



RESEARCH ARTICLE

A suite of polymerase chain reaction-based peptide tagging plasmids for epitope-targeted enzymatic functionalization of yeast proteins

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Abstract

The budding yeast and model eukaryote *Saccharomyces cerevisiae* has been invaluable for purification and analysis of numerous evolutionarily conserved proteins and multisubunit complexes that cannot be readily reconstituted in *Escherichia coli*. For many studies, it is desirable to functionalize a particular protein or subunit of a complex with a ligand, fluorophore or other small molecule. Enzyme-catalysed site-specific modification of proteins bearing short peptide tags is a powerful strategy to overcome the limitations associated with traditional nonselective labelling chemistries. Towards this end, we developed a suite of template plasmids for C-terminal tagging with short peptide sequences that can be site-specifically functionalized with high efficiency and selectivity. We have also combined these sequences with the FLAG tag as a handle for purification or immunological detection of the modified protein. We demonstrate the utility of these plasmids by site-specifically labelling the 28-subunit core particle subcomplex of the 26S proteasome with the small molecule fluorophore Cy5. The full set of plasmids has been deposited in the non-profit plasmid repository Addgene (<http://www.addgene.org>).

KEYWORDS

budding yeast, epitope tagging, fluorescence, PCR, pFA6a plasmid, proteasome

1 | INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is frequently used as a source material or expression host for purification of proteins and protein complexes due to its rapid growth, low cost of growth media, eukaryotic protein folding machinery, and the abundance of strains genetically optimized for expression or purification. Budding yeast has been especially powerful for the purification and analysis of proteins that cannot be recombinantly expressed in *Escherichia coli* and for large, evolutionarily conserved eukaryotic multisubunit complexes such as the spliceosome (Wan, Bai, Yan, Lei, & Shi, 2019; Whittaker, Lossky, & Beggs, 1990), the RNA polymerase complex (Koleske, Chao, & Young, 1996), the anaphase-promoting complex (Passmore et al., 2003), and the 26S proteasome (Eisele et al., 2018; Leggett et al., 2002; Leggett, Glickman, & Finley, 2005; Li, Tomko, & Hochstrasser, 2015). It is

often necessary to covalently modify such purified proteins and protein complexes with ligands, fluorophores or other small molecules for downstream applications. For example, site-specific modification of protein-based pharmaceuticals with polyethylene glycol is frequently used to extend the biological half-life of the drug (Harris & Chess, 2003). For in vitro functional studies, biotinylation of proteins at a single position is frequently used to immobilize proteins on solid supports (Cho et al., 2007) or to stably recruit the protein to a second biomolecule of interest (Valadon et al., 2010). Similarly, attachment of fluorescent molecules to proteins at a single position is necessary for many fluorescence-based studies (Toseland, 2013).

Traditional approaches for site-specific protein modification exploit conjugates of a desired small molecule to a chemical moiety that reacts specifically with the side chains of a particular amino acid. Although these approaches can be readily implemented to modify any

protein containing a suitable amino acid, they indiscriminately modify any surface-exposed residue containing that side chain. The most common amino acid targets for such traditional modification are lysine and cysteine. However, lysine and cysteine are highly abundant amino acids in most proteins, constituting approximately 7% and 2%, respectively, of protein residues in *S. cerevisiae* and with comparable levels in other eukaryotes (Echols et al., 2002). As a result, this approach cannot be applied to modify most proteins at a single site without extensive mutagenesis to remove other reactive amino acids, and there is a risk that the amino acid substitutions will disrupt the structure or function of the protein. Moreover, large multisubunit complexes can contain hundreds of lysines or cysteines, rendering such mutagenesis essentially impossible.

A second approach commonly used to incorporate a desired molecule at a single position in a protein or complex introduces a non-standard amino acid (nsAA) bearing a chemically reactive side chain into a protein of interest using amber suppression and an orthogonal tRNA-synthase pair (Lang & Chin, 2014; Young & Schultz, 2010). The reactive side chain can then be modified with an appropriate small molecule or conjugate. This approach has been utilized successfully in yeast (Chin et al., 2003). However, there are several limitations that have restricted this approach. These include the high cost of nsAAs, off-target nsAA incorporation into other cellular proteins bearing amber codons, and site-specific variability in nsAA incorporation efficiency that is difficult to predict (Yin et al., 2017). Further, for essential genes, introduction of an amber codon at a desired incorporation position often results in lethality due to premature termination of the protein product in the absence of the nsAA. Thus, a simple and efficient means to functionalize a particular yeast protein or complex at a single, defined site would be particularly valuable.

