Leveraging yeast sequestration to study and engineer post-translational modification enzymes.

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Abstract

Enzymes that catalyze post-translational modifications of peptides and proteins (PTM-enzymes) – proteases, protein ligases, oxidoreductases, kinases, and other transferases - are foundational to our understanding of health and disease and empower applications in chemical biology, synthetic biology, and biomedicine. To fully harness the potential of PTM-enzymes, there is a critical need to decipher their enzymatic and biological mechanisms, develop molecules that can probe and reprogram them, and endow them with improved and novel functions. These objectives are contingent upon implementation of high-throughput functional screens and selections that interrogate large sequence libraries to isolate desired PTM-enzyme properties. This review discusses the principles of *S. cerevisiae* organelle sequestration to study and engineer PTM-enzymes. These include methods that modify yeast surface display and employ enzyme-mediated transcription activation to evolve the activity and substrate specificity of proteases and protein ligases. We also present a detailed discussion of yeast endoplasmic reticulum (ER) sequestration for the first time. Where appropriate, we highlight the major features and limitations of different systems, specifically how they can measure and control enzyme catalytic efficiencies. Taken together, yeast-based high-throughput sequestration approaches significantly lower the barrier to understanding how PTM-enzymes function and how to reprogram them.

Introduction

Protein post-translational modifications (PTMs) largely regulate the functional proteome. Thus, the enzymes that catalyze these transformations (PTM-enzymes) are central to understanding cellular processes in health and disease. Aside from their physiological roles, PTM-enzymes are foundational to biotechnology and synthetic biology because they catalyze chemo-, regio-, and sequence selective PTMs of user-defined proteins and peptides. Synthetic PTMs can impart new functions to biomolecules, introduce probes and labels, and establish customized genetic and protein circuits in living cells. With their diverse sequence specificities, PTM-enzymes enable technologies in proteomics, biorthogonal chemistry, and biochemical and cellular imaging. For instance, proteases with broad specificity have transformed mass spectrometry-based proteomics (Giansanti et al., 2016; Schräder et al., 2017; Tran et al., 2016). Contrariwise, proteases with narrow specificity are heavily used in recombinant protein purification (Waugh, 2011), drive protein-based synthetic circuits in cells (H. Kay Chung & Michael Z. Lin, 2020; Fink & Jerala, 2022; Gao et al., 2018), and are being engineered as potential enzyme therapeutics (Shankar et al., 2021). Transpeptidases such as sortases (Pishesha et al., 2018) and asparaginyl endopeptidases allow one to generate multifunctional protein conjugates that can serve as therapeutics and imaging agents (Morgan et al., 2022). Finally, enzymes that modify specific amino acid side chains, including ligases, transferases, oxidoreductases, and Spycatcher/Spytag systems (Reddington & Howarth, 2015), realize the formidable possibilities of protein composability and interfacing with a variety of materials (Rashidian et al., 2013).

With this armamentarium of possibilities, PTM-enzymes represent a treasure trove for chemical biologists seeking to leverage them for fundamental and applied research. From a biomedical and drug discovery

point of view, molecules and other modalities that can control these enzymes can lead to therapeutics and diagnostics reagents. From a chemical biology and biotechnology point of view, there remains a need to discover new PTM-enzymes and improve the properties of existing ones for specific applications. In this vein, high-throughput protein engineering and screening platforms are essential to harness the possibilities PTM-enzymes can offer fully. Specifically, high-throughput platforms allow one to profile PTM-enzyme activities, identify their physiological substrates, screen inhibitors against specific PTM-enzymes, and use protein engineering to improve existing properties or introduce novel activities.

Many proteomic (Johnson et al., 2023; Schilling & Overall, 2007) and genetic platforms (Dyer & Weiss, 2022) are now available to study and engineer PTM-enzymes. Mass spectrometry-based methods based on protein labeling are essential to assigning PTM-enzyme cellular substrates and have been primarily applied to proteases and kinases. Genetic platforms typically introduce the PTM-enzyme(s) of interest in a heterologous host and devise a screening or selection method to assay enzyme activities. Notable examples in bacterial hosts include phage-assisted continuous evolution (Blum et al., 2021) and an OmpT-linked bacterial surface display platform to engineer protease specificity (Ramesh et al., 2019; Varadarajan et al., 2009; Varadarajan et al., 2005), and an *E. coli* platform wherein cell survival is linked to protease inhibition (Lopez et al., 2019). Over the past two decades, yeast, particularly*Saccharomyces cerevisiae* , has been a prominent organism in PTM-enzyme investigations, in addition to its contribution as a model of human diseases involving aberrant PTMs. *S. cerevisiae* has countless genetic tools for controlling gene and protein expression and editing its genome. Furthermore, our ability to generate large DNA libraries in this organism enables protein engineering and directed evolution campaigns. Yeast offers distinct advantages over bacterial systems for studying PTM enzymes. These include expressing active human PTM-enzymes and building spatially and temporally controlled genetic and protein circuits empowered by organelle compartmentalization. By taking advantage of yeast's many protein sequestration mechanisms, one can build high-throughput screening and selection platforms to engineer and profile the substrate specificity of PTM-enzymes and screen for their inhibitors.

