

## Applications of Yeast Surface Display for Protein Engineering

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### Abstract

The method of displaying recombinant proteins on the surface of *Saccharomyces cerevisiae* via genetic fusion to an abundant cell wall protein, a technology known as yeast surface display, or simply, yeast display, has become a valuable protein engineering tool for a broad spectrum of biotechnology and biomedical applications. This review focuses on the use of yeast display for engineering protein affinity, stability, and enzymatic activity. Strategies and examples for each protein engineering goal are discussed. Additional applications of yeast display are also briefly presented, including protein epitope mapping, identification of protein-protein interactions, and uses of displayed proteins in industry and medicine.

**Key words** Yeast surface display, Protein engineering, Random mutagenesis, DNA shuffling, Affinity maturation, Protein stability engineering, Enzyme engineering

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### 1 Introduction

The ability to create engineered proteins with enhanced properties including increased binding affinity, stability, and catalytic activity, has had significant impact on biological research, medicine, and biotechnology. Despite an improved understanding of protein chemistry and folding, it remains challenging to design proteins from first principles. Thus, most strategies rely on combinatorial methods, such as directed evolution, to engineer optimized proteins, by applying random or site-directed mutagenesis techniques to generate “libraries” of up to  $10^{14}$  variants of an individual protein. These protein libraries are then screened in a high-throughput manner to identify amino acid mutations that confer the desired phenotype.

Numerous molecular display platforms have been specifically developed for protein engineering, including tethering libraries of protein variants to ribosomes and mRNA [1–5], or to the surface of phage [6, 7], bacteria [8], mammalian [9, 10], insect [11], or yeast [12] cells. In the case of cell surface display, each individual

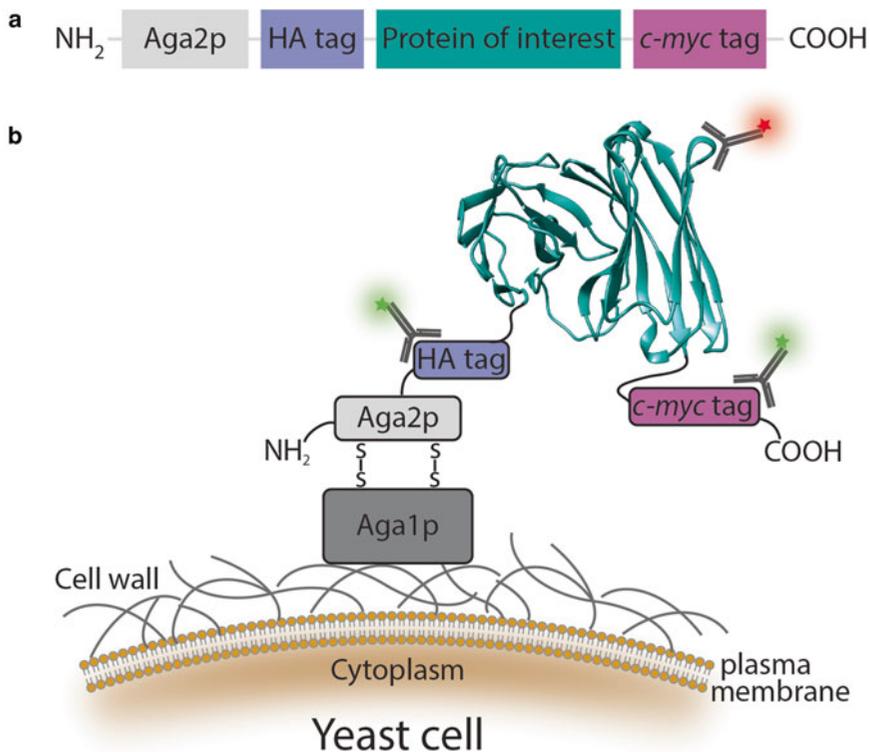
cell is transformed with a single vector encoding a protein variant of interest that is genetically fused to a cell-surface anchor protein. The anchor protein contains a signal sequence that directs efficient transport of the fusion protein to the cell surface, where it is immobilized and accessible to the extracellular space. Yeast surface display has become a leading platform for protein engineering due to its collective advantages, including (1) a eukaryotic expression system capable of incorporating posttranslational modifications such as disulfide bond formation; (2) low technical and time requirements relative to other eukaryotic display systems; (3) inclusion of epitope tags, which allows normalization of protein function (e.g., ligand binding) to surface expression and, thus, identification of proteins that both express at high levels and bind with high affinity to a target protein; and (4) compatibility with flow cytometric analysis, which allows quantitative measurements of equilibrium binding constants, dissociation kinetics, stability, and specificity of the displayed proteins without the laborious requirements of soluble protein expression and purification.

This review primarily focuses on the applications of yeast display from a protein engineering perspective, including examples of protein affinity maturation, stability engineering, and enzyme engineering. Other applications of yeast display are briefly reviewed, such as protein epitope mapping, identification of protein-protein interactions, and display of proteins and enzymes on yeast cells for biotechnology and biomedical applications.