Enzymatic modification of small peptide tag sequences has recently emerged as a valuable means to covalently modify target proteins (Toseland, 2013). Two families of enzymes in particular have gained significant popularity for such protein derivatizations. The first are bacterial 4'-phosphopantetheinyl transferases such as *E. coli* AcpS and *Bacillus subtilis* Sfp. These enzymes covalently transfer the 4'-phosphopantetheinyl group from Coenzyme A (CoA) to a conserved serine residue in bacterial acyl or peptidyl carrier proteins (Yin, Lin, Golan, & Walsh, 2006). Recently, optimized short peptide sequences have been developed that are efficiently modified by AcpS (A1 tag) or Sfp (S6 tag) with minimal cross-modification by the other enzyme (Zhou et al., 2007). Importantly, these enzymes efficiently transfer a wide variety of readily synthesized small molecule-4'-phosphopantetheinyl conjugates to the serine present in the target peptide sequence (Yin et al., 2006). These proteins have been utilized to modify purified proteins (Yin, Straight, et al., 2005) and surface proteins on living cells (Yin, Lin, et al., 2005) with small molecules, such as chemical fluorophores and biotin. Although Ppt2, Fas2 and Lys5 are known to have 4'-phosphopantetheinyl transferase activity in *S. cerevisiae* (Ehmann, Gehring, & Walsh, 1999; Fichtlscherer, Wellein, Mittag, & Schweizer, 2000; Stuitable, Meier, Wagner, Hannappel, & Schweizer, 1998), the A1 and S6 sequences are divergent from the target sequences of these three enzymes, and we have not observed

any evidence that they are modified by these endogenous enzymes (data not shown).

The second family, the Gram-positive bacterial sortases, are transpeptidases that normally anchor surface proteins to the bacterial cell wall (Mazmanian, Liu, Ton-That, & Schneewind, 1999). Sortase A from *Staphylococcus aureus* recognizes a conserved LPXTG motif (where X is any amino acid) present in the cell surface protein, catalyses cleavage of the LPXTG threonyl-glycyl peptide bond, and promotes subsequent peptide bond formation between the released threonine carboxylate and the primary amine of glycine present in peptidoglycan (reviewed in Pishesha, Ingram, & Ploegh, 2018). Purified sortase A can be used for in vitro peptide bond formation between any protein bearing this C-terminal sortase A recognition sequence (SRS) and a protein or peptide with an N-terminal glycine (Guimaraes et al., 2013). Because short peptides containing N-terminal glycines can be readily synthesized with a variety of chemical modifications, it is possible to covalently modify the protein bearing the SRS at a defined site with a desired peptide or small molecule-peptide conjugate. Indeed, sortase has been used to derivatize polypeptides with fluorophores (Bard, Bashore, Dong, & Martin, 2019), biotin (Shi et al., 2014), lipids (Tomita et al., 2013), antibiotics (Samantaray, Marathe, Dasgupta, Nandicoori, & Roy, 2008), folates (Mao, Hart, Schink, & Pollok, 2004), and cell-penetrating peptides (Pritz et al., 2007). It has also been utilized to modify proteins on the surface of living cells (Shi et al., 2014), to cyclize polypeptides (Parthasarathy, Subramanian, & Boder, 2007), and to covalently link two proteins together (Stinson, Baytshok, Schmitz, Baker, & Sauer, 2015).

In this study, we developed a set of 24 plasmids designed for polymerase chain reaction (PCR)-based C-terminal tagging with short peptides containing the recognition sequences of AcpS, Sfp, and sortase A. We demonstrate the utility of several of these plasmids by tagging, purifying, and fluorescently labelling two different subunits of the proteasomal core particle subcomplex with no detectable off-target labelling.

2 | MATERIALS AND METHODS

2.1 | Strains and media

All yeast manipulations were carried out according to standard techniques. Yeast strains used in this study are listed in Table S1. Primers used for construction of C-terminally tagged strains are shown in Table S2. For growth assays, the indicated strains were diluted to $OD_{600} = 0.1$ with sterile water and spotted as sixfold serial dilutions onto the indicated media. *E. coli* strain TOP10 F' (Life Technologies) was used for DNA manipulations and cloning. Standard bacterial culture media and growth conditions were used.