This review discusses the principles and latest applications of *S. cerevisiae* organelle sequestration to study and engineer PTM-enzymes, particularly proteases and protein ligases. These include methods that modify yeast surface display and employ enzyme-catalyzed transcription activation. Lastly, we discuss yeast endoplasmic reticulum (ER) sequestration, one of the more powerful methods to engineer PTM-enzymes. Where appropriate, we highlight the major differences between these approaches, including how they can measure and control enzyme catalytic efficiencies.

Yeast surface display repurposed for PTM-enzyme engineering.

1.1 Directed evolution of bond-forming enzymes.

Since its initial development in 1997, yeast surface display (YSD) has been a staple technology for studying, measuring, and evolving biomolecular recognition (Gai & Wittrup, 2007; Raeeszadeh-Sarmazdeh & Boder, 2022). YSD is attractive because many eukaryotic proteins can be functionally expressed, secretory pathway engineering and protein expression optimizations can achieve surface display of complex proteins and assemblies (Lamote et al., 2023), and surface-displayed phenotypes can be selected by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS). The most mature YSD method is the AGA1p-AGA2p system, which takes advantage of the naturally occurring disulfide bonded interactions between the a-agglutinin 1 and 2 subunits on the yeast cell surface. Originally developed for engineering protein binding affinity, YSD has been modified in several ways to measure, control, and evolve enzyme activities (reviewed extensively elsewhere). However, assaying PTM-enzymes on the yeast surface poses specific challenges compared to other biocatalysts. First, proteases, transglutaminases, and sortases, among others, are often synthesized as inactive zymogens, requiring post-translational activation. Expressing PTM-enzymes in their active forms can be toxic if they modify essential host proteins. Second, pro-sequences can sometimes be necessary for enzyme folding (Chang et al., 2018), and their activation may depend on other enzymes or conditions not present in the yeast secretory pathway (Zhao et al., 2014). Lastly, since PTM-enzymes react on proteins and peptides and may require two or more substrates, it is necessary to deliver these substrates in a way that safeguards the genotype-to-phenotype linkage.

Creative designs circumvent and, at times, leverage these conditions to engineer bond-forming enzymes on the yeast surface, particularly sortases. The common strategy involves co-localizing a sortase and one of its substrates on the yeast surface and exogenously supplying the second substrate, which carries a handle for fluorescence detection or magnetic selection (Figure 1A). For example, one can fuse a sortase and its substrate to the 5' and 3' end of the *aga2* gene, respectively (Lim et al., 2017). When supplied with an azide-functionalized primary amine nucleophile, sortase conjugates this molecule to its sorting signal LPETGG. A copper-free Click reaction between the incorporated azide and biotin-DBCO and subsequent labeling with streptavidin conjugated to phycoerythrin allows them to visualize sortase activity on the yeast surface by flow cytometry.

A more elaborate strategy to engineer bond-forming enzymes on the yeast surface (Chen et al., 2011) involves fusing the enzyme to the C-terminus of AGA2p, and connect an S6 peptide to the C-terminus of AGA1p (Chen et al., 2011). The S6 peptide reactive handle is a substrate for Sfp phosphopantetheinyl transferase from *Bacillus subtilis* , which catalyzes the transfer of a CoA-activated peptide to the S6 peptide on the yeast surface (Figure 1B). Liu and coworkers developed this system to engineer *Staphylococcus aureus* sortase A (SrtA) variants with increased activity on the native substrate LPETGG. In this case, a biotinylated oligoglycine nucleophile is supplied to yeast cells expressing sortase A variants and an S6 peptide-attached LPETGG substrate, allowing sortase variants to perform the transpeptidation reaction. This method obtained evolved sortases with up to 140-fold increased catalytic efficiencies in LPETG-coupling reactions. These variants showed significant improvements in target recognition and kinetics of LPETGG-containing CD154 on the surface of HeLa cells. In a subsequent study, *S. aureus* sortase A was evolved towards non-canonical substrates LAXTG and LPXCG, resulting in two variants with 51,00-fold (eSrtA(2A-9), LAXTG) and 120-fold (eSrtA(4S-9), LPXCG) switches in specificity (Dorr et al., 2014). Importantly, thek_{cat}/K_M of the two eSrtA variants were comparable to that of the original eSrtA on LPXTG. This shows that even under single turnover conditions, sortase directed evolution is possible on the yeast surface.

More recently, Liu took the bold step of engineering *S. aureus*sortase A to recognize an LMVGG sequence found in amyloid-β, which differs from the canonical LPXTG at three positions (Podracky et al., 2021). Starting from a variant with relaxed specificity (4S.6), they tailored a directed evolution scheme that included lowering the substrate concentration, altering selection conditions, and introducing various decoy off-target substrates. The resulting variant SrtAβ, obtained after 16 rounds of evolution, contained an additional 25 mutations compared to 4S.6 and exhibited over a 1,400-fold change in substrate preference from LPESG to LMVGG. This modified preference toward this target substrate provides the basis for using sortase variants, particularly SrtAβ, as potential targeting agents to prevent protein aggregation and related neurological diseases.