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## 2 Yeast Surface Display Platform

Yeast offer multiple options for cell surface anchor proteins, including Aga1p, Aga2p, Cwp1p, Cwp2p, Tip1p, Flo1p, Sed1p, YCR89w, and Tir1 [13]. Fusion of a protein of interest to the C- or N-terminus of an anchor protein typically results in the display of up to 100,000 copies of the fusion protein on the cell surface of *Saccharomyces cerevisiae* [14]. The choice of the anchor protein and fusion terminus depends on the protein to be engineered; generally the terminus farthest from the functional portion of the protein should be tethered to the anchor protein to avoid disrupting activity. The most common yeast display system employs fusion of the protein of interest to the C-terminus of the *a*-agglutinin mating protein Aga2p subunit, a technology pioneered by Boder and Wittrup [12] (Fig. 1). The yeast surface display construct designed for this system includes two epitope tags: a hemagglutinin (HA) tag between Aga2p and the N-terminus of the protein of interest, and a C-terminal *c-myc* tag (Fig. 1a). Induction of protein expression results in surface display of the fusion protein through disulfide bond formation of Aga2p to the  $\beta$ 1,6-glucan-anchored Aga1p domain of *a*-agglutinin [15–17] (Fig. 1b). The epitope tags allow quantification of fusion protein expression, and thus



**Fig. 1** Schematic representation of yeast surface display. **(a)** The protein of interest is flanked by two epitope tags: a 9-amino acid hemagglutinin antigen (HA) tag and a 10-amino acid *c-myc* tag, and is fused to the C-terminus of the *a*-agglutinin Aga2p subunit. **(b)** Protein display on the yeast cell surface. Following translation, the 69-amino acid Aga2p subunit associates with 725-amino acid *a*-agglutinin Aga1p subunit via two disulfide bonds. The fusion protein is subsequently secreted to the extracellular space where Aga1p is anchored to the cell wall via a  $\beta$ 1,6-glucan covalent linkage. As a result, the protein of interest is displayed on the cell surface where it is accessible by soluble ligands. Functional display of the protein of interest (shown here as a scFv [129] modified from PDB 1X9Q using the UCSF Chimera package [130]) can be detected by a fluorescently labeled antibody or ligand (*red star*) specific to the native fold. The epitope tags are used to normalize protein function to surface expression level through either labeled anti-HA or anti-*c-myc* antibodies (*green stars*). These features allow flow cytometric sorting of a heterogeneous mixture of yeast cells, each displaying up to 100,000 copies of a individual protein variant, based on the biophysical and biochemical properties of the displayed proteins

normalization of protein function to expression level by flow cytometry using fluorescently labeled antibodies. However, detection of epitope tags yields no information on the fold or function of the protein of interest. Therefore, a ligand or antibody specific for the native fold of the displayed protein must be used to interrogate these properties.

Protein engineering applications using yeast display involve expression of a protein library, which is generated from the underlying genetic material that codes for the protein variants. This connection is known as a genotype-phenotype linkage and must be maintained throughout the protein engineering process so that the desired protein variants can be identified by sequencing following library screening. To create a protein library, diverse genetic material,

which can be obtained directly from organisms or generated by mutagenic PCR, is transformed into yeast cells and induction of expression through a GAL promoter leads to surface display of the protein variants. The resulting library can then be screened using flow cytometric sorting (also known as fluorescence-activated cell sorting, or FACS) to isolate yeast displaying proteins with the desired properties. For this purpose, yeast are incubated with fluorescent probes that differentially label the cells based on the biochemical and biophysical properties of their displayed protein (e.g., affinity, stability, specificity). The yeast cells are then passed single-file through the fluidics stream of a FACS instrument, which analyzes and sorts them based on cell size, granularity, and/or fluorescence measurements. Detailed protocols for library creation and screening have been well described in the field [14, 18–21].

Phage display and ribosome display techniques take advantage of panning-based methods to efficiently screen libraries as large as  $10^{12}$ – $10^{14}$  variants. In comparison, yeast display offers lower throughput due to limitations in yeast transformation efficiency and current cell screening technology. The upper limit of library sizes that can currently be screened by FACS is  $\sim 10^8$ – $10^9$  yeast cells, determined by the maximum sampling rate ( $\sim 50,000$  cells/s) of the leading flow cytometry instruments. Moreover, libraries are typically sampled by FACS at higher coverage (e.g.,  $10\times$ ) to increase the probability of sampling each variant at least once. Thus, to increase throughput, yeast-displayed libraries of greater than  $10^8$  variants can first be screened using bead-based magnetic activated cell sorting (MACS) to reduce the library diversity before screening with FACS [22, 23]. In this method, magnetic beads are coated with a soluble target of interest, for example, an antibody or ligand. The beads are then incubated with the yeast library, and yeast displaying non-binding protein variants are removed by washing, after which the yeast binding the desired target are eluted and recovered. This method is advantageous for removing truncated, misfolded, and weak affinity proteins from the library, thereby reducing library diversity to a size that is amenable to quantitative screening by FACS. Finally, while differences between human and yeast glycosylation patterns have typically not prevented functional display of glycosylated human proteins, yeast strains that express human glycosylation machinery have been engineered and similar technology could potentially be developed for yeast display applications if human glycosylation is desired (*see refs. 24–26 for reviews*).

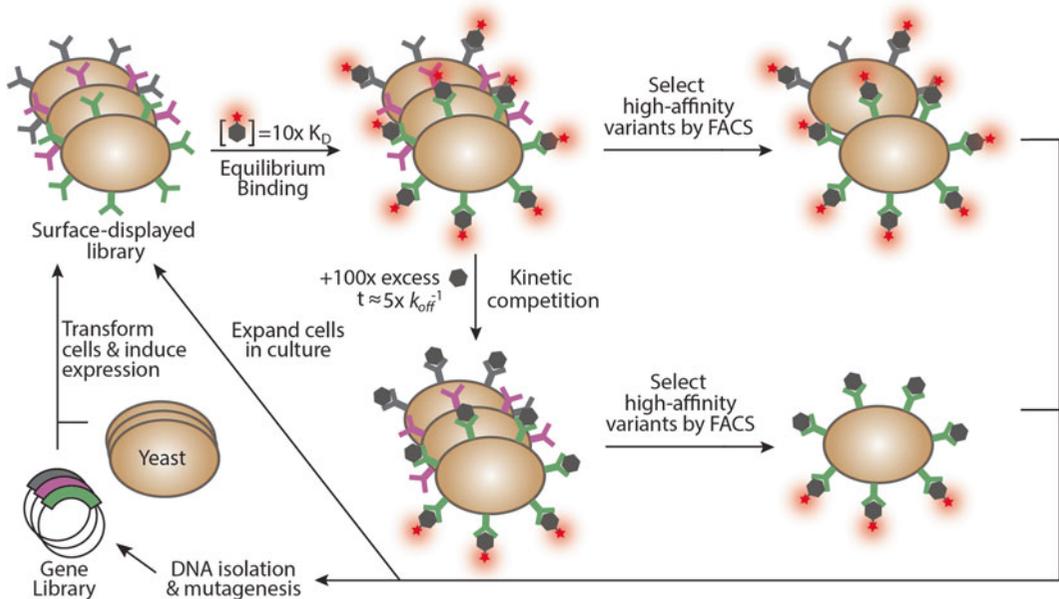
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### 3 Engineering Proteins for Increased Affinity