2.2 | Plasmids

Oligos encoding the A1, A1-FLAG, S6, S6-FLAG, SRS, or SRS-FLAG sequences were annealed and inserted into the PacI and AscI sites of

pFA6a-6xGly-FLAG:hphMX4 (Funakoshi & Hochstrasser, 2009), replacing the sequence encoding 6xGly-FLAG. For generation of kanMX6- and HIS3MX6-based tagging plasmids, the respective selection cassettes and the *ADH1* transcriptional terminator were excised from pFA6a-6xGly-FLAG:kanMX6 or pFA6a-6xGly-FLAG:HIS3MX6 (Funakoshi & Hochstrasser, 2009) using *AscI* and *SacI* and were inserted in the same sites of the appropriate hphMX4-based plasmid. For generation of natMX4-based tagging plasmids, the *Bgl* II – *Sac* I fragment from pAG25 (Goldstein & McCusker, 1999) was inserted into the same sites of the appropriate hphMX4-based plasmid. The plasmids and tag sequences are listed in Table S3. Full plasmid sequences are available upon request from the authors or from Addgene (Addgene #134425-134448).

2.3 | Native polyacrylamide gel electrophoresis

Cell extracts (60 µg) were separated by non-denaturing polyacrylamide electrophoresis as described previously (Eisele et al., 2018; Nemeč, Howell, Peterson, Murray, & Tomko, 2017; Nemeč, Peterson, Warnock, Reed, & Tomko, 2019). Briefly, cells were grown to $OD_{600} \approx 2.0$, harvested by centrifugation at 5,000× *g* for 5 min, washed in 25 ml of ice-cold water, and centrifuged at 5,000× *g* for 5 min. The supernatant was discarded; the pellet was frozen in liquid nitrogen and was ground into powder using a mortar and pestle. Cell powder was rehydrated in one volume of extraction buffer (50-mM Tris-HCl, pH 7.5, 5-mM MgCl₂, 10% glycerol, 0.5-mM ATP, 0.015% w/v xylene cyanol) and incubated on ice with frequent vortexing. Cell lysates were cleared by centrifugation at 21,000× *g* for 10 min at 4°C. Protein levels were quantified by BCA assay, and equal amounts of protein were loaded onto 4% native polyacrylamide gels containing 0.5-mM ATP and with a 3.5% polyacrylamide stacker containing 2.5% sucrose and 0.5-mM ATP. Samples were separated at 100 V, 4°C for 3 h or until the dye front escaped.

2.4 | Western blotting

Proteins separated by native PAGE or SDS-PAGE were transferred to PVDF membranes at 100 V for 1 h at 4°C. Membranes were probed with antibodies against 20S (1:2,500) (Enzo Life Sciences), FLAG (1:2,500) (Sigma), or G6PD (1:10,000) (Sigma). Membranes were imaged with a Bio-Rad ChemiDoc MP using HRP-conjugated secondary antibodies (GE Healthcare) and ECL reagent.

2.5 | Measurement of peptidase activity

For measurement of suc-LLVY-AMC hydrolysis in non-denaturing gels, 60 µg of cell extract was separated as described above. The gel was then incubated in overlay buffer (50-mM Tris-HCl, pH 7.5, 5-mM MgCl₂, 10% glycerol, 1-mM ATP, 0.04% SDS) containing 50-µM suc-LLVY-AMC for 30 min at 30°C with gentle rocking. After 30 min, the

gel was imaged using a Bio-Rad ChemiDoc MP using the filter set for ethidium bromide.

2.6 | Purification of core particle from yeast

Proteasomal CPs were purified either via FLAG affinity exactly as described previously (Li et al., 2015; Nemeč et al., 2019) or via a hybrid conventional/affinity purification scheme described here. Briefly, the appropriate yeast strain was inoculated into 6 L of YPD medium and grown for 48 h. The cells were harvested via centrifugation at 8,200× *g*, 4°C for 5 min, washed with 1 L of ice-cold water, recentrifuged, and the pellet was snap-frozen in liquid nitrogen. The frozen cells were ground into powder using a SPEX 6850 freezer mill, and the powder was stored at –80°C. The cell powder was hydrated in one volume of CP lysis buffer (50-mM Tris-HCl, pH 7.5, 50-mM NaCl, 50-mM KCl, 0.5-mM EDTA, 0.05% NP-40) followed by centrifugation at 30,000× *g* for 20 min at 4°C. The lysate was first fractionated by slow addition of ammonium sulfate to a final concentration of 40% with stirring at 4°C. The mixture was stirred for 1 h and pelleted at 30,000× *g* for 30 min at 4°C. The supernatant (containing CP) was transferred to a new beaker, and ammonium sulfate was slowly added to a final concentration of 70% with stirring at 4°C. After stirring for 1 h, the CP was pelleted at 30,000× *g* for 30 min at 4°C. The pellet was resuspended in 100 ml of CP Ni binding buffer (50-mM Tris-HCl, pH 7.5, 500-mM NaCl, 10-mM imidazole) and was incubated with pre-equilibrated Ni-NTA resin for 45 min at 4°C. Following one wash with Ni CP binding buffer, the resin was poured into a disposable Bio-Rad Econo-column, washed with CP Ni low salt wash buffer (50-mM Tris-HCl, pH 7.5, 50-mM NaCl, 10-mM imidazole) and eluted with Elution buffer (50-mM HEPES-NaOH, pH 7.5, 100-mM NaCl, 100-mM KCl, 5% glycerol, 500-mM imidazole). Eluates were further purified by anion exchange using a MonoQ column in buffer IX-A (50-mM Tris-HCl, pH 7.5, 5-mM MgCl₂). CP was eluted with a linear gradient of buffer IX-B (50-mM Tris-HCl, pH 7.5, 1-M NaCl, 5-mM MgCl₂) and concentrated using a 30,000 kDa MWCO filter (Amicon). The concentrated CP was further purified by gel filtration using a Superose 6 10–30 column equilibrated in CP buffer (50-mM Tris-HCl, pH 7.5, 50-mM NaCl, 50-mM KCl, 0.5-mM EDTA). Pure fractions were pooled, concentrated as above, snap-frozen in small aliquots and stored at –80°C.