To engineer microbial transglutaminases (mTGs) and mitigate cytotoxicity associated with their active forms, Kolmar and coworkers developed a method to display an inactive mTG followed by activation via synthetic pro-sequence cleavage (Deweid et al., 2018). mTGs can crosslink a side chain of glutamine and primary amine (usually the ε-amino group of lysine) via a transamidation reaction and are useful for site-specific protein conjugations (Dickgiesser et al., 2019). To enhance the activity of mTG, a synthetic pro-mTG was fused to the N-terminus of AGA2p. Once on the surface, mTG could be activated with an enterokinase. When supplementing a biotinylated glutamine-donor peptide, the activated mTG could use its surface lysines as acyl acceptors, catalyzing their labeling, thus enabling the selection of tagged cells by FACS (Figure 1D). This method could isolate mTG variants with up to 36% increased catalytic efficiencies. One could argue that this method also selected for mTG variants that remain stable with their surface lysines increasingly modified.

1.2 Protease engineering on the yeast surface requires a unique approach.

Assaying bond-forming enzymes on the yeast surface is practical because these reactions yield a product with a labeled moiety. In contrast, engineering proteases in a similar manner to sortases is not a straightforward assay since proteolytic cleavage results in signal loss. To circumvent this obstacle and enable protease engineering and substrate profiling directly on the yeast surface, Hollfelder and coworkers developed a bait-

and-capture YSD approach using the bacterial macromolecule alpha-2-macroglobulin (A2M) (Knyphausen et al., 2023). A2M is a naturally occurring broad-spectrum protease inhibitor. The mechanism of inhibition relies on a protease bait region and a buried receptor binding domain (Figure 1C). When a protease cleaves the bait region, an A2M conformational rearrangement results in the physical trapping of the protease and the exposure of a reactive thioester bond which may result in the formation of a covalent bond between A2M and protease lysine residues. Hollfelder showed that an active target protease on the yeast surface could be trapped by an A2M protein modified to present the protease's target sequence, $A2M^{cap}$. This approach allowed them to evolve an SpIB variant that does not require removal of an N-terminal pro-sequence for activation, generating an enzyme that can more easily integrate synthetic biology and other applications. Lastly, a SCHEMA-based subdomain shuffling library of SplA-F followed by MACS and FACS selections led to a chimeric scaffold that supports specificity switching via subdomain exchange. Out of the 7 subdomains, subdomain 5 was found to confer most of the substrate specificity in Spl chimeras. Therefore, a diversification strategy focused on block 5 may be a good starting point for substrate specificity engineering in the Spl scaffold. Moreover, A2M capture is mechanism agnostic, meaning that it could be applied to many proteases and drive forward protease engineering for therapeutic applications.

Proteolysis-mediated protein activation.

Proteases with bespoke specificities are essential reagents in biotechnology and could usher in a new wave of enzyme therapeutics. Moreover, the principles of proteolysis-mediated nuclear translocation of transcription factors or protease-mediated activation of a toxic protein, common in protease-based synthetic circuits (H. K. Chung & M. Z. Lin, 2020), are used to build high-throughput screening and selections in yeast. To construct a protease-based transcriptional output, one can sequester a transcription factor away from the nucleus by confining it to the cytoplasm or attaching it to the inner plasma membrane via a linker harboring a protease-cleavage sequence. Once cleaved by the protease of interest, the transcription factor localizes to the nucleus to activate a reporter or an antibiotic resistance gene.

This concept was first illustrated with the Genetic Assay for Site-specific Proteolysis (GASP) system (Sellamuthu et al., 2011). Here, a lexA-b42 transcription is fused to the *Ste2* transmembrane domain via a peptide linker containing a polyQ sequence found in the Huntingtin protein (Figure 2A). This setup was used to engineer the substrate specificity of Hepatitis A virus 3C Protease to cleave polyQ. Active site saturation mutagenesis isolated mutants around the S2 and S1' subsites with improved activity towards polyQ. While these mutants largely showed relaxed rather than switched specificity, the Var26 variant prevented polyglutamine-induced neuronal cell death. Ting and coworkers built upon this concept to establish a significantly more advanced strategy to engineer proteases using blue light-induced protein-protein complementation between CRY and CIBN (Sanchez & Ting, 2020). Their design anchors a fusion protein composed of STE2Δ-CIBN-LOV-ProteaseCleavageSite-VP16 to the inner membrane (Figure 2C). The protease is expressed in the cytosol as a C-terminal fusion to the CRY protein. Under blue light, the CRY and CIBN proteins interact, bringing the protease closer to its substrate. Furthermore, access to the substrate is only possible because the LOV domain undergoes a conformational change, exposing the protease cleavage site. Protease cleavage releases the VP16 transcription factor, allowing it to migrate to the nucleus to activate the transcription of a fluorescent protein. Optogenetic protein circuits provide increased temporal resolution from seconds to hours, allowing one fine control over protein-protein interactions. This approach was applied to engineering TEV proteases with increased catalytic activity. After several rounds of evolution and selection stringency manipulations, Ting evolved high-affinity TEV proteases, including truncated TEV variants, uTEV1Δ, and uTEV2Δ, with turnover rates over 5-fold higher (compared to WT TEVΔ) and a full-length TEV protease, uTEV3 (2.5-fold faster than the WT enzyme). The increased catalytic efficiency of truncated variants improved SPARK (Kim et al., 2017) and FLARE (Wang et al., 2017), two transcription-based time-gated transcriptional readouts previously established by the Ting lab. Integration of new variants into these systems resulted in increased temporal control (uTEV1Δ responding to stimuli in <1 minute in SPARK, compared to the original design requiring >10 minutes) and overall enzymatic performance (uTEV1Δ observed to have increased signal-to-noise ratios 27-fold higher than original FLARE system).

A common limitation of protease-activatable transcriptional activation for protease engineering is the lack of a counterselection substrate. When evolving proteases for switched specificity, selecting against undesired cleavage sequences is imperative to avoid variants with relaxed specificity. Tucker and coworkers aimed to overcome this limitation by designing a proteolysis-dependent transcription factor inspired by hormone-inducible synthetic TFs (Cleveland et al., 2022). Their best design incorporated a counterselection substrate in the following fusion protein: estrogen receptor ligand binding domain-Gal4 binding domain-counterselection substrate-VP16 activation domain-selection substrate-estrogen receptor ligand binding domain, abbreviated as ER-LBD-GAL4BD-CS-VP16-SS-ER-LBD (Figure 2B). Without protease activity, this fusion protein resides in the cytosol as the ER-LBD remains bound to cytosolic Hsp90. Protease cleavage of the CS separates GAL4BD from VP16, resulting in an inactive transcription factor. Conversely, cleavage of the SS leads to the removal of one ER-LBD. This activity is enough to translocate the ER-LBD-GAL4BD-CS-VP16 to the nucleus, where it can activate a GAL-driven URA3 marker, leading to cell survival on Sc-URA plates. This approach was applied to evolve the cleavage specificity of Botulinum Neurotoxin Serotype B1 proteases (BoNT/B) away from its native sequence (VAMP3) towards an orthogonal substrate, specifically a VAMP3 sequence with a mutation at residue 59. They co-transformed a structure-guided triple mutant NNK library with 14 VAMP3 Q59 mutants. After URA3 selection, five mutants were obtained, including the promising RRG variant, which showed 10% cleavage of WT VAMP3, but 75% cleavage of VAMP3(Q59Y). However, the stringency of the counterselection cannot be tuned independently from the selection substrate, lowering the dynamic range this strategy can achieve.

Yeast ER sequestration accurately quantifies PTM-enzyme catalytic turnovers.

Engineering PTM-enzymes on the yeast surface or through transcriptional ON switches can suffer from design constraints that limit the extent to which PTM-enzyme catalytic properties can be modified. Transcription turn-on systems may require significant optimizations to establish a high dynamic range since a single protease turnover turns on a TF that catalyzes transcription exponentially. Similarly, a single turnover of a PTM-enzyme on the yeast cell surface is enough to generate a fluorescent signal. Therefore, in these platforms, increases in catalytic efficiency are driven mainly by improvements in substrate binding affinity. In cases where an increase in catalytic turnover (k_{cat}) is the goal, there is a need for a modified approach. In this vein, two technologies have been developed that leverage ER sequestration to engineer PTM-enzymes for multiple turnovers: the Yeast Endoplasmic Reticulum Sequestration and Screening (YESS) and the FRET-based protease evolution via cleavage of an intracellular substrate (PrECISE) systems. ER sequestration approaches, particularly ones based on the YESS approach, can give fine-tuned control in protein investigations.

3.1 YESS for protease engineering.

The Yeast Endoplasmic Reticulum Sequestration and screening (YESS) system allows one to accurately control and quantify catalytic turnovers and select variants solely based on these activities (Figure 3A). The YESS system leverages yeast surface display in a clever way, where the activity of the PTM-enzyme can be measured on the yeast cell surface, while the enzyme remains in the ER. One can visualize the YESS system as a flow reactor with an enzyme anchored in the ER, and the substrate cassette, destined for cell surface display, is modified as it travels through the ER and secretory pathway. Once on the surface, substrate modifications can be visualized using fluorescently labeled antibodies. Cells harboring the desired modifications can be isolated by FACS or MACS. Originally reported ten years ago, the YESS system consists of two components. The first is to target and partially retain two or more transcriptional cassettes, one for the enzyme, and the other for the enzyme's substrate(s), by appending an ER signal peptide to the 5' and an ER retention sequence (ERS) to the 3' end of each coding sequence, respectively. The second is to design a substrate cassette, typically fused to the C-terminus of AGA2p. The substrate cassette is the activity reporter and can be a short sequence flanked by epitope tags or a full protein. For protease engineering, YESS allows one to incorporate both counterselection and selection substrates, a vital attribute to avoid engineering protease generalists. Lastly, polypeptide retention in the ER can be manipulated by changing ERSs with weak or strong binding affinities to ER receptors. This way, the stoichiometry and

contact time between an enzyme and its substrate can be readily titrated. While the YESS system presents many moving parts, it enables unprecedented control of ER-localized manipulation of enzyme activities at the transcriptional and post-translational levels.

In its initial development, YESS was used to evolve TEV proteases with orthogonal P1 specificities (Yi et al., 2013). Yi and coworkers employed a library-against-library screening approach, where an S1 pocket saturation mutagenesis of TEV protease and a substate cassette containing the native substrate as counterselection ENLYFQS and ENLYFXS as a selection substrate library (saturation mutagenesis at P1 position) were interrogated simultaneously. After several rounds of sorting and further error-prone engineering, screening, and analysis homed in on two TEVp protease variants, PE10 and PH21. TEV-PE10 and TEV-PH21 showed 5,000-fold and 1,100 switches in substrate specificity toward a P1 Glu and P1 His, respectively. Furthermore, the authors show that removing the ERSs from the substrate and protease cassettes allowed them to evolve a faster TEV protease on its canonical substrate, TEV-Fast, with a 4.6-fold increase in catalytic efficiency.

3.2 Protease substrate specificity profiling using YESS.

Because all its components are DNA-encoded, the YESS system offers a platform capable of performing three high-throughput experiments: enzyme engineering, substrate specificity profiling, and mutational scanning. Performing each experiment would typically require three different technologies. For instance, one can engineer a protease and profile the substrate specificity of evolved variants during the engineering campaign. Furthermore, when combined with next-generation sequencing and deep learning, YESS can map the substrate specificity landscape of proteases (Figure 3B).

To optimize YESS for protease substrate specificity profiling, Qing and coworkers sought to analyze and remove major endogenous proteolytic events in the yeast secretory pathway, which could convolute analysis of cleavage specificities of recombinantly expressed proteases (Li et al., 2017). Screening a DNA-encoded pentapeptide library revealed that a secretory pathway protease cleaved many arginine and lysine-containing sequences. This protease was identified as the Golgi resident*kex2* protease, with a major cleavage pattern of Ali/Leu-X-Lys/Arg-Arg. These results helped generate a *kex2*knockout yeast strain, a superior strain to profile the substrate specificity of proteases, particularly ones with trypsin-like cleavage patterns.

Predicting PTM-enzyme substrate specificity is essential for designing specific activity probes and inhibitors, inferring physiological substrates, and guiding PTM-enzyme substrate specificity engineering. The main obstacle to overcome in enzyme-substrate specificity profiling is undersampling. Substrate specificity is relative, and for promiscuous enzymes, it is better defined when more substrates are interrogated. Unfortunately, even the largest substrate libraries generated with yeast or phage display ($>10^9$ unique sequences) only sample a fraction of possible amino acid combinations in a heptapeptide library. Machine learning can overcome this bottleneck, and the DNA-encoded substrate libraries in the YESS system provide the sequence-function datasets to build ML models for substrate specificity prediction. Khare and coworkers judiciously showed that combining the YESS system, computational modeling, and machine learning allows one to entirely map the P6-P2 substrate specificity and energetic landscape of HCVp (Pethe et al., 2019). They sorted a naïve pentapeptide library spanning the P6 to P2 sites of HCVp and selected three distinct populations by FACS: uncleaved, partially cleaved, and completely cleaved sequences. They showed that fully and partially cleaved sequences form separate clusters and that one can map sequence preference trajectories by single substrate mutation tracking within the data. To predict the cleavability of the entire pentapeptide library diversity (3.2 million sequences), they implemented a support vector machine method trained on energetic features of experimentally derived sequences obtained from Rosetta modeling. This approach allowed them to reconstruct the pentapeptide substrate landscape completely. Most importantly, they discovered and characterized a novel cleavage pattern (PSTVF) in addition to the four previously known HCVp cleavage specificities. This deep analysis could be tailored to any PTM-enzyme and its variants (including drug-resistant mutations) to explore sequence and structure landscapes of enzyme-substrate interactions not possible with experiments alone. One obvious next step would be to leverage machine learning and substrate profiling to infer physiological substrates as a complement to more expensive proteomics approaches such as SILAC.

3.3 PTM-enzyme assays beyond proteases.

In principle, any PTM-enzyme whose activity can be detected on the yeast cell surface and whose activity is minimally obstructed by an endogenous enzyme can be assayed in YESS. Thus, YESS-based enzyme substrate profiling has extended to tyrosine kinases and histone acetyltransferases. Taft and coworkers first showed that tyrosine phosphorylation could be measured in YESS and performed two interesting experiments (Taft et al., 2019). First, they profiled the substrate specificity of human SRC, LYN, and ABL kinases and implemented a machine-learning algorithm utilizing amino acid covariances to predict ABL1 kinase peptide substrates. Second, and most importantly, they used YESS to screen a randomized library of ABL1 kinase domain and isolated ABL1 mutants resistant to the clinically used inhibitors dasatinib and ponatinib. This drug resistance screening strategy recapitulated all validated BCR-ABL1 mutations leading to clinical resistance to dasatinib, in addition to identifying other mutations previously observed in patients. Importantly, Taft showed that ponatinib remained effective against most single mutants of ABL1 kinase, with drug resistance starting only with rare compound mutations. Stern and colleagues recently harnessed ER sequestration to measure tyrosine phosphorylation cascades using full-length protein substrates (Ezagui et al., 2022). Using a modified version of the YESS plasmid containing a ribosomal skipping sequence (T2A), the protein-tyrosine kinase (LCK) is expressed and activates ZAP-70, an intermediate enzyme, through phosphorylation of its catalytic loop. Activated ZAP-70 is shuttled through the ER sequestration signal, where it interacts and phosphorylates LAT, the peptide sequence that is shuttled to the cell's surface for YSD analysis. This cascade of phosphorylation-activated enzymatic activity provides an experimental framework for understanding the kinase interactome in an orthogonal host.

Mapping epigenetic changes in a cell typically involves expensive and complicated recombinant proteins and cell-based assays followed by chromatography and mass spectrometry. Furthermore, crosstalk between epigenetic modifications, even catalyzed by the same enzyme, remains unclear. Keung and Rao adapted the YESS system to show that one can study human histone acetyltransferases (HAT) in yeast and investigate inter-residue communication in histone lysine modification (Waldman et al., 2021). They chose the HAT p300 as the writer and histones H3 and H4 as the substrates. Using yeast in this capacity proved a reliable platform to test the binding affinity and specificity of several commercial anti-lysine acetylation antibodies. Furthermore, this assay proved robust in mapping residue crosstalk between histone lysine residues, particularly the H3 and H4 sites. Among other interesting findings, it was observed that the strong acetylation preference of p300 at H4K20, H4K8, and H4K16 was significantly diminished when H4K20 was mutated to arginine. Interestingly, this was not observed when an arginine mutation was introduced at H4K8 or H4K16. Tuning interaction time between the epigenome writers and histone regions provides a robust, adaptable avenue to gain insights into epigenetic mechanisms. In theory, YESS can extend to processes such as protein methylation and other PTMs.

3.4 Engineering strategies to optimize the YESS system.

With any new engineering platform, several opportunities remain to improve the system through host cell and circuit engineering. The YESS system controls enzyme activities at the transcriptional and post-translational levels. Practically, achieving this control requires optimizing promoter strengths and testing ERSs with various retention strengths on the protease and substrates. Due to its lack of modularity, the original YESS plasmid faced a bottleneck when constructing plasmids to optimize activity and expression. Furthermore, enzyme transcription could not be turned off or controlled using a bidirectional GAL promoter. Titrating enzyme transcription is important to modulate enzyme: substrate stoichiometry in the ER, and the ability to turn off the enzyme is crucial in protease profiling experiments. Since cleaved substrates are selected for by a loss of fluorescence signal, any mutation in the substrate cassette's C-terminal tag will appear as a false positive on FACS. To counter these challenges, Denard and coworkers tackled two aspects of the YESS system in YESS 2.0 (Denard et al., 2021). First, they used a two-step golden gate approach to enable rapid assembly of YESS plasmid parts, marking a first step towards a fully modular YESS platform (YESS 2.0). Second, they introduced a synthetic transcription factor in the EBY100 kex2Δ strain, enabling titratable β-estradiol induction of the protease, thus achieving decoupled transcription (Figure 3C). To showcase this advancement,

they further engineered a TEV protease variant (eTEV) with an 8-fold faster catalytic efficiency than wild-type TEVp. Because YESS 2.0 could achieve low enzyme: substrate ratios in the ER, this evolved TEV showed a 3-fold increase in catalytic turnover.

Engineering the contact time between an enzyme and its substrate in the ER influences enzyme activity. Mei and coworkers studied how ERS sequences engaged ER receptors ERD1 and ERD2 (Mei et al., 2017). Their investigation discovered that the phenylalanine residue in the FEHDEL ERS sequence played a significant role in ERS: ERD2 interactions. By performing saturation mutagenesis at this residue, they discovered that the non-natural ERS sequence WEHDEL exhibited the strongest affinity for ERD2. Using this ERS on both the enzyme and substrate cassettes, they established activity for matrix metalloprotease 7 (MMP7) on an IgG-derived hinge sequence PAPELLGG, a previously intractable activity in the YESS system. This new ERS sequence now opens the possibility of engineering IgG-hinge cleaving metalloproteases. These improvements to the YESS system place this platform at the forefront of many biochemical assays of PTM-enzymes.

3.5 Protease evolution via cleavage of an intracellular substrate (PrECISE).

In parallel to the YESS system, Guerrero and coworkers developed an ER sequestration approach that relies on intracellular FRET release, which they named protease evolution via cleavage of an intracellular substrate (PrECISE) (Guerrero et al., 2016). In PrECISE, FRET is released upon protease cleavage of a linker sequence that keeps a quenched two-protein FRET pair (Figrue 3D). To showcase PrECISE, they evolved the cleavage specificity of human kallikrein 7 (hK7) towards the amyloid-β8 sequence KLVFFAED (Aβ8). Cleavage of Aβ after phenylalanine within Aβ8 disrupts its aggregation. However, wild type hK7 shows little activity towards Aβ8. Using error-prone PCR, hK7 was engineered to yield variant hK7-2.7, harboring three mutations compared to WT hK7. Variant hK7's switched specificity was primarily driven by a decreased preference towards tyrosine at the P1 position compared to WT hK7 and not an increased preference for phenylalanine at P1. Curiously, hK7-2.7's catalytic efficiency Aβ8 was only ˜80% of that of WT on that same sequence. The selection of a variant with lower catalytic efficiency on the positive selection substrate is unexpected. Without a dedicated counterselection substrate, the evolution campaign selected against promiscuous variants that impacted yeast cell growth. As evidence, the authors find that yeast cells harboring evolved variants outgrew those with the wild-type enzyme. The growth selection aspect of cell-based screens may be leveraged to narrow the substrate specificity of toxic enzymes.

Conclusion

PTM-enzymes enable many advances in chemical biology, biotechnology, and biomedicine. Although many technologies exist to investigate PTM-enzymes, *S. cerevisiae* is one of the most versatile organisms for PTM-enzyme biochemical assays due to its genetic tractability and comprehensive synthetic biology toolbox. This success is contingent on organelle sequestration strategies where PTM-enzyme activities can be tied to a measurable signal. Here, we described three general approaches to measure the activities of PTM-enzymes and profile and engineer their substrate specificity in a high-throughput manner.

Modified YSD approaches have been used to engineer the substrate specificity of bond-forming enzymes. The sortase examples showcase both the power and limitations of YSD in this regard. Of note, the A2M bait-and-capture method may provide a significant advance for engineering proteases on the yeast surface. It will be interesting to test this method on narrowing the substrate specificity of promiscuous and toxic proteases. Furthermore, protease-dependent synthetic protein circuits that drive a transcriptional output are quite useful for protease substrate specificity engineering. These approaches often allow one to engineer a PTM-enzyme in the same context of its application. Lastly, sequestration in the ER, particularly with YESS, has useful advantages over YSD and cytosolic sequestration, beyond YESS' ability to operate under multiple catalytic turnovers. The versatility of YESS in specificity profiling, engineering and inhibitor screening and its growing PTM-enzyme compatibility places it at the forefront of technologies. The YESS system allows the user to easily introduce one or more counterselection substrates without drastically changing the system's performance. However, a unique challenge in protease engineering in yeast in general is to include

counterselection substrates in a higher stoichiometric ratio than the selection substrate to impose higher selection stringencies. Expressing additional copies of counterselection substrates may not match competition assays achieved by exogenous addition (Podracky et al., 2021) or through orthogonal transcriptional nodes (Blum et al., 2021). In this vein, modifications that leverage ER retention signal binding affinities may provide a solution to this limitation. In conclusion, using yeast as a chassis for PTM-enzyme high-throughput assays significantly lowers the barrier to understanding how these enzymes work and how to reprogram them. In the coming years, we expect to see more PTM-enzymes integrate systems such as YESS, including protein arginine methyltransferases, formylglcine-generating enzymes, and sortases, as well as more complicated protein circuits that enable reprogramming through complex protein interactions. Finally, the integration of high-throughput sequence function data with machine learning lends itself perfectly to the yeast assays described here and will continue to push this field forward.

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Conflicts of interest

The authors have no conflict of interest to declare.

Figure legends

Figure 1. Leveraging Yeast Surface Display for PTM-enzyme engineering. (A) Using AGA1-AGA2 surface binding proteins to tether sortase variants and substrate targets to the yeast surface using N and C terminal binding and screened by either FACS or MACS. (B) Selection of tethered sortase enzymes first by the acetylation of the substrate peptide (S6) sequence, a reaction that selectively activates the tethered sortase. Cell populations are incubated with a nucleophile and then stained with a specific fluorescent antibody for that nucleophile, followed by selection by either FACS or MACS. (C) Protease variants tethered to the N-terminus of AGA2 screened for cleavage efficiency of a substrate sequence blocking protease trapping via A2M. Active proteases are trapped, labeled with fluorescent markers, and screened by FACS. (D) Screening of tethered mTG variants to determine the efficiency of binding glutamine-donor peptides to free lysine, screened by FACS.

Figure 2. Transcription-based screens of engineered PTM-enzymes via cytosolic sequestration. (A) Screening of active enzyme variants against non-canonical substrates using a membrane-tethered transcription factor (TF), tethered by desired substrate sequence, for growth-based selection. B) Dual selection screening of enzyme efficiency and cleavage-site recognition of substrate orthologs via TF tethered to estrogen-receptor ligand binding domains (LBD) by substrate sequences. (C) Engineered PTM-enzyme circuit in yeast that selects for active engineered variants through blue light-mediated substrate exposure and enzyme-substrate proximity facilitated by the recognition of the CRY and CIBN domains

Figure 3. Yeast ER sequestration accurately quantifies PTM-enzyme catalytic turnovers. (A) YESS enzyme (blue) and substrate (purple) cassettes present on a plasmid that can be transformed into yeast for enzyme-substrate activity assays, including signal peptides (SP), ER retention signals (ERS), and unique epitope tags for fluorescence staining. (B) YESS can perform library screening of (1) protease variants, (2) substrate variants, or (3) both. Enzyme-substrate interactions can be assayed for enzymatic activity, substrate selectivity, and selectivity modulation using FACS, MACS, NGS, and ML. (C) Two-promoter design of YESS 2.0 with -estradiol induction driving protease expression and substrate expression remaining under galactose induction. (D) PrECISE: Selection of active variants against a desired substrate motif within a large library achieved using a protease-mediated protein FRET-based assay.

Figure 1

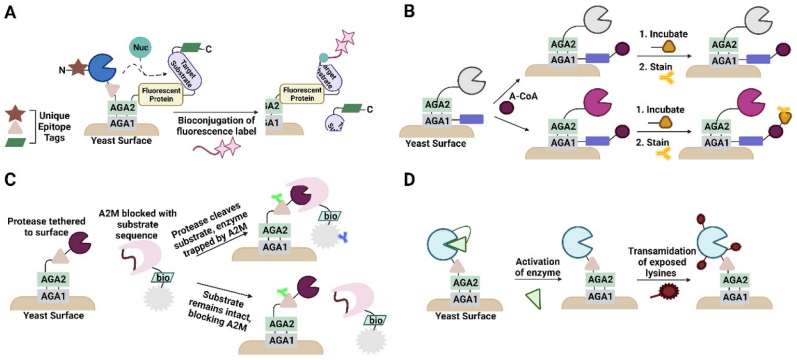


Figure 2

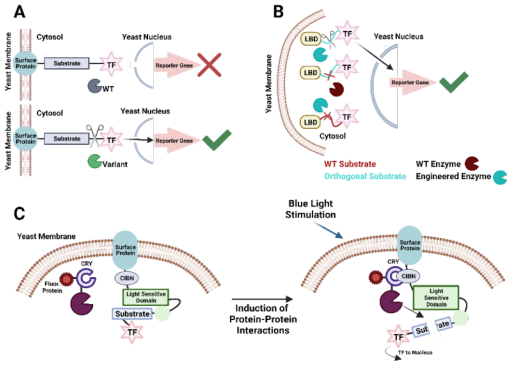
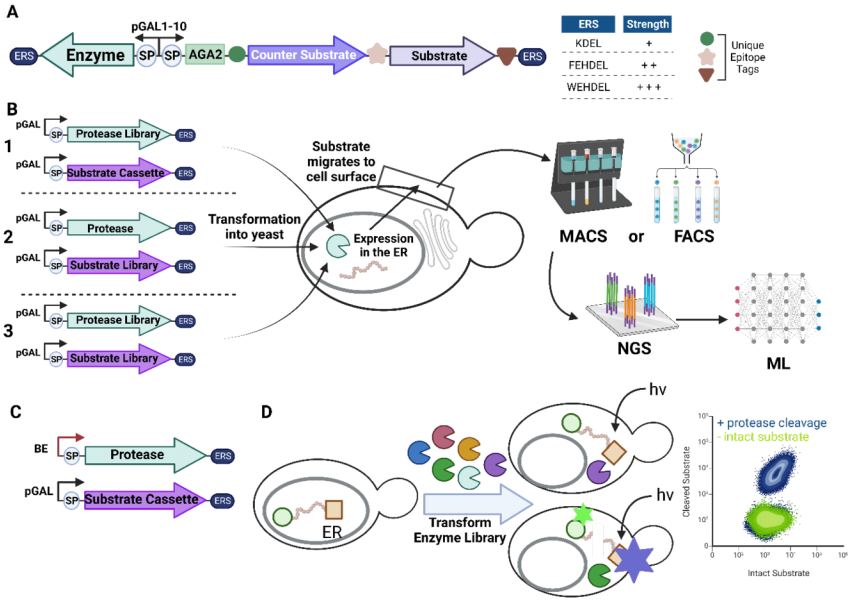


Figure 3



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