The affinity a protein has for its binding partner is a key parameter that often regulates the biological function of the bound complex. High binding affinity is a desired characteristic of proteins used for

research, therapeutic, and diagnostic applications, and thus multiple strategies for increasing protein affinity (termed “affinity maturation”) have been developed, with the most common involving directed evolution and molecular display technologies. Over the last decade, yeast display has become a leading platform for affinity maturation; in addition to the aforementioned advantages, yeast display can discriminate between proteins with only twofold differences in affinity [21, 27], further illustrating the sensitivity of this approach.

A general strategy of affinity maturation using yeast display involves creation of a library on the order of  $10^7$ – $10^9$  protein variants by random mutagenesis, followed by display of these variants on the surface of yeast as fusions to the Aga2p cell wall protein (Fig. 2).



**Fig. 2** Isolating high-affinity protein variants from a yeast-displayed library by FACS. Following transformation of yeast cells with a gene library and induction of surface expression, two main strategies are used to differentially label the displayed library prior to screening: (1) an equilibrium binding strategy where the library is incubated with a ligand concentration 5–10 times greater than the expected  $K_D$  value of the highest affinity variant, resulting in near saturation of tight binding variants and partial labeling of weaker affinity variants at equilibrium, and (2) a kinetic binding strategy where the library is incubated with ligand as described for the equilibrium binding strategy, but unbound ligand is removed by washing and the library is then incubated either with a 100-fold excess of unlabeled ligand or in a sufficiently large volume of buffer to prevent rebinding of dissociated ligand. During this second incubation step, the excess unlabeled ligand or large incubation volume prevents dissociated labeled ligands from rebinding. Proteins are thus differentiated based on their dissociation rate constants ( $k_{off}$ ), with variants having the slowest  $k_{off}$  retaining the largest percentage of pre-bound labeled ligand. Addition of a fluorescently labeled anti-epitope tag antibody (not shown) permits normalization of yeast surface expression levels with binding, allowing the highest affinity variants to be isolated by FACS. Sorted pools of yeast clones can be expanded in culture for either analysis or a subsequent round of sorting, or DNA from these clones can be isolated, subjected to mutagenesis, and used to transform a new batch of yeast for further directed protein evolution. Components of the yeast display platform, including Aga1p, Aga2p, HA, and *c-myc* epitope tags, and detection antibodies depicted in Fig. 1, are omitted for clarity

Subsequently, two main strategies are used to label the yeast-displayed library prior to sorting by FACS. In the first approach, library members are screened based on their equilibrium dissociation constants ( $K_D$ ) [27, 28]. The yeast-displayed library is incubated with the soluble binding partner (ligand) at a concentration of approximately five- to tenfold greater than the expected  $K_D$  of the highest affinity variants (typically near the  $K_D$  of the wild-type interaction), and binding is allowed to reach equilibrium. This method requires at least a tenfold excess of ligand relative to the number of yeast-displayed protein variants in the binding reaction. If a lower ratio is used, binding may significantly alter the concentration of free ligand in solution, which would result in ligand depletion and non-compliance with rules that govern equilibrium binding isotherms. The second approach uses kinetic competition and screens yeast-displayed variants based on their dissociation rate constants ( $k_{\text{off}}$ ) [12, 29, 30]. The library is incubated with a saturating concentration of fluorescently labeled ligand, washed, and then either incubated with 100-fold excess of unlabeled ligand, if available, or incubated in a sufficiently large volume of buffer to prevent rebinding of the labeled ligand after dissociation. This method is primarily used when evolving variants of a protein with a strong starting affinity ( $K_D < 1$  nM) that would require inconveniently large incubation volumes to meet the tenfold excess ligand requirement of the former method, or when the dissociation kinetics are the most important functional characteristic of the desired protein. Regardless of which labeling method is used, high-affinity variants are selected using FACS to isolate cells that exhibit high levels of binding for a given amount of cell surface expression, as measured by a fluorescent antibody against the C-terminal epitope tag and a fluorescently labeled soluble ligand, respectively (Fig. 1).

Following each round of cell sorting, two paths can be taken: (1) the selected cells can be amplified in culture and sorted again to further reduce the library diversity to a smaller subset of clones with the highest affinity, or (2) DNA from the selected cells can be extracted and subjected to another round of mutagenesis (e.g., random mutagenesis or DNA shuffling [31]) to introduce additional diversity or combine potentially favorable mutations, followed by display of the new library and another round of cell sorting. The latter approach is termed “directed evolution,” and incorporates multiple rounds of mutagenesis and library screening to iteratively evolve proteins with the desired binding characteristics. After the library size is reduced to a smaller pool of high-affinity proteins, the concentration of soluble ligand can be lowered (while still avoiding ligand depletion) to adjust the resolution between the weaker and tighter binding proteins and further aid in selection of variants with the highest affinity. Typically, multiple rounds of mutagenesis and/or library sorting are applied to isolate high-affinity variants with equilibrium dissociation constants in the low nanomolar to picomolar range.