2.7 | Synthesis and purification of small molecule-CoA conjugates

To synthesize sulfo-Cy5-CoA, 3.18 µmol (2.5 mg) of coenzyme A trilithium salt (Millipore-Sigma) was added to 100 µl of phosphate-buffered saline, pH 7.0. Once dissolved, the entire volume was used to dissolve 1.245 µmol (1 mg) of sulfo-cyanine5-maleimide (Lumiprobe). The reaction was allowed to proceed at room temperature for 2 h in the dark, at which point unreacted maleimide was quenched with 6-mM dithiothreitol for 30 min at room temperature.

The dye-CoA conjugate was then separated via reverse phase liquid chromatography on a Phenomenex Jupiter 4u Proteo 90A 250 × 4.6 mm column on a Shimadzu Prominence HPLC. The mobile phase consisted of 5% acetonitrile, 0.1% trifluoroacetic acid in deionized water (Eluent A), and the dye-CoA conjugate was eluted with a linear gradient of Eluent B (90% acetonitrile, 0.08% trifluoroacetic acid in deionized water) at a flow rate of 1 ml/min. The eluate was manually collected and lyophilized in single-use aliquots that were stored at −20°C.

2.8 | Expression and purification of AcpS

N-terminally 6His-tagged *E. coli* AcpS was expressed from a pET28b-6His-AcpS plasmid in LOBSTR (DE3) cells (Kerafast). Briefly, cells were grown at 37°C with aeration in terrific broth containing 40 µg/ml kanamycin until $OD_{600} \approx 1.0$, at which point IPTG was added to 0.5 mM, and the culture was shaken for four additional hours. Cells were harvested and resuspended in binding buffer (50-mM Tris-Cl, pH 7.5, 500-mM NaCl, 10% glycerol, 0.2% Tween-20, 40-mM imidazole in deionized water), lysed using an Avestin Emulsiflex C-5 high pressure homogenizer, and insoluble debris was removed via centrifugation at 30,000× *g* for 20 min at 4°C. The supernatant was applied to Ni-NTA resin, which was then washed with 100 column volumes of binding buffer before elution with elution buffer (50-mM Tris-Cl, pH 7.5, 500-mM NaCl, 10% glycerol, 0.2% Tween-20, 250-mM imidazole in deionized water). Eluted 6His-AcpS was dialysed into storage buffer (50-mM Tris-Cl, pH 7.5, 500-mM NaCl, 10% glycerol, 0.2% Tween-20 in deionized water) overnight at 4°C, diluted to 50 µM using storage buffer, and snap-frozen in single-use aliquots in liquid nitrogen before storage at −80°C.

2.9 | Expression and purification of Sfp

C-terminally 6His-tagged *B. subtilis* Sfp was expressed from a pET29-Sfp-6His plasmid (Yin et al., 2006) and purified exactly as for AcpS. Eluted Sfp-6His was dialysed into storage buffer (50-mM Tris-Cl, pH 7.5, 500-mM NaCl, 10% glycerol, 0.2% Tween-20 in deionized water) overnight at 4°C, diluted to 50 µM using storage buffer, and snap-frozen in single-use aliquots in liquid nitrogen before storage at −80°C.

2.10 | Site-specific labelling of CP

For A1 labelling, purified CP (2 µM) bearing or lacking a subunit with the A1-FLAG tag as described was incubated with 5-µM sulfo-Cy5-CoA and 5-µM AcpS in labelling buffer (50-mM Tris-HCl, pH 7.5, 50-mM NaCl, 50-mM KCl, 0.5-mM EDTA, 10-mM MgCl₂) at 25°C for 90 min. For S6 labelling, purified CP (0.5 µM) bearing or lacking an S6 tag on the α7 subunit was incubated with 5-µM sulfo-Cy5-CoA and 5-µM Sfp in labelling buffer at 25°C for 90 min.

3 | RESULTS

3.1 | Construction of a collection of plasmids for C-terminal tagging

We have designed a series of plasmids for PCR-based single-step C-terminal tagging of yeast proteins with short amino acid sequences that permit targeted functionalization with small molecules of interest. These plasmids, based on the pFA6a series (Wach, Brachat, Pohlmann, & Philippsen, 1994), encode short peptide recognition sequences for enzymatic functionalization, followed by the *ADH1* transcriptional terminator (Figure S1). Each also encodes a dominant selectable marker for selection of transformants.

We selected peptide recognition sequences based on the following considerations: first, the recognition sequences would be short (~10 amino acids) to minimally perturb protein function. Second, when possible, we selected tags whose protein sequences were absent from the yeast proteome to minimize the possibility of modifying any co-purified contaminant proteins. Third, we selected tags modified by enzymes for which many small molecule substrates were either commercially available or could be easily synthesized by standard molecular biology labs. Finally, we selected tags that could in principle be combined to modify several subunits within a particular protein complex without significant risk of cross-reactivity, owing to the use of orthogonal chemistries and/or divergent recognition sequences. With these criteria in mind, we selected the A1, S6 and SRS tags (Guimaraes et al., 2013; Zhou et al., 2007). Importantly, plasmids for the high-yield bacterial expression and purification of the enzymes to modify each of these tags are commercially available (Sfp: Addgene #75015; AcpS: Addgene #145376; Sortase A: Addgene #51141), allowing any standard molecular biology lab to utilize these labelling strategies.

We constructed pFA6a-A1-*hphMX4*, pFA6a-S6-*hphMX4* and pFA6a-SRS-*hphMX4* plasmids by replacing the 6xGly-FLAG coding sequence from pFA6a-6xGly-FLAG-*hphMX4* (Funakoshi & Hochstrasser, 2009) with the A1, S6 and SRS peptide coding sequences. We also created versions in which the peptide tag coding sequence was followed by sequence encoding the FLAG epitope tag to permit immunodetection and affinity purification. Finally, we replaced the *hphMX4* cassette in each of these six plasmids with *HIS3MX6*, *kanMX6* or *natMX4* to provide additional marker options for selection of transformants. In each case, the coding sequence for the tag is followed by a stop codon, the *ADH1* transcriptional terminator and a selectable marker cassette. Tag sequences and plasmid names are shown in Table S3.

3.2 | Validation of new tagging plasmids

To validate the utility of the plasmids, we integrated sequences encoding the A1, S6 and SRS target peptides, with and without additional C-terminal FLAG tags, into the native loci of the *PRE9* and *PRE10* genes. The *PRE9* and *PRE10* genes respectively encode the α3

and $\alpha 7$ subunits of the proteasomal core particle (CP). The CP is a sub-complex of the 26S proteasome that harbours the proteasome's peptidase sites (Howell, Tomko, & Kusmierczyk, 2017; Tomko & Hochstrasser, 2013). *PRE9* is dispensable for viability in budding yeast (Emori et al., 1991), whereas *PRE10* is essential. After denaturing SDS-PAGE and immunoblotting of whole cell extracts from each strain, a single FLAG-reactive band was evident at the appropriate molecular weight and only for the A1-FLAG, S6-FLAG and SRS-FLAG fusions. This indicated that integration was successful and that the A1, S6 or SRS peptide sequences were intact. Of note, the FLAG signal was weaker (albeit still detectable) for all proteins tested when it followed the S6 tag (Figure 1a and not shown), suggesting that the upstream peptide sequence may influence antibody reactivity (and

see below). For the untagged A1, S6 and SRS fusions, proper integration was confirmed via colony PCR (not shown).

C-terminal tagging of proteins in yeast, especially essential proteins, can sometimes lead to functional defects that manifest as growth phenotypes under stress conditions (Sabourin, Tuzon, Fisher, & Zakian, 2007). Proteasome mutants are often hypersensitive to elevated temperature (Gerlinger, Guckel, Hoffmann, Wolf, & Hilt, 1997; Tomko & Hochstrasser, 2011), which induces protein unfolding and increases the cellular proteolytic burden. To test whether introduction of the tags had adverse effects on proteasome function, we performed growth assays at elevated temperatures on the tagged strains and a corresponding untagged WT strain. A strain harbouring a deletion of *PRE9*/ $\alpha 3$ was included as a control. As shown in Figure 1b,

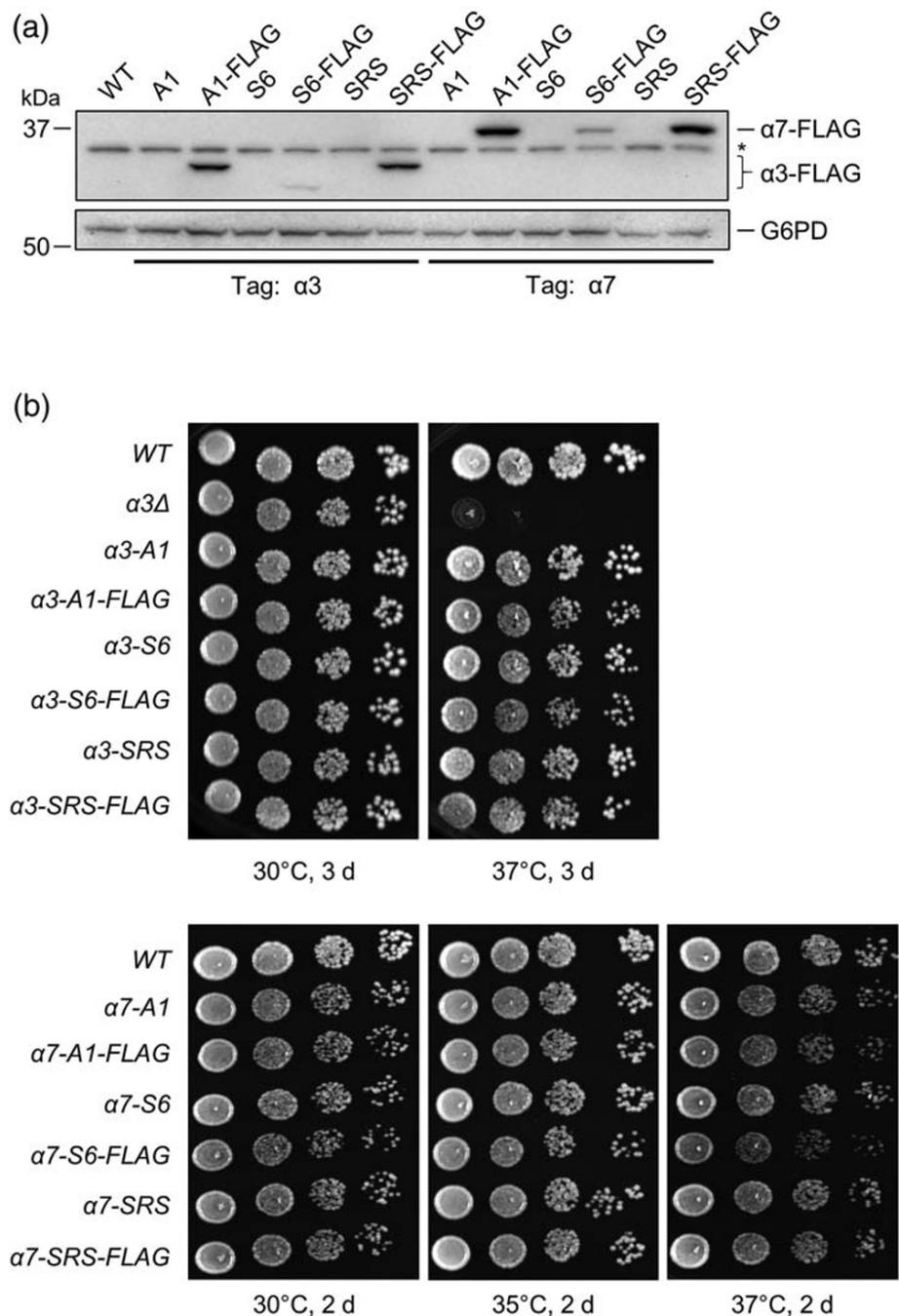


FIGURE 1 Chromosomal tagging of proteasome subunits $\alpha 3$ and $\alpha 7$. The chromosomal loci of proteasome subunits $\alpha 3$ and $\alpha 7$ were modified to encode the indicated subunit fusions. (a) Protein extracts from the indicated yeast strains were separated by SDS-PAGE and immunoblotted with antibodies against the FLAG tag or G6PD (loading control). An asterisk indicates a nonspecific band recognized by the FLAG antibody. (b) Equal numbers of cells from the indicated yeast strains were spotted in sixfold serial dilutions on YPD plates and incubated at the indicated temperatures and times

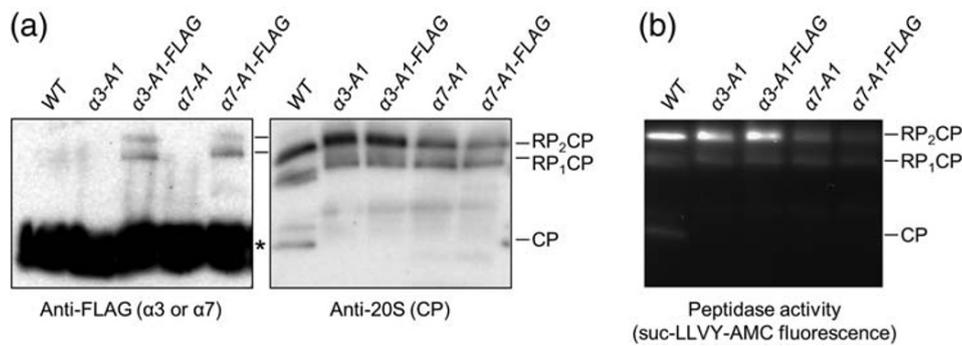


FIGURE 2 Peptide tags are present in fully assembled proteasomes. (a) Extracts from the indicated yeast strains were separated by non-denaturing PAGE and immunoblotted with antibodies against the core particle (CP) or against FLAG. RP₂CP and RP₁CP indicate CP capped with two or one regulatory particles (RP), respectively. An asterisk indicates a nonspecific band recognized by the FLAG antibody. (b) The chymotryptic-like peptidase activity of proteasomes in the indicated whole cell extracts was measured after non-denaturing PAGE via incubation with the fluorogenic substrate suc-LLVY-AMC. The fluorescence intensity correlated with the abundance of proteasomes observed in panel (a)

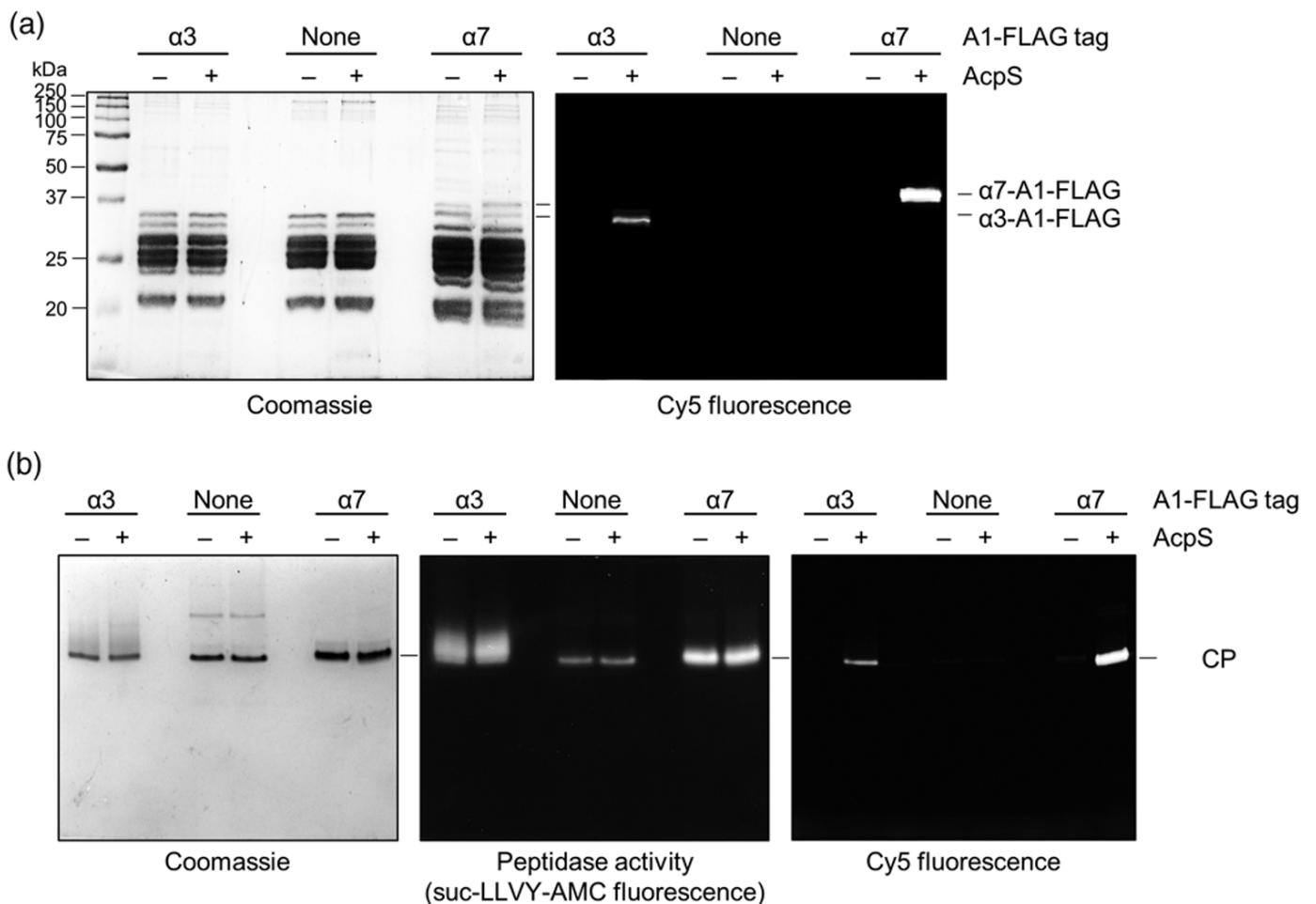


FIGURE 3 Labelling of purified, A1-tagged proteasomal CPs. (a) Purified CP containing either no A1 tag or containing the A1 recognition motif in the form of α 3-A1-FLAG or α 7-A1-FLAG subunit fusions were incubated with sulfo-Cy5-Coenzyme A in the presence or absence of recombinant AcpS. After labelling, the indicated CPs were separated by SDS-PAGE and Cy5 fluorescence (right panel) was imaged. The gel was then stained with Coomassie and imaged again (left panel). (b) Purified CPs labelled as in (a) were resolved by native PAGE before staining with Coomassie (left panel), imaging to detect peptidase activity (suc-LLVY-AMC hydrolysis, middle panel) or Cy5 fluorescence (right panel)

there was little to no impact on cell growth with the A1 and SRS tags, even at 37°C, indicating the tags are generally well tolerated. Although the S6-FLAG tag appeared to be well tolerated when fused to $\alpha 3$, there was a modest impact on cell growth at 37°C when this tag was fused to $\alpha 7$. Considering the lower apparent expression of the S6-FLAG-tagged $\alpha 3$ and $\alpha 7$ alleles in Figure 1a, we recommend that this tag be used with caution.

To confirm that tagged subunits efficiently incorporated into fully assembled proteasomes without causing structural or functional defects, we separated whole cell extracts from strains expressing A1- or A1-FLAG-tagged forms of $\alpha 3$ and $\alpha 7$ by non-denaturing polyacrylamide electrophoresis (native PAGE), which allows separation of protein complexes of different compositions. We then investigated the assembly state of proteasomes via immunoblotting. Fully assembled proteasomes typically run as two major species via native PAGE: a CP with a regulatory particle (RP) on each end (RP₂CP) and a CP with a single RP (RP₁CP). Typically, only a minor amount of free CP is observed. Consistent with this, we observed primarily RP₂CP and RP₁CP in both the WT extracts and each of the tagged strain extracts in anti-CP immunoblots. In FLAG immunoblots, there was a strong cross-reactive band present even in cell extracts lacking FLAG-tagged proteins that, in our hands, is more prevalent under non-denaturing conditions (Nemec et al., 2019). Despite this, FLAG reactivity was evident as bands with migrations consistent with that of RP₂CP and RP₁CP only in extracts from strains bearing A1-FLAG tags (Figure 2a). We ensured that proteasomes remained functional upon incorporation of tagged subunits by measuring cleavage of the fluorogenic peptide substrate suc-LLVY-amidomethylcoumarin (suc-LLVY-AMC). This highly specific proteasome substrate can be soaked into native PAGE gels, and proteasome activity can be visualized as an increase in AMC fluorescence. As shown in Figure 2b, suc-LLVY-AMC fluorescence remained proportional to the proteasome abundance observed in Figure 2a in all cases, indicating that these tags had little to no impact on proteasomal peptide hydrolysis. Together, this indicates that tagged subunits are efficiently incorporated into functional proteasomes.

Finally, we tested whether purified CPs bearing C-terminal A1 or S6 tags could be functionalized. We did not verify the functionality of the SRS tag in yeast, although the only requirement for labelling of proteins by sortase is an accessible LPXTG motif. Purified CPs bearing A1-FLAG tags on the $\alpha 3$ or $\alpha 7$ subunits could be functionalized by enzymatically attaching the small molecule fluorophore Cy5 from a Cy5-CoA conjugate to the A1 tag using recombinant AcpS. Despite provision of a molar excess of Cy5-CoA, no Cy5 labelling was detected in SDS-PAGE-separated proteasomes incubated without AcpS, