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Yeast surface display of CD154

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ABSTRACT

The CD154 (CD40L) is a member of tumor necrosis factor (TNF) family that plays a crucial role in regulation of both cell-mediated and humoral immunity by activating antigen presenting cells (APCs). Previously, studies in the laboratory demonstrated that yeast surface display system allows the expression of biologically functional antigen, such as subunit HA, against influenza virus. Yeast surface display system seems to be an excellent system to be utilized to develop anti-influenza virus vaccine. Thus, it is of interest to study the adjuvant function of CD154 on the subunit HA vaccine against influenza virus. In this project, the CD154 encoding gene was isolated from mouse spleen tissue and sub-cloned into a yeast cell surface protein display vector. Display of CD154 on yeast surface was investigated and confirmed through immunofluorescence microscopy, Western blot, and flow cytometric analysis. To elevate high level display of CD154 on yeast surface, the time course of CD154 surface display was determined and used for optimizing the yeast expression system. This work laid out the foundation for using CD154 as an adjuvant for recombinant yeast influenza vaccines. The adjuvant effect of yeast surface displayed CD154 is now tested in animal studies in this laboratory.

This thesis is approved for recommendation
to the Graduate Council.

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Chapter I. LITERATURE REVIEW

1.1. Introduction

Avian influenza, an infectious disease caused by highly pathogenic avian influenza (HPAI) A virus, has given rise to millions of deaths among poultry and wild birds around the world. Some mutated avian influenza viruses can also infect humans with 60% mortality. Vaccines, antiviral drugs and personal protective cares are the most common methods for influenza treatment and prevention. Nowadays there are only two major classes of influenza antiviral agents certified in the United States–M2 ion channel inhibitors and neuraminidase inhibitors. Single point mutations in the critical residues of the neuraminidase and M2 proteins result in the viral resistance to the drugs [1-2]. Data show that influenza A viruses become resistant to amantidine and rimantidine (M2 channel protein inhibitors) as soon as 2-3 days after the beginning of drug therapy [3]. In December 2008, the Centers for Disease Control and Prevention published the new data displaying that a heavy percentage of the circulating A/H1N1 viruses are already resistant to the neuraminidase inhibitor—oseltamivir [4]. Additionally, these resistant strains were reported to be persistent and transmissible from person to person [5]. Thus, vaccination remains the only reliable method that can effectively cease the spreading of influenza virus among the world.

Traditional flu vaccine development is based on mixing a target virus with a non-hazardous strain in chicken eggs followed by screening for a suitable vaccine candidate. However, this method does not work for the H5N1 strain, as it is lethal for chicken embryos [6]. Furthermore, the screening of suitable vaccine candidates could take up to ten months with the limited

capability of massive production, while during the outbreak it should be constructed and released as soon as possible.

Among all technologies DNA immunizations seem very promising. They are the simplest manifestation of vaccines that provide genes encoding an antigen, instead of using the antigen itself. One of its potential advantages is the induction of both major histocompatibility complex (MHC) class I and II responses by DNA vaccines [7-8]. DNA vaccines are widely used and proclaimed to be safe and simple in use. However, they have some disadvantages. For instance, there is a possibility of integration of foreign DNA into a host's genome. Thus, risk of DNA integration into genome of fetus of pregnant woman injected with such vaccine is a big concern.

Another promising vaccine technology is to use adenoviral vectors for influenza antigens delivery. Adenoviral vectors have been shown to be very efficient agents for gene delivery and protein expression *in vitro* and *in vivo*. They can stimulate both innate and adaptive immune responses in mammalian hosts. Nevertheless, there are some serious drawbacks associated with adenoviral systems. One of the issues is its safety due to its distribution throughout the body when being inoculated. This risk needs to be thoroughly monitored. Removal of key transacting genes of adenoviruses, such as E1 or E4, could potentially prevent their replication in host cells. The production of these recombinant viruses can be achieved by using a helper virus with complement for the defective genome or by sub-cloning the equivalent region of the adenoviral genome into the chromosomes of cells that produce the viruses. The removal of helper viruses from these recombinant viruses before they can be used for vaccination has been a significant challenge. In addition, there is a chance of recombination between the replication-defective and

wild-type adenoviruses owing to the co-dissemination [9]. Another disadvantage is that even after the deletion of E1A, some cells still express viral genes, leading to an immune response against the infected cells and resulting in their fast destruction. Finally, adenoviruses have limited cloning capacity as compared to other viral systems [9].

One should keep in mind that two-thirds of the world population is living in developing countries. Infectious diseases (including influenza) cause most deaths in children under age 5 , and represent massive health problems in older children and adults as well in these countries. High cost, troublesome distribution and administration logistics of current influenza vaccines are the main reasons that prevent most people in these countries from reaching vaccines [10]. It is, thus, highly desirable for developing a new technology that allows for rapid, massive and low cost production of influenza vaccines. The study presented in this thesis explored the feasibility of utilizing yeast as a carrier to deliver immunogenic proteins for developing new types of influenza vaccines.

Safety is one of the major concerns in pharmaceutical industries and vaccine production in particular. One of the most suitable microorganisms for formulating subunit flu vaccines is the *Saccharomyces cerevisiae (S.cerevisiae),* as it has obtained a "generally regarded as safe" (GRAS) status. *S.cerevisiae* has been used orally for *Clostridium difficile* diarrhea treatment, and shown to be protective in mice against influenza infection [11]. However, most studies were carried out with *S.boulardii,* but together obtained data provide strong evidence that *Saccharomyces* expressing antigens on its surface potentially can be a good candidate for use as a vaccine platform.

1.2. Yeast Surface Protein Display

The cell surface is a barrier between the inner and outer space of a cell. There are a number of specific systems on the cell surface for anchoring proteins. Cell surface protein expression systems were firstly established by fusing a peptide of interest to a docking protein (pIII) of a filamentous phage without any influence on its ability to infect *E. coli* [12]. This led to the development of phage peptide/protein display systems. These surface peptide/protein display systems allow researchers to isolate antibodies from a combinatorial library of antigens or specific ligands. The problem with these protein display systems is that the fused peptide or proteins cannot be immersed completely into the phage surface unit [13].

Bacterial surfaces appeared to be more appropriate for displaying a large size of proteins. Both Gram-negative and -positive bacteria have been used for displaying heterologous proteins, but the use of these surface display systems in food or pharmaceutical industries has been challenging due to its safety concern [14-19].

To address these issues, yeast surface protein display systems have been developed recently. These systems permit the expression of a heterologous protein of interest on the surface of *S.cerevisiae* by elongation the protein with α-agglutinin that serves as a cell wall anchor. The development of such systems is believed to be practical in different technologies, including production of microbial biocatalysts; separation of expressed polypeptides; identification of desired proteins from a combinatory library; whole-cell adsorbents, and live vaccines [20-22].

Unlike *E. coli, S.cerevisiae* has protein folding and secretory mechanisms similar to those in mammalian cells, making it suitable for vaccine development. Yeast has a rigid wall located outside of its plasma membrane. It is approximately 200 nm thick, mostly composed of mannoproteins and β-linked glucans. The cell wall is characterized by a bilayer structure: its inner layer consists of glucan composed of β-1,3- and β-1,6- linked glucose, whereas its outer layer is mainly composed of mannoproteins attached to glucans by covalent bonds [23-24]. There are two types of mannoproteins found on yeast surface, i.e., mannoproteins that are loosely linked to the cell wall *via* noncovalent bonds and can be released by SDS (some 60 such proteins with low molecular weight can be extracted after treating the separated cell wall with hot SDS [25] and mannoproteins that can be released by solublizing the glucan layer of the cell wall with β-1,3- or β-1,6 – glucanase[26]. There are many glucanase-extractable proteins on yeast surface. Most contain a specific glycosylphosphatidylinositol (GPI) anchor that is critical for the surface display of a protein of interest. Among such proteins are agglutinin (Agα1 and Aga1), flocculin Flo1, Cwp1, Cwp2, Tip1, Sed1, and Tir1/Srp1. The organization of the yeast GPI anchor is comparable to that found in eukaryotes. Its core structure is conserved among various organisms. A GPI-anchored protein can be directed to yeast surface through a secretory pathway. Two different yeast surface display systems have been developed so far: one utilizes the agglutinin system and the other employes the floculin system [27].

Agglutinin-based yeast cell surface protein display systems

The *S.cerevisiae* mating-type-specific agglutinine are glucanase-extractable mannoproteins anchored on the outer surface of yeast. They are responsible for direct cell-cell adhesion between cells of opposite mating types during the replication process [28]. There are two mating cell

types: cells with **a-type** expressed a-agglutin and cells with **α-type** –α-agglutinin respectively. The α-agglutinin is encoded by AGα1. It binds to the a-agglutinin complex. The a-agglutinin contains two subunits. Its core subunit is encoded by AGA1. It attaches to a smaller binding subunit encoded by AGA2 through disulfide bonds. α- and the core subunit of the a-agglutinin share a very similar structure: a secretion-signal region, an active region, a support region high in threonine and serine, and an assumed GPI anchor-attachment signal, suggestively in heavily O-glycosylated forms [27]. Both a- and α-agglutinin anchoring mechanisms have been adopted for displaying many heterologous proteins to the outer glycoprotein layer of yeast surface. The general scheme of this protein display system is depicted in **Fig. 1.1A**. In this molecular design, the C-terminal part of the a-agglutinin, which contains a GPI-anchor signal, is fused with a protein of interest, leading to the covalent binding of the protein to glucan on the outer surface of yeast. Another design is to link a protein of interest to the Aga2p protein (**Fig. 1.1B**). The binding of Aga2p to the Aga1p through an S-S bond leads to the display of the protein on yeast surface [29-30].

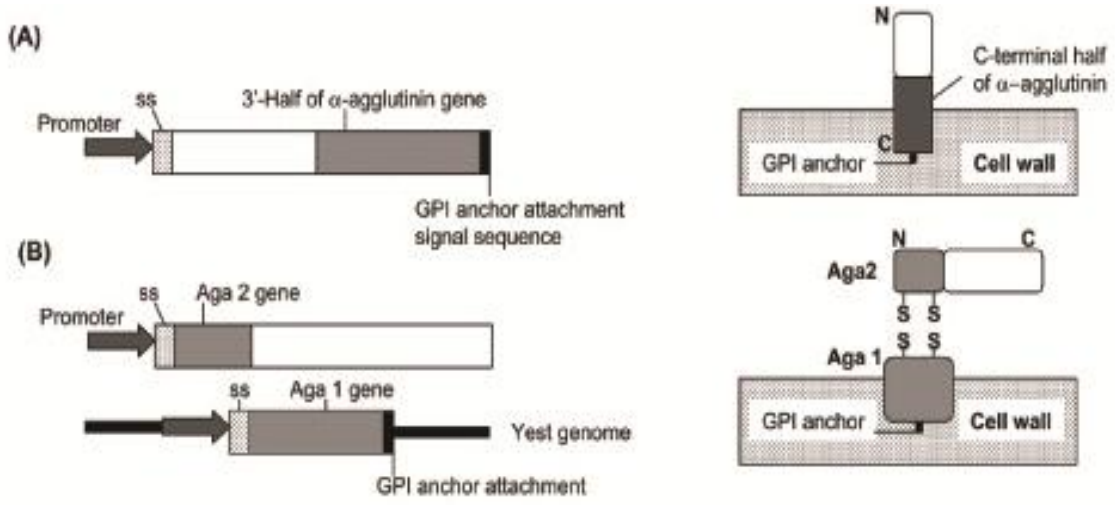


Fig.1.1. Yeast surface display systems using agglutinin GPI-anchoring mechanisms, **adapted from [27].** (A) Cell-surface display of an enzyme using **α-agglutinin system**; (B) Cell-surface display of an enzyme using **a-agglutinin system**.

Flocculin protein surface display systems

Flo1p is found among *S. cerevisiae* cell-wall proteins and encoded by the FLO1 gene [31-32]. It is a lectin-like protein that plays a major role in the flocculation process. Its high level O- and N-glycosylation allows it to form stem-like structures and cover the distance of 300 nm, equivalent to the thickness of yeast cell wall. Consequently, it leads to the design of anchors with varied lengths.

The Flop1 protein consists of several domains: a secretion signal, a flocculational functional domain, a GPI-anchor attachment signal, and a membrane-anchoring domain [32]. Two Flo1p surface protein display systems have been developed (**Fig. 1.2**.). The first is the GPI anchoring system, consisting of six types of the cell-surface system with the Flop1 C-terminal part containing a GPI-attachment signal and anchors of different lengths: 42,102,146, 318,428, and 1326 amino acids. The C-terminus of a target protein can be fused to the anchor signal peptide. The length of the reduced Flop1 is chosen based on the nature of the target protein and also the purpose of display [33]. The second system manages the adhesive ability of the flocculation functional domain of Flop1p to generate a surface protein display system that involves FS and FL protein of functional domain of Flop1 with the insertion site and secretion signal for a target protein [34]. The fusion of N-terminal of a target protein to the functional domain of Flop results in non-covalent interaction of its flocculation functional domain with the mannans of the yeast cell wall.

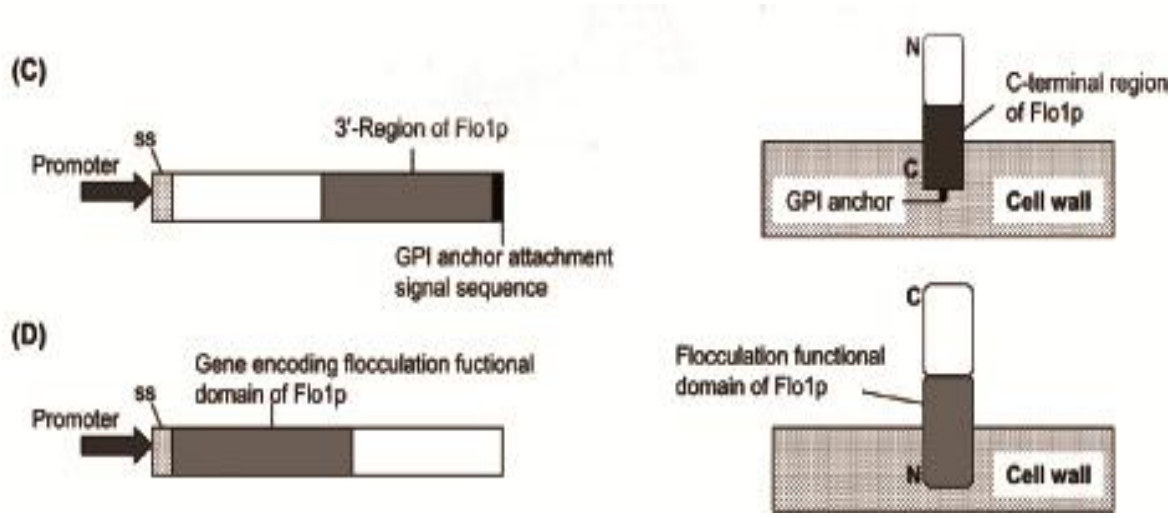


Fig. 1.2. Yeast surface display systems using flocculin GPI-anchored protein, adapted from [27]. (C) The GPI system constructed using the C-terminal region of Flo1p, and the C-terminus of the target protein fused to the anchor; (D) The surface-display construct consisting of the flocculation functional domain of Flo1p, where the N-terminus of the target protein is fused to it.

1.3. Recent applications of yeast surface protein display systems

The ability of yeast cells to express mammalian proteins with high fidelities allows the favorable screening of cDNA libraries derived from eukaryotic organisms. For instance, the yeast surface display of a human cDNA library has been used to screen libraries for antigens correspond to proteins that react with breast cancer serum. The resulting library allowed isolating a previously unknown small breast epithelial mucin. Compartment of its content in healthy and diseased tissues has shown that the diseased samples contain a deletion mutation [35]. A human cDNA library has also been screened to determine the interacting partners for phosphorylated, synthetic peptides derivative of the epidermal growth factor receptor (EGFR) or focal adhesion kinase (FAK). Five clones (two expressing proteins with the binding affinity to phosphorylated EGFR and three with the binding affinity to phosphorylated FAK) were isolated from the library eventually [36].

Yeast cells afford an exclusive platform for cell adhesion assays due to their simple manipulation and easy to control cell adhesive behaviors. Measurement of angular and translational rate have showed that yeast expressing E-selectin roll rather than slide on the sialyl-Lewis-x surfaces, verifying that selectin interactions intercede a true "rolling" movement [37]. Yeast surface display method has been applied for the mapping of antibody epitopes by using either an antigen or the fragment of an antibody. For example, epitopes for a number of EGFR antibodies have been determined using yeast surface protein display approaches [38-39]. Display of firmly folded EGFR fragments or spontaneously mutated full-length EGFR on the yeast surface provides an easy way to determine antibody binding to specific domains or epitopes through flow cytometric analysis. Moreover, heat denaturation of the EGFR fragments helped to define linear but not conformational epitope binding [38-39]. On the other hand, the yeast surface displayed scFvs can also be used to identify their binding epitopes. Three monoclonal antibodies with earlier determined epitopes have been employed for characterizing the epitopes of EGF binding scFvs that were isolated from a library. Yeast surface displayed EGF-binding scFvs were soaked with EGF and consequently incubated with all three antibodies. The overlapping of the scFv epitopes with the epitopes of monoclonal antibodies resulted in blockage of EGF binding to the scFvs. Thus, it became possible to group the scFvs in accordance with their epitopes and arrange epitope-specific scFvs against EGF [40].

1.4. Characteristics of CD154

CD154 (also known as CD40 L, or gp 39) is a member of tumor necrosis factor (TNF) family that serves as a ligand for the cell surface receptor CD40 on antigen presenting cells (APCs).

Initial studies predicted its molecular mass of 39 kDa, but later it was shown that majority of cells express CD154 as a 32-33 kDa protein. Moreover, the amino acid backbone of the protein suggests 29 kDa of its molecular weight, which means that the posttranslational modification takes place. It appears that, human-, murine-, and cattle-CD40L all have a single glycosylation region in its extracellular domain [41].

CD154 has a structure of a sandwich consisting of two β-sheets with Swiss roll topology. Despite its type II transmembrane protein structure, it may be present on the cell surface as a heteromultimeric complex. Excluding its 33 kDa form, the molecule interacts with the shorter soluble structures of the protein of 31 and 18 kDa. These shorter proteins still keep their capability to form trimers, interact with CD154 and lead the biological signals, which indicates that CD154 can also serve as a bona fide cytokine [42-44]. Activated, but not resting T cells, express CD154. Its expression can be induced on T_{h0}, T_{h1} and T_{h2} cells; but mostly it is expressed on activated $CD4^+$ T helper cells, although a small portion of $CD8^+$ T cells is also able to express the CD40 L protein. Other cells have been found to express the CD40 L as well. For example, basophils, mast cells and eosinophils can all react to anti-CD40L or CD40-Ig antibodies. In the same way the expression of CD40 L on B cells, dendritic cells (DC), natural killer (NK) cells and monocytes/ macrophages has been detected under some certain conditions [45-46].

CD40-CD40L interaction is believed to play a critical role in immune response regulation. It was discovered in 1993 that the CD40L is responsible for the X-linked hyper-IgM syndrome, which is characterized by the deficit of circulating IgA and IgG and the deficiency of germinal center [47]. B cells recognize a specific antigen, receiving a transmembrane signal throughout a B-cell receptor (BCR), which contains membrane immunoglobulin (mIg) and extra transmembrane

polypeptides. The signal through the BCR provokes early events in the B-cell activation, enhancing expression of MHC class II molecules. Without antigen-reactive T helper cells, activated B cells proliferate and differentiate at a very poor level and produce a small amount of antigen-specific immunoglobulin M (IgM). An antigen is digested by B cells in endosomes and the digested antigenic peptides are presented to $CD4^+$ T cells in the way of MHC class II molecules. It results in $CD4^+$ T-cell activation and up-regulation of significant cell-surface molecules, such as CD40L, further activating antigen-specific B cells through the CD40-CDL interaction. This co-stimulatory signal is required for B-cell activation, isotype switching, germinal centers formation and the generation of B cell memory [48]. The importance of CD40-CD40L interaction against the viral infection was discovered in the study of intranasal influenza vaccine construction where influenza A virus nucleoprotein (NP) served as an antigen. Anti-CD40 monoclonal antibody (anti-CD40 mAb) and synthetic NP336-374 peptide, matching the cytotoxic T lymphocytes (CTLs) epitope on NP, were encapsulated in liposomes. Intranasal immunization of mice induced mucosal immunity and reduced virus replication in the lung, confirming the importance of CD40-CD40L signaling on generation of memory CLTs. It is also found that the dendritic cells (DCs) activated through CD40-CD40L signaling are able to induce the production of influenza A virus-specific memory $CD8^+$ T cells *in vitro*. Interestingly, immunization of mice lacking MHC class II gene could not prevent the viral replication in the lung, implying that there is another mechanism of protection, besides the priming of $CD8^+$ T cells by activated DCs with the anti-CD40 mAb, so it is possible that $CD4^+$ T cells non-specifically cooperate with B cells in antibodies production [49]. Infection of $CD40L^{-/-}$ mice with lymphocytic choriomeningitis virus (LCMV) and vesicular stomatitis virus (VSV) disease models has helped to detect that CD40-CD40L interaction is not required for T-help-independent

anti-viral IgM responses. However, CD40-CD40L interaction was showen to be critically important for T-dependent anti-viral Ig class switching of virus-specific B cells. Furthermore, it was understood that the importance of CD40-CD40L pair in the induction of T_h cell effector functions is different from cognate T help for B cells [50].

The ability of CD40L expressing DNA vectors to affect the type and the level of humoral and cellular immune responses *in vivo* has been evaluated in numerous studies. For example, plasmid DNA itself includes specific immunostimulatory sequences (ISS) containing unmethylated CpG motifs with confirmed adjuvant effects in the murine models [51-54]. It was reported that the expression of CD40L could improve the ability of $CD40^+$ cells to activate T cells in response to presented antigens [55]. The overexpression of CD40 ligand/trimer (CD40LT) showed much higher Ag-specific production of INF-γ, cytotolic T cell activity, and IgG2a antibody in BALB/c mice [56].

The expression of CD40L and green fluorescent protein (GFP) fusion proteins in mice showed a significant activation of B cells by providing the coincident stimulation of BCR and CD40 [57]. This study demonstrated that the immunization with the plasmids encoding the fused protein led to synergistic induction of GFP-specific antibodies, whereas the control plasmids for GFP, CD40LT, or GFP with CD40LT did not. Immunization with the single dose of the GFP-CD40LT protein induced a strong GFP-specific immunoglobulin IgG1 antibody response, demonstrating that the GFP-CD40LT fusion protein stimulates a GFP-specific B-cell activation and synergistic antibody response in an antigen-guided way [57].

The CD154 protein and CD40-CD154 signaling pathway have been shown to play an important role in tumor studies as well. Extracellular domain of the CD40L protein was fused to idiotype (Id) protein – a weakly immunogenic tumor-specific antigen, the murine B-cell lymphoma derivative (Id). Id-CD40L, free Id, and IgG-CD40L protein, structurally identical to Id-CD40L, but without Id part, were injected in mice with consequent measurement of anti-Id response [58]. Results indicated significantly higher anti-Id response in mice injected with the fused protein Id-CD4OL compared to the other two groups treatment, and so it was confirmed that fusing CD40L to an appropriate antigen can notably improve the adjuvant capability of CD154 protein.

Experiments with respiratory syncytial virus (RSV) in BALB/c mice indicated that CD40L expression enhances the levels of pulmonary nitric oxide (NO) synthesis and also increases the levels of IL-2 and INF-γ and the anti-RSV antibody response with the direction of this response towards a T_{h1} phenotype [59]. Several studies have been focused on the avian influenza vaccine construction. Thus, influenza hemagglutinin protein (HA) was fused to extracellular domain of chicken CD154 protein and sub-cloned into adenoviral vector to make a veterinary vaccine that could potentially be effective against HPAI. Immunization with adenoviral vector encoding chimeric HA-CD154 protein induced much higher cellular immune responses and HA-specific antibody titer as compared to the vector encoding only HA. Results also indicated that chimeric molecule HA-CD154 is able to multimerize *in vivo*, which allows it to interact more efficiently with a CD40 receptor [60]. The chimerically fused HA-CD154 has been used as an antigen. Study showed that the HA-CD154 can elicit much higher HI antibody titers and IFN-γ secretion as compared to the HA antigen [61]. Finally, the generation of duck subunit DNA vaccine by

targeting of avian influenza HA with CD154 allowed achievement of the partial protection against the genetically isolated HPAI.

Chapter II. MATERIALS AND METHODS

2.1. Introduction

The yeast surface protein display system used in this study is based on the a-agglutinin receptor of *S.cerevisiae*. The a-agglutinin receptor is made up of two proteins encoded by AGA1 and AGA2 genes. The Aga1 protein is directed to the extracellular surface where it binds to β-glucan, whereas the Aga2 protein is secreted to the extracellular space and then binds to the Aga1 proteins through two disulfide bonds, leading to the display of the Aga2 protein on the cell surface. The N-terminal part of Aga2 is necessary for its attachment to Aga1, while its C-terminus can be replaced by a protein of interest, resulting in the display of the protein (such as CD154 in this study)on the cell surface along with the half of Aga2, as shown in **Fig. 2.1**.

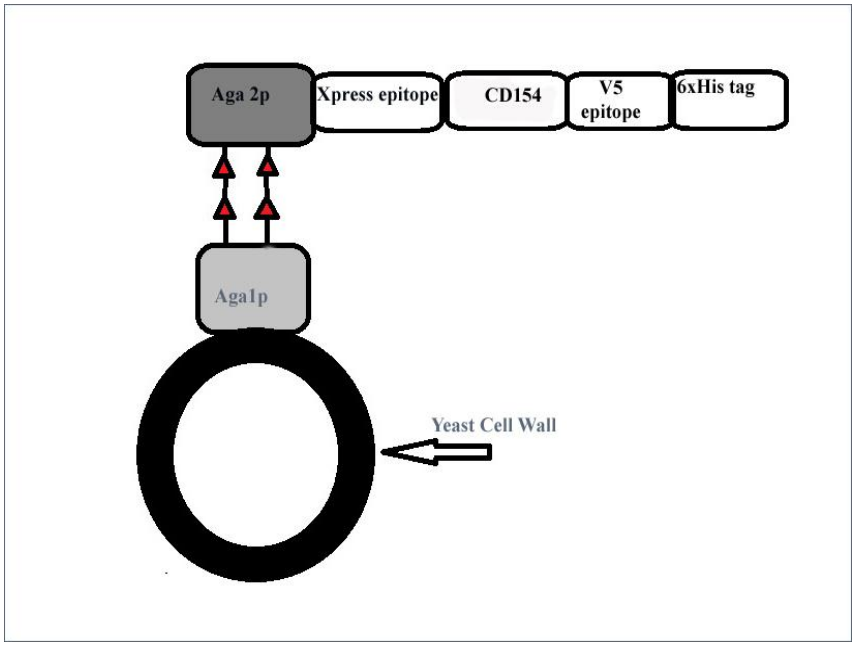


Fig. 2.1. A schematic diagram of CD154 display on yeast surface. Aga1, Aga2 – two subunits of α-agglutinin receptor. Xpress epitope, V5 epitope and 6xHis tag : tags that can be used for detecting surface display of CD154.

The pYD1 plasmid (Invitrogen, Carlsbad, CA) chosen for this study is a 5.0 kb shuttle vector for displaying a protein on the surface of *S. cerevisiae* (Fig. 2.2).

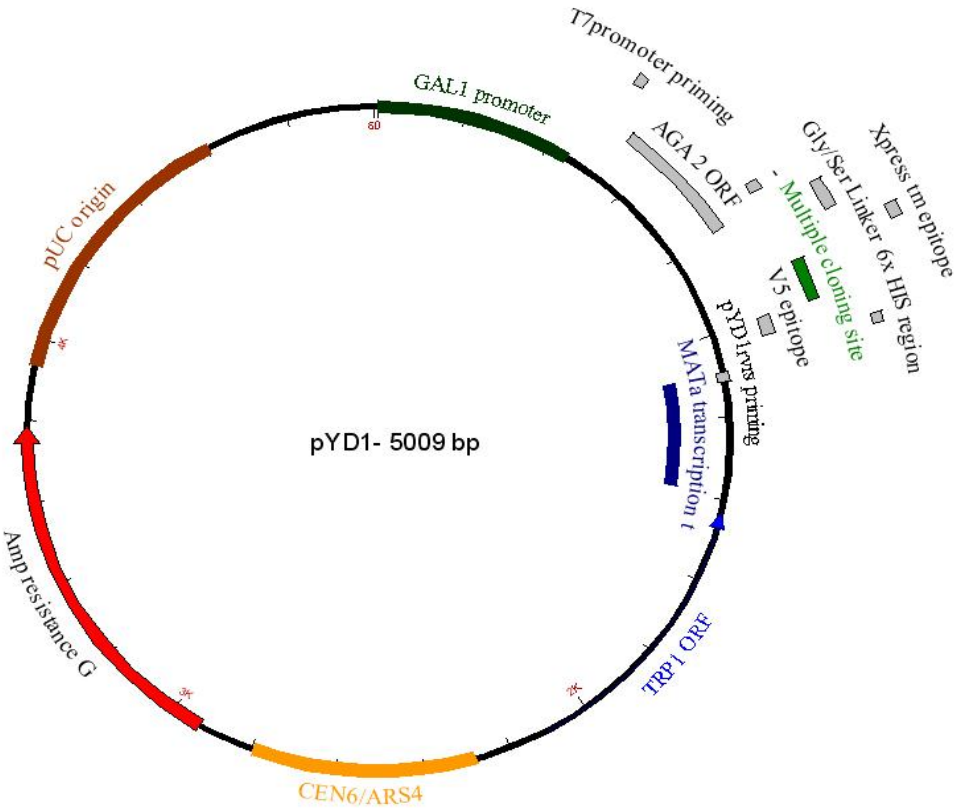


Fig. 2.2. A plasmid map of the pYD1 vector

Features of the pYD1 plasmid components:

GAL1 promoter is one of the most powerful tightly-regulated promoters of *S.cerevisiae* involved in metabolizing galactose. Many genes are involved in its regulation but the important interaction is between the trans-activator encoded by GAL4, the repressor encoded by GAL80, and the GAL UAS [62]. Binding of GAL4 protein to the UAS is critical for induction; GAL80 protein binds GAL4 and acts as a repressor unless galactose is added. The promoters are repressed by glucose and so the maximal induction can only be reached following reduction of glucose. There are

three ways for galactose-inductions: 1) growing the culture on a non-inducing sugar, when very rapid induction follows addition of galactose; 2) growing the culture in glucose medium with the next removing the glucose by centrifugation and washing the cells before re-suspension in galactose medium. This leads to a pause of 3 to 5 h in induction; 3) growing the yeast cells in medium containing both glucose and galactose, when the glucose is preferentially utilized before galactose-induction can take place [62].

- **T7 promoter/priming site** is responsible for *in vitro* transcription in the sense orientation**;**
- **AGA2 gene** from *S.cerevisiae* encodes the N-terminal *s*ubunit of a-agglutinin receptor. The fusion of a protein of interest to the AGA2 permits the display of the protein on the yeast surface;
- **pYD1 Forward priming site** *i*s used for sequencing of insert;
- **Gly-Ser Linker** separates the protein of interest from the Aga2 protein;
- **Xpress™**, **V5** and **Polyhistidine (6xHis tag) epitopes** permit the detection of the displayed protein consequently with the anti- Xpress™, anti-V5 or anti-His (C-term) antibodies.
- **Multiple cloning site** permits insertion of the gene of interest;
- **pYD1 Reverse priming site** allows sequencing of the insert;
- **MATα transcription termination region** allows complete termination and polyadenylation of mRNA;
- **TRP1 gene** permits the selection of yeast transformants;
- **CEN6/ARS4** permits constant episomal replication and yeast budding;

- **Ampicilin resistance gene (β-lactamase)** permits selection in *E. coli*;

- **pUC origin** results in high-copy number replication and propagation of plasmids in *E. Coli*

2.2. Plasmids construction

Two CD154 surface display vectors were constructed. In the first vector, the CD154 gene was inserted between the AGA2 and the V5 epitope/polyhistidine epitope gene, whereas in the second vector, a stop codon was placed at end of the AGA2-CD154 fusion gene, which terminates the translation of the fusion gene. The removal of V5 epitope and $(His)_6$ tag from the Aga2-CD154 fusion protein eliminates any possible interference of these tags with CD154 expression and folding.

The Kpn I and Xho I restricted sites in the pYD1 vector were selected for subcloning of CD154 that carries no stop codon, whereas the Nhe I and EcoR I sites were chosen for subcloning of CD154 that bears a stop codon.

The mouse CD154 gene was amplified by PCR from a CD154 expression plasmid provided by Dr. Jin's lab. The cytoplasmic and transmembrane domain sequences of the CD154 were removed from the CD154 gene during PCR amplification. The truncated CD154 gene was amplified by PCR with Phusion^{TM} polymerase (New England Biolabs Inc.) in the presence of Phusion^{TM} HF buffer (1.5 mM $MgCl_2$) (Table 2.1).

Table 2.1. The composition of the PCR reaction solution for CD154 gene amplification

Component	Volume/ 50 µl reaction	Final concentration
DNase-free Water	Add to 50 µl	
5x Phusion™ HF buffer	10 µl	1x
10 mM dNTPs	1 µl	200 µM each
Forward primer (10 µg/ µl)	2. 5 µl	0.5 µM
Reverse primer (10 µg/ µl)	2.5 µl	0.5 µM
pLC-CD154 plasmid	x µl	10 ng
Phusion™ DNA Polymerase, 2,000 U/ ml	0.5 µl	0.02 U/ µl
Total volume	50 µl	

For construction of the first CD154 surface display vector, the following set of PCR primers was used: **CD154-F1 (forward)** (5'-GTTTAGGTACCACATAGAAGATTGGATAAGGTC-3') and **CD154-R1 (reverse)** (5'– TCATTCCTCGAGGAGTTTGAGTAAGCCAAAAG -3'). The PCR was carried out under the following conditions: initial denaturation at 98^0C for 30 s, denaturation at 98^0C for 10 s, annealing at 51^0C for 25 s and extension at 72^0C for 1 min, repeated for 30 cycles and final extension of 72^0C for 7 min.

To construct the second CD154 display vector, the following PCR primer set was employed: **C-1 (forward)** (5'-CTAGCTAGCCATAGAAGATTGGATAAGG-3') and **C-2 (reverse)** (5'-CCGGAATTCTTAGAGTTTGAGTAAGCCAA -3'). The PCR program was set as follows: initial denaturation at 98^0C for 30 s, denaturation at 98^0C for 10 s, annealing at 55^0C for 25 s and extension at 72^0C for 1 min, repeated for 30 cycles and final extension of 72^0C for 7 min.

A portion of 5 µl of each PCR reaction solution was mixed with 6x gel loading dye, Blue (2.5% Ficolli-400, 11 mM EDTA, 3.3mM Tris-HCl (pH 8.0), 0.017% SDS and 0.015% bromophenol blue) (New England Biolabs Inc., Ipswich, MA) in the ratio 1:5 (v/v), and analyzed by DNA electrophoresis in 0.8 % TAE agarose gel (0.8 g agarose, 100 ml 1x TAE buffer , 0.5 µg/ml ethidium bromide). Positive samples were purified using a PCR purification Kit QIAquick from Qiagen (Valencia, CA) by following a protocol provided by the manufacturer. After PCR DNA cleaning, both the CD154 inserts and pYD1 vectors were double-digested with a pair of Kpn I and Xho I, or a pair of Nhe I and EcoR I enzymes (New England Biolabs Inc., Ipswich, MA) as shown in Table 2.2.

Table 2.2. Enzyme digestion of CD154 inserts and pYD1 vectors.

Component	**PCR product**	**Vector pYD1**
DNase-free water	Add to 20 µl	Add to 20 µl
Buffer, 10x	2.0 µl	2.0 µl
BSA, 100x	0.2 µl	0.2 µl

DNA	Half of the volume of purified PCR product	Volume in equivalent to1 µg pYD1 vector
Enzyme 1, 10,000 U/ml	1 µl	1 µl
Enzyme 2 , 10,000U/ml	1 µl	1 µl
Total volume:	**20 µl**	

After enzyme digestion, the entire enzyme digestion solution (20 µl) was mixed with 4 µl of 6x aforementioned gel loading dye and subject to DNA gel electrophoresis with 0.8% TAE agarose gel (0.8 g agarose, 100 ml 1x TAE buffer, 0.5µg/ ml ethidium bromide,). After electrophoresis, the digested DNA fragments were extracted using a gel extraction kit from Qiagen (Valencia, CA) by following the manufacture's instruction.

To perform the vector dephosphorylation, 20 pmol of enzyme digested pYD1 vectors were mixed with 5 µl of 10x alkaline phosphatase buffer (500 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$) and 1 µl of alkaline phosphatase (30,000 U/ml) provided by Takara Bio Inc (Mountain View, CA). The volume of the reaction mixture was brought to 50 µl with sterilized distilled water and incubated at 37^0C for 30 min, followed by extraction with phenol/chloroform (1:1, v/v) by centrifugation at 13,000 rpm for 5 min. Upper layer of supernatant was carefully removed, and procedure was repeated two more times, but the third time extraction was performed only with chloroform. Supernatant was carefully removed without touching the protein pellet and mixed with sodium acetate (3 M), in an amount equal to 1/10 volume of supernatant; 1/20 volume of glycogen (20 mg/ml) and 2.5 volume of chilled ethanol (100%). Sample was kept at -80^0C

overnight. Next day mixture was centrifuged for 15 min at 13,000 rpm. DNA pellets were rinsed with -20^{0}C cold ethanol (70%) and air-dried, after what it was dissolved in 20 µl of diethylpyrocarbonate (DEPC) treated water.

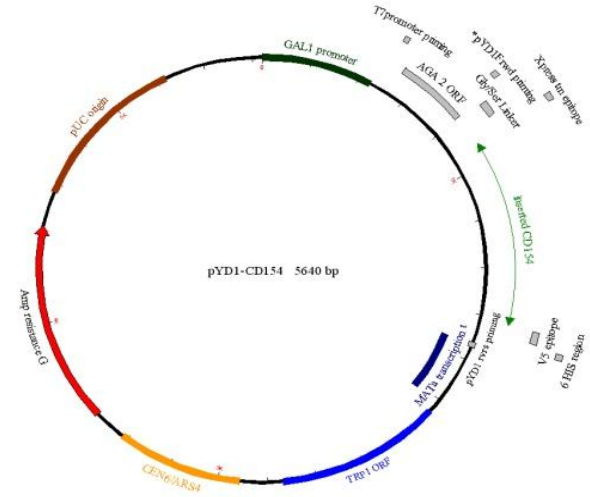
The purified CD154 amplicons were sub-cloned into the dephosphorylated pYD1 vector in the presence of T4 ligase buffer 5x (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP) provided with the T4 DNA Ligase Kit (Fermentas Inc., Glen Burnie, MD). Ligation was allowed to occur with T4 DNA ligase for 20 min at room temperature. The volume ratios of components are presented in Table 2.3.

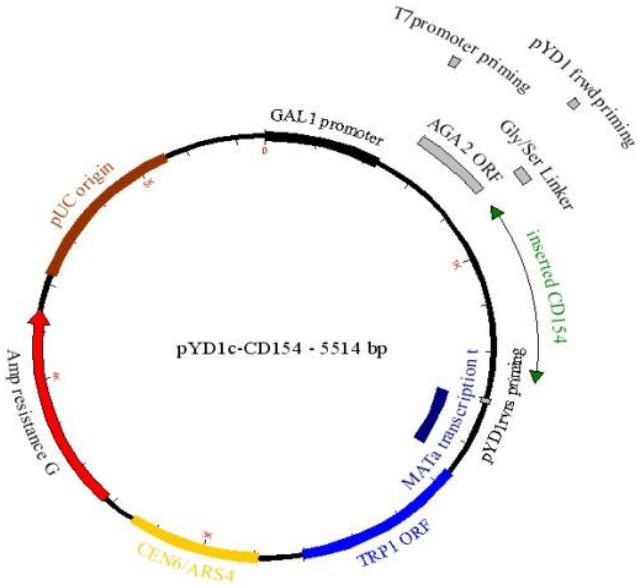
Table 2.3. Volumes of components used for ligation reaction with T4 DNA ligase

Component	**Volume**
Sterilized nuclease free water	9 µl
T4 ligase buffer, 5x	4 µl
Vector	1 µl
CD154 Insert	5 µl
T4 ligase, 5,000 U/ml	1 µl
Total volume	**20 µl**

The CD154 inserts with or without a stop codon were sub-cloned into pYD1 vector and the resultant expression plasmids were referred to as pYD1c-CD154 (with a stop codon) and pYD1-CD154 (without a stop codon), as shown in **Fig. 2.3**.

A)





B)

Fig. 2.3. Plasmid maps of CD154 display vectors. The pYD1-CD154 (A) encodes the fusion protein Aga2-CD154-X-express-V5-$(His)_6$, whereas the pYD1c-CD154 (B) encodes the fusion protein Aga2-CD154, where the translation of the fusion protein is terminated due to the induction of a stop codon at of the end of the AGA2-CD154 fusion gene.

We also constructed a GFP surface display vector as a positive control for the study. the enhanced green fluorescence protein encoding (EGFP) gene was amplified from pEGFP plasmid (Clontech, Mountain View, CA) using Phusion^{TM} polymerase (New England Biolabs Inc, Ipswich MA) in the presence of Phusion^{TM} HF buffer (1.5 mM $MgCl_2$), and primers containing Nhe I and EcoR 1 restriction enzymes sites as follows: **E-1** (5'-CTAGCTAGCGTGAGCAAGGGCGAGGAGCT-3') and **E-2** (5'-CCGGAATTCTTA CTTGTACAGCTCGTCCAT-3'). The PCR reaction solution was prepared as listed in **Table 2.1**. The PCR program was set as follows: initial denaturation at 98^0C for 30 s, denaturation at 98^0C for 10 s, annealing at 55^0C for 25 s and extension at 72^0C for 1 min, repeated for 30 cycles and final extension of 72^0C for 7 min. A portion of 5 µl of EGFP amplicons was mixed with 1 µl of 6x Gel loading dye, Blue (2.5% Ficolli-400, 11 mM EDTA, 3.3mM Tris-HCl (pH 8.0), 0.017% SDS and 0.015% bromophenol blue) (New England Biolabs Inc., Ipswich, MA), and detected through DNA electrophoresis using 0.8% TAE agarose gel (0.8g agarose, 1x TAE buffer, 5 µg/ml ethidium bromide). The rest of the PCR products was purified using a PCR purification kit from Qiagen (Valencia, CA) by following the manufacturer's instruction. The PCR amplified EGFP inserts and the pYD1 vectors were digested using both Nhe I and EcoR I enzymes in the presence of Buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1mM dithiothreitol) and BSA Buffer (20 mM KPO_4, 50 mM NaCl, 0.1 mM EDTA, 5% glycerol) provided by New England Biolabs Inc (Ipswich, MA), as listed in **Table 2.2**. The enzyme reaction was performed at 37 ^{0}C for 1 h. Completely digested DNA fragments in the volume of

20 µl were mixed with 4 µl of 6x gel loading dye (Blue (2.5% Ficolli-400, 11 mM EDTA, 3.3mM Tris-HCl (pH 8.0)) and subjected to DNA gel electrophoresis with 0.8 % TAE agarose gel (0.8 g agarose, 1x TAE, 5µg/ml ethidium bromide). After electrophoresis, the DNA fragments were extracted using an aforementioned gel extraction kit. The enzyme digested EGFP inserts and pYD1 vectors were ligated in the presence of T4 ligase buffer 5x (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP) and T4 DNA ligase (5,000 U/ml) provided by the T4 DNA Ligase Kit (Fermentas Inc., Glen Burnie, MD), as listed in **Table 2.3**. Ligation was carried out at room temperature for 20 min. The resultant plasmid was referred to as pYD1c-EGFP (where "c" refers to "cut") (**Fig. 2.4**) where a stop codon was placed right after the EGFP gene in order to terminate the translation of the fusion protein AGA2-EGFP.

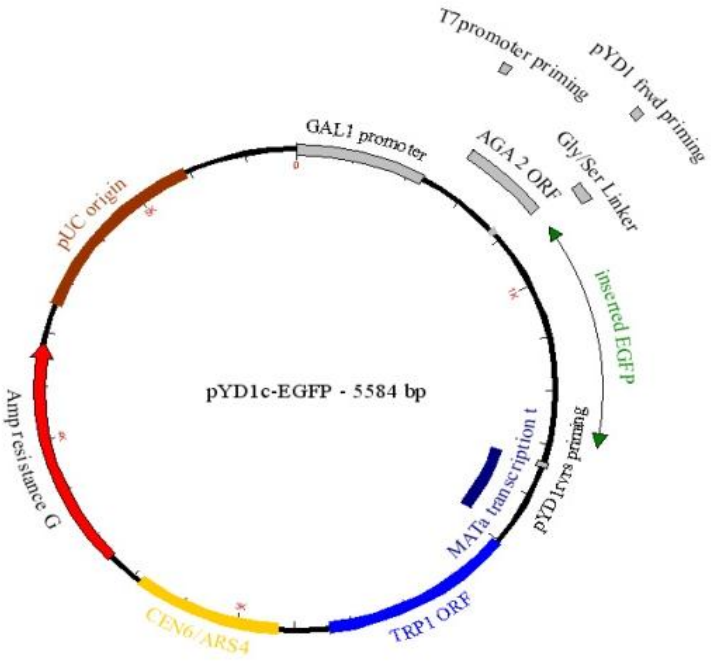


Fig. 2.4. Plasmid map of constructed pYD1c-EGFP vector, **encoding the fusion protein Aga2-EGFP without V5 and Polyhistidine regions.**

2.3. *E. coli* transformation

E. coli DH-5α (New England Biolabs Inc., Ipswich, MA) was chosen as a host strain for all of the plasmid amplifications described above. This strain contains the following components, making it suitable for recombinant DNA sub-cloning:

- The **endA1 mutation** results in inactivation of intracellular endonuclease and consequently enhancement of DNA plasmid propagation;
- The **hsdR17 mutation** deactivates the restriction endonuclease of the EcoKI restriction-modification system, and so DNA deficient in the EcoKI methylation will not be degraded;
- **Δ*(lacZ)*M15** is responsible for blue-white screening needed for many lacZ based vectors;
- **recA** reduces homologous recombination. This might results in some weakness of the strain, but at the same time it decreases deletion formation and multimerization of plasmid.

pYD1c-EGFP, pYD1-CD154, pYD1c-CD154, and pYD1 vectors were transformed into *E. coli* DH-5α chemically-competent cells through "heat shock". Here is the procedure for heat shock. Firstly, tubes with the competent cells were kept on ice until thawing, followed by addition of 7 µl of ligation mixture to each tube with careful flicking of to mix cells and DNA briefly. Tubes were placed on ice for 30 min, followed by heat shock at 42^0C for exactly 30 s. Tubes were placed on ice immediately and incubated for another 5 min, followed by addition of 950 µl of room temperature SOC medium (0.5% Yeast extract, 2% Tryptone , 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 4% glucose) provided along with the competent cells by New England Biolabs Inc (Ipswich, MA). Afterwards, tubes were transferred to an environment

incubator and incubated at 37^0C for 1 h with a vigorous shaking at 150 rpm. Cells were centrifuged at 13,000 rpm for 1 min. Collected cell pellet were re-suspended in 50 µl of room temperature SOC medium and spread on the LB-plates (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, 1.5 % agar) containing ampicillin (100 µg/ml) and cultivated at 37^0C overnight. Colonies were picked from each plate next day and re-suspended in 10 µl of sterile water. Only 1 µl of each suspension was used for PCR, leaving the rest 9 µl for overnight cultivating in the case of positive results. PCR was performed with 0.2 µl of Taq DNA Polymerase (5,000 U/ml) supplemented with 2.5 µl of 5x Standard Taq Polymerase Buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3) (New England Biolabs Inc., Ipswich, MA), 2.0 µl of 10 mM dNTPs (Agilent Technologies, Inc., Santa Clara, CA) and appropriate primers: CD154-F1, CD154-R1; C-1, C-2, and E-1, E-2 (see Section 2.1-2.2) in the reaction volume of 20 µl. The PCR program consisted of initial denaturation at 94^0C for 5 min, denaturation at 94^0C for 30 s, annealing at 55^0C for 30 s and extension at 68^0C for 1 min, repeated for 25 cycles and final extension of 68^0C for 7 min. A portion of 5 µl of each PCR product, mixed with 1 µl of 6x gel loading dye (Blue (2.5% Ficolli-400, 11 mM EDTA, 3.3 mM Tris-HCl (pH 8.0)) were subject to DNA electrophoresis using 0.8 % TAE agarose gel (0.8 g agarose, 100 ml 1xTAE buffer, 5µg/ml Ethidium Bromide)and analyzed on the presence of a band with a correct size using 1 kb DNA Ladder (NEB Biolabs Inc., Ipswich, MA) as a marker.

Positive colonies were cultivated in 5 ml of LB Broth (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, pH 7.0) containing ampicillin (100 µg/ml) overnight at 37^0C with shaking at 250 rpm. Glycerol stocks of all positive clones were prepared next day: 850 µl of each *E. coli* culture were

carefully mixed with 150 µl of sterile glycerol (100%) in 1.5 ml tube. All tubes are stored at -80^0C.

Plasmids were extracted from overnight grown *E. coli* transformants using a Plasmid Miniprep Kit (Promega, Madison, WI) by following the manufacturer's instruction. A portion of 1 µg of each isolated plasmid was digested with Xho I and Kpn I restriction enzymes (NEB Biolabs Inc., Ipswich, MA) in order to confirm the successful subcloning of pYD1-CD154 or Nhe I and EcoR I enzymes (NEB Biolabs Inc., Ipswich, MA) for the confirmation of successful subcloning of pYD1c-CD154 and pYD1c-EGFP, as listed in **Table 2.2**. The presence of the inserts in the extracted plasmids was confirmed by DNA electrophoresis in 0.8 % TAE agarose gel (0.8 g agarose, 1x TAE buffer, 5 µg/ml ethidium bromide) using 1 kb DNA ladder (New England Biolabs Inc., Ipswich, MA) as a DNA marker.Positive pYD1-CD154, pYD1c-CD154, and pYD1c-EGFP plasmids were sequenced with the sequencing primers pYD1-Forward (5'-AGTAACGTTTGTCAGTAATTGC-3') and pYD1-Reverse (5'-GTCGATTTTGTTACATCTACAC-3') in the DNA Sequencing Center (University of Arkansas, Fayetteville, AR) and confirmed by analysis with Gene 1.5 Software.

2.4. Yeast transformation

After confirmation of positive sub-cloning, the CD154 and EGFP yeast surface display vectors were electroporated into *S.cerevisiae* EBY100 (Invitrogen, Carlsbad, CA). EBY100 was created by assimilating the vector pIU211 into the AGA1 locus of the *S.cerevisiae* strain BJ5465. Plasmid pIU211 contains the AGA1 gene regulated by the GAL1 promoter and an URA3

selection marker. In order to integrate the AGA1 gene into the yeast chromosome, the vector was linearized using the BsiW1 restriction enzyme. We chose electroporation to transform the DNA vectors into yeast.

2.4.1. Preparation of competent cells for electroporation

Yeast electroporation competent cells were prepared as follows:

Firstly, the glycerol stock of EBY100 was streaked and grown on a minimal dextrose plate (0.67% YNB with ammonium sulfate, without amino acids; 2% glucose,0.01% leucine,0.01% tryptophan, 1.5% agar). The plate was incubated at 30^0C until colonies appeared (2-3 days).

Subsequently, two days before the actual experiment the single yeast colony was inoculated with 5 ml of autoclaved YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30^0C with shaking at 250 rpm. After 12 h, a portion of 4 ml of cell cultures was added to the fresh YPD medium with the final volume of 100 ml, and cultivated at the same conditions until cell density reached $1x10^8$ cells/ml (OD_{600} = 3.0-3.5). Then cells were chilled in an ice-water bath for 15 min to stop cell growth. A portion of 100 ml of the yeast culture were transferred into two bottles – 50 ml each, and centrifuged at 7,200 rpm for 5 min at 4^0C. Cell pellets were re-suspended with 16 ml of sterile water, 2 ml of 10x TE buffer (1M Tris-Cl, pH 7.5, 0.5M EDTA pH 8.0) and 2 ml of Lithium acetate (1M) for the cell wall permeabilization. Cells were swirled and carefully mixed before adding every reagent.

The yeast culture was incubated at 30^0C with shaking at 85 rpm. After 15 min of incubation 0.5 ml of DTT (1M) were added, cells were swirled, mixed and kept for incubation at 30^0C for additional 15 min with shaking at 85 rpm. After incubation the volume of each cell pellet was

brought to 50 ml with the sterile water and centrifuged at 7,200 for 5 min at 4^0C. Supernatant was discarded and each cell pellet was re-suspended with 10 ml of ice-cold water by vortex. Then volume was brought to 50 ml with sterile, ice-cold water and cells were washed again by centrifugation at 7,200 rpm for 5 min at 4^0C. Supernatant was removed; cell pellet was re-suspended in 4 ml of sterile, ice-cold sorbitol (1 M) and transferred to a chilled tube. Cells were pelleted by centrifugation at 7200 rpm at 4^0C for 10 min; supernatant was discarded. On the last step cells were re-suspended in 0.1 ml of sterile, ice-cold sorbitol (1 M) and kept on ice until electroporation was performed.

Competent cells that were not used for electroporation were kept at -80 ^{0}C for long storage by adding glycerol to 15% (v/v). The transformation efficiency, however, was expected to drop more than 10-fold with freezing.

2.4.2. Electroporation

A portion of 50 µl of freshly-prepared yeast competent cells were diluted with ice-cold sorbitol (1 M) in the ratio 1:100 (v/v). A portion of 5 µl of DNA (with the concentration of 100 ng) was mixed with 80 µl of diluted yeast competent cells, transferred into tubes and incubated on ice for 5 min. Four tubes containing competent cells mixed with plasmids pYD1, pYD1-CD154, pYD1c-CD154, and pYD1c-EGFP were prepared. The fifth tube contained only competent cells, served as a control for checking the selective marker – tryptophan. Samples were transferred to chilled 0.4 cm electroporation cuvettes (Bio-Rad Laboratories, Inc., Hercules, CA), and each suspension was tapped to the bottom. Cuvette was placed in the ShockPod of Bio-Rad Gene

Pulser Xcell Electroporation System (Bio-Rad Laboratories, Inc., Hercules, CA), where electroporation was performed at such conditions: C= 25 μF; PC = 200 ohm; V = 2.5 kV, t = 5 msec. Cuvettes were removed from the chamber, followed by addition of 1 ml of ice cold sorbitol (1 M) to the chamber immediately. The diluted cells were gently transferred into a sterile 17 x 100 mm tube, and 150 μl of competent cells were plated on the selective plates (0.67% YNB with ammonium sulfate, without amino acids; 2% glucose, 0.01% leucine, 1.5% agar). Plates were incubated at 30^0C until colonies appeared (2-3 days).

2.5. Verification of transformed plasmids.

To confirm the positive transformed colonies formed after DNA electroporation, plasmids were extracted from overnight culture of: single colony picked from the selection plates in containing YNB-CAA medium (0.67% YNB, 0.5% Casamino acids, 0.01% leucine) at 30^0C with shaking at 250 rpm overnight. After OD_{600} reached 0.2-0.6, the cell pellets were collected and subject to plasmid extraction using Zymoprep™ II-Yeast Plasmid Miniprep (Zymo Research, Irvine CA) by following the manufacturers' instruction. Each of the extracted plasmids was amplified by PCR in the reaction volume of 20 μl with the 1.25 μl of 10 mM pYD1 sequenced primers: pYD1-Forward (5'-AGTAACGTTTGTCAGTAATTGC-3') and pYD1-Reverse (5'-GTCGATTTTGTTACATCTACAC-3') using 0.2 μl of Taq DNA Polymerase (5,000 U/ml) in the presence of 1.25 μl of 5x Standard Taq Polymerase Buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3) provided by New England Biolabs Inc. (Ipswich, MA) and 2.0 μl of 10mM dNTPs (Agilent Technologies, Inc., Santa Clara, CA). The PCR program was set as follows: initial denaturation at 94^0C for 5 min, denaturation at 94^0C for 30 s, annealing at 55^0C for 30 s and

extension at 68^0C for 1 min, repeated for 25 cycles and final extension of 68^0C for 7 min. Amplified products were mixed with 6x gel loading dye (Blue (2.5% Ficolli-400, 11 mM EDTA, 3.3mM Tris-HCl (pH 8.0), 0.017% SDS and 0.015% bromophenol blue)) in a ratio 1:5 (v/v), and analyzed by DNA electrophoresis in 1.3 % TAE agarose gel (1.3 g agarose, 100 ml 1xTAE buffer, 5µg/ml Ethidium Bromide), using 100 bp DNA ladder (New England Biolabs Inc., Ipswich, MA) as a DNA marker. Positive plasmids identified in above procedures were subject to DNA sequencing to determine whether there were any mutation introduced during PCR and sub-cloning.

2.6. Protein expression

To display the recombinant proteins on yeast surface, a picked single colony was inoculated into 10 ml of Yeast Nitrogen Base - Casamino Acids (YNB-CAA) medium (0.67% YNB with ammonium sulfate, without amino acids; 0.5% Casamino acids, 2% glucose) and grown at 30^0C with shaking at 250 rpm overnight. Samples were taken after 16 h of cultivation in order to determine the cell density at 600 nm. The expected value of OD_{600} should be in the range between 2 and 5. In general, value less than 2 will decrease the number of cells displaying the proteins, while value greater that 5 is expected to postpone the induction of protein expression. To completely remove glucose from the cell culture medium in order to induce the expression of proteins using galactose, cells were collected with centrifugation 7,200 rpm for 5 min at room temperature after OD_{600} reached between 2 and 5. Cell pellets were then re-suspended in YNB-CAA medium containing galactose (0.67% YNB with ammonium sulfate, without amino acids; 0.5% Casamino acids, 2% galactose) to an OD_{600} of 0.5to ensure the continuous growth of the

cells in the log-phase. On the other hand, galactose (2%) was directly added to the cell culture medium without complete removal of glucose from the cell culture medium when OD_{600} reached between 2 and 5. After switching to a galactose induction medium, recombinant yeasts were incubated at 20^0C with shaking at 250 rpm. Immediately after switching to the galactose medium two samples of 1 OD_{600} unit were taken as zero time point samples. These samples were centrifuged at 7,200 rpm at 4^0C for 5 min and kept at 4^0C (for immunofluorescence assay) and -20^0C (for western blot assay). Subsequently, samples were taken at 24, 50 and 72 h post induction to determine the optimal induction time for protein surface display. For each time point, volume of cells that is equivalent to 1 OD_{600} unit was collected, centrifuged at 7,200 rpm at 4^0C for 5 min and kept either at -20^0C until use or at 4^0C for no longer than two weeks.

2.7. Immunofluorescence microscopy

An immunofluorescence microscopy was performed in order to detect yeast surface displayed proteins. In brief, samples collected at different each time point were centrifuged and stored at 4^0C as mentioned above. After re-suspension in 1x ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) and washing three times by centrifugation at 5,000 *x* g for 5 min at 4^0C with BSA/PBS (1% BSA, 1xPBS) , the cells were re-suspended in 500 µl of goat anti-CD154 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-1594) diluted with BSA/PBS (1% BSA, 1xPBS) at the ratio of 1:500 (v/v). Samples with the primary antibodies were incubated at 4^0C with occasional shaking overnight and then were centrifuged at 5,000 *x* g for 5 min at 4^0C, followed by washing three times with 1x ice-cold PBS as described above. Subsequently, cells pellets were re-suspended in 500 µl of donkey anti-goat

IgG FITC conjugated antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-2024) diluted with BSA/PBS (1% BSA, 1x PBS) at a ratio of 1:2000 (v/v) and incubated for 1 h at room temperature with occasional shaking in the dark (tubes were wrapped in the foil). Afterwards, cells were washed three times with 1xPBS by centrifugation at 7,200 rpm for 5 min at 4^0C. Cells were re-suspended in 40 µl 1x PBS after the last washing. A portion of 5 µl of each sample were spotted onto a slide and covered with cover slip for detection of surface displayed proteins under an inverted phase contrast fluorescence microscope Olympus 1x70 (MVI, Avon, MA) equipped with a highly sensitive CCD camera (Qimaging, 32-0139-104) and Slidebook imaging analysis software 4.2.version (Olympus Imaging America Inc., Center Valley, PA).

2.8. Reverse-transcription PCR (RT-PCR)

RT-PCR was carried out to determine the expression of recombinant proteins in yeast. Recombinant yeasts EBY100/pYD1, served as negative control, and EBY100/pYD1-CD154 were cultivated in 10 ml YNB-CAA medium (0.67% YNB with ammonium sulfate, without amino acids; 0.5% CAA, 2% glucose) at 30^0C, 250 rpm shaking, until the OD_{600} reached 4 (about 14 h). Cell pellets were collected by centrifugation at 7,200 rpm for 5 min at room temperature. The cell pellets were re-suspended in YNB-CAA medium (0.67% YNB with ammonium sulfate, without amino acids 0.5% CAA, 2% galactose) to an OD_{600} of 0.8 to ensure that the cells continue to grow in log-phase. Cells were then incubated at 25^0C, 250 rpm shaking. Samples of 5 OD_{600} units were collected at 48 and 70 h post induction, respectively. The total RNAs were extracted using YeaStar^{TM} RNA Kit (Zymo Research, Irvine, CA) in a

designated RNase free area by following manufacturer's instruction. All microcentrifuge tubes and pipette tips were DNase and RNase free.

In order to eliminate genomic DNA contamination in these RNA samples, samples containing 1 µg of RNA were mixed with 1 µl of RQ_1 RNase-Free DNase (10,000 U/ml) along with 1 µl of RQ_1 RNase-Free DNase 10 x buffer (400mM Tris-HCl (pH 8.0), 100 mM $MgSO_4$ and 10 mM $CaCl_2$). The final volume of the mixture was brought to 10 µl with nuclease-free water. The mixture solutions were incubated at 37^0C for 30 min, followed by the addition of 1 µl of RQ_1 RNase-Free DNase stop solution (20 mM EGTA (pH 8.0)) to the mixture. The mixtures were then inactivated at 65^0C for 10 min to stop the reaction. Reagents for genomic DNA removal were all acquired from Promega (Madison, WI). First Strand cDNA Synthesis was performed by using Protoscript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs Inc., Ipswich, MA) as instructed by the manufacturer. PCR was performed with Phusion ™ DNA Polymerase (New England Biolabs Inc., Ipswich, MA) in the presence of Phusion HF buffer (1.5 mM $MgCl_2$), using the following forward and reverse primers that contain KpnI and XhoI restriction sites: CD154-F1 (5'-GTTTAGGTACCACATAGAAGATTGGATAAGGTC-3') and CD154-R1 (5'– TCATTCCTCGAGGAGTTTGAGTAAGCCAAAAG -3'). The PCR program was set as: initial denaturation at 98^0C for 30 s, denaturation at 98^0C for 10 s, annealing at 51^0C for 25 s and extension at 72^0C for 1 min, repeated for 30 cycles and final extension of 72^0C for 7 min. PCR products were analyzed through DNA gel electrophoresis on 0.8 % TAE agarose gel (0.8g agarose, 100 ml TAE, 5 µg/ml ethidium bromide) using 1kb DNA ladder (New England Biolabs Inc., Ipswich, MA) as a marker.

2.9. Western blotting

1 OD_{600} equivalent samples collected at different time points (24, 50 and 72 h post induction) were washed once with 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) by centrifugation at 7,200 rpm for 5 min at 4^0C. Washed samples were diluted with 20 µl of Laemmli loading buffer (Bio-Rad Laboratories, Inc., Hercules, CA), containing 5% of β-mercaptoethanol, re-suspended and heated at 100^0C for 10 min. After heating the samples were centrifuged at 15,000 rpm for 10 min. The supernatants were carefully removed from the cell pellets and loaded onto a 12% polyacrylamide Tris-glycine gel wells (Bio-Rad Laboratories, Inc., Hercules, CA) with special gel loading tips. Electrophoresis was performed in the TGS running buffer (25 mM Tris, 192 mM Glycine, 0.1% w/v SDS, pH 8.3) (Bio-Rad Laboratories, Inc., Hercules, CA) at 200 V for 35 min. The Precision Plus Protein WesternC Standards was used as a marker (Bio-Rad Laboratories, Inc., Hercules, CA).

After electrophoresis, gel was carefully removed from the cassette. A "sandwich" blot assembly was prepared by packing components in the following order: sponge, filter paper (Bio-Rad Laboratories, Inc., Hercules, CA), gel, 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA), filter paper, and sponge. Membrane was pre-wet in transferring buffer (Tris, 192 mM Glycine, 0.1% w/v SDS, 20% v/v methanol, pH 8.3) at room temperature for 10 min. Each layer was pre-wet with transferring buffer, and all the bubbles between gel and membrane layers were carefully removed by pipette. The "sandwich" assembly was placed into a transferring tray, filled with an ice-cold transferring buffer. The tray was placed into a box filled with ice-cold water and transferred in an ice-cold transferring buffer at 150 V for 1 h. Membrane

was carefully removed from the cassette with tweezers and incubated in a blocking buffer (5% non-fat dry milk, 1x PBS, 0.05 % Tween-20) for 2 h with shaking at room temperature. After blocking the membrane was incubated with aforementioned goat anti- CD154 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-1594) diluted in non-fat dry milk/PBST (5% non-fat dry milk, 1x PBS, 0.05 % Tween-20) at a ratio of 1: 200 (v/v) at 4^0C with shaking overnight. The membrane was then washed three times with PBST (1x PBS, 0.05 % Tween-20), 5 min each time, and incubated with donkey anti-goat IgG (Fab specific) horse peroxidase (HRP) conjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-2020) diluted at a ratio of 1:2000 (v/v) in non-fat dry milk/PBST (5% non-fat dry milk, 1x PBS, 0.05 % Tween-20). For detection of the marker bands was included 1µl of Precision Protein™ StrepTactin-HRP conjugate (Bio-Rad Laboratories, Inc., Hercules, CA). After shaking at room temperature for one hour, the membrane was washed three times with PBST, 5 min each. Subsequently, equal portions of the enhancer and stable peroxidase solutions (Thermo Fisher Scientific Inc., Rockford, IL) were mixed and carefully loaded onto the membrane and incubated in a dark for 5 min. The membrane was imaged using Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories, Inc., Hercules, CA) and analyzed using PDquest Analysis software (Bio-Rad Laboratories, Inc., Hercules, CA).

2.10. Deglycosylation assay

2 OD_{600} equivalent Cell samples were collected and centrifuged at 7,200 rpm at 4^0C for 5 min, followed by two washes with 1 ml of 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4). Cell pellets were then mixed with 1 µl of 10 x glycoprotein

denaturating buffer (5% SDS, 0.4 M DTT) and 3 µl of sterilized water in order to make a 10 µl total reaction volume. Glycoproteins were denaturated by heating at 100^0C for 10 min. The reaction volume was adjusted to 20 µl by mixing 2µl of 10 x G7 reaction buffer (0.5 M Sodium Phosphate), 2 µl NP-40 (10%), 4 µl of sterilized water and 2 µl of PNGaseF (500,000 U/ml) and incubated at 37^0C for 1 h. All reaction components were acquired from the New England Biolabs Inc. (Ipswich, MA). Deglycosylated samples were detected through Western blot as described above.

2.11. Flow cytometric analysis

Samples of recombinant yeasts EBY100/pYD1, EBY100/pYD1-CD154, pYD1c-CD154 were prepared as described above. Yeasts harboring pYD1 plasmid served as a negative control.

Cell samples collected at different time points were centrifuged and stored at 4^0C as mentioned above. After re-suspension in 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) and washing three times by centrifugation at 7,200 rpm for 5 min at 4^0C with BSA/PBS (1% BSA, 1xPBS), the cell pellets were re-suspended in 500 µl of goat anti-CD154 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-1594) diluted with BSA/PBS (1% BSA, 1xPBS) at a ratio of 1:500 (v/v). Samples with the primary antibodies were incubated at 4^0C overnight with occasional shaking, followed by centrifugation at 7,200 rpm at 4^0C for 5 min. After three washes with 1x PBS, cells were re-suspended in 500 µl of donkey anti-goat IgG FITC conjugated antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-2024) diluted with BSA/PBS (1% BSA, 1x PBS) at a ratio of 1:2000 (v/v) and incubated at room temperature with occasional shaking in the dark (tubes were wrapped in the foil) for 1 h.

Afterwards, cells were washed three times with 1x PBS by centrifugation at 7,200 rpm at 4^0C for 5 min. Cells were then diluted with 300 µl of 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) and subject to flow cytometric analysis by BD FACSCanto II, using FACSDiva software .

Chapter III. RESULTS AND DISCUSSION

3.1. Display of EGFP on yeast surface

EGFP is a small protein (238 amino acids) that radiates bright green fluorescence when excited at 475 nm. The EGFP is very useful in DNA subcloning, as it is easy to be subcloned and also less toxic to cells. Based on these characteristics, we decided to use it as a positive control for testing the protein surface display vector used in this study. The EGFP yeast surface display vector, pYD1c-EGFP was constructed and transformed into yeast EBY100, as described above.

The expression of EGFP is controlled tightly by the GAL1 promoter that requires the activation with galactose. As galactose is not the best carbon source for yeast to grow, we used glucose instead of galactose before induction so yeast can grow much fast. Once yeast growth entered an exponential growth phase (Fig. 3.1.), we switched the carbon source in the cell culture medium from glucose to galactose in order to induce the expression of EGFP.

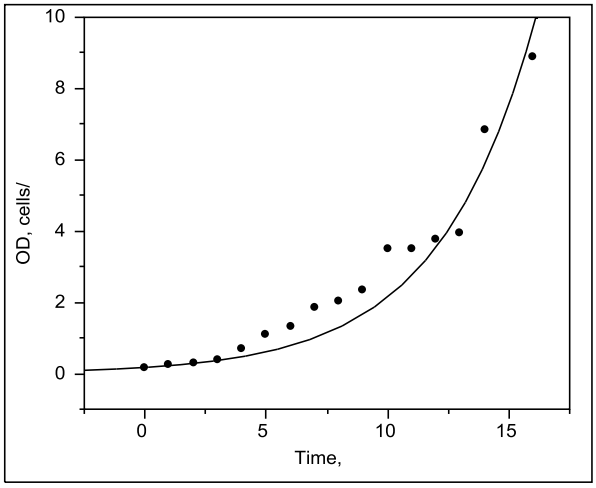


Fig. 3.1. The growth curve of yeast S.cerevisiae harboring pYD1c-EGFP plasmid. Cells were grown in YNB-CAA medium containing 2% glucose. The yeast growth was observed over 16 hour culture period.

Here two strategies were tested. One is to completely remove glucose from the cell culture medium using centrifugation right before the galactose induction; whereas the other strategy is to add the galactose directly to cell culture for induction by assuming the depletion of glucose in the cell culture medium at the time when the galactose is added to the medium. As yeast could suffer from stress caused by centrifugation, the addition of galactose to the cell culture medium without centrifugation could potentially prevent cells from damaging caused by centrifugation. Now, the question is whether the glucose has been completely consumed before induction or whether trace amount of glucose in the cell culture medium affects the expression of EGFP when induced with galactose. On the hand, the initial glucose concentration in the cell culture medium can be controlled to insure the exhaust of glucose before galactose is added to the cell culture medium for induction. We tested both strategies. 1 OD_{600} equivalent samples were taken before and after galactose induction. The samples taken right before the galactose induction were referred to as zero time samples. Cells were collected at 24 and 50 h post induction to determine when the EGFP expression levels after galactose induction. Recombinant EBY100/pYD1 served as a negative control for these assays. The surface displayed EGFP was determined by detecting the green fluorescence emitted from the yeast surface through fluorescence microscopy. As shown in Fig. 3.2, the display of EGFP on yeast surface can be observed as early as at 24 h post induction Figs. 3.2A and C and the expression level of EGFP reached much higher at 50 h post induction (Figs. 3.2B and D). It seemed that the centrifugation is not necessary before galactose induction. The EGFP was expressed when galactose was added directly to the cell culture medium (Fig. 3.2C). This might be due in part to the exhaust of glucose in the cell culture medium, although we did not measure glucose concentration before galactose was added to the cell culture medium. Compared to the method 1 where centrifugation was carried out before addition of

galactose, the expression of EGFP was much higher when the galactose was added to the cell culture without centrifuging the cells (Fig. 3.2D). The reason that EGFP was expressed much higher when centrifugation was not applied. It has been well known that cells suffer from stress during centrifugation. Based on these experimental results, we decided that no centrifugation is necessary for inducing the expression of a protein in the cell culture system that we used for displaying the protein on the surface of yeast.

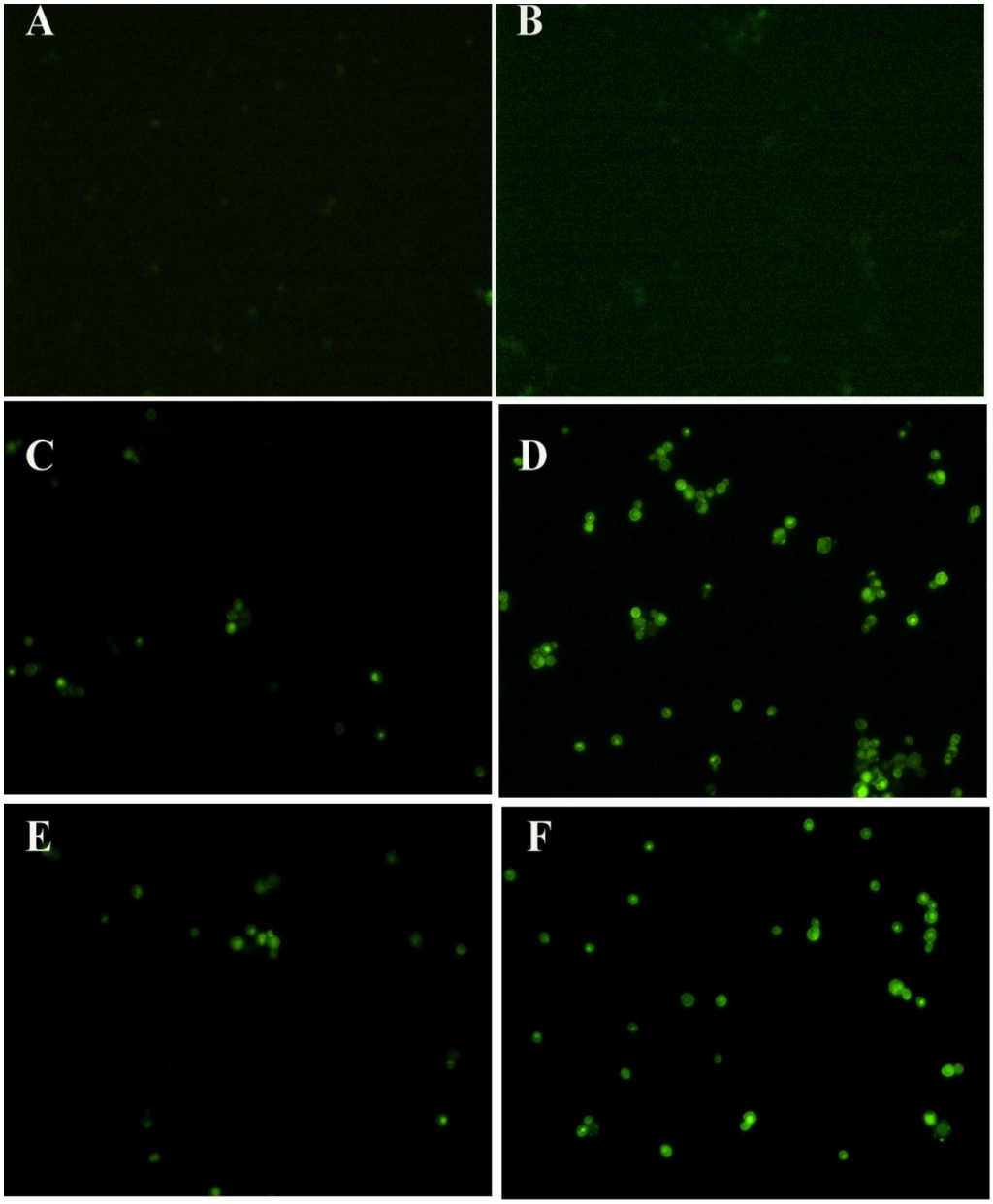


Fig. 3.2. Display of EGFP on the yeast surface. The expression of EGFP was induced by galactose (2%) with complete removal of glucose (C) and (E) or without removal of glucose (D) and (F) from the medium before galastose was added to the medium. Samples were taken at 0 h (A)-(B), 24 h (C)-(D) and 50 h (E)-(D) post induction. 1 OD_{600} equivalent samples were taken. The cells were transferred to a cover slip and examined under a phase contrast fluorescence microscope with an 400 x objective.

3.2. Display of CD154 on yeast surface

To display a CD154 on yeast surface, we inserted the CD154 gene between the Aga2 and the V5 gene. The tagging of CD154 with an V5 epitope and $(His)_6$ allows for detecting the surface displayed CD154 with antibodies against the V5 epitope or the $(His)_6$ tag.

We also constructed another CD154 display vector by placing a stop codon just before the V5 epitope, leading to the display of CD154 without bearing any tag, avoiding any structural interference from these tags during protein expression and maturation.

The murine CD154 has a transmembrane domain at its C-terminus. This transmembrane domain was deleted from the CD154 protein during subcloning in order to avoid its possible adverse effect on CD154 surface display.

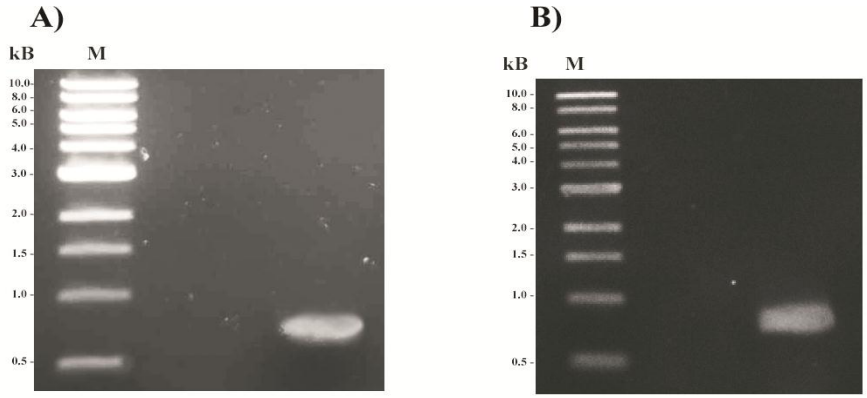


Fig. 3.3. PCR products of amplified CD154 fragments using different pairs of PCR primers. (A), CD154 fragment with V5 and $(His)_6$. (B), CD154 fragment without V5 and $(His)_6$ tag. The PCR products were analyzed using 0.8 % agrose gel.

The PCR products were digested with the correspond restriction enzymes, and sub-cloned into pYD1. Ligated plasmids were transformed to chemically-prepared competent *E. coli* ΔH5α. Several transformants were picked from each plate and grown overnight for validation. Figs. 3.4 and 3.5 exhibit the validation results. The pair of enzymes Kpn I and Xho I was used to validate pYD1-CD154, whereas the pair of enzymes Nhe I and EcoR I was used to validate pYD1c-CD154. As shown in Fig. 3.4 two bands, one 4974 bp (backbone of the vector) and the other at 665 bp (the CD154 insert) were observed after enzyme digestion of pYD1c-CD154 plasmid. Similarly, two bands, one at 4975 bp (the backbone of the vector) and the other at 664 bp were observed for pYD1-CD154 (Fig.3.5).

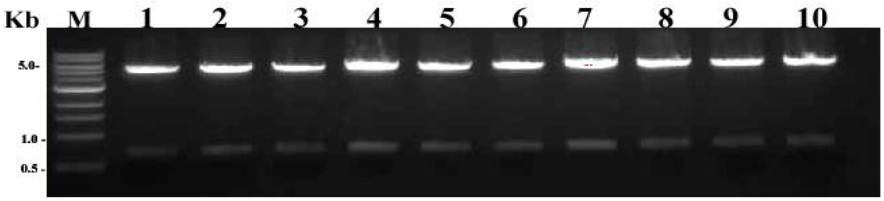


Fig. 3.4. Validation of pYD1c-CD154 through enzyme digestion. The pYD1c-CD154 was digested with Nhe I and EcoR 1 restriction enzymes and analyzed through electrophoresis using 0.8 % agrose gel. M – 1 kb DNA marker; 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 - plasmids extracted from ten different colonies

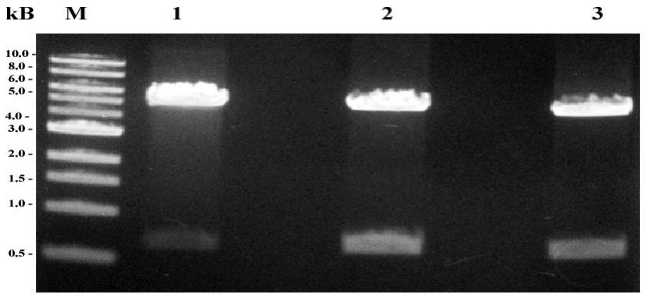


Fig.3.5. Validation of pYD1-CD154 through enzyme digestion. The pYD1-CD154 was digested with Kpn I and Xho I restriction enzymes and analyzed through electrophoresis using 0.8 % agrose gel. . M – 1 kb DNA marker; 1, 2, 3, - plasmids extracted from three different colonies.

DNA sequencing further confirmed the positive colonies identified through enzyme digestion.

Before we detected the display of CD154 on yeast surface, we first determined the expression of mRNA of CD154 in the recombinant EBY100/pYD1-CD154. Total RNA was extracted from galactose induced recombinant EBY100/pYD1-CD154 collected at 24, 50, and 72 h post induction. Cell samples collected right before the galactose induction was referred to as zero time samples, serving as a control. After reverse transcription, a primer set of CD154-F1 and CD154-F1 was used to amplify cDNA of CD154 generated in reverse transcription reaction. The results are shown in Fig. 3.5

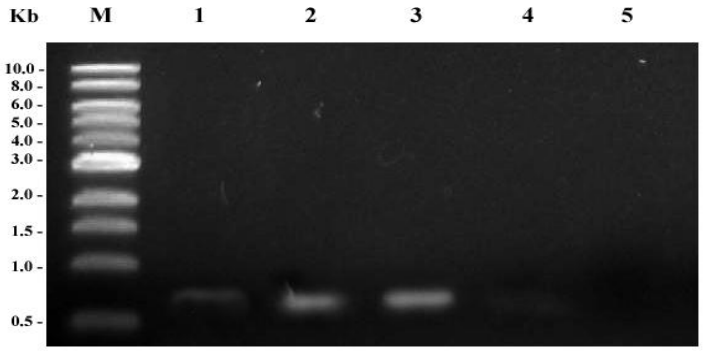


Fig. 3.6. RT-PCR detection of CD154 mRNA expression in recombinant yeast EBY100/pYD1-CD154. Lane **M -** 1 kb DNA marker; 1 -24 h post induction; 2- 50 h post induction; 3- 72 h post induction; 4 – sample collected before galactose induction; and 5 – negative control, strain EBY100/pYD1.

From Fig. 3.6, it seemed that we could detect the expression of the CD154 mRNA as early as at 24 h post induction. The expression of CD154 mRNA appeared to reach higher expression level at 50 and 72 h post induction. No expression of CD154 mRNA was observed before galactose induction and in the negative cell samples collected from the culture of recombinant EBY100/pYD1. It should be pointed out that all the RNA samples were not treated with DNase. It was possible that these RNA samples were contaminated with genomic DNAs, leading to false

positive results. To rule out this possibility, we performed RT-PCR after treating the RNA samples with DNase I to eliminate genomic DNAs from the RNA samples taken after 50 and 72 hours post induction. The results were shown in Fig. 3.7. After treating the RNA samples with DNase, the expression of CD154 mRNA could not be detected at 50 h post induction, although there was detectable signal generated from DNase untreated RNA samples. This experiment suggested that the RNA preparation approach that we used in this study does not prevent the contamination of genomic DNAs in the RNA samples. Therefore, the DNAse treatment is essential to rule out any false positive results in RT-PCR analysis.

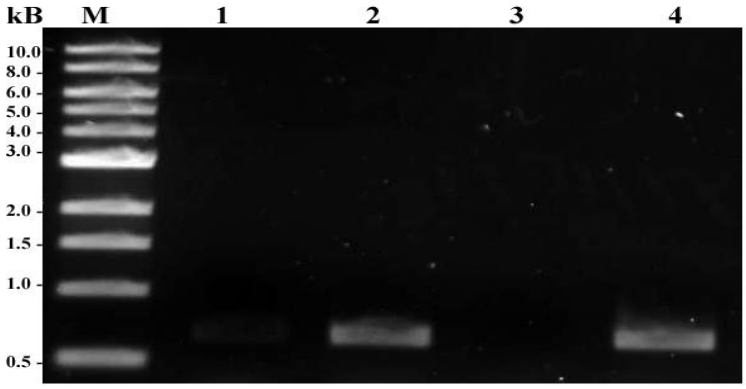


Fig. 3.7. Effect of DNAse (1 U/µl) treatment on RT-PCR assay. RT-PCR detection of CD154 mRNA in the DNAse treated RNA samples collected at 50 h (lane 1) and 70 h (lane 2) post induction. Lane 3: RNA samples collected from yeast EBY100/pYD1 at 50 h post induction. Lane 4, DNAse untreated RNA samples from yeast EBY100/pYD1-CD154 collected at 50 h post induction. Lane M, 1 kb DNA.

Upon the confirmation of CD154 mRNA expression, we next determined the display of CD154 on yeast surface through immunofluorescence microscopy. Goat Anti-CD154 antibody was used as primary antibody and donkey anti-goat IgG FITC conjugate was employed as secondary antibody for this assay.

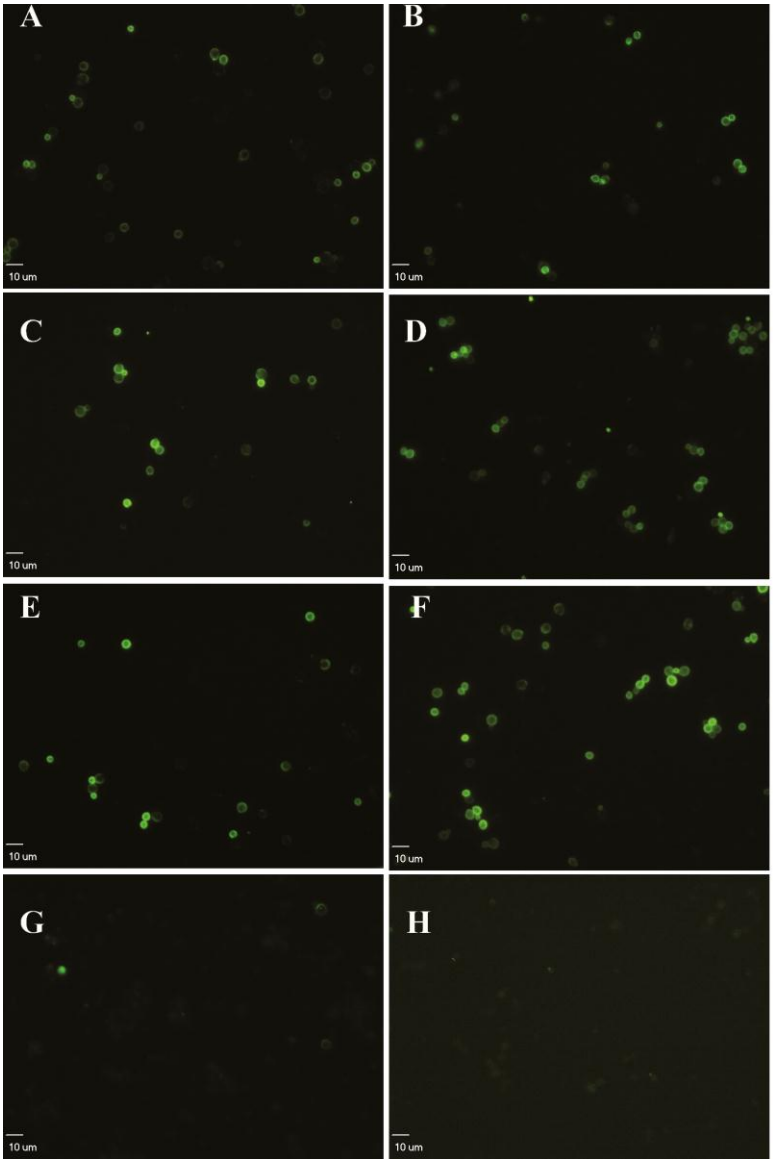


Fig. 3.8. Immunofluorescence microscopy of surface displayed CD154 in recombinant yeast EBY100/pYD1c-CD154. Cells were induced in a galactose medium for 72 h at 20^0C, incubated with goat anti-CD154 primary antibody (1:100), and then labeled with fluorescein-conjugated donkey anti-goat IgG secondary antibody (1:200). (A), (C), (E), the surface displayed CD154 detected at 24 h, 50 h, and 72 h post induction where the glucose was completely removed through centrifugation before the addition of galactose to the medium for induction. (B), (D), and (F), surface displayed CD154 at 24 h, 50 h, and 72 h post induction where the galactose was added to the cell culture medium without removal of glucose. **(**G) (H) – negative controls, where EBY100/pYD1c-CD154 samples taken right before galactose induction (G); EBY100/pYD1 sample taken at 50 h post induction (H).

Galactose induced samples were collected at 0, 24, 50 and 72 h post induction. Recombinant yeasts EBY100/pYD1 grown under the same conditions served as a negative control for this assay. Fig. 3.8 showed the detection of surface displayed CD154 in culture of recombinant EBY100/pYD1c-CD154. The surface display of CD154 could be detected as early as at 24 h post induction (Figs. 3.8 A and B). Similar to what we observed when displaying EGFP on yeast surface, the centrifugation is not required before galactose induction. The expression of CD154 increased significantly over the time. No expression of CD154 could be detected in the negative control (Fig. 3.8G) and before the galactose induction (Fig. 3.8H). Higher level expression of CD154 was observed at 50 and 72 h post induction (Figs. 3.8C-F). The fluorescence intensity between 50 h and 72 h collected samples did not differ too much, indicating the expression of CD154 became plateau at 50 h post induction. The flow cytometric analysis confirmed this observation. As shown in Fig. 3.9, the CD154 displayed yeast cultured with method 2 where no centrifugation was performed before galactose induction was 53.5%, 60.6%, and 63.4% at 24, 50, 72 h post induction, respectively. Interestingly, the percentage of CD154 expressing cells was much lower in cells cultured with Method 1 where centrifugation was carried out to completely remove glucose from cell culture medium before galactose was added to the medium. This indicates that cells suffered from stress during centrifugation, leading to low expression of CD154 in these cells. The mean values of FITC channel detected in CD154 expressing cells cultured with Method 2 (Figs. 3.9B, D, F) were 6,649; 5,912; 5,679 at 24, 50, and 72 h post induction, whereas the mean values of FITC channel detected in CD154 expressing cells cultured with Method1 (Figs. 3.9A, C, and E) were 4,685; 5,396; 5,671 at 24, 50, 72 h post induction. The mean value of FITC channel represents the average display level of CD154 on surface of individual yeast cells, thereby indicating the expression level of CD154 in these cells. This assay

clearly suggests the stress exerted by centrifugation negatively impacts the expression of CD154 on yeast cell surface.

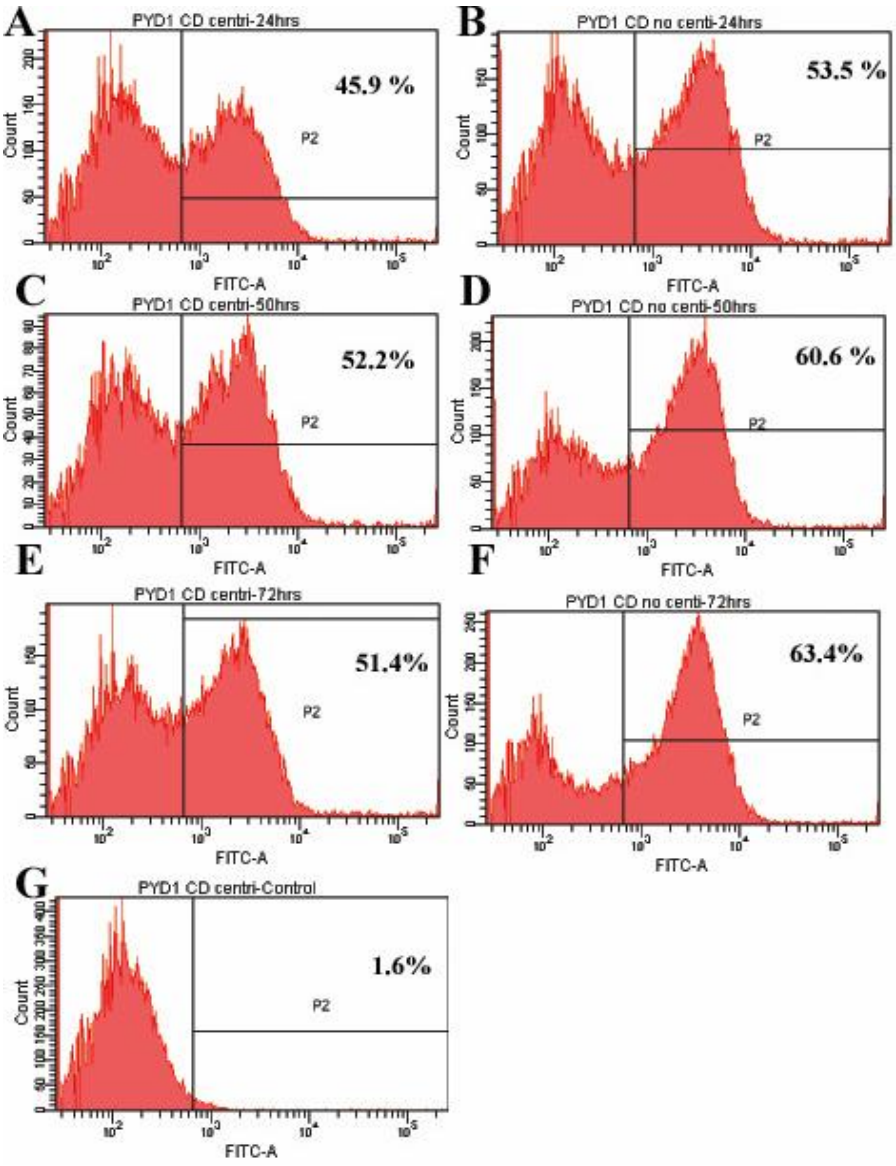


Fig. 3.9. Flow cytometric analysis of CD154 display on yeast surface. Recombinant yeasts **EBY100/pYD1c-CD154** were induced with galactose for 72 hours at 20^0C. The induction was performed by removing glucose (A, C, E**)** or without removing glucose (B, D, F) from the cell culture medium before the addition of galactose for induction. The samples were taken at 24 h (A, B), 50 h (C, D), and 72 h (E, F) and stained with goat anti-CD154 as primary antibody (1:100) and donkey anti-goat IgG FITC conjugates as the secondary antibodies (1:200). Yeast cells harboring pYD1 plasmid served as a negative control in this analysis (G)

Next, we determined the effect of tags that linked to the CD154 on its display on yeast surface. The display of CD154 tagged with V5 epitope and $(His)_6$ on recombinant EBY100/pYD1-CD154 surface was performed as described above. The results were shown in Fig. 3.10. The surface display of CD154 could be detected in these cells, suggesting that the tagging CD154 with V5 epitope and $(His)_6$ does not seem to affect the surface display of CD154 on yeast surface

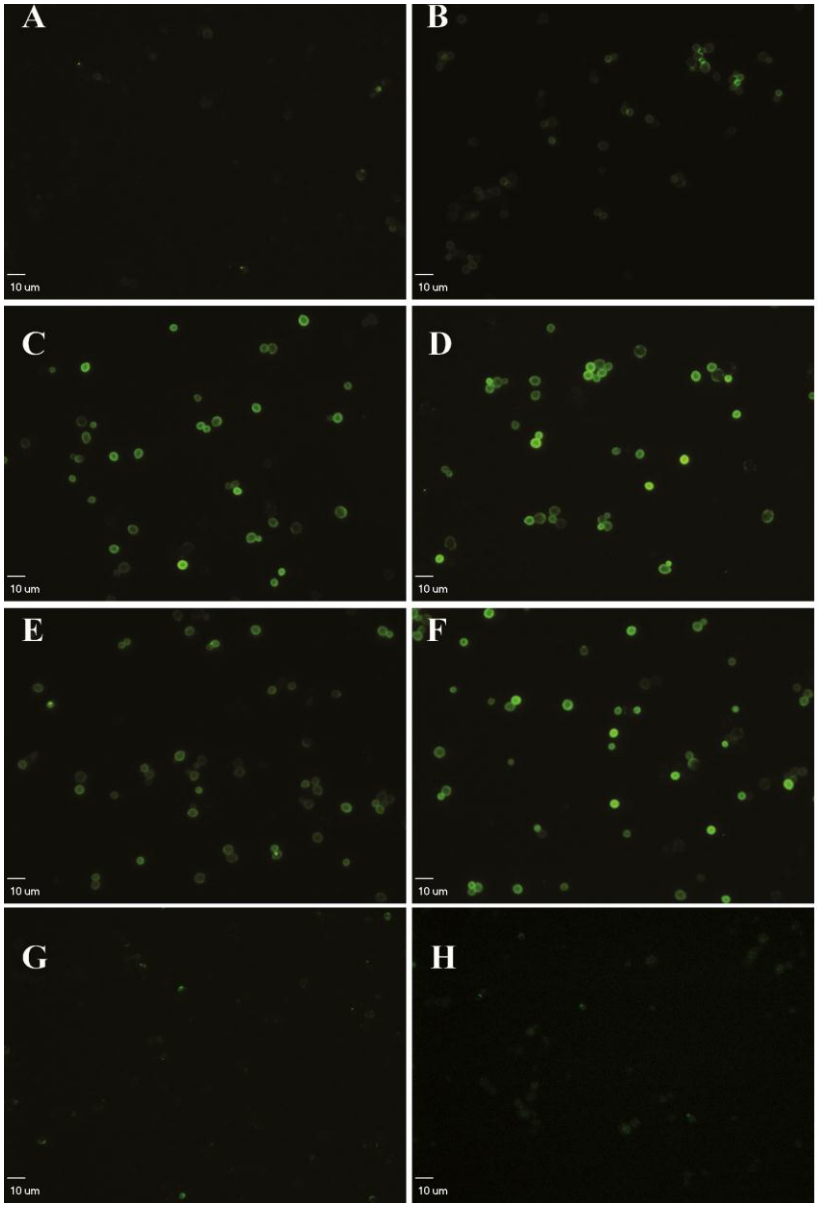


Fig. 3.10. Immunofluorescence microscopy of surface displayed CD154 in recombinant yeast EBY100/pYD1-CD154. Cells were induced in a galactose medium for 72 h at 20^0C, incubated with goat anti-CD154 IgG primary antibody, and then labeled with fluorescein-conjugated donkey anti-goat IgG secondary antibody. (A), (C), (E), the surface displayed CD154 detected at 24 h, 50 h, and 72 h post induction where the glucose was completely removed through centrifugation before the addition of galactose to the medium for induction. (B), (D), and (F), surface displayed CD154 at 24 h, 50 h, and 72 h post induction where the galactose was added to the cell culture medium without removal of glucose. **(**G), EBY100/pYD1-CD154 samples taken right before galactose induction and (H), EBY100/pYD1 sample taken at 50 h post induction.

The expression of CD154 in yeast was further confirmed through Western blot assay, as shown in Figs. 3.11 and 3.12. As observed in both immunofluorescence microscopy and flow cytometric analyses, the expression of CD154 could be detected by Western blot as early as at 24 h post induction and expression of CD154 became much higher at 50 h post induction.

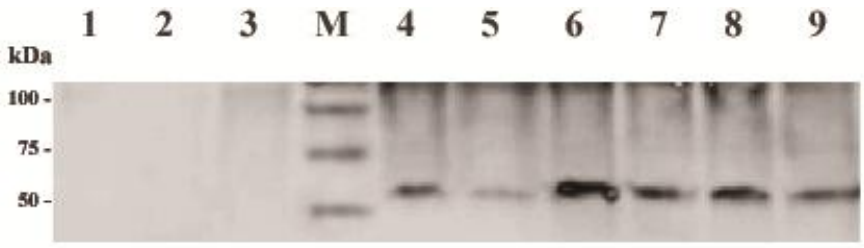


Fig. 3.11. Western blot assay of CD154 expression in recombinant EBY/pYD1-CD154. Goat anti-CD154 was used as primary antibodies (1:100) and donkey anti-goat HRP conjugates were employed as secondary antibodies (1:200) for detecting CD154 expression in recombinant EBY/pYD1-CD154 culture. The expression of CD154 was induced with either method 1 where centrifugation was performed (Lane 2, 4, 6, and 8) or method 2 where no centrifugation was carried out (Lane 3, 5, 7, and 9) before induction. Cell samples were collected before (Lanes 2 and 3) and after induction for 24 (Lanes 4 and 5), 50 (Lanes 6 and 7) and 72 h (Lanes 8 and 9). Lane M, protein marker and lane 1, recombinany EBY100/pYD1 collected at 50 h after addition of galactose to the medium.

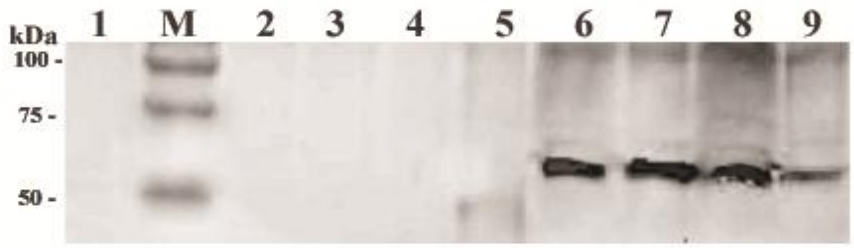


Fig. 3.12. . Western blot assay of CD154 expression in recombinant EBY/pYD1c-CD154. Goat anti-CD154 was used as primary antibodies (1:100) and donkey anti-goat HRP conjugates were employed as secondary antibodies (1:200) for detecting CD154 expression in recombinant EBY/pYD1-CD154 culture. The expression of CD154 was induced with either method 1 where centrifugation was performed (Lane 2, 4, 6, and 8) or method 2 where no centrifugation was carried out (Lane 3, 5, 7, and 9) before induction. Cell samples were collected before (Lanes 2 and 3) and after induction for 24 (Lanes 4 and 5), 50 (Lanes 6 and 7) and 72 h (Lanes 8 and 9). Lane M, protein marker and lane 1, recombinany EBY100/pYD1 collected at 50 h after addition of galactose to the medium.

The estimated molecular weight of the Aga2-CD154--V5-$(His)_6$ fusion protein is about 41 kDa, whereas the Aga2-CD154 fusion protein is about 36 kDa. This is not in consistence with those observed in Western blot assay. The protein bands shown in Western blot close to 60 kDa for Aga2-CD154- V5-$(His)_6$ and 55 kDa for Aga2-CD154. The difference between the estimated and detected molecular weights could be due to the glycosylation of CD154 during its posttrranlation modification. Yeast has been known to have ability of glycosylating eukaryotic proteins. To determine this possibility, we used PNGase F enzyme to remove sugars before performing Western blot assay. As shown in Fig. 3.13, the treatment of samples with PNGase F enzyme did not reduce the molecular weight of the fusion protein.

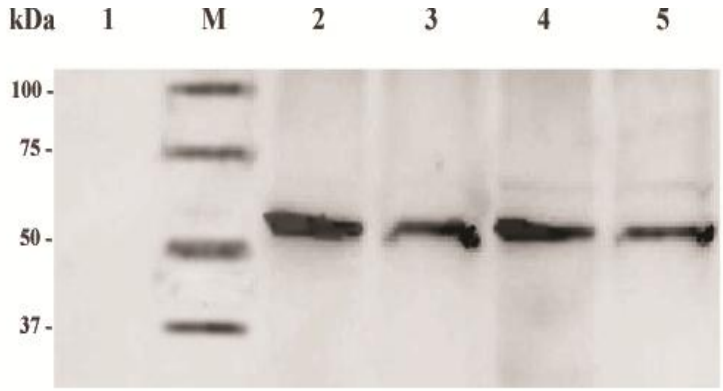


Fig. 3.13. Western blotting of yeast surface displayed CD154 treated with PNGase F enzyme. Lane: M, Protein marker; 1, recombinant EBY/pYD1 collected at 50 h after the addition of galactose to the medium; 2, Aga2-CD154 treated with PNGase F enzyme ; 3,Aga2-CD154 ; 4, Aga2-CD154-V5-$(His)_6$ treated with PNGase F enzyme; 5, Aga2-CD154-V5-$(His)_6$. All the samples were collected at 72 h post induction.

The presence of N-glycosylation in human CD154 protein was reported [41]. However, after analyzing the amino acid sequence of mouse CD154, we found that the number of serine and threonine is higher than the number of asparagine or arginine amino acids, which allows for suggesting that the O-linked, but not N- linked glycosylation takes place. This type of glycosylation cannot be detected by PNGase F enzyme, but by an exoglycosidases, for example enzyme O-glycosydase. Another reason of increased protein mass could be that protein CD154 expressed as a dimer or heteromultimeric complex [42], as earlier studies reported that this protein is easier expressed as dimer or even trimer, than monomer [42-43].

3.3. Conclusion and future works

The CD154 is known to play an important role in cellular and humoral immune response. In this study we reported the sub-cloning and display of mouse CD154 on yeast surface. Our

experimental results suggested that the centrifugation is not necessary before galactose induction, as the glucose might have already been exhausted at that time point. The elimination of centrifugation step significantly improved the display of the CD154 on yeast surface. We also showed that the tagging of CD154 with some tags such as V5 epitope or a $(His)_6$ to the CD154 does not interfere with the surface display of CD154. Moreover, we demonstrated that the CD154 is expressed as early as at 24 h post induction, and it reaches plateau after 50 h induction. The future work includes the further confirming the O-linked glycosylation of CD154 when expressed in yeast. Further we will explore the use of CD154 as adjuvant to enhance influenza subunit vaccines that are currently developed in our lab. Animal studies of yeast surfaced displayed CD154 should be performed to investigate its adjuvant effects on eliciting high immune response of flu subunit vaccines developed in our lab. The human CD154 would be used when we developed human flu vaccines. The work presented here lays the foundation for future research projects.

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APPENDIX

5.1. PCR amplification with Phusion DNA Polymerase (New England Biolabs)

1. Mix the following components on ice:

Component	Volume, µl	Final concentration
Water	Up to 50	
5x Phusion HF Buffer	10	1x
Forward primer	x	0.5 uM
Reverse primer	x	0.5 uM
10 mM dNTP	1 ul	200 uM each
Template DNA	x	10 ng
DNA Polymerase (2U/ul)	0.5 ul	0.02 U/ul

2. Mix gently. Overlay with mineral oil if the thermal cycler lacks a heated lid.

3. The following PCR cycling conditions are recommended:

Cycle step	Temperature	Time
Initial denaturation	98 ^{0}C	30 seconds
	98 ^{0}C	5-10 seconds
25-35 cycles	72 ^{0}C	15-30 second per 1 Kb
Final extension	72 ^{0}C	5-10 minutes

Hold	4^0C	∞

4. Analyze 5 µl of PCR product on by gel electrophoresis.

5.2. PCR purification protocol (PCR purification Kit, QIAquick)

1. Add 4 volumes of buffer PB to 1 volume of the PCR sample and mix

2. Place a QIAquick spin column in a provided 2 ml collection tube

3. To bind DNA, apply the sample to the QIAquick spin column and centrifuge for 30-60 seconds

4. Discard flow-through. Place the QIAquick column back into the same tube

5. To wash add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30-60 seconds

6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an addition 1 minute**.** **Important**: residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

7. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

8. To elute DNA, add 50 ul Buffer EB or water to the center of the QIAquick membrane and centrifuge the column for 1 minute. for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 minute, and then centrifuge.

5.3. Gel extraction protocol (Gel extraction kit, QIAquick)

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 minutes during the incubation**.** **Important:** Solubilize agarose completely. For > 2% gels, increase incubation time.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

5. Add 1 gel volume of isopropanol to the sample and mix. Do not centrifuge the sample at this stage.

6. Place a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 minute. The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.

8. Discard flow-through and place QIAquick column back in the same collection tube.

9. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 minute. Note: If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 minutes after addition of Buffer PE, before centrifuging.

10. Discard the flow-through and centrifuge the QIAquick column for an additional 1 minute at 13,000 rpm **Important:** Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

11. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

12. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 minute. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 minute, and then centrifuge for 1 minute. **Important:** Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl.

5.4. Transformation of *E.Coli* (5-α competent cells, New England Biolabs)

1. Thaw a tube on ice until the last ice crystals disappear. Mix gently and carefully pipette 50 µl of cells into two transformation tubes on ice.

2. Add 3 µl of ligation mixture to the cells. Carefully flick the tube 4-5 times to mix cells and DNA. Do not vortex.

3. Place the mixture on ice for 30 minutes. Do not mix.

4. Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.

5. Place on ice for 5 minutes. Do not mix.

6. Pipette 950 µl of room temperature SOC into the mixture.

7. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.

8. Warm selection plates to 37°C.

9. Centrifuge culture for 1 minute at 10,000 rpm. Discard the supernatant.

10. Re-suspend cell pellet in 50 µl of SOC medium.

11. Spread mixture on a selection plates and incubate overnight at 37°C.

5.5. Plasmid miniprep protocol (Pureyield Plasmid Miniprep system, Promega)

1. Transfer 600µl of bacterial culture grown in LB medium to a 1.5ml microcentrifuge tube.

2. Add 100µl of Cell Lysis Buffer, and mix by inverting the tube 6 times. The solution should change from opaque to clear blue, indicating complete lysis.

Note: Proceed to Step 3 within 2 minutes. Excessive lysis can result in denatured plasmid DNA. If processing a large number of samples, process samples in groups of ten or less. Continue with the next set of ten samples after the first set has been neutralized and mixed thoroughly.

3. Add 350µl of cold (4–8°C) Neutralization Solution, and mix thoroughly by inverting the tube. The sample will turn yellow when neutralization is complete, and a yellow precipitate will form. Invert the sample an additional 3 times to ensure complete neutralization.

4. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.

5. Transfer the supernatant (~900µl) to a PureYield™ Minicolumn. Do not disturb the cell debris pellet. For maximum yield, transfer the supernatant with a pipette.

6. Place the minicolumn into a PureYield™ Collection Tube, and centrifuge at maximum speed in a microcentrifuge for 15 seconds.

7. Discard the flow-through, and place the minicolumn into the same PureYield™ Collection Tube.

8. Add 200µl of Endotoxin Removal Wash to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds.

9. Add 400µl of Column Wash Solution to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 seconds.

10. Transfer the minicolumn to a clean 1.5ml microcentrifuge tube, and then add 30µl of Elution Buffer directly to the minicolumn matrix. Let stand for 1 minute at room temperature.

Note: Nuclease-free water at neutral pH can also be used to elute DNA. For large plasmids (>10kb), warm the Elution Buffer to 50ºC prior to elution, and increase elution volume to 50µl. Also incubate the column at room temperature (22–25°C) for 5–10 minutes before proceeding to Step 11.

11. Centrifuge at maximum speed in a microcentrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube, and store eluted plasmid DNA at –20°C.

5.6. Electroporation of *S. cerevisiae* cells (strain EBY100, Invitrogen)

5.6.1. Preparation of electrocompetent cells

1. Two days before the experiment, inoculate 5 ml of YPD medium with a single yeast colony, Grow overnight to saturation at 30 ℃ (stored at 4 ℃).

2. Inoculate 250 ml of YPD in a 1L Fernbranch Flask with the 2.5 ml of yeast colony of S.cerevisiae. Grow overnight at 30 ^{0}C, shaking at 250 rpm, to a density of ~1x10^{8} cells/ml (OD_{600} (1:10 dilution) = 0.30 – 0.35).

3. Chill the cells in an ice water bath for 15 minutes to stop growth.

4. Decant the 100 ml of cells into a sterile 100 ml centrifuge bottle. All the media should be divided for 5 bottles. Pellet the cells by centrifugation at 4000 X g for 5 minutes at 4 ^{0}C.

5. Carefully pour off and discard the supernatant; place the centrifuge bottles with the cells on ice.

6. Add 16 ml of sterile water to each bottle and vortex to re-suspend the cell pellet

7. Add 2 ml of 10X TE buffer; swirl and mix. Incubate 15 minutes at 30 ^{0}C shaking at ~ 85 RPM.

8. Add 0.5 ml of 1M DTT; swirl to mix. Incubate 15 minutes at 30 ^{0}C shaking at ~ 85 RPM.

9. Bring the volume to 100 ml with the sterile water. Pellet the cells by centrifugation at 7,200 rpm for 5 minutes at 4 ^{0}C; pour off and discard the supernatant.

10. Add ~ 10 ml of sterile, ice-cold water to each bottle and vortex to resuspend the cell pellet; bring the volume to 100 ml with sterile, ice-cold water. Pellet the cells by centrifugation at 7,200 rpm for 5 minutes at 4 ^{0}C; pour off and discard the supernatant.

11. Re-suspend the cell pellet in 5-6 ml of sterile, ice-cold 1M sorbitol and transfer to a chilled 50 ml Oakridge tube. Pellet the cells by centrifugation at 7,200 rpm for 5 minutes at 4 ^{0}C; pour off and discard the supernatant.

12. Re-suspend the cell pellet in 0.1 ml of sterile, ice-cold 1M sorbitol; the final cell volume should be 0.3 ml and the cell concentration should be ~ 1x 10^{10} cells/ml. Keep the cells on ice and use ASAP for electroporation.

5.6.2. Electroporation of *S.cerevisiae* (Gene Pulser Xcell^{TM} , BioRad)

1. For each sample to be electroporated, prepare a 17x100 mm sterile tube with 1 ml of 1 M Sorbitol and place on ice; also place a 0.4 cm cuvette on ice.

2. Pipette the DNA samples (5-100 ng in a volume of 5 µl) to be electroporated into sterile 1.5 ml microfuge tubes. Place tubes on ice.

3. Add 80 µl of the competent cells to each DNA sample in a 0.4 cm cuvette. Mix gently and incubate on ice for ~ 5 minutes.

4. From the home screen on Gene Pulser Xcell open the Pre-set Protocols screen, then the Fungai Protocol screen (press 2, Enter to open the S.cerevisiae, 4 mm Protocol detail screen). Press Enter to open the S.cerevisiae, 2 mm cuvette Protocol Detail screen (for using 0.2cm).

5. Transfer the DNA- cell samples to the appropriate electroporation cuvettes that have been chilled in ice and tap the suspension to the bottom. Place the cuvette in the ShockPod. Push the lid down to close. Pulse once.

6. Remove the cuvette from the chamber, immediately add 1ml of ice cold 1 M sorbitol to the cuvette, and then gently transfer the diluted cells into a sterile 17x100 mm tube.

7. Check and record the pulse parameters. The time constant should be about 5 milliseconds. The voltage should be approximately 2.5 kV when pulsing the 0.4 cm cuvettes.

8. Plate aliquots of the electroporated cells (150 ul) on selective agar plates. Incubate at 30 ℃ until colonies appear

5.7. Yeast plasmid extraction protocol (Zymoprep Yeast Plasmid Miniprep II, Zymoresearch)

1. Aliquot 0.1-1.5 ml of the yeast cells into 1.5 ml microfuge tubes and spin down the cells at 600 x g for 2 minutes.

2. Add 200 µl Solution 1 to each pellet.

3. Add 3 µl of Zymolyase™ to each tube. Resuspend the pellet by flicking with finger or mild vortexing.

4. Incubate at 37°C for 15-60 minutes (15 minutes is the minimal incubation time. Longer incubation is optional, but is suggested for stationary phase or older cells).

5. Add 200 µl Solution 2 to each tube. Mix well.

6. Add 400 µl Solution 3 to each tube. Mix well.

7. Centrifuge at maximum speed for 3 minutes.

8. Transfer the supernatant to the Zymo-Spin-I column.

9. Spin the Zymo-Spin I column for 30 seconds.

10. Discard the flow-through in the collection tube. Make sure the liquid does not touch the bottom part of the column.

11. Add 550 µl of Wash Buffer (ethanol added) onto the column with the collection tube and spin for 1-2 minutes. Discard the wash buffer. Place column into a new 1.5 ml microfuge tube (not provided).

12. Add 10 µl of water or TE and spin for 30 seconds-1 minute to elute plasmid off the column into a new 1.5 ml microfuge tube. For plasmids larger than > 15 Kb, incubate the column and elution buffer (water or TE) for 5 minutes before centrifugation to increase plasmid yields.

5.8. Preparation of minimal dextrose plates with amino acids

1. For 1 liter, dissolve 6.7 g YNB and 15 g agar in 900 ml deionized water.

2. Autoclave on liquid cycle for 20 minutes.

3. After autoclaving, allow the medium to cool to ~55-60°C and add 100 ml of 20% glucose (filter-sterilized).

4. Add 10 ml of 10 mg/ml leucine (filter-sterilized) and/or 10 ml of 10 mg/ml tryptophan (filter-sterilized). Alternatively, plates can be made ahead of time and amino acids spread onto the plate prior to use. Spread 100 µl of a 10 mg/ml amino acid solution onto the plate and let the solution soak into the plate before plating cells.

5. Store at +4°C. Plates are stable for 1-2 months.

5.9. Expression and display of protein fusion

5.9.1. Induction

1. Inoculate a single yeast colony into 10 ml YNB-CAA containing 2% glucose and grow overnight at 30 ^{0}C with shaking.

2. Read the absorbance of the cell culture at 600nm. The OD_{600} should be between 2 and 5. In general, OD_{600} readings less than 2 will decrease the number of cells display the fusion proteins. OD_{600} readings greater than 5 will delay induction of expression of the displayed protein.

3. Centrifuge the cell culture at 7,200 rpm for 5 minutes at room temperature.

4. Re-suspend the cell pellet in YNB-CAA medium containing 2% galactose to an OD_{600} of 0.5 to 1. This is to ensure that the cells continue to grow in log-phase. (e.g. if the OD_{600} is 2 from Step 2, resuspend the cells in 20 to 40 ml of medium).

5. Immediately remove a volume of cells equivalent to 2 OD_{600} units. For an OD_{600} of 0.5, remove 4 ml and place on ice. This is your zero time point.

6. Incubate the cell culture at 20^{0}C with shaking.

7. Assay the cell culture over 72-hour time period.

8. Proceed to staining of displayed proteins.

5.9.2. Staining of displayed proteins

For each time point, assay untransformed EBY100/pYD1, and EBY100/pYD1-CD154. Time points may be processed as they are collected or placed on ice and stored at +4°C until all time points are collected. Do not freeze cells.

1. Wash the cell pellet with 1 ml of iced cold PBS/1.3% BSA, centrifuge at 7,200 rpm, 5 minutes, +4 ^{0}C. Repeat three times.
2. Apply the 250 µl of 100x diluted goat anti- CD154 antibody. Re-suspend by tapping. Incubate at +4 ^{0}C overnight, shaking.
3. Wash 3x times with 1ml of iced-cold PBS buffer, and incubate cells with 250 µl of 200x diluted FITC conjugated donkey anti-goat IgG in a dark for 1 hour at room temperature (wrap it in a foil), shaking.
4. Wash the cells 3x times with 1 ml of iced-cold PBS buffer, 7,200 rpm, 5 minutes, +4 ^{0}C
5. Re-suspend the cell pellet in 50 µl of PBS.
6. Analyze the sample using fluorescence microscope.

5.9.3. Western blotting of displayed proteins

For each time point, assay untransformed EBY100/pYD1, and EBY100/pYD1-CD154. Time points may be processed as they are collected or placed on ice and stored at -20°C or – 80 20°C until all time points are collected.

1. Re-suspend the cell pellets in ice-cold 1×PBS, centrifuge at 7,200 rpm, 5 minutes, and 4 ^{0}C, discard the supernatant.

2. Dilute the pellet using 19 µl loading buffer with 1 µl of β-ME, and boil the mixture at 95-100 ^{o}C for 10 minutes. Mix the sample before and after heating step by vortexing. Centrifuge at 15,000 rpm, 10 minutes, keep the supernatant as protein samples.

3. Load the samples and the marker, 15µl per mini-gel well.

4. Start the electrophoresis at 200 V for 35minutes.

5. Pre-wet membrane in transfer buffer 10 minutes before use at room temperature.

6. Cut the top right corner of a membrane and label the top left corner with the blot number.

7. Prepare the transfer apparatus: fill the box half full with pre-cold transfer buffer. Wet sponges and filter paper in transfer buffer.

8. Carefully transfer the gel to the filter paper, such that the top right corner is on the right and faces away from the hinge.

9. Transfer the membrane on top of the gel to match the orientation of the gel (nicked corner of gel to nicked corner of membrane). This ensures transfer of protein from left to right on the membrane, with marker on the left and samples numbering up.

10. Ensure that the membrane and gel remain wet, and remove any bubbles in between them. Complete the transfer sandwich with filter paper and sponge, then clamp the tray closed. Close the transfer box, place it in a box filled with an ice.

11. Transfer 1.5 hours at 100V.

12. Perform blocking with PBST/5% non-fat dry milk and incubate for 2 hours, shaking at room temperature.

13. Incubate with goat anti-CD154 antibody (1:200 in PBST/5% non-fat dry milk, v/v) overnight

14. Wash the membrane 3x times with 1×PBST, 5 minutes each time

16. Incubate the membrane with donkey Anti-goat IgG (Fab specific) Antibody Peroxidase Conjugated (1:2000 in PBST/5% non-fat dry milk, v/v) for 1 hour

17. Wash the cells 3x times with PBST, 5 minutes each

18. Mix equal volumes of the Enhanced Luminol Reagent and the Oxidizing Reagent. Incubate the membrane in the chemiluminescence reagent for 5 minutes with gentle agitation.

19. Analyze the membrane and take images.

5.10. Yeast RNA extraction (Yeastar^{TM} RNA Kit, Zymoresearch)

1. Inoculate a single yeast colony into 10 ml YNB-CAA containing 2% glucose and grow overnight at 30 ^{0}C with shaking.

2. Read the absorbance of the cell culture at 600nm. The OD_{600} should be between 2 and 5. In general, OD_{600} readings less than 2 will decrease the number of cells display the fusion protein. OD_{600} readings greater than 5 will delay induction of expression of the displayed protein.

3. Centrifuge the cell culture at 7,200 rpm for 5 minutes at room temperature.

4. Re-suspend the cell pellet in YNB-CAA medium containing 2% galactose to an OD_{600} of 0.5 to 1. This is to ensure that the cells continue to grow in log-phase. (e.g. if the OD_{600} is 2 from Step 2, resuspend the cells in 20 to 40 ml of medium).

6. Incubate the cell culture at 20^{0}C with shaking.

7. Assay the cell culture over 72-hour time period.

Next steps should be performed in RNase/DNase free area. All the tubes and tips should be RNase/DNase free.

8. Pellet 1-5 x10^7 cells (1-1.5 ml culture) by centrifugation at 3,000 rpm for 2 minutes. Carefully remove all of the supernatant.

9. Add 80 µl of the YR Digestion Buffer and 5 µl of the Zymolyase to the cell pellet and re-suspend the pellet completely by pipetting. Incubate the suspension at 30-37°C for 40-60 minutes.

10. Add 160 µl of the YR Lysis Buffer and mix thoroughly by vortexing.

11. Centrifuge the mixture at 10,000 rpm for 2 minutes

12. Transfer the supernatant to the Zymo-Spin™ IIIC Column in a Collection Tube and centrifuge at ≥10,000 rpm for 1 minute.

13. Add 200 µl RNA Wash Buffer to the column and centrifuge at ≥10,000 rpm for 1 minute. Discard the flow-through. Repeat the wash step.

14. Remove the Zymo-Spin IIIC Column carefully from the Collection Tube and transfer it into an RNase-Free Tube. Add 60 µl of DNase/RNase-Free Water directly to the column matrix

15. Centrifuge at ≥10,000 rpm for 30 seconds. The eluted RNA can be used immediately or stored at -70ºC.

5.11. DNase Treatment of RNA Samples Prior to RT-PCR (RQ1 RNase-Free DNase, Promega)

1. Set up the DNase digestion reaction as follows:

RNA in water or TE buffer……1–8µl

RQ1 RNase-Free DNase 10X Reaction Buffer…… 1µl

RQ1 RNase-Free DNase……1u/µg RNA

Nuclease-free water…….. to a final volume of 10µl

Note: Use 1 unit of RQ1 RNase-Free DNase per microgram of RNA. For smaller amounts of RNA, use 1 unit of RQ1 RNase-Free DNase per reaction.

2. Incubate at 37°C for 30 minutes.

Note: If analyzing RNA samples by gel electrophoresis, perform a phenol:chloroform extraction and ethanol precipitation before loading the samples on the gel because salts in the RQ1 DNase Reaction Buffer and Stop Solution may cause aberrant migration or smearing of RNA on gels. Steps 3 and 4 may be omitted if a phenol:chloroform extraction is performed.

3. Add 1µl of RQ1 DNase Stop Solution to terminate the reaction.

4. Incubate at 65°C for 10 minutes to inactivate the DNase.

5. Add all or a portion of the treated RNA to the RT-PCR

5.12. First strand cDNA synthesis (Protoscript M-MuLV

RT-PCR Kit, New England Biolabs)

1 . Make the RNA/primer/dNTP mix by combining the following components in a sterile RNase-free microfuge tube:

Total RNA ……...….1–10 µl (1 ng–1 µg)

Primer $dT_{23}VN$ …….. .....2 µl

dNTP Mix ……...4 µl

Nuclease-free H_20 …. ……..…….…….variable

Total volume…….…….……16 µl

2. Heat for 5 minutes at 70°C. Spin briefly and promptly chill on ice.

3. Add the following components to the 16 µl RNA/primer/dNTP solution and mix well by pipetting up and down:

10X RT Buffer ……...……..…….2 µl

Murine RNase Inhibitor……...……0 .5 µl

M-MuLV Reverse Transcriptase……...……...……. 1 µl

Nuclease-free H_20 …….…… to a final volume of 20 µl

4. Incubate the 20 µl cDNA synthesis reaction at 42°C for 1 hour. If random primers are used, an incubation step at 25°C for 5 minutes is recommended prior to the 42°C incubation.

5. Inactivate the enzyme at 80°C for 5 minutes.

6. Bring the reaction volume to 50 µl with water. The cDNA product should be stored at –20°C.

PCR amplification

Use 2–5 µl of the diluted cDNA product per 50 µl PCR reaction.

1. Mix the following in a PCR tube on ice:

5x Phusion HF Buffer…….10 µl

dNTPs (10mM)…….1 µl

Forward Primer (10mM) …….2.5 µl

Reverse Primer (10 mM) ……2.5 µl

cDNA ……..2-5 µl

Phusion HF DNA Polymerase……0.5 µl

Water ……to a final volume of 50 µl

2. Mix gently. Overlay with mineral oil if the thermal cycler lacks a heated lid.

3. The following PCR cycling conditions are recommended:

Cycle step	**Temperature**	**Time**
Initial denaturation	98 ^{0}C	30 seconds
	98 ^{0}C	5-10 seconds
25-35 cycles	72 ^{0}C	15-30 second per 1 Kb
Final extension	72 ^{0}C	5-10 minutes
Hold	4^{0}C	∞

4. Analyze 5 µl of the PCR product by agarose gel electrophoresis.