Following library sorting, individual variants can be quantitatively analyzed for their binding properties while still tethered to the yeast cell surface [27, 28]. This is a major advantage of yeast display technology as it allows fast, quantitative comparisons of binding properties without the need for laborious soluble expression and purification of each protein. For this purpose, the enriched library pool is amplified by culturing and is then plated to isolate individual yeast clones that each expresses a single variant. These individual clones are induced for cell surface expression and tested for binding to varying concentrations of ligand typically ranging from tenfold above to tenfold below the expected  $K_D$  of the displayed variant. Equilibrium binding constants or dissociation kinetics are then determined by flow cytometry [27, 28]. Importantly, many studies have demonstrated that the  $K_D$  of a protein-protein binding interaction measured on the surface of yeast is essentially equal to the  $K_D$  measured using soluble proteins [32].

Using the general strategies outlined above numerous proteins have been engineered with enhanced affinities for their binding partners, including T cell receptors with greater than 100-fold enhanced affinity for a peptide/MHC ligand [33], epidermal growth factor (EGF) with 30-fold enhanced affinity for the EGF receptor [34], interleukin-2 (IL-2) with up to 30-fold enhanced affinity for the IL-2 receptor alpha subunit [35, 36], leptins with up to 60-fold enhanced affinity for the leptin receptor [37], an Axl receptor variant with 12-fold enhanced affinity for its ligand Gas6 (final  $K_D=2.7$  pM) [38], and a signal-regulatory protein  $\alpha$  (SIRP $\alpha$ ) variant with ~50,000-fold enhanced affinity for CD47 (final  $K_D=11.1$  pM) [39, 40]. Yeast display has also been applied to engineer and affinity mature numerous antibodies, including antibodies against cholera toxin [41], FITC [22], HIV-1 gp120 [42], hemagglutinin surface glycoprotein of the H1N1 virus [43], HER2/neu [44], T cell receptors [28], TNF- $\alpha$  [45], and a host of other targets (*see* ref. 46 for a review). In a classical demonstration of the technological capabilities afforded by yeast display, a fluorescein-binding antibody was engineered with a  $K_D$  equal to 48 fM and a dissociation rate greater than 1,000-fold lower than the parent antibody [30], representing one of the strongest protein binding interactions ever engineered and among the strongest found in nature.

Yeast display technology has also been used to engineer novel non-antibody protein binders against targets of interest. These so-called alternative scaffold proteins have been chosen for protein engineering applications based on positive attributes including stability, amenability to mutation, ease of expression and purification, and binding epitope surface area. Typically, the amino acid sequence of a contiguous solvent-exposed region of the scaffold is randomized to generate a “naïve” library, and the library is displayed on the surface of yeast and screened for binding to a target protein using FACS [47, 48]. A number of novel ligands have been engineered using this strategy, including cysteine knot peptides (knottins) that

bind to various integrins with  $K_D$  values in the picomolar to nanomolar range [47, 49–53], or that inhibit human matriptase-1 with picomolar to nanomolar inhibition constants ( $K_i$ ) [54]; human fibronectin 10th type III domain scaffold [55] variants that bind to a variety of protein targets [18, 56, 57], such as lysozyme, with  $K_D$  values in the nanomolar to picomolar range [29, 32]; green fluorescent protein variants that bind streptavidin-phycoerythrin, biotin-phycoerythrin, glyceraldehyde 3-phosphate dehydrogenase, and a neurotrophin receptor with  $K_D$  values of 70 nM, 190 nM, 18 nM, and 3.2 nM, respectively [58]; human kringle domain variants that bind death receptor 4 (DR4), DR5, or TNF- $\alpha$  with  $K_D$  values of 680 nM, 172 nM, and 29 nM, respectively [59]; and Sso7d protein variants from the hyperthermophilic archaeon *Sulfolobus solfataricus* that bind fluorescein, a peptide fragment from  $\beta$ -catenin, hen egg lysozyme, streptavidin, and chicken and mouse immunoglobulins with  $K_D$  values in the nanomolar to micromolar range [60].

