but not with the control baits Gal4p or IKB-ct. 15 of 21 preys tested were bait dependent and are marked with an asterisk. (B) Network graph of the Urrlp-interactome identified in the cytoY2H screen. The fifteen novel Urrlp-interactors (framed in red) are shown as color-coded ellipses according to their functional categories listed in Table 1 (yellow, protein translation; green, protein folding; brown, metabolic protein; dark blue; transcription, bright blue, DNA silencing; grey, miscellaneous functions; white, unknown.). Black lines connecting nodes represent physical protein-protein interactions and red dashed lines represent synthetic genetic interactions. The previously known Urrlp-interactors are shown as black closed circles. The graph was produced using Cytoscape software. (C) Confirmation of the interaction between Urrlp and the preys Ssblp, Fprlp, Tcflp or Tef4p by co-immunoprecipitation. Yeast expressing the HA-tagged preys and endogenously c-myc-tagged Urrlp were immunoprecipitated with an anti-HA antibody (+Ab) or with Protein G sepharose only (-Ab). Precipitates were subjected to immunoblot analysis using anti-myc or anti-HA antibody. The arrowhead indicates the position of the Urrlp band.
can be difficult to apply in cDNA library screens and may produce a high number of false positives (20). Furthermore, a replica plating step must be used for selection, which makes the method laborious. In contrast, the cytoY2H does not rely on 5-FOA selection, but takes advantage of a very stringent reporter system, encompassing two different auxotrophic reporter genes and a lacZ marker. The Pol III system, despite not using 5-FOA selection, is difficult to handle since it requires simultaneous introduction of three different plasmids, resulting in a significantly reduced transformation efficiency in cDNA library screens. In addition, growth of yeast takes place at 37°C, which puts additional stress on the yeast cells and increases the risk of false negative and false positive protein interactions. In contrast, the cytoY2H system utilizes the split-ubiquitin complementation mechanism (6), which is well established and has been shown to work under a variety of different conditions and with a diverse range of protein classes (reviewed in (7)).

In summary, the cytoY2H system is able to detect pairwise interactions between a wide range of different proteins, including classical nuclear transcription factors, proteins which shuttle between the cytosol and the nucleus, and cytosolic proteins. The assay is highly specific, as none of the baits interacted with any of the numerous controls tested. We believe that the cytoY2H system will be a valuable tool for working with those classes of proteins which have not yet been covered by existing methods.
References


4. Chapter  Uri1p structure – function analysis

Introduction

mRNA translation
Translation of mRNA into proteins represents the final step in the gene-expression pathway, which mediates the formation of the proteome from genomic information. The translation process is divided into three distinct phases: initiation, elongation and termination.

Translation initiation
The eukaryotic translation initiation is a complex process, which is mediated by numerous different initiation factors, depicted by their according numbers in Figure 4.1. All three phases of translation can be subject to regulatory mechanisms. Translation initiation provides the rate-limiting step in translational regulation.

The initiation of translation requires a pool of dissociated ribosomal subunits, which is maintained with the aid of eIF3 and eIF1A. Both factors are associated with the small (40S) ribosomal subunit. The ternary complex, consisting of methionine-loaded initiation tRNA (tRNAiMet) bound to GTP-coupled eIF2, joins the 40S-eIF3-eIF1A complex to form the 43S pre-initiation complex. The assembly of the ternary complex is regulated by eIF2B. This pre-initiation complex recognizes the mRNA by the binding of the scaffold proteins eIF3 to the eIF4G subunit of the cap-binding complex. In addition to eIF4G, the cap-binding complex contains eIF4E, which directly binds to the cap, and eIF4A, an RNA helicase that unwinds secondary structure during the subsequent step of scanning. eIF4G also contacts the poly(A)-binding protein (PABP) and this interaction is thought to circularize the mRNA. The 43S pre-initiation complex scans the mRNA until it identifies the initiator codon AUG. Scanning is assisted by the factors eIF1 and eIF1A. Stable binding of the 43S pre-initiation complex to the AUG codon yields the 48S initiation complex. After the formation of the 48S initiation complex eIF5 facilitates the hydrolysis of GTP carried by eIF2 and the subsequent discharge of the initiation factors. eIF5B is required for the joining of the large (60S) ribosomal subunit, resulting in the formation of the 80S initiation complex, at which point the polypeptide-elongation step of translation commences (Gebauer and Hentze, 2004; Holcik and Sonenberg, 2005).
Translation elongation and termination

During translation elongation, amino acids are successively added to the nascent polypeptide chain. This process requires two important elongation factors, which are termed eEF1A and eEF2B in eukaryotes. Whereas the tRNA$\text{Met}^*$ is delivered by the GTP-bound eIF2, all other standard aminoacyl (aa)-tRNA are recruited by the eEF1A to the ribosome. eEF2 mediates the translocation steps, in which the ribosome moves relative to the mRNA by the equivalent of one codon.
Elongation is briefly summarized and illustrated in Figure 4.2. eEF1A binds the aa-tRNA in a GTP-dependent manner and transfers it to the vacant ribosomal A-site (association). The codon-anticodon pairing triggers GTP-hydrolysis by eEF1A and causes its dissociation from the aa-tRNA at the A-site (Merrick and Nyborg, 2000). Guanidine nucleotide exchange is performed by eEF1Bα (catalytic subunit) and seems to be regulated by eEF1Bγ (Anand et al., 2001). The peptidyl transferase center of the ribosome catalyses the formation of a peptide bond between the amino acid of the delivered aa-tRNA and the growing peptide chain found in the peptidyl-tRNA binding site (P-site). GTP-bound eEF2 enters the ribosome in the pre-translocation state of the ribosome. The translocation of the peptidyl-tRNA from the A-site to the P-site is mediated by eEF2 upon GTP-hydrolysis and the deacylated tRNA is shifted to the E-(exit) site. Translation is terminated by the binding of the releasing factors eRF1 (or eRF2, depending on the stop codon) and GTP-bound eRF3 to an A-site bearing a stop codon. Upon hydrolysis on GTP-bound eRF3, the completed polypeptide is released. A ribosome recycling step is responsible for the disassembly of the post-termination ribosomal complex (Alberts et al., 2002).

All subunits of the eEF1 complex can affect translation fidelity. Intriguingly, eEF1A is involved in a variety of cellular functions aside from protein synthesis, namely protein degradation (Chuang et al., 2005) and the actin organization. The latter showed to be a function of eEF1 that is independent from translation (Gross and Kinzy, 2005).

Figure 4.2. Translation elongation comprises the binding of the aa-tRNA to the ribosomal entry site (A-site), codon recognition, the peptidyl transfer from the aa-tRNA to the growing polypeptide chain at the P-site and translocation of tRNA from the A- to the P-site and the P- to the E-(exit)-site respectively. Adapted from Alberts et al., Molecular Biology of the Cell, 2002.
Protein folding - partially a cotranslational process
The majority of small, newly synthesized polypeptides which are released from the ribosome fold without further assistance in their three-dimensional structure. Around 30% of chains reach their native state with help of molecular chaperones. Since it takes several minutes to synthesize a protein of average size, a great deal of the folding process is complete by the time the ribosome releases the C-terminal end of a peptide chain (Fedorov and Baldwin, 1997).

Molecular chaperones in the cytosol
The cellular chaperonc machinery consists of several protein families that facilitate polypeptide folding in vivo and prevent misfolding and aggregation. Chaperones have two important functions, one: during de novo folding following translation, and the second upon denaturation during conditions of environmental stress (Parsell and Lindquist, 1993; Morimoto et al., 1997; Hartl and Hayer-Hartl, 2002).

Protein folding upon biogenesis/ Protein flux through the chaperone system
Two distinct mechanisms exist for de novo protein folding of cytosolic proteins, which are conserved in all three domains of life and can cooperate in a topologically and timely ordered manner (Siegers et al., 1999). Chaperones, such as the trigger factor and proteins of the Hsp70 family (Table 4.1), act by holding nascent and newly synthesized chains in a state competent of folding upon release into the medium (Fig. 4.3). Some of these chaperones including the yeast Hsp70 protein Ssb1/2p, bind directly to the ribosome near the polypeptide exit site and are positioned to interact with nascent chains (Fig. 4.3). Ssb1/2p acts in concert with RAC, the ribosome associated complex, in stabilizing peptide chains. While the majority of small proteins are thought to fold without further assistance, larger chains interact subsequently with members of a second class of nascent polypeptide binding chaperones, including the yeast Hsp70 Ssa1p and prefoldin (see below) (Deuerling et al., 1999; Teter et al., 1999; Hartl and Hayer-Hartl, 2002). These kinds of chaperones stabilize elongating chains, assist in co- or posttranslational folding or help to transfer them to downstream chaperones.
Another well defined (evolutionary conserved) complex, which helps to fold proteins is the chaperonin complex. In contrast to nascent chain binding chaperones, chaperonin complexes define compartments inside which a protein or protein domain can fold, protected from aggregation. Both prokaryotes and eukaryotes chaperonins interact with a subset of slow-folding and aggregation-sensitive proteins for folding (Houry et al., 1999; Thulasiraman et al., 1999). The eukaryotic chaperonin TRiC was shown to be responsible for the folding of tubulin and actin (Gao et al., 1992), some distinct proteins like the Hippel-Lindau tumor suppressor in mammals (Young et al., 2004) and for folding of several WD40-repeat proteins (Camasses et al., 2003). Ssb1/2p seems to bind WD40-repeat proteins during their translation and until they have completed TRiC-mediated folding. Also yeast Hsp70 Ssa proteins, with their co-chaperones the Hsp40 Sis1p and Ydj1p, work together with TRiC. Ssa, Ssb proteins and prefoldins (see below) all interact with TRiC.
The Hsp70 chaperones

The Hsp70 family is represented in all domains, bacteria, archae and eukarya (Fig. 4.3, Table 4.1). Together with co-chaperones of the Hsp40 (DnaJ), Hsp70s function by binding and releasing native polypeptide chains, in an ATP-dependent manner (Deuerling et al., 1999; Teter et al., 1999; Hartl and Hayer-Hartl, 2002). *Saccharomyces cerevisiae* has four Hsp70 in the cytosol which are not ribosome bound, Ssa1p to Ssa4p. Eukaryotes contain both constitutively expressed (Ssb-proteins) and stress-induced (Ssa-proteins) Hsp70s (Albanese et al., 2006).

Table 4.1. Cytosolic members of the Hsp70 chaperone family

<table>
<thead>
<tr>
<th>Groups or species</th>
<th>Hsp70</th>
<th>Hsp40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria, archae</td>
<td>DnaK</td>
<td>DnaJ</td>
</tr>
<tr>
<td>Mammals</td>
<td>HSC70</td>
<td>HDJ1 and HDJ2</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Ssa</td>
<td>Ydj1 and Sis1</td>
</tr>
<tr>
<td></td>
<td>Ssb1/2p</td>
<td>Zuotin</td>
</tr>
</tbody>
</table>

Chaperones linked to protein synthesis (CLIPS)

In prokaryotes, overlapping sets of chaperones mediate both types of protein folding mentioned above, the folding of newly translated as well as stress-denatured proteins. It has been suggested, that eukaryotes have different sets of chaperone proteins for the two types of folding. On the one hand the heat shock chaperones (HSPs) like the classical Hsp104, on the other hand a chaperone network that is specialized in protein biogenesis (CLIPS) (Albanese et al., 2006) (Fig. 4.4). The latter comprises the NAC subunit Egd2p, Ssb1/2p, prefoldins and the chaperonin TRiC amongst others. The authors revealed these two subgroups by promoter analysis of ribosome-specific and stress-specific transcription elements in chaperone genes. Since cellular stress, including heat shock, leads to the general downregulation of ribosomal genes, they also tested the effect of various stress conditions on the regulation of a large set of chaperones. Whereas the mRNA of HSPs proved to be stress-induced, they found the CLIPS mRNA to be stress-repressed. Next, CLIPS were shown to associate physically with the ribosome and to be sensitive towards translational inhibitory drugs. These results, corroborated by additional genetic studies, illustrated that CLIPS are transcriptionally, functionally, and physically linked to the translational apparatus.
Ssb1/2p seems to play a central role in CLIPS-mediated *de novo* folding, whereas Ssa proteins might have an analogous function in the stress induced HSP proteins.

Consistent with a function in *de novo* protein folding, impairment of the translation-linked chaperone network renders cells sensitive to misfolding in the context of protein synthesis but not in the context of environmental stress.

Figure 4.4. Distinct organization of chaperone action in prokaryotic and eukaryotic cells. In prokaryotes, most protein folding of newly translated and stress-denatured polypeptides is carried out posttranslationally by a chaperone network containing the stress-inducible DnaK/Hsp70 and GroEL/Hsp60. Only the trigger factor, which lacks direct homologs in eukaryotes, is ribosome-associated. Eukaryotes have two distinct chaperone networks: The CLIPS network, denoted in green, functions co- and posttranslationally to mediate *de novo* folding. The HSP chaperone network, denoted in red, functions to refold stress-denatured proteins or direct them to the ubiquitin-proteasome system for degradation. Under some conditions, the HSP chaperone network can partially alleviate the loss of CLIPS chaperones such as Ssb1/2p, suggesting there is some overlap between the two chaperone networks. Taken from Albanèse et al., *Cell*, 2006.

**Prefoldin**

The prefoldin, also known as Gim complex, is a hexameric chaperone complex with a jellyfish like structure, built from two related classes of subunits which are present in eukaryotes and archae (Vainberg et al., 1998). The prefoldin complex in yeast contains six different subunits, four \( \alpha \)- and two \( \beta \)-subunits (Fig. 4.5). Interestingly, prefoldin can functionally substitute for the Hsp70 chaperone system in stabilizing non-native proteins also of the WD40-repeat substrates for subsequent folding in the chaperonin, whereas other chaperones, including Ssa, cannot replace Ssb-function. (Deuerling et al., 1999; Teter et al., 1999; Hartl and Hayer-Hartl, 2002). This demonstrates the redundancy among nascent chain-binding chaperones.
Effects of Ssb1p and Sis1p in protein translation

Interestingly, translation fidelity is impaired in the absence of functional RAC and Ssb1/2p (Rakwalska and Rospert, 2004). The mutants suffer primarily a defect in translation termination and to a lower extent in translation fidelity. Recent studies showed a programmed -1 ribosomal frameshifting in the RAC and Ssb mutants (Muldoon-Jacobs and Dinman, 2006). The authors suggested that impaired chaperoning causes nascent peptides to back up into the ribosomal exit tunnel, where they misposition the peptidyl-tRNA 3’end and thus inhibit accommodation of the aa-tRNA in the A-site. Intriguingly, the Hsp40 chaperone Sis1p, affects protein translation initiation by an unknown mechanism (Zhong and Arndt, 1993). These studies demonstrate the tight connection between protein synthesis and the chaperone system. However, it is elusive today if chaperones effect translation indirectly or directly on distinct steps.
URI, an unconventional prefoldin protein

*S. cerevisiae* Uri1p/ Bud27p (YFL023w) is a rather novel protein whose molecular function is unknown. Human URI was identified by mass spectrometry in a complex including the prefoldins STAP1, PFD2, PFD4, the ATPases TIP48/49 and RPB5, the shared subunit of the RNA-polymerase I, II, and III. Since URI contains a prefoldin (PFD) domain, binds to RPB5 and is much larger than known PFD subunits, the novel protein was termed URI for unconventional prefoldin Rpb5-binding protein (Gstaiger et al., 2003). Structure comparison of human URI revealed orthologs in *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Saccharomyces cerevisiae*. The PFD-, the RPB5-binding site and the last 30 amino acids of the Uri1p C-terminus, termed Uri-box, seem to be rather conserved among these species, whereas the central acidic part differs significantly and is more than twice as long in fly and yeast than in the residual organisms.

Phenotypic studies of URIΔ deleted cells, revealed hypersensitive cells to high temperature and showed invasive growth, which is typically for cells living under starvation conditions. Upon treatment with rapamycin and in yeast cells starved for amino acids, protein levels of Uri1p decrease and the transcriptional response is altered (Gstaiger et al., 2003). The majority of genes for which mRNA levels are affected by loss of URI are transcriptional targets of the transcription factor Gcn4p. Further analyses revealed that Uri1p was capable to repress the translation of the *GCN4* mRNA, partially independent of the Gcn2p kinase, which phosphorylates eIF2 (Gstaiger et al., 2003). Intriguingly, only those *GCN4* target genes are induced in uril1Δ cells that are also repressed by the TOR pathway. Thus, it was suggested that Uri1p is required for TOR-dependent nutrient signaling.

The activator protein Gcn4p and TOR signaling

Regulation at the level of translation allows for an immediate and rapid response to changes in physiological conditions. Such regulation is of increased importance under cellular stress conditions, such as heat shock, hypoxia or nutrient deprivation. In response to these and further types of cellular stress the global translation is reduced. This leads to a crucial saving of cellular energy, since translation is supposed to consume up to 50% of the cellular energy (depending on the organism) (Warner, 1999; Rudra and Warner, 2004). Stress-induced attenuation of the general translation is often accompanied by a switch to the selective trans-
4. Ur1p structure – function analysis

lation of proteins that are required for cell survival under stress (Harding et al., 2000; Holcik et al., 2000). This kind of regulation was shown to intervene commonly at two different steps of translation. The first step concerns the regeneration of the GTP-bound ternary initiation complex and the second step deals with the ribosome recruitment to the mRNA at the pre-initiation complex (Holcik and Sonenberg, 2005).

The best-studied example for translational control under stress conditions is the regulation of the transcriptional activator GCN4. Internal amino acid concentrations are sensed by the general amino acid control (GAAC) (Schneper et al., 2004). When *S. cerevisiae* starves for a certain amino acid, the GAAC signal transduction cascade is activated, which leads to a general inhibition of translation. The signal for activation of the pathway is uncharged tRNAs, which are bound by the kinase Gcn2p (Fig. 4.6). Phosphorylation of eIF2α by Gcn2p leads to an overall decrease in translation efficiency. However, the translation of GCN4 mRNA is selectively derepressed via a mechanism dependent on the four upstream open reading frames (uORFs) in its 5'-leader region, which usually function as cis-elements to prevent translation of the GCN4 ORF (Hinnebusch, 1997). Gcn4p acts as a transcriptional activator for over 500 genes including amino acid and purine biosynthesis genes.

![Amino acid starvation](diagram.png)

Figure 4.6. Uncharged tRNA accumulates under amino acid starvation and activates the Gcn2p kinase, which in turn phosphorylates eIF2-GDP. Phosphorylation prevents nucleotide exchange by eIF2B. Consequently, levels of the ternary complex (TC), which consists of GTP-bound eIF1A and the initiator tRNA (tRNA_Met) decrease and the general translation is downregulated. Following translation of the GCN4 uORF1, 50% of the 40S ribosomes remain attached to the mRNA and resume scanning. Under nonstarvation conditions, the 40S subunit quickly rebinds the TC and reinitiates at uORF4 because the TC concentration is high. Under amino acid starvation conditions, 50% of the rescanning 40S ribosomes fail to rebind the TC until scanning past uORF4, because the TC concentration is low, and reinitiate at GCN4 instead. The remaining 50% rebind the TC before reaching uORF4, translate uORF4, and dissociate. Adapted from Hinnebusch, Annu Rev Microbiol., 2005.
GCN4 function and ribosomal biogenesis is influenced by the TOR (target of rapamycin) pathway, which controls cell growth and proliferation in eukaryotic cells and responds to the availability of amino acids and nitrogen (Schneper et al., 2004). The central components of the pathway are the TOR kinases which are organized in two complexes, TORC1 and TORC2, with different functions. TORC1 appears to be activated by nutrient cues and is inhibited by stresses and rapamycin (De Virgilio and Loewith, 2006).

Various characteristics and phenotypes of Δuril cells suggested an important physiological role for Uri1p in yeast and its orthologs. Some of these features linked Uri1p with GCN4 translation and nutrient signaling, whereas others indicated a possible function for Uri1p in chaperoning. Since the precise function of Uri1p was not known, we wanted to elucidate by which molecular mechanism Uri1p was able to repress translation of GCN4. The second question we addressed was whether Uri1p might feature chaperone activity or could contribute to proper protein folding. Finally, we were wondering if and how Uri1p could provide a link between mRNA translation and protein folding.
Methods & Materials

Construction of bait and prey vectors
The coding DNA sequence (CDS) of the *S. cerevisiae* proteins studied in this section were cloned into the bait vectors pASO (described in chapter 2) or pBT3-Ste (Dualsystems), and the prey vectors pDSL-Nx and pADSL-Nx (Dualsystems). Baits in pASO express an N-terminal Ost4p fusion and a C-terminally fused Cub-LexA-VP16 reporter construct. Baits were transferred from pASO into the vector pBT3-Ste to remove the Ost4p membrane anchor protein. Baits in pBT3-Ste express a 15 amino acids leader sequence and the C-terminally fused Cub-LexA-VP16 reporter cassette. The CDS of the following genes were clone full-length via *Sfi I* sites into pASO: *SSB1, SIS1, TEF1, PFD6, URII* and the truncated forms of *URI* listed in Table 4.2. The CDS of *fl-Uri1p* and the truncated versions of *Uri1p* listed in Table 4.2. were cloned by *Sfi I* sites into pBT3-Ste. The CDS of *TEF3* was cloned by *Sfi I* sites into pADSL-Nx.

Table 4.2. DNA encoding truncated *S. cerevisiae* protein subfragments, inserted into pASO and the pBT3-Ste

<table>
<thead>
<tr>
<th>CDS of bait proteins</th>
<th>Vector</th>
<th>amino acids</th>
<th>bait vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fl-Uri1p</td>
<td>-fl-Uri1</td>
<td>2-795</td>
<td>pASO, pBT3-Ste</td>
</tr>
<tr>
<td>N-terminal Uri1p region</td>
<td>-Uri1-NT</td>
<td>2-266</td>
<td>pASO</td>
</tr>
<tr>
<td>C-terminal Uri1p region</td>
<td>-Uri1-CT</td>
<td>176-796</td>
<td>pASO</td>
</tr>
<tr>
<td>Prefoldin-domain</td>
<td>-Uri1-PFD</td>
<td>2-126</td>
<td>pASO, pBT3-Ste</td>
</tr>
<tr>
<td>Rpb5-binding site</td>
<td>-Uri1-RPB</td>
<td>131-242</td>
<td>pASO, pBT3-Ste</td>
</tr>
<tr>
<td>Complete acidic region</td>
<td>-Uri1-AC1</td>
<td>258-766</td>
<td>pASO, pBT3-Ste</td>
</tr>
<tr>
<td>Acidic region 2</td>
<td>-Uri1-AC2</td>
<td>300-766</td>
<td>pASO</td>
</tr>
<tr>
<td>Acidic region 3</td>
<td>-Uri1-AC3</td>
<td>258-700</td>
<td>pASO</td>
</tr>
<tr>
<td>URI-box</td>
<td>-Uri1-box</td>
<td>767-796</td>
<td>pASO, pBT3-Ste</td>
</tr>
</tbody>
</table>
Yeast strains

The reporter strain NMY51, expressing endogenous Uri1p and the URII-deleted reporter strain YNM7, were used for detection of defined protein interactions in the cytoY2H system. S288C and URII-deleted S288C (YAD213) were used for studying sensitivities against temperature, cycloheximide, hygromycin B, rapamycin and hydroxurca. YBL320 and YBL323 were used to study the indirect expression of GCN4 via its lacZ fused target gene HIS4.

Table 4.3. Yeast strains used for studies in chapter 4

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMY51</td>
<td>MATa trp1-901 leu2-3,112 his3Δ200 LYS2::(lexAop)4-HIS3 ade2::(lexAop)2-ADE2) ura3::(lexAop)4-lacZ</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>YNM7</td>
<td>MATa trp1-901 leu2-3,112 his3Δ200 LYS2::(lexAop)4-HIS3 ade2::(lexAop)2-ADE2) ura3::(lexAop)4-lacZ uri::KanMX</td>
<td>NMY51</td>
</tr>
<tr>
<td>Jel1</td>
<td>MATa leu2 trp1 ura3-52 prb1-22 pep4-3 Δhis3::PGAL10-GAL4</td>
<td>(Lindsley and Wang, 1991)</td>
</tr>
<tr>
<td>S288C</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ1</td>
<td>(Mortimer and Johnston, 1986)</td>
</tr>
<tr>
<td>YAD213</td>
<td>leu2Δ0 met15Δ0 ura3Δ1; uri1::Kan</td>
<td>S288C</td>
</tr>
<tr>
<td>YBL320</td>
<td>HIS4::lacZ, gcn2Δ</td>
<td>KT2205</td>
</tr>
<tr>
<td>YBL323</td>
<td>HIS4::lacZ, urilΔ, gcn2Δ</td>
<td>(Tatchell et al., 1984)</td>
</tr>
</tbody>
</table>

Co-immunoprecipitation

300ml yeast cultures were grown until mid-log phase, centrifuged and washed in 50ml H2O. The cell pellet was resuspended in 2ml IP-buffer (20mM Tris pH7.5, 100mM NaCl, 5mM MgCl2, 2% NP40 0, 10% glycerol, 20mM β-glycerophosphate, 1mM NaF, 0.5mM DTT, protease inhibitor cocktail (Roche Diagnostics) and broken using a one shot cell disrupter (Constant Systems) followed by centrifugation at 14'000g for 10 min at 4°C. For co-immunoprecipitations 10μg monoclonal anti-HA antibody were added to 2mg of cell-lysate and gently mixed for 2h at 4°C. 50μl of pre-washed proteinG sepharose beads (Amersham) were added and mixed for another hour. The beads were washed 5 times 5min in IP-buffer containing 300-600mM NaCl and eluted in 30μl 3x Laemmli-buffer.
Defined protein-protein interactions

Bait constructs were co-expressed with the corresponding preys in the reporter strain NMY51 expressing endogenous Uri1p or in the URII-deleted reporter strain YNM7. The cytoY2H interaction assays were performed as described in chapter 2, “yeast transformation and spotting”.

Uri1p-network

Defined protein interactions between baits and preys were detected by the cytoY2H system as described above. Four dilution series of spots per protein-interaction grown on selective plates were evaluated qualitatively, by giving zero points for zero growth corresponding to no interaction up to four points for intense growth, if all four spots of a serial dilution were grown. β-gal enzymatic activity was determined by the pellet X-gal assay (Mockli and Auerbach, 2004). Color development was recorded by using a flatbed scanner and evaluated quantitatively by Scion image processing and analysis. Background β-gal activity on selective plates or in the pellet X-gal assay from baits or preys expressed with the corresponding empty prey or bait vector was subtracted. The mean values for selective growth and β-gal activity from two independent experiments were evaluated by Excel (Microsoft) and illustrated as protein interaction network.

Sensitivities of urⅰΔ-cells against drugs and temperature

pASO, pASO-fl-Uri1p, pASO-Uri1-NT, pASO-Uri1-CT, pASO-Uri1-PFD, pASO-Uri1-RPB, pASO-Uri1-AC1, pASO-Uri1-AC2, pASO-Uri1-AC3, pASO-Uri1-UB were transformed in the yeast strain AD213 and pASO into the yeast strain S288C using the lithium acetate method (Gietz and Woods, 2005) and plated on SD-Leu. pASO was transformed into S288C as control. Transformants were grown over night in SD-Leu to OD₅₄₆ 0.8. 5 μl of undiluted culture and 5 μl of four tenfold serial dilutions were spotted on SD-Leu. Plates were supplemented by 0.05ug/ml and 0.07ug/ml cycloheximide, 0.4mM hygromycin B, 5mM and 7.5 mM rapamycin or 0.2mM hydroxyurea and grown for 2-3 days at 30°C to test sensitivities against the different drugs. Not supplemented plates were grown for two days at 30°C, 35°C and 38°C to test temperature sensitivity.
**HIS4-lacZ assay**

pASO, pASO-fl-Uri1p, pASO-Uri1-NT, pASO-Uri1-CT, pASO-Uri1-PFD, pASO-Uri1-RPB, pASO-Uri1-AC1, pASO-Uri1-AC2, pASO-Uri1-AC3, pASO-Uri1-UB and the corresponding pBT3-Ste bait constructs were transformed using the lithium acetate method (Gietz and Woods, 2005) in the yeast strain YBL323. pASO and pBT3-Ste were transformed into YBL320. Transformants were plated on SD-Leu and grown for 3 days at 30°C. Three independent overnight cultures of each transformant were grown to OD$_{546}$ 0.8-1.0. 1ml culture was subjected to *lacZ* activity by the pellet X-gal assay (Mockli and Auerbach, 2004). Color development was recorded by using a flatbed scanner and evaluated quantitatively by Scion image processing and analysis.
Results

Severe phenotypes in *URI1*-deleted cells of different organisms such as human, worm and yeast and its involvement in the TOR nutrient signaling pathway implicate a significant role of the evolutionarily conserved URI1 in biological processes (Gstaiger et al., 2003; Parusel et al., 2006). To clarify the function of Ur1p, we decided to search for physical interactors of Ur1p in a general approach, since the identification of protein binding partners for a given protein can provide a clue for its molecular role.

**Ur1p cDNA library screen using the cytoY2H system**

The conventional yeast two-hybrid method was not applicable to find protein interaction partners of yeast Ur1p, since Ur1p autonomously activated the y2h reporter system in absence of interacting preys. Instead, we used the cytosolic y2h system (cytoY2H), which is based on the split-ubiquitin system and employs a transcriptional readout (Stagljar et al., 1998). *S. cerevisiae* Ur1p was anchored at the membrane of the endoplasmic reticulum in order to prevent auto-activation and stimulation of a transcriptional response in the cytoY2H system. We selected 16 putative novel interactors from screening a *S. cerevisiae* cDNA library with Ur1p. Most prey proteins could be correlated with protein translation or protein folding (Table 4.4). Since Ur1p was linked to these closely related processes by several features and phenotypes, we focused in the subsequent analyses on the candidates either involved in translation or protein folding. Figure 4.8 shows a local protein interaction network centered on Ur1p which consists of the new interactors. We then searched SGD (Cherry et al., 1998) and BioGrid (Stark et al., 2006) for previously known physical interactions among these proteins and assembled this information into a local interaction network of Ur1p. Since none of the *in vivo* confirmed Ur1p-candidates was previously known to interact with Ur1p, it was important to verify data by an independent *in vitro* method. Using co-immunoprecipitation, Tef1p, Tcf4p, Tef5p, Ssb1p, Sis1p and Fpr1p were shown to interact specifically with Ur1p (A. Deplazes, manuscript in preparation), (Fig. 4.7 and Table 4.4).
Table 4.4. Bait-dependent clones resulting from a cytoY2H screen with Uri1p.

1) *in vitro* verification by co-immunoprecipitation.

<table>
<thead>
<tr>
<th>Class</th>
<th>Identity</th>
<th>Clones</th>
<th>Function</th>
<th><em>in vitro</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Translation</td>
<td>TEF1/2</td>
<td>2</td>
<td>Translation elongation factor 1 A</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>TEF4</td>
<td>1</td>
<td>Translation elongation factor 1Bγ</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>TEF5</td>
<td>1</td>
<td>Translation elongation factor 1Bβ</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>TIF51A</td>
<td>12</td>
<td>Translation initiation factor 5A</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>TIF11</td>
<td>1</td>
<td>Translation initiation factor 1A</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>RPS4A</td>
<td>1</td>
<td>40S ribosomal protein</td>
<td>no</td>
</tr>
<tr>
<td>Protein folding</td>
<td>SSB1</td>
<td>2</td>
<td>Hsp70 family</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>SIS1</td>
<td>1</td>
<td>Hsp40 family, dnaJ homolog</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>CPR1</td>
<td>1</td>
<td>Cyclophilin, chaperone</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>FPR1</td>
<td>1</td>
<td>Rapamycin binding protein</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>EGD2</td>
<td>5</td>
<td>NAC subunit</td>
<td>ND</td>
</tr>
<tr>
<td>Others</td>
<td>3-PGK</td>
<td>5</td>
<td>3-Phosphoglycerate kinase</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>DEFI</td>
<td>1</td>
<td>RNA pol II degradation factor</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VMA4</td>
<td>1</td>
<td>Vacuolar ATPase</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PEP1</td>
<td>1</td>
<td>Vacuolar sorting protein</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ERG1</td>
<td>1</td>
<td>Squalene monoxygenase</td>
<td>ND</td>
</tr>
</tbody>
</table>

Figure 4.7. Confirmation of the interaction between Uri1p and the preys Ssb1p, Fpr1p, Tef1p or Tef4p by co-immunoprecipitation. Yeast expressing the HA-tagged preys and endogenously c-myc-tagged Uri1p were immunoprecipitated with an anti-HA antibody (+Ab) or with Protein G sepharose only (-Ab). Precipitates were subjected to immunoblot analysis using anti-myc or anti-HA antibody. The arrowhead indicates the position of the Uri1p band. Co-immunoprecipitation of Uri1p with Sis1p will be published elsewhere.
Figure 4.8. (A) Protein interaction map of Urilp. Interactions detected by the cytoY2H system are red framed; previously described interactions, black framed. Twelve previously described distinct Urilp kinases are for simplicity not depicted (Ptacek et al., 2005). Urilp interactors are color-coded according their functions. Yellow, protein translation; green, chaperones; brown, metabolic protein; dark blue; transcription, bright blue, DNA silencing; grey, miscellaneous functions; white, unknown. Ironnersjö et al., 2006 performed a y2h-screen with two domains of the transcription factor Gislp, thereby detecting 19 proteins including truncated Urilp. The truncated Urilp, consisting of the whole Rpb5 binding site reciprocally interacted as a bait with 16 of the found Gislp interactors (italic). (B) Functional categorization of the Urilp interactors, resulting from the interaction map in (A). 50 percent of the Urilp interactor function in transcription, translation or protein folding.

In the following, I will focus first on the Urilp interactors and their relationships among each other. Second, structure-function analyses of Urilp and its potential interactors are presented. Studies on Urilp were performed in collaboration with A. Deplazes. Her most important results concerning the molecular function of Urilp in connection with the novel interactors are integrated in this work.
A molecular function for Uri1p in translation initiation

Interestingly, all subunits of the elongation factor 1 complex, TEF1/2, TEF4 and TEF5, were detected in the Uri1p screen. Incorporating their prey expression levels, we identified a strong interaction between Uri1p and eEF1A (Tef1p) and a very weak interaction between Uri1p and eEF1Bβ (Tef5p), as well as Uri1p and eEF1Bγ (Tef4p) (Table 4.5). Since TEF3 and TEF4 share 70% sequence homology, the Uri1p/Tef3p interaction was probed as well. Again, a very weak interaction was identified for this protein pair, consistent with the weak Uri1p/Tef4p interaction. Since all the EF1 subunits build a complex, the interactions between Uri1p and Tef3p, Tef4p and Tef5p is suggested to be indirect and mediated by Tef1p.

Furthermore, we identified the initiation factors eIF1A (TIF11) and eIF5A (TIF51) as well as a protein of the 40S ribosomal unit (RPS4A) in the Uri1p screen. Since Uri1p was thought to regulate GCN4 on a translational level (Gstaiger et al., 2003) the association of Uri1p with the ribosome was assayed. Indeed, Uri1p was found partially and loosely associated with ribosomes. Furthermore, URI1-deleted yeast cells revealed hypersensitivity to the translational inhibitors cycloheximide and hygromycinB, which corroborated that Uri1p affected protein synthesis on the translational apparatus. Analysis of actively translating polyribosomes on sucrose density gradients (polysome profiles) indicated a translation initiation defect in uri1Δ cells (A. Deplazes, manuscript in preparation). Taken together, these data strongly suggested an indirect or direct molecular role for Uri1p in translation initiation.

Table 4.5. Interaction of Uri1p with the full-length elongation factor 1 subunits in yeast

<table>
<thead>
<tr>
<th>eEF1</th>
<th>Genes</th>
<th>Function</th>
<th>Uri1-prey clone</th>
<th>bait-dependent</th>
<th>in vitro confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>TEF1</td>
<td>Binding reaction of aa-rRNA to ribosomes</td>
<td>yes</td>
<td>strong</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>TEF2</td>
<td></td>
<td>yes</td>
<td>not determined</td>
<td>not determined</td>
</tr>
<tr>
<td>Bβ</td>
<td>TEF5</td>
<td>Regeneration of eEF1A-GTP</td>
<td>yes</td>
<td>very weak</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>TEF4</td>
<td>Regulation of eEF1A</td>
<td>no</td>
<td>very weak</td>
<td>not determined</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>yes</td>
<td>very weak</td>
<td>yes</td>
</tr>
</tbody>
</table>
The potential Uri1p interactors involved in protein folding included the Hsp70 protein Ssb1p and the Hsp40 Sis1p. Both proteins show not only chaperone activity but also affect protein translation (Zhong and Arndt, 1993; Albanese et al., 2006; Muldoon-Jacobs and Dinman, 2006). Thus, these are parallels to features of Uri1p, which is an uncommon prefoldin chaperone, known to interact with prefoldins and Hsp70s and now was linked to translation as well. Moreover, Uri1p revealed many features of the stress resressed CLIPS-chaperones, such as the sensitivity to translation inhibitors, the association with ribosomes and the presence of ribosome specific transcriptional elements in its promoter region. In addition, mRNA profiles under varying environmental stress, revealed the repression of Uri1p not only under amino acid and nitrogen starvation, but also under heat, hydrogen peroxide, osmotic and reductive stress (Gasch et al., 2000). Both Ssb1p and prefoldins are representatives of CLIPSs. Sis1p, in contrast, belongs to heat shock induced proteins, but seems like Uri1p to be involved in translation initiation (Zhong and Arndt, 1993).

Thus, a molecular function of Uri1p at the interface of translation and protein folding is likely. Present studies could not elucidate the precise molecular mechanism of action between Uri1p, Sis1p and Ssb1p in chaperone activity, most probably due to high redundancy among nascent chain-binding chaperones including Hsp70 proteins and prefoldins (Hartl and Hayer-Hartl, 2002).

Egd2p, a subunit of the nascent polypeptide-associated complex (NAC), cyclophilin (Cpr1p) and the rapamycin binding protein 1 (Fpr1p) are all involved in de novo protein folding in the cytosol and were found as potential Uri1p interactor in the cytoY2H screen. Cpr1p and Fpr1p are two of 13 known prolyl isomerasers in yeast which are sensitive to immune suppressive drugs and are suggested to functionally overlap (Arevalo-Rodriguez, 2004). Interestingly, Egd2p, Cpr1p and Fpr1p are also localized in the nucleus under different conditions (Franke et al., 2001; Arevalo-Rodriguez et al., 2004). Tef1p, Cpr1p and Fpr1p were previously linked by Zpr1p (Ansari et al., 2002). Upon starvation the zinc finger protein Zpr1p translocates from the nucleus into the cytosol via a process mediated by Cpr1p. In addition Zpr1p was suggested to participate in translation and was shown to interact physically with the elongation factor 1A (Tef1p) (Arevalo-Rodriguez et al., 2004). Both cpr1Δ and a mutated form of zpr1, as well as cpr1Δ/fpr1Δ cells were shown to be synthetically lethal. On the basis of these interconnections, we speculate that Uri1p might serve a role in correlation with Fpr1p, Cpr1p, Tef1p and Zpr1p, which was not proved so far.
**Ur1p protein interaction network**

The large number of novel Ur1p protein interactors suggested Ur1p to be a component of a protein complex. Thus, we were interested if proteins binding to Ur1p would interact also among each other. The fact that many novel Ur1p interactors, including Ur1p, do associate with the ribosome or the nascent polypeptide chain and work in closely related biological processes prompted the existence of a protein complex additionally.

The following full-length proteins, Ur1p, Tef1p, Pfr1p, Ssb1p, Sis1p, Egd2p, Pfd6p and Rpb5p were crosswise tested for interactions, including also other related proteins like, Pfd1p, Pfd2p, Pfd4p or the unrelated Gal4p as a negative control. Several previously not described interactions were detected, which link translation with the chaperone system. The elongation factor 1A (Tef1p) was found to strongly interact with the chaperone Ssb1p (Fig. 4.8). High affinity was also observed between a Pfd6p bait and full-length Hsp40 Sis1p. The previously detected strong interactions between Ur1p and Ssb1p, Tef1p, Pfd6p and Rpb5p were confirmed in this network. The identified interactions suggested a protein complex consisting of Ur1p, Tef1p, Pfd6p, Sis1p and Ssb1p. According growth intensity and β-galactosidase activity, the affinity among these proteins seems to vary. Tef1p was the only protein found to associate with Rpb5p, besides Ur1p. Fpr1p and Egd2p generated background growth or unspecific lacZ activity in these experiments. After subtracting the background and applying a stringent practice only selecting moderate and strong interactions, Fpr1p and Egd2p were excluded from the complex. The weaker Ur1p/fl-Sis1p interaction was included in the suggested complex, since the truncated Sis1p prey clone, but not fl-Sis1p, was shown to interact specifically with Ur1p in numerous cytoY2H assays. Interestingly, the Ur1p prey did not interact with other baits than Pfd6p and itself. The specificity of these interactions was stressed by the fact that the affinity and directionality (when a protein served as bait or prey) was very different, indicated by the arrows in Figure 4.9. In addition we detected known interactions such as the dimerization of Sis1p (Lee et al., 2002) and Ur1p (Tronnersonjo et al., 2006) and the elongation factor 1A (Bunai et al., 2006) and the association of Pfd6p with Pfd4p (Siegert et al., 2000).
4. Uri1p structure – function analysis

Figure 4.9. Small Uri1p interaction network. Interactions between fl-Uri1p and five of its interactors (all full-length), were crosswise tested employing the cytoY2H system. Interactions were detected by growth selection and lacZ assay and evaluated as described in material and methods. (A) Numbers correspond to mean values for a given interaction and were color-coded according their affinity. Note: Uri1p interacts with the C-terminal domain of Sis1p, but only weakly with fl-Sis1p used in this assay. (B) Only moderate to strong interactions were selected for the Uri1p network. Arrowheads point to prey interaction for a given bait. Thick arrows, strong interaction; thin arrows, moderate interaction.

The Uri1p – prefoldin interaction

To date one heterohexameric chaperone complex is known in yeast, consisting of the subunits Pfd1p to Pfd6p (Vainberg et al., 1998). We addressed the question whether Uri1p interacts with other subunits of the prefoldin complex besides, Pfd6p. However, we could not detect an interaction between Uri1p and the alpha-subunits Pfd2p and Pfd4p or the beta-subunit Pfd3p (Fig. 4.10). Pfd1p created a weak background with Uri1p which was not interpreted as an interaction considering the low endogenous expression of Pfd1p, its expression in Western blottings and comparing it with the Uri1p/Pfd6p interaction.

Figure 4.10. Probing the Uri1p/PFD interactions in the cytoY2H system. Besides Pfd6p, none of the PFD subunits did interact with Uri1p. The Uri1p/Rpb5-interaction was used as positive control.
Uri1p structure-function analysis

The deletion of URI1 in yeast cells causes several strong phenotypes, such as sensitivity to high temperature and translational inhibitors and the induction of GCN4 translation. Gstaiger and coworkers divided Uri1p into four distinct regions, the N-terminal prefoldin domain, followed by an RPB5-binding site, a central acidic region and the C-terminal Uri-box, a small conserved sequence stretch (Gstaiger et al., 2003) (Fig. 4.11A). In the following, the question is addressed, which regions of Uri1p could be responsible for the different uri1Δ phenotypes and would recognize the different Uri1p interactors.

Uri1p, an intrinsically unstructured protein

At first, to learn more about the Uri1p structure, its primary sequence was explored by application of several different algorithms of DISPROT (Radivojac et al., 2003; Obradovic et al., 2005; Peng et al., 2006), PONDR and FoldIndex (Prilusky et al., 2005). Uri1p was predicted with 80% accuracy to be a largely intrinsically unstructured protein (IUP) (Fig. 4.11A). The behavior of Uri1p in biochemical experiments supported the in silico findings, as Uri1p showed to be very sensitive to degradation in homogenates and displayed a molecular weight in gel electrophoresis that exceeded the calculated one by 55-70kDa. Both features are characteristic for IUPs (Dyson and Wright, 2005). The classification of Uri1p as an IUP does mean that Uri1p most probably does not fold spontaneously into a well-organized globular structure in absence of stabilizing interactions. IUPs are thought to have a structural continuum and to be functional in different states of disorder and order (Dunker and Obradovic, 2001; Dyson and Wright, 2005). Disordered protein regions can bind partners with both high specificity and low affinity and many IUPs were shown to interact with numerous different proteins. A recent study broadened the current understanding of IUPs by showing the dimerization of an IUP without any transition of the disordered into the ordered state (Sigalov et al., 2006). To date IUPs are known to be mainly involved in signaling and regulative cell processes, such as the regulation of translation, transcription, the self-assembly of large multiprotein complexes such as the ribosome and chaperone activity. These functions fit well to a suggested role of Uri1p.
Figure 4.11. Ur1p structure-function analysis. (A) Prediction of unstructured regions from the Ur1p amino acid sequence. (B) Sensitivity towards high temperature and the translational inhibitor cycloheximide (0.7 μg/ml medium) of ur1/Δ cells after three days of growth. (C) HIS4-lacZ assay: Indirect measurement of GCN4 expression. (D) Normalized Western blottings of pASO-Ur1p-truncations (α-actin loading control not shown).

Functionality of Ur1p subregions

Next, we tried to rescue temperature sensitivity in ur1/Δ-cells with bait constructs in which full-length Ur1p or its truncations were fused to the membrane anchor Ost4p and the reporter cassette Cub-LexA-VP16. In fact, the fl-Ur1p-bait construct did rescue temperature sensitivity at 38°C completely. Thus the N- and C-terminal fusion and the association of Ur1p at the ER-membrane did not sterically hinder the function of Ur1p (Fig. 4.11 B). ur1/Δ cells, transformed with the N-terminal region of Ur1p, the Rpb5-binding site, the PFD domain or the Uri-box proved to be as sensitive to high temperature as the negative control. However, most surprisingly the acidic region alone did fully rescue temperature sensitivity. We further narrowed down the truncated region by 42 amino acids at the N- and 66 amino acids at the C-terminal end of the acidic region. The residues 258-300 of the acidic region seem to be critical in causing temperature sensitivity (ts), whereas the effect of the residues 700-766 contributes less to the “ts-phenotype” (Fig. 4.11 B).
Next, we tested if the Uri1p-truncations could rescue the sensitivity to the translational inhibitors cycloheximide and hygromycin B. A fl-Uri1p-bait construct did almost rescue the sensitivity of urilΔ-yeast cells against these translational inhibitors (Fig. 4.11B and Fig. 4.12). Although this phenotype was less pronounced when compared to the temperature sensitivity, we found again the acidic region alone to rescue the susceptibility towards cycloheximide and hygromycin B. Finally, urilΔ-cells were weakly susceptible to hydroxyurea, an inhibitor of DNA-synthesis, and also very weakly sensitive to rapamycin (Fig. 4.12). Rapamycin artificially induces a starvation-like state in yeast. In all experiments the residues 258-700 of the Uri1p-acidic region showed to be sufficient to rescue the sensitivity against the applied drugs (Fig. 4.12), which strongly indicates that this central acidic sequence provides the functional region of S. cerevisiae Uri1p.

The transcription factor Gcn4p that is repressed under normal growth conditions, was shown to be induced on translational level in urilΔ-yeast cells (A. Deplazes, manuscript in preparation). The translational induction of GCN4 can be monitored indirectly via its target gene HIS4 in a reporter strain, expressing a HIS4-lacZ fusion (Mueller and Hinnebusch, 1986).

The effect of the Uri1p truncations on HIS4-lacZ induction was monitored by measuring β-galactosidase activity in an urilA background. The acidic region of Uri1p was capable to repress HIS4-lacZ activity similar to the fl-Uri1p bait construct (Fig. 4.11C, 4.12).

Figure 4.12. Rescuing of the sensitivity of UR11- deleted cells against high temperature (ts) and various drugs. Fl-Uri1p or Uri1p truncations were expressed in urilΔ yeast strains. CX, cycloheximide; HygB, HygromycinB; GCN4, HIS4-lacZ assay; HU, Hydroxyurea; Rapa, Rapamycin. Sensitivity towards ts, drugs and repression of the HIS4-lacZ reporter is indicated with increasing amounts of crosses. Note: Residues 258-706 of the acidic region rescues nearly all phenotypes and derepresses HIS4-lacZ. The Rpb5-binding domain slightly increases sensitivity against HygB and induces HIS4-lacZ in urilΔ cells.
Further truncation of the acidic region did not lead to derepression of \( GCN4 \) translation. In contrast, the PFD domain and the Uri-box were not capable to repress Gen4p activity and the transcription factor was induced comparably to the \( URII \)-deleted reporter strain transformed with empty bait vector. Surprisingly, a slight additional induction of \( GCN4 \)-translation was observed by the Rpb5-binding site (Fig. 4.11C, 4.12). Note, that expression levels did not influence the outcome of these functional assays (Fig. 4.11D).

Whereas the long acidic region showed to be the functional portion of Uri1p, responsible for the most important \( uri\Delta \)-phenotypes, we hypothesized that a different region might be responsible for the association of Uri1p with the ribosome. Rescuing of temperature sensitivity and measurements of \( HHS4\text{-}\text{lacZ} \) activity in \( uril\Delta \)-cells were repeated with anchorless baits, which were still C-terminally fused to the Cub-LexA-VP16 reporter cassette. However, removal of the ER-anchor did not alter the previous results for \( GCN4 \) translation (data not shown).

**Derepression of \( GCN4 \) translation by the Rpb5-binding site?**

The Rpb5-binding domain did not generate unspecific growth in cyto2H-interaction assays, but we noticed that cells transformed with this bait construct were white-colored in the \( URII \) wild type- and the \( URII \)-deleted reporter strain, independent of an interaction. The white color of yeast indicated activation of the \( ADE2 \) reporter gene, in contrast to the red shaded cells. To find an explanation for this effect, the individual Uri1p-truncations were transformed in \( URII \)-deleted cells and plated on minimal medium selecting for the \( ADE2 \) or the \( HIS3 \) marker gene only. We found the RPB5-binding domain to activate \( ADE2 \) and \( HHS3 \) transcription without any selection for the bait plasmid and in absence of an interacting prey partner (Fig. 4.13A). The PFD domain and the acidic region showed no such effect. Strikingly, the 30 amino acids Uri-box was capable to activate \( ADE2 \) as well, but not \( HIS3 \) transcription.

**Uri1p is able to repress \( ADE2 \) transcription under heat stress**

The downregulation of the general translation and induction of a subset of specific genes is a response to heat shock, nutrient deprivation and other cellular stress, which allows the cell to save energy and enables cell survival (Holcik and Sonenberg, 2005). \( GCN4 \) is one of these specific genes which is induced on translational level upon amino acid starvation. The gene product Gen4p stimulates the transcription of genes involved in the biosynthesis of amino
acids or purines, such as ADE2 (Hinnebusch, 1984). Moreover, it was shown that also tRNA undercharging and prolonged growth at 37°C induce the general control of amino acids (GAAC) and GCN4 (Messenguy and Scherens, 1990).

Deletion of URII causes not only derepression of GCN4 translation, but also strong temperature sensitivity. It is not clear yet, how these two phenomena are linked. Since ADE2 is a target gene of GCN4, the effect of the Uri1p truncations on ADE2 gene expression was tested at physiological temperature and under mild heat stress conditions at 35°C in ur1Δ cells. Indeed, the white color of cells indicated that ADE2 was induced in ur1Δ cells at 35°C, suggesting the activation of the GAAC by heat stress (Fig. 4.13B). Interestingly, ADE2 transcription was repressed at 35°C when expressing full-length Uri1p or the C-terminal region of Uri1p, corresponding to the acidic region and the Uri-box. Consistent with previous results, the full-length and the acidic region of Uri1p could rescue the slight temperature sensitivity caused at 35°C. The residual Uri1p-truncations were not capable to repress ADE2 transcription under heat stress and slight temperature sensitivity was visible (Fig. 4.13B).

Uri1p is degraded under amino acid starvation and the GCN4 mRNA translated. Uri1p was therefore implicated with the GAAC by amino acid starvation (Gstaiger et al., 2003). These results provide a first hint that Uri1p might be able to repress GCN4 also upon heat stress and could link temperature sensitivity of ur1Δ cells with the general control of amino acids, as discussed below.

Figure 4.13. Effect of the Rpb5-binding domain on transcription under different conditions. Fl-Uri1p or the Uri1p truncations were expressed in the URII-deleted reporter strain YNM7. (A) Transformants spotted on minimal medium lacking Ade or His, incubated at 30°C. (B) Transformants spotted on SD-Leu, selecting for the bait constructs, incubated at 30°C or 35°C. White colored cells indicate transcription of the ADE2 reporter gene, in contrast to red shaded cells. NT or CT, N- or C- terminal domain of Uri1p, respectively; PFD, prefoldin domain; RBP, Rpb5 binding site; AC1, complete acidic region, SV40LT, large T antigen, serving as control together with the empty bait vector pASO.
Mapping of the Uri1p-prey interactions

The acidic region was shown to be the functional relevant part of Uri1p. Hence, we analyzed which regions of Uri1p were responsible for binding the Uri1p protein binding partners.

In a cytoY2H approach, the interactions between multiple Uri1p interactors and the prefoldin-like region (PFD), the Rpb5-binding site, the acidic region and the short Uri-box (Gstaiger et al., 2003) were probed. Surprisingly we found that several of the preys, Teflp, Egd2p, Fprlp and Tif51Ap interacted with all four regions; predominantly with the acidic region and the Rpb5-binding domain and to a weaker extend with the PFD-domain and the Uri-box (Table 4.6). In contrast, the Hsp40 Sislp interacted with the acidic region and the Rpb5-binding site, but hardly with the residual domains. Interestingly, also the association of the Hsp70 Ssb1p with the PFD-domain was weak, but interacted strongly with the residual domains. Since we questioned the specificity of these interactions, the individual Uri1p-domains were probed for interaction with twelve preys, including the related prefoldins. Furthermore some preys were tested for interaction with four yeast and mammalian control baits. No interaction or negligible background was detected with these control proteins (data not shown). Pfd6p, a β-subunit of the heterohexameric prefoldin complex and Rpb5p, a shared subunit of the RNA polymerase I - III, are two known Uri1p interaction partners. They interacted specifically and strongly with the corresponding Uri1p-domain and weaker with the acidic region, but not with the residual domains (Table 4.6). This shows that Uri1p associates with distinct regions of its interactors Sislp, Pfd6p and Rpb5p, but over an extended surface to the preys Teflp, Ssb1p, Fprlp, and Tif51A.

Table 4.6. Mapping of the Uri1p-prey interactions using the cytoY2H method. Bait constructs correspond to fl-Uri1p and the four distinct regions of Uri1p. Bait and preys were co-expressed in NMY51. Bait-prey interactions were selected on SD-Ade-His-Leu-Trp, supplemented with 15mM 3-AT. Yeast transformation was controlled by spotting the same volume on SD-Leu-Trp. Rpb5-bdg., Rpb5-binding site. Empty bait corresponds to bait vector pASO, expressing Ost4p-Cub-LexA-VP16. For simplicity Uri1p interactions with Fprlp, Egd2 and Tif51Ap are not shown.
No bridging between Uri1p bait constructs and the Uri1p-preys

In spite of the interactions with distinct Uri1p domains, the question raised if endogenous full-length Uri1p could dimerize with some of the episomally expressed Uri1p truncations, thereby bridging an Uri1p truncation with a prey and leading to a transcriptional readout in the cytoY2H assay. To exclude this possibility, URI1 was deleted in the yeast reporter strain and the above preys were retested for their interaction with Uri1p domains. The interaction assays showed the identical result as seen with the host strain NMY51, endogenously expressing Uri1p (data not shown). Thus, interactions due to a bridging effect can be excluded.
4. Uri1p structure – function analysis

Discussion

Conclusions drawn from Uri1p protein interactions

The new Uri1p protein interactors identified by the cytoY2h system and the established protein interaction network support a function of Uri1p at the translational apparatus and couple translation and protein folding through eEF1A, the elongation factor that recruits the aminoacyl-tRNA to the ribosomal A-site.

It is now believed that a majority of proteins in a given organism are involved in complex formation at some point in life and that each protein may have on average six to eight interacting partners (Tong et al., 2004). The total of known and novel protein interactors for Uri1p exceeds the suggested mean by far (Fig. 4.8). Diverse classes of proteins were revealed by the current Uri1p interactome, pointing to a Uri1p function in more than one biological process. I therefore conclude that Uri1p is able to function in different cellular protein complexes, at different time points, under different conditions and most probably at varying subcellular locations. The question was posed how a single protein might be competent to interact with a multitude of different proteins. A prominent feature of IUPs is the adaptation of different structures upon different stimuli or with different partners, which enables their versatile interaction with various targets (Tompa, 2002). Thus, the large size of Uri1p and its intrinsically disordered structure allows the interaction with numerous proteins by low affinity, but high specificity.

Is Uri1p a subunit of an alternative chaperone complex?

From cytoY2H studies it was concluded that Uri1p does not substitute for any subunit in the hexameric prefoldin complex. However, it is possible that Uri1p builds an alternative chaperone complex with unknown yeast proteins sharing little or no sequence homology. This idea is supported by recent studies showing striking similarities between the structures of the bacterial Skp, the mitochondrial Tim9-Tim10 complex and prefoldin (Fig. 4.14). Despite the lack of sequence homology, the three chaperone protein complexes share an “octopus-like” prefoldin structure, suggesting a convergent evolution for diverse chaperones (Stirling et al., 2006).
4. Uri1p structure – function analysis

Mapping interactions

Mapping the interactions of distinct Uri1p sequence regions and the novel interactors provided at first a puzzling result, revealing an association for most prey proteins with several Uri1p regions. The proof that this is not an artifact was given by probing the Uri1p truncations and the preys under investigation with multiple unrelated and even related proteins such as prefoldin subunits, which did not interact with the proteins under investigation. Moreover, a very strong interaction of the known Uri1p interactors Rpb5p and Pfd6p and the corresponding Uri1p sites demonstrated, that Uri1p can both, interact with distinct regions of its sequence as well as interacting over its nearly entire sequence with a given protein as seen for the Uri1p/eEF1A interaction. Although these results were surprising, it has to be underlined again that Uri1p is predicted with high probability to be an intrinsically unstructured protein which behaves very different from folded proteins. The lack of a folded structure offers multiple advantages to Uri1p and IUPs in general. Low mean hydrophobicity and high net charge, associated with the disordered state preclude the formation of a hydrophobic cluster and promote an extended conformation by electrostatic repulsion (Tompa, 2002). Upon interaction, IUPs often undergo a disorder-order transition, which results in a large decrease in conformational entropy that uncouples binding strength from

Figure 4.14. Structural comparison of prefoldin, Skp and Tim9-Tim10; molecular chaperones which do not share sequence homology, but evolved convergently their structures. It is imaginable that Uri1p forms an alternative chaperone complex with different unknown subunits. Taken from Stirling et al., Nat Struct Mol Biol., 2006.
specificity and renders highly specific interactions reversible. Their open structure generates a disproportionately large binding surface and multiple contact points over their entire length, as illustrated in Fig. 4.15. For effectors, it provides specificity because of interaction with distant regions on the target, as illustrated in Fig. 4.15A. Thus, it is not astonishing that even a small region of Uri1p such as the 30 amino acid Uri-box could associate with a distinct Uri1p interactor and give a positive signal in the very sensitive cytoY2H assay.

How can Uri1p act in related but mechanistically distinct processes?

The steps in protein synthesis such as translation initiation, elongation, termination and co-translational protein folding at the ribosome are tightly coupled and require proteins to act in concert. Scaffolds, like the elf3 complex, organize a web of interactions to assemble proteins at the ribosome and participate in different reactions involved in protein translation (Hinnebusch, 2006). To my knowledge a scaffold protein which acts in translation as well as in protein folding was not described so far. However, as we found several characteristics which link Uri1p with chaperones - especially with CLIPS - and simultaneously observed an effect for Uri1p in general and specific translation, I propose Uri1p to be a scaffold which influences both processes. The proposed Uri1p function is consistent with the predicted largely disordered structure of Uri1p, which allows assembling numerous proteins. Interestingly, the frequency of disordered structures in chaperones is very high compared to the other classes (Tompa and Csermely, 2004). This further supports the idea that Uri1p could act as molecular chaperone.

Impaired chaperone activity can alter protein translation as a whole. Thus, deletion of CLIPS resulted in cells susceptible for translation inhibitors (Albanese et al., 2006). The same was shown for urilA cells. This might be conform to the model that nascent peptides back up
into the ribosomal exit tunnel and interfere finally with the accommodation of the aminoacyl-tRNA in the A-site upon impaired protein folding (Muldoon-Jacobs and Dinman, 2006).

**Simultaneous regulation of the general translation and the specific** *GCN4* **translation**

Binding of the ternary complex (TC) to the 40S ribosome is stimulated by initiation factors including the eIF3 complex and eIF1A (*TIF11*), (Fig. 4.1). The latter is encoded by an essential gene and was identified in the cytoY2H screen as novel Uri1p interactor. Interestingly, a truncated version of eIF1A reduced the rate of TC binding to 40S subunits scanning downstream from uORF1 on *GCN4* mRNA and derepressed *GCN4* translation in the absence of eIF2α phosphorylation by Gcn2p (Hinnebusch, 2005). This mimicked the results of reduced TC formation elicited by eIF2α phosphorylation or mutation in eIF2B genes. Impaired TC recruitment was consequently suppressed by overexpression of the TC (Hinnebusch, 2005). Interestingly, derepression in *urilΔ* cells was also rescued by TC overexpression (A. Deplazes, unpublished results), which suggests Uri1p to stimulate analogously the binding of the TC to the 40S subunit by association or in cooperation with eIF1A or an unknown factor. This molecular function of Uri1p is depicted as a model in Figure 4.16. Reduced rates of TC binding to the 40S subunit not only induce the *GCN4* specific translation, but impair translation initiation in general, which explains how the loss of *URII* affects both processes and causes *urilΔ* phenotypes like slow growth under physiological conditions.

*URII* is not essential in yeast cells, in contrast to the genes encoding the initiation factor 3 scaffold, which performs a similar function as suggested for Uri1p and for which a similar phenotype is proposed (Hinnebusch, 2005; Hinnebusch, 2006). Therefore, Uri1p might have an ancillary role which is analogous to eIF3 and promote in addition translation elongation as indicated by a strong association with the key elongation factor 1 A.

The recruitment of the ternary complex (GTP-eEF1A bound to aminoacyl-tRNA) in translation elongation to the ribosome is a process which is analogous to translation initiation (sequestration of GTP-eIF2 bound Met-tRNA<sub>Met</sub> to the 40S subunit). Therefore it is plausible that Uri1p operates in both processes (Fig. 4.16). Moreover, Uri1p might facilitate the cooperation of eEF1A with Ssb1p and other chaperones.
Does an unmasked Rpb5-binding domain induce GCN4?
The Rpb5-binding region showed not only derepression but even slight activation of HIS4-lacZ, indicating GCN4 induction. Moreover, uri1Δ cells expressing the Rpb5-binding region showed to be more sensitive to various drugs than in absence of this domain (Fig. 4.12).

Both genes, ADE2 and HIS3, are targets of the key regulator Gcn4p (Natarajan et al., 2001). Thus, in accordance with the HIS4-lacZ assay, the RPB5-binding domain might have been able to co-activate GCN4 translation resulting in the expression of the Gcn4p target genes ADE2 and HIS3 in the in uri1Δ reporter strain YNM7 (Fig. 4.13A). However, since ADE2 is under the control of LexA-responsive elements and the GAL2 promoter, it is not clear how activation of ADE2 transcription has occurred in the cytoY2H strain. In contrast, the reporter gene HIS3 is driven by its own endogenous promoter and yeast strain KT2205 harboring the HIS4-lacZ reporter has no LexA-responsive elements. Since the HIS4-lacZ assay is an indirect measurement of GCN4 translation, the potential Rpb5-site activity has to be examined by a direct assay using a GCN4 uORF-lacZ reporter to confirm the described effect of the Rpb5-binding domain.
Figure 4.17. Model - Schematic illustration of a possible Uri1p regulation. 1. Upon amino acids and nitrogen starvation *UR11* is repressed on transcriptional level (Gasch et al., 2000) by promoter specific repressors such as Rap1p or Abf1p and gets likely phosphorylated or dephosphorylated. As a consequence the molecular function of Uri1p in translation initiation is slowly reduced and stopped, leading to an impairment of the general translation and derepression of *GCN4* specific translation. Thus, a modification of Uri1p under starvation might influence its binding abilities and its function as an assembling protein. As a result, it is imaginable that Uri1p dimers dissociate into non-functional monomers and a masked Rpb5p site might be freed. The Rpb5-binding region of Uri1p now is able to activate *GCN4* on translational level or Uri1p might be directed into the nucleus due to an unmasked NLS in the Rpb5- binding site (3.). In the nucleus Uri1p could act as a transcriptional co-activator of stress-dependent genes maybe in cooperation with Gis1p or Rpb5p (4.). The expressed target genes may function in a negative feedback loop in additional induction of *GCN4* or in the degradation of Uri1p (5.,6.).

**Activation of transcription by the Rpb5-binding site?**

A nuclear localization signal (NLS), predicted in the Rpb5-binding site of scUri1p (amino acids 188 to 205) provides a different explanation for *ADE2* activation by the Rpb5-binding domain. The NLS possibly prevented the association of this domain fused to the Ost4p anchor at the endoplasmic reticulum. Instead, the Rpb5-binding domain could have been translocated into the nucleus where it activated *ADE2* and *HIS3* transcription due to the fused transcription factor LexA-VP16.
It will be helpful to clarify the raised questions around the Rpb5-binding site. If this site is indeed responsible for independent \textit{GCN4} activation, it is conceivable that it is masked in active full-length Uri1p and \textit{GCN4} translation is repressed due to the functional acidic region. Uri1p is slowly degraded under starvation and Gcn4p expressed. Thus, in starved cells, Uri1p might be modified by phosphorylation or dephosphorylation, which could trigger inactivation of Uri1p. The regulation of Uri1p by phosphorylation can be concluded from several independent lines of evidence (Venturi et al., 2000; Ptacek et al., 2005; Parusel et al., 2006). By disabling Uri1p, the dominant Rpb5-binding site might be unmasked, i.e. triggered by dissociation of Uri1p dimers, as proposed in Fig. 4.17. This in turn could lead to additional activation of \textit{GCN4} translation. In a negative feedback loop Gcn4p would subsequently induce degradation of Uri1p, directly or indirectly. Alternatively, Uri1p might be degraded rapidly due to its extreme proteolytic sensitivity, which is typically for IUPs (Wright and Dyson, 1999).

Different conclusions can be drawn if a strong NLS of the Rpb5-binding domain prevented its membrane association in genetic studies and caused nuclear localization of this domain. It is imaginable, that Uri1p is directed into the nucleus as a rapid response to amino acid and nitrogen starvation and acts as a transcriptional co-regulator of nutrient starvation dependent genes (Fig. 4.17). Support for this assumptions is given by a well characterized interaction between Uri1p and Gis1p, a transcription factor which is involved in the expression of genes during nutrient limitation (Tronnersjo et al., 2006) and the Uri1p/Rpb5p interaction. Moreover, Uri1p might stimulate its own degradation on transcriptional level.

It was reported that the human URI, an ortholog of yeast Uri1p, is directed into the nucleus upon masking of a cytosolic localization signal (Delgermaa, et al. 2004). The NLS of human URI, responsible for the subsequent nuclear translocation, was localized in the acidic region. In contrast the NLS of \textit{sc}Uri1p is predicted in the Rpb5-binding domain (residues 188 to 205). Furthermore yeast Uri1p differs from other orthologues significantly in its long acidic region, for which no considerable homologues are found beyond fungi. The acidic region composes more than half of \textit{sc}Uri1p and is twice as long as in human URI. This implies divergent functions of yeast and human URI.
**Uri1p, a versatile protein**

Published data including the recent study on Gislp (Tronnersjo et al., 2006) do not only indicate a function for Uri1p in protein synthesis, but also in nuclear processes like transcription. Parusel and coworkers propose a role for *C. elegans* URI-1 in the maintenance of DNA integrity by affecting directly or indirectly DNA metabolism (Parusel et al., 2006). The current protein interaction map shows the interactions between yeast Uri1p and 36 different proteins of which ten proteins have nuclear functions (Fig. 4.8). This corroborates that Uri1p, which is only partially associated with the ribosome, might be localized under different physiological conditions at varying subcellular locations and also in the nucleus. Thus, Uri1p might shuttle between the cytosol and the nucleus and co-regulate diverse processes, possibly depending on the nutrient state of the cell.

**Uri1p, involved in the cellular heat stress response and general cellular stress response?**

Expression of *ADE2* in *uri1Δ* cells under heat stress and the rescue of this phenotype by expressing full-length Uri1p or its C-terminal domain, suggest Uri1p to be implicated in the heat stressed induced GAAC. These are preliminary results which have to be proved by additional experiments and more direct analyses. However, this outcome is not surprising, since mRNA profiles have shown that *UR11* is repressed under varying environmental stress conditions (Gasch et al., 2000), analogous to CLIPS (Albanese et al., 2006). Since the Uri1p function was thought to be restricted to amino acid and nitrogen starvation (Gstaiger et al., 2003), this might extent the range of the molecular role for Uri1p.

Uri1p is therefore suggested to provide a downstream target in adaptive cellular programs responding to general environmental stress (Fig. 4.18). Recently, Uri1p was shown to be phosphorylated by twelve different kinases (Ptacek et al., 2005) and an interaction between Uri1p and the serine/threonine phosphatase I, Glc7p, was well described (Tu et al., 1996; Venturi et al., 2000; Ito et al., 2001), supporting the idea that Uri1p might be highly regulated. Under physiological conditions, the Uri1p function appears to be important in the maintenance of the global translation by recruitment of the TC to the 40S ribosomal subunit and the repression of stress induced genes, such as *GCN4*. By doing so, Uri1p preserves the cellular homeostasis and impedes stress adaptive programs. General cellular stress might lead
to the downregulation and inactivation of Uri1p and result thereby in the reduced global translation and activation of the cellular stress response, which allows cell survival or the initiation of apoptotic programs. The different phenotypes observable in urilÀ yeast cells, such as sensitivity towards high temperature, various drugs, nitrogen starvation and slow growth, are coherent with this model.

Figure 4.18. Model - Uri1p involved in the general cellular stress response. For simplicity only features in correlation with Uri1p are depicted.
5. Chapter - General discussion

In this section, the significance of the cytoY2H system in the context of alternative methods aiming at the identification of protein interactions is discussed. In addition, a possible future application is presented.

Why studying transactivating factors in vivo?

The cytoY2H system was primarily designed for proteins which show intrinsic transcriptional activity in the conventional y2h system. In principle transactivating proteins can be studied by other methods than in vivo two-hybrid based technologies. Co-AP/MS and the proteomic chip technique provide suitable tools. Recently, tissue specific interactions between human transcription factors (TFs) were predicted on a larger scale by computational analysis of combinatorial gene regulation (Yu et al., 2006). TF gene expression profiles and promoter specific DNA binding sites for TFs were utilized for their studies. Results were validated by considering known TF-TF interactions, based on y2h data sets, and tissue specific co-expression of target genes.

So, why do numerous attempts exist to study transcription factors in vivo? Different experimental approaches have diverse advantages and answer different kinds of questions. In contrast to Co-AP/MS, two-hybrid based systems detect binary interactions which can be important for TFs. TFs provide a downstream target of cell signaling and the flow of information can be traced by identifying binary interactions. Moreover, as indicated initially, distinct systems are biased against different proteins. Contrarily to in silico and in vitro systems, an advantage of the conventional y2h system is provided by the fact that it is hardly biased against proteins of high abundance, most likely because transient interactions can be detected, which is not the case for example in Co-AP/MS (von Mering et al., 2002). Since many two-hybrid based systems, and particularly the split-ubiquitin system, are capable of sensing transient interactions, it can be concluded that alternative systems offer this advantage of a low bias towards high abundant proteins as well.

Functional categories of interacting proteins are not even covered by the existing methods. For example proteins which are involved in transcription and transcriptional control are well covered by the y2h method, whereas the same method detects relatively few proteins involved in translation (von Mering et al., 2002). Thus, distinct interactions can be detected by differing technologies. Consequently, they complement each other. Often, in silico
methods use data sets from various experimental sources to predict a protein interaction, including y2h-generated results. An example for this is given by the studies of Yu and coworkers above, predicting TF interactions by computational analyses (Yu et al., 2006). Surprisingly, not merely alternative proteomic systems, but also results produced by the same method, can have a very low overlap as shown for the large scale studies of the yeast proteome of Uetz and Ito (Uetz et al., 2000; Ito et al., 2001). They both utilized the y2h system, but several parameters and their screening strategy differed, which explains partially the small overlap. In addition, most large-scale studies do not reach saturation and produce a considerable number of false positives, which holds true for most methods.

Two-hybrid-based systems are in the main very versatile. Thus, besides allowing the detection of interactions, the same assays are also suitable to characterize protein-interactions under dynamic conditions and are capable of screening for disrupters of a designated protein-protein interaction (Serebriiskii and Kotova, 2004; Eyckerman et al., 2005). The latter is an essential tool for drug discovery.

For these reasons none of the established methods is indispensable and the availability of a huge variety of different proteomic technologies is fascinating and precious. Finally, the highest accuracy for predicting a protein interaction is achieved when an interaction is supported by more than one method.

**Pro and cons of alternative y2h based screening systems**

The cytoY2H system aimed both, the characterization and the identification of protein interactions. Since the latter is the most challenging item in studying protein interactions, I focus on closely related techniques of the cytoY2H system, which are suitable to find novel interaction partners of transactivating bait proteins through cDNA library screenings. The most important features of these methods are summarized in Table 5.1.

Both, the RTA system (Hirst et al., 2001) and the rUra3 based split-ubiquitin (rUra3-SU) system (Wittke et al., 1999) were capable of identifying novel interaction pairs. Of the y2h-based techniques, the rUra3-SU system provides the most versatile system for the identification of new protein interactions. This method was used to characterize proteins of the translocation machinery (Dunnwald et al., 1999; Wittke et al., 1999) and identified proteins interacting with the transcriptional regulators Gal4p and Tup1p (Laser et al., 2000).
Moreover it was shown that the rUra3-SU method was able to sense interactions between a glucocorticoid receptor (fused to Cub) and a small molecule, which was attached to a DHFR-Nub fusion (Dirnberger, 2006). In contrast, the use of the RTA system is very limited to soluble, strongly transactivating baits and soluble preys. Nonetheless, putative binding partners of the transcription factor VP16 and the androgen receptor were isolated by the RTA technique (Hirst et al., 2001; Wafa et al., 2003; Ray et al., 2006).

Despite their success, the RTA technique and the rUra3-SU system bear some technical constraints. Both systems use resistance to 5-fluoro-orotic acid (5-FOA) as the selection mechanism. 5-FOA selection is difficult to apply in cDNA library screenings as it produces a high number of false positives due to non-specific growth in the presence of 5-FOA. Furthermore, a replica plating step must be used for selection which makes the method very laborious and which is not conducive for large scale applications.

The SRS- (Aronheim et al., 1994), RRS- (Broder et al., 1998) and the Pol III- (Petrascheck et al., 2001) systems also avoid 5-FOA- selection by utilizing strains which are temperature-sensitive for Cdc25p or U6snRNA, respectively. However, screenings are not performed under physiological conditions since interacting bait and preys are selected at 36°C or 37°C. Temperature changes generate considerable alterations in the pattern of the yeast protein biosynthesis (Miller et al., 1979). Consequently, the proteome, protein interactions and interactions which can be covered by these methods differ from normal conditions. This bias has to be considered when evaluating results from SRS-, RRS- or Pol III- screenings, since it is usually not the intention of researchers to find interaction partners under special cellular conditions. Moreover, selection on increased temperature puts additional stress on the yeast cells and increases the risk of obtaining false positives, which appear in any event in RRS- and SRS- screenings, since members from the Ras family present in a given cDNA library can bypass rather easily the selection system (Huang et al., 2001). The SRS system was improved slightly (Huang et al., 2001). However, the same authors qualified the SRS system as not a useful tool for broad applications, due to several technical constraints.
Despite these limitations, the SRS technique has been successfully applied for identification of c-Jun (Aronheim et al., 1997), BRCA1 (Yu et al., 1998) and p73 (Hanamoto et al., 2005) binding partners. The RRS system was able to find two valid interactors of a c-Jun bait (Broder et al., 1998). Although the principle of the Pol III system is very elegant, this technique is not technically mature. This is also reflected by only one report of a screen with a BRCA1 bait, yielding 183 clones which were not validated for their biological significance.

**Split-ubiquitin specific benefits**

Transient interactions can be detected by the original y2h system. However, if a bait-prey interaction is occurring in the cytoplasm, this DBD-bait – prey-AD complex has first to be directed into the nucleus for activation of the reporter system. Thus, interactions taking place outside from the nucleus need to be relatively stable to detect them in the conventional Y2H system and some of the y2h alternatives, the RTA and the Pol III system. In contrast, several y2h-derivatives require only a very short bait-prey interaction to trigger the experimental readout of a protein interaction. This is particularly true for the split-ubiquitin system, since reassociation of split-ubiquitin is fast. Therefore, split-ubiquitin based techniques allow detecting short transient protein interactions which are fundamental to many cellular processes.

An additional important advantage in split-ubiquitin techniques is provided by a specific functional test which probes whether a bait construct is able to activate the split-ubiquitin reporter system, independent of an interacting prey (Fig. 2.9). Especially heterologously expressed proteins can be instable or toxic in yeast, which leads to degradation or insufficient protein expression. Since the functional assay is performed at an initial step of the screening procedures (Table 2.14), bait constructs which are not suitable for screening can be sorted out immediately and resources can be saved.
The cytoY2H method in comparison with alternative screening systems

Intriguingly, an obvious issue of the y2h-based (Table 5.1) and of most other technical or computational methods in the identification of protein interactions is the occurrence of false positive interactors.

Numbers of false positives occurring in the cytoY2H system varied considerably, similar to conventional y2h-screenings. They were shown to depend on a given bait and were not of systematic nature, as false positives appearing under 5’ FOA selection or in Ras recruitment-based systems. Interaction partners of the cytoY2H membrane anchor Ost4p were hardly selected in screenings. Most of the false positives in the cytoY2H system were identified easily by a bait-dependency test in which unrelated bait proteins were used as controls. The cytoY2H reporter strain harbors two different marker genes for prototrophic growth and a lacZ marker. Therefore stringency is rather high and can be further increased by the use of additional marker genes, which I consider an important optimization for this novel method. The fine-tuning of the system stringency by applying different reporter genes and varying the number of the corresponding operator sites is an advantage of genetic over alternative techniques.

Compared to the techniques which select protein interactions at high temperature (RRS, SRS, Pol III), cytoY2H screenings are performed under mild conditions, since a protein interaction is allowed to occur at physiological temperature. Consequently, the temperature can be adjusted according the experimental question of an investigator and the conditions required.

A cytoY2H bait protein is at each terminus attached to a fusion protein (either Ost4p or Cub-LexA-VP16). In contrast, baits in the alternative systems bear one fusion protein at one of their termini. It is not clear yet, if baits in the cytoY2H system are sterically hindered by this additional fusion protein in associating with prey proteins. However, a high percentage of previously described protein interactions which were probed by the cytoY2H system could be detected. Therefore, the occurrence of false negatives in screenings is rather referred to the different biases between alternative proteomic methods than to impeded bait-prey association by sterical hindrance caused by the two bait fusions.
The cytoY2H approach is based on the split-ubiquitin system (Johnsson and Varshavsky, 1994; Stagljar et al., 1998), for which numerous successful studies have been reported (Thaminy et al., 2003; Wang et al., 2004; Matsuda et al., 2005), including a high-throughput screen (Miller et al., 2005). Besides the rUra3-SUB, the cytoY2H represents the most versatile system, which is only restricted to soluble baits, but in principle can identify any soluble, membrane associated or integral membrane protein. Consequently, it can be deduced that the coverage of protein interactions by the cytoY2H system is high. The novel method is a straightforward approach, which requires no replica plating steps in contrast to 5-FOA selection and can be adapted easily to large scale applications, in contrast to many of its alternative systems.

We could not identify previously described interaction partners by screening a cDNA library with p53 and Gal4p, two different transcription factors. However, the developed system was very useful in identifying multiple interactors of the transactivating protein Uri1p. Several of these novel binding partners were further confirmed by co-immunoprecipitation.

Subsequently, the cytoY2H method has proven to be a valuable tool for the characterization of the novel interactors. It was capable of mapping interactions between truncated forms of Uri1p and its interactors (Table. 4.6), allowed us to find novel interactions between Uri1p binding partners and to establish a small protein interaction network (Fig. 4.9B). Finally, the Uri1p bait (fused to the N- and C-terminal cytoY2H-reporter cassette) was used for functional analyses. These analyses demonstrated that the Uri1p bait was functional, despite its attached reporter constructs, and led to the identification of the functional region of the Uri1p protein (Fig. 4.12). Experience with the cytoY2H method showed that this system is very sensitive and suitable to sense weak interactions and interactions of low expressed proteins such as the SV40LT-dimerization.
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<td>all kinds of proteins</td>
<td>- Successfully used for a transactivating protein</td>
<td>- Varying numbers of false positives</td>
<td>Stagljar et al., 1998, this work</td>
</tr>
<tr>
<td>rUra3p split-ubiquitin</td>
<td>Split-ubiquitin, destabilized Ura3p</td>
<td>all kinds of proteins</td>
<td>all kinds of proteins</td>
<td>- Successfully used for different baits including TFs</td>
<td>- False positives by 5-FOA selection</td>
<td>Johnsson and Varshavsky, 1994; Winke et al., 1999</td>
</tr>
<tr>
<td>Repressed transactivator system (RTA)</td>
<td>Tup1-RD fused bait, Gal4-DBD-fused prey</td>
<td>soluble, transactivating</td>
<td>soluble</td>
<td>Successfully used for TF baits</td>
<td>- False positives by 5-FOA selection</td>
<td>Hirst et al., 2001</td>
</tr>
<tr>
<td>SOS- recruitment system (SRS)</td>
<td>Ras signal transduction pathway Human SOS instead of Cdc25p</td>
<td>soluble</td>
<td>soluble, membrane associated</td>
<td>Successfully used for TF baits</td>
<td>- Systematic false positives</td>
<td>Aronheim et al., 1994</td>
</tr>
<tr>
<td>RAS recruitment system (RRS)</td>
<td>Ras signal transduction pathway Activated human Ras</td>
<td>soluble</td>
<td>soluble, membrane associated</td>
<td>Successfully used for TF baits</td>
<td>- Systematic false positives</td>
<td>Broder et al., 1998</td>
</tr>
<tr>
<td>RNA pol III system (Pol III)</td>
<td>Essential snRNA U6 SNR6-UAS6, - reporter</td>
<td>soluble</td>
<td>soluble</td>
<td>RNA pol II independent</td>
<td>- Unphysiological conditions</td>
<td>Marsolier et al., 1997; Petrascheck et al., 2001</td>
</tr>
</tbody>
</table>

RD, repressor domain; TF, transcription factor
Our original intention was to generate a method which is convenient for screening transactivating proteins such as TFs. Defined protein interactions with multiple transactivating bait proteins were characterized successfully. The usefulness of the cytoY2H screening system for the identification of novel binding partners for nuclear transcriptional regulators remains uncertain at that time. More transcription factors need to be tested to answer this question. However, since the cytoY2H system proved to be robust in identifying defined interactions of all classes of soluble proteins, this goal could achieved likely by use of a matrix approach.

Potential of the cytoY2H assay

One of the major achievements in cytoY2H screenings was the identification of diverse interaction partners of U11p, which could be ascribed to ribosome associated processes such as protein translation and protein de novo folding. The U11p bait was anchored at the endoplasmic reticulum (ER) by the Ost4p membrane protein. Therefore it is not surprising that it was possible to detect interactions with these classes of proteins, since a fraction of co-translating ribosomes are associated with the ER-membrane. Intriguingly, proteins involved in translation are not sufficiently covered by the conventional Y2H screening (von Mering et al., 2002). The same is true for mitochondrial proteins. Since the cytoY2H bait can be targeted to any arbitrary cellular membrane by choosing the according organelle specific membrane anchor (Fig. 5.1), it can be searched for protein interactions at a designated cytoplasmic subcellular localization. Therefore the cytoY2H system has the possibility to answer specific questions of different nature. This selection of a bias towards a subcellular setting is suggested to be a large potential of the cytoY2H system, which is not given by the RTA-.
RRS-, SRS- and Pol III- alternatives, but is possible for the rUra3-SU technique as well. In addition and contrarily to the rUra3-SU technique, the cytoY2H system is a promising tool for large scale applications, since it allows a straight forward strategy.

We are confident that further experience, gained by probing other diverse proteins in cytoY2H screenings, optimization of the current cytoY2H version and adaptations for special needs will establish the cytoY2H system as a useful tool to study protein interactions in yeast.
References


Sigalov, A.B., Zhuravleva, A.V. and Orekhov, V.V.: Binding of intrinsically disordered proteins is not necessarily accompanied by a structural transition to a folded form. Biochimie (2006).


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PUBLICATIONS

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It is a journey, never really an arrival."

Karl Popper