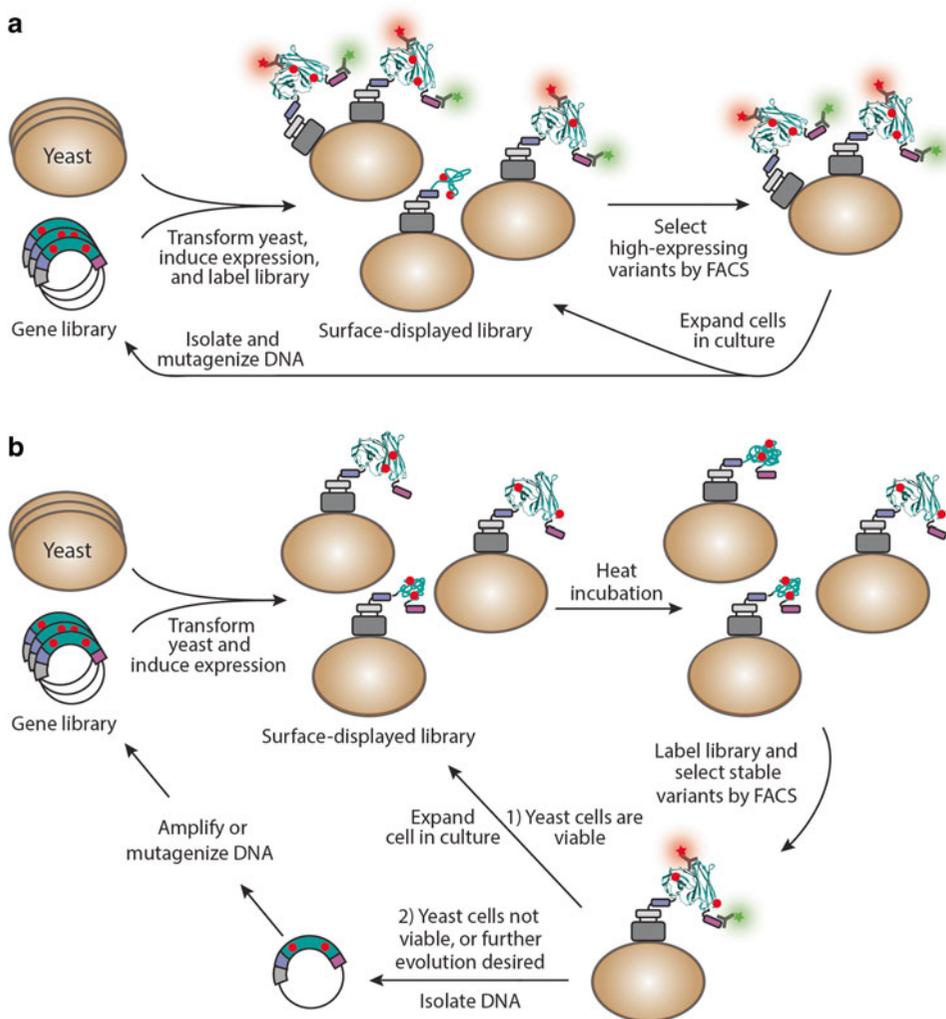
In many of the scaffold examples described above, the screening strategy identified a number of high-affinity ligands that bound to different epitopes of the target protein. Novel ligands have also been engineered to bind to a specific epitope of the target protein by first selecting all library variants that bind to a wild-type target protein, and then screening the selected pool of binders for variants that do not bind to an epitope-altered form of the target protein. As a recent example, a dengue virus-neutralizing antibody was engineered using yeast display by selecting antibodies from a library that bound to the wild-type viral envelope protein domain III, but not to a form of the target protein with a specific epitope mutated [61]. Importantly, this strategy is contingent on proper design of the mutant target epitope used for library screening. First, the target epitope must be sufficiently mutated such that ligands that bind to the wild-type epitope will not bind to the mutated form. Second, the mutation(s) must only affect the structure of the protein at the site of the target epitope and must not affect the global fold of the protein, as screening for epitope-specific binders using a completely misfolded mutant competitor would be futile.

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## 4 Engineering Proteins for Increased Stability

The stability of a protein generally refers to its ability to resist thermal and chemical denaturation and proteolytic degradation. High stability is a desired characteristic of proteins that are used for research, industrial, and therapeutic applications, and translates to longer shelf life, duration of activity, and in vivo activity. As with binding affinity, thermal stability can be analyzed while a protein variant is still tethered to the yeast cell surface, allowing for rapid, quantitative measurement of half-maximal denaturation ( $T_M$ ) values.

Three general strategies have been applied to engineer proteins with increased stability (Fig. 3). In each approach, a library on the order of  $10^7$  to  $10^9$  protein variants is generated by random mutagenesis and displayed on the surface of yeast as a fusion to the Aga2p cell wall protein.



**Fig. 3** Isolating high-stability protein variants from a yeast-displayed library by FACS. **(a)** Screening of stable protein variants based on their level of surface expression. Transformation of yeast with a mutant gene library generally results in display of properly folded and truncated protein variants that range in expression level, which can be used as a proxy for protein stability [62–64]. Cells are labeled with a fluorescent antibody (*green star*) against the *c-myc* epitope tag (*purple box*) and a ligand (*red star*) specific to the native fold of the displayed protein, and cells expressing the highest levels of properly folded variants are selected by FACS. **(b)** Screening of stable protein variants based on their ability to resist irreversible thermal denaturation. Following heat incubation and sorting of yeast displaying stable protein variants, viable cells can be expanded in culture for additional screening and/or analysis, whereas DNA from nonviable cells must be isolated and amplified for analysis or an additional round of yeast transformation and screening. Point mutations are shown as red circles, and colors indicating protein identity match those shown in Fig. 1. The displayed protein depicted here is an scFv [129] modified from PDB 1X9Q using the UCSF Chimera package [130]

The first strategy for stability engineering exploits a correlation between the yeast surface expression levels of properly folded proteins and their thermal stability [62–64] (Fig. 3a). For example, a library of single-chain T-cell receptor (scTCR) variants was expressed on the yeast surface and enriched for cells displaying the highest levels of properly folded protein as determined by binding to a conformationally specific antibody [65]. When individual protein mutants from this enriched pool of yeast were recombinantly expressed in soluble form and assayed, the most stable scTCR variant retained 80 % activity after incubation at 50 °C for 30 min, whereas the parent scTCR protein retained less than 10 % activity under the same conditions. In another example, yeast surface display and library screening were used to identify an epidermal growth factor receptor (EGFR) mutant with a  $T_M$  of  $61.0 \pm 1.3$  °C compared to a  $T_M$  of  $52.5 \pm 0.7$  °C for wild-type EGFR [66]. Similarly, yeast display was used to identify a single-chain class II major histocompatibility complex protein (scDR1 $\alpha\beta$ ) with a  $T_M$  of  $73.3 \pm 1.8$  °C, whereas display of the properly folded wild-type scDR1 $\alpha\beta$  protein was barely detectable [67]. This general strategy has been applied to enhance the stability of numerous other proteins and is reviewed in detail elsewhere [68].

Despite the successes described above, using surface expression level as a proxy for protein stability may be better suited for proteins with low inherent thermal stabilities. The correlation between expression level and protein stability is due, in part, to the quality control process that occurs in the endoplasmic reticulum (ER) during protein synthesis and posttranslational processing. The ER quality control mechanism ensures efficient export of properly folded proteins, whereas misfolded proteins are retro-translocated across the ER membrane and degraded in the cytosol [69, 70]. This process generally results in inefficient expression of unstable proteins that adopt a higher ratio of misfolded to native structures. However, the observed correlation between yeast surface expression level and stability is likely limited to proteins of low stability, as proteins above a certain stability threshold are generally expected to escape the ER quality control mechanism. In support of this assumption, variants of a highly thermostable three-helix bundle protein  $\alpha$ 3D with varying  $T_m$  values all above 80 °C showed no correlation between yeast surface expression level and thermal stability [71].

A second strategy for increasing protein stability involves application of heat stress (up to 85 °C for 10 min) directly to a yeast-displayed library prior to cell sorting (Fig. 3b) [65, 72, 73]. This approach may be better suited for proteins with higher inherent thermal stabilities, assuming that irreversible denaturation will occur under these experimental conditions. Yeast-displayed variants that resist thermal denaturation are discriminated by FACS, based on their ability to bind to a fluorescently labeled antibody or ligand specific to the native protein fold [67]. As an example, a library of

IgG1-Fc scaffold variants was displayed on yeast and subjected to heat stress (79 °C for 10 min) [21]. Stable IgG1-Fc variants that bound to a conformationally specific antibody or a soluble Fc $\gamma$  receptor after heat stress were enriched using multiple rounds of FACS, and isolated variants were analyzed for thermal stability. IgG1-Fc variants were identified with increased  $T_M$  values up to  $91.0 \pm 0.1$  °C, compared to a  $T_M$  of  $82.6 \pm 0.0$  °C for wild-type IgG1-Fc. Using this general strategy, a Her2/neu-binding antibody fragment variant was engineered with an increase in  $T_M$  from ~70 °C (parent IgG1-Fc) to ~75 °C and an increased resistance to aggregation [74]. Similarly, variants of a monomeric yeast-enhanced green fluorescent protein (GFPM) were engineered with three- to sixfold increased resistance to thermal denaturation at 70 °C [72]. Notably, increased thermal stability did not confer increased yeast-surface expression levels for the GFPM variants, which further supports the addition of a heat stress step to the library screening protocol when engineering proteins with high intrinsic thermostabilities. An important consideration is that although yeast cells remain intact and can be efficiently sorted by FACS even after heat stress (e.g., 72 °C for 90 min or 85 °C for 10 min) [73], their viability is compromised at temperatures above 42 °C [73]. Thus, after each round of heat stress and sorting, plasmid DNA from yeast cells should be isolated, amplified by PCR, and used to transform viable cells for a subsequent round of screening [20, 21]. Additionally, this strategy involving heat stress is only applicable to proteins/domains that denature irreversibly; refolding after heat stress would prevent discrimination between variants of different thermal stabilities.

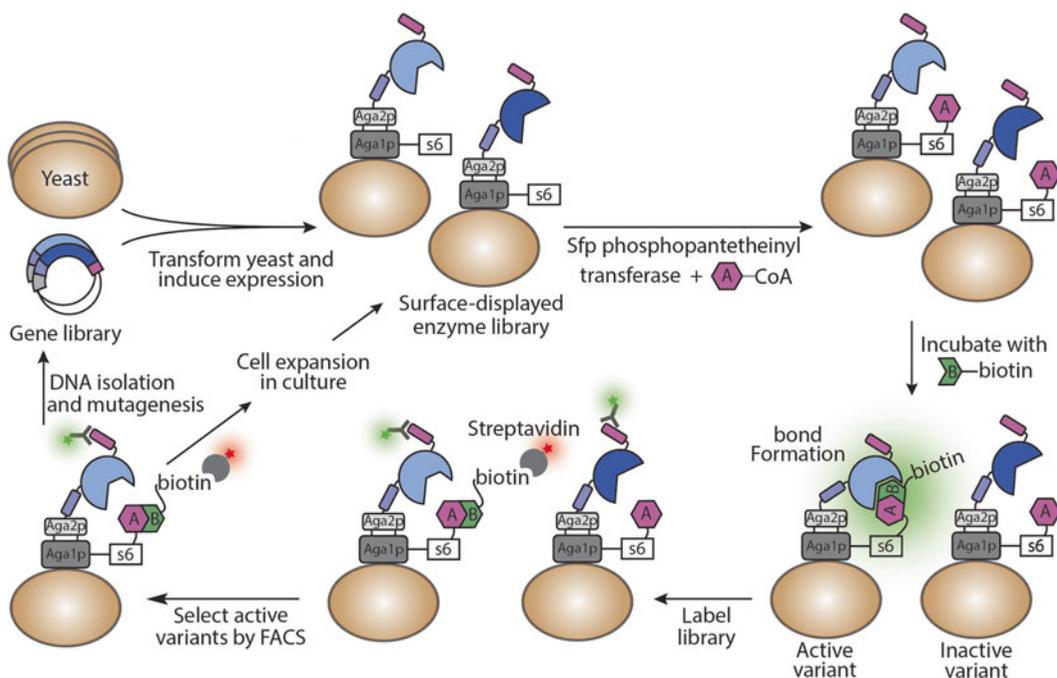
Alternatively, a third strategy exists for increasing protein stability that harnesses both the advantages of increased temperature and the quality control mechanisms of the ER to select stable variants. In this method, expression of surface-displayed protein is induced for 24 h at temperatures up to 37 °C (compared to 20 °C or 30 °C which is typically used) to shift the equilibrium of protein structures toward the misfolded state during protein synthesis and posttranslational processing in the ER while maintaining yeast cell viability. Generally, only proteins that are efficiently folded and processed at the elevated induction temperatures will avoid the ER quality control machinery and be efficiently exported to the cell surface. The library can then be sorted as described in the first strategy (Fig. 3a) to select variants with increased stabilities. For example, this strategy was applied to enhance the stability of an scTCR [65], and to engineer a hepatocyte growth factor fragment with a 15 °C increase in  $T_M$  and a 40-fold increase in expression yield relative to the wild-type protein fragment [75]. In many cases, increased thermal stability also correlates to increased recombinant expression of the soluble form of the protein relative to the wild-type protein [66, 67, 76–79], highlighting an additional benefit of these techniques for protein engineering.

## 5 Enzyme Engineering

Directed evolution is a powerful technique for enzyme engineering. However, developing a strategy for linking the genotype of an enzyme mutant to its phenotype (e.g., catalytic activity or substrate specificity) poses a difficult challenge since in most cases substrate turnover results in a product that is diffusible and not covalently bound to the surface of a phage or cell. Thus, lower throughput microtiter or colony-based screening methods ( $10^3$ – $10^4$  variants) have historically been used for enzyme engineering [80–82]. Several alternate techniques have recently been developed to address these challenges, including oil-water emulsion methods that encapsulate genetic material, the translated enzyme, and its catalyzed product into droplets that can be sorted by flow cytometry [83, 84].

More recently, unique strategies incorporating yeast display have allowed libraries of up to  $10^8$  enzyme variants to be screened for increased activity and substrate specificity using FACS. In this approach, yeast cells are fluorescently labeled as a result of enzymatic activity, which allows discrimination of enzyme variants based on their level of activity and/or specificity. In one example, a library of horseradish peroxidase (HRP) enzyme variants was differentially labeled based on substrate specificity [85]. The library was incubated with a fluorescently labeled substrate, which produced a functionalized fluorophore by-product that covalently attached to tyrosines on the yeast cell surface. As a result, cells displaying the most active HRP variants were labeled with higher levels of fluorophore and subsequently selected by FACS. Using this strategy, an HRP variant with eightfold altered enantioselectivity was evolved using a combination of positive and negative selection [85]. A similar strategy was applied to evolve HRP variants with increased selectivity toward either substrate enantiomer by up to 2 orders of magnitude [86]. In another example, a general strategy for evolving bond-forming enzymes was developed (Fig. 4) and applied to identify a bacterial transpeptidase sortase A enzyme with a 140-fold enhancement in catalytic activity [87]. Yeast display has been applied to enhance the activity and/or substrate selectivity of a variety of other enzymes, including firefly luciferase [88], *Rhizomucor miehei* lipase [89], the adenylation domain of a nonribosomal peptide synthetase [90], *E. coli* lipoic acid ligase [91], and a tobacco etch virus protease [92]. In general, the success of these strategies was contingent on the enzyme substrate being labeled with an affinity handle or fluorescent probe. Thus, yeast display methodology is currently limited to engineering a subset of enzymes, as not all will be tolerant to such substrate modifications.

Finally, many enzyme engineering examples employ site-directed saturation mutagenesis [93] rather than random



**Fig. 4** A general strategy for selecting bond-forming enzyme variants with increased catalytic activity from a yeast-displayed library [87]. A library of enzyme variants is generated and displayed as a fusion to Aga2p, and a reactive peptide handle (s6) is fused to Aga1p. Sfp phosphopantetheinyl transferase covalently links CoA-conjugated enzyme substrate A to the s6 peptide handle, where it is accessible by the displayed enzyme. Subsequently, enzyme substrate B linked to an affinity handle (shown here as biotin) is incubated with the library, resulting in A–B bond formation catalyzed by active enzyme variants. Addition of a fluorescently labeled anti-epitope tag antibody (*green star*) and an affinity agent that binds to the handle on substrate B permits discrimination of enzyme variants with increased catalytic activity by FACS. Next, selected pools of yeast can be amplified for analysis or an additional round of screening, or their DNA can be extracted, subjected to mutagenesis, and used to transform new cells for further directed evolution. Application of this strategy is limited to bond-forming enzyme-substrate systems that remain functional when the substrates are tethered to the s6 peptide and an affinity handle

mutagenesis to generate protein libraries. This directed mutagenesis restricts the amino acid search space to a particular region of interest of the enzyme (typically proximal to the active site), and allows the investigation of every possible combination of mutations at selected amino acid positions. However, the maximum number of these positions one can exhaustively investigate is still limited by the throughput of the FACS instrument used to screen the library. For example, a library comprising every possible combination of amino acids at 6 or 7 sites in an enzyme ( $20^6$  or  $20^7$  variants total) would require  $\sim 0.4$  or 7 h to sort at 50,000 cells/s, respectively.

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## 6 Additional Applications

Yeast display has been used for other purposes besides protein engineering, briefly described below, including protein epitope mapping, identification of protein-protein interactions, and generation of “armed” yeast cells for a variety of applications.

Knowledge of the critical contact sites of a protein binding pair that govern their affinity can be advantageous. Two methods for identifying these contact sites, called domain-level [94] or fine [95] epitope mapping, have been developed using yeast display and applied to a wide range of protein binding pairs. For domain-level epitope mapping, individual domains from one of the binding proteins are displayed on yeast and screened for binding to a soluble version of the other binding partner [94]. In addition, the competitive binding of two ligands has been tested to identify ligands that share overlapping binding epitopes. In contrast, fine epitope mapping has been used to identify specific amino acids at the binding interface that directly contribute to the binding affinity [95]. For this method, a protein library is generated using random mutagenesis, displayed on the surface of yeast, and incubated with its wild-type binding partner. Cells displaying weak-binding proteins are then selected and their encoding DNA is sequenced to identify consensus amino acid sites that substantially influence the affinity of the protein pair, and thus are suggestive of the binding interface location. These strategies have been applied to map the binding epitopes of EGFR-specific antibodies [94, 95] and engineered EGFR-specific scaffold proteins [96], antibodies against H1N1 [43] and H5N1 [97], virus hemagglutinin surface glycoprotein, gp120-binding antibodies [98], and other binding pairs as reviewed elsewhere [99, 100].

Yeast display has also been applied to identify new protein-protein interactions. For example, an adult human testis cDNA library was displayed on yeast and screened for binding to phosphorylated peptides derived from autophosphorylation sites of EGFR and focal adhesion kinase (FAK) [101]. As a result, binding interactions were discovered between autophosphorylated EGFR sites and the SH2 domains of adapter protein APS and phosphoinositide 3-kinase regulatory subunit 3, as well as between autophosphorylated FAK sites and the SH2 domains of SH2B, tensin, and adapter protein APS [101]. Similarly, screening of a yeast-displayed human proteome library for binding to a mesothelioma-targeting single-chain variable antibody fragment (scFv) identified the specific cell surface antigen targeted by the scFv [102]. Yeast display has also been applied to identify interactions between proteins and small molecules. For example, a human

cDNA library comprising  $2 \times 10^7$  cDNA fragments from multiple human tissue samples was displayed on yeast and screened for binding to biotinylated phosphatidylinositides [103]. As a result, known interactions with pleckstrin homology domains and a phosphotyrosine-binding domain were identified, and a novel interaction with a fragment of apolipoprotein H was discovered [103]. These and other protein-protein interactions identified using yeast display have been reviewed elsewhere [99, 100].

Divergent from protein engineering, yeast display technology has been used to functionalize yeast cells for a variety of biotechnology and biomedical applications, including generation of whole-cell biocatalysts, antimicrobial agents oral vaccines, and for biosorption of various metals. In one prominent example, microbial conversion of cellulosic biomass into fuels gained substantial interest as a means of establishing a renewable energy source and an alternative to petroleum-based fuel production. Yeast display is an attractive technology for generating biofuel from cellulosic material, as it enables enzyme production, cellulose hydrolysis, and fermentation all in one step by localization of cellulolytic, amylolytic, and xylanolytic enzymes at the yeast cell surface. *S. cerevisiae* has been engineered using yeast display technology to convert cellulosic material into bioethanol (for reviews, see refs. 104, 105). Specifically, yeast cells were engineered to co-display endoglucanase II and  $\beta$ -glucosidase enzymes, and directly fermented 45 g of  $\beta$ -glucan per liter of media to produce 16.5 g of ethanol per liter in approximately 50 h [106]. The ratio of grams of ethanol produced to grams of  $\beta$ -glucan utilized was 0.48 g/g (or 93.3 % of the theoretical yield). Yeast co-displaying xylanase and  $\beta$ -xylosidase directly fermented xylan from sulfuric acid hydrolysate of wood chips [107], and yeast co-displaying glucoamylase and  $\alpha$ -amylase directly fermented raw cornstarch [108]. Display of minicellulosomes on the surface of yeast for bioethanol production has also been achieved by simultaneously binding dockerin-tagged endoglucanase, exoglucanase, and  $\beta$ -glucosidase enzymes to Aga2p-scaffoldin protein fusions [109–112]. Yeast cells have also been engineered as whole-cell biocatalysts for various other applications. For example, yeast displaying *Rhizopus oryzae* lipase were used as whole-cell biocatalysts to generate biodiesel from methanol and soybean oil [113]; yeast displaying *Geotrichum* sp. lipase were used to enrich docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) from fish oil [114]; yeast displaying *Corynebacterium diphtheriae* sialidase were used to transfer sialic acids for glycoprotein remodeling [115]; and yeast displaying glucose oxidase were used for electrochemical glucose sensing [116]. Yeast have also been functionalized with antimicrobial peptides [117, 118], pathogenic proteins for oral vaccine delivery [119–121], and metal-binding proteins for bioadsorption of various metals [122–128]. These and other examples have recently been reviewed [69].

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## 7 Conclusion

Yeast surface display is an effective tool for protein and cellular engineering, and has facilitated countless applications in research, biotechnology, and medicine. In contrast to other technologies such as ribosome and phage display, yeast display offers compatibility with flow cytometric analysis, enabling quantitative on-cell measurements of protein expression level, stability, affinity, and specificity without the need for soluble protein expression and purification steps. Additionally, unlike bacterial and phage display, yeast display provides a eukaryotic expression system capable of producing complex mammalian proteins containing multiple disulfide bonds. This unique combination of advantages has established yeast display as a leading technology for engineering protein stability, expression, and binding interactions, and as an emerging technology for high-throughput enzyme engineering and yeast cell engineering. Furthermore, other than requiring a FACS instrument for quantitative library screening and analysis, yeast display employs standard laboratory equipment and materials for microbial transformation and culture, and libraries can be readily generated and screened by users within a matter of weeks. As the use and applications of yeast display continue to rapidly expand, it will be exciting to see the advances this powerful technology delivers in the years to come.

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## Acknowledgements

Gerald M. Cherf is supported by the National Cancer Institute of the National Institutes of Health under Award Number F31CA186478, and funding from the Stanford Bioengineering Department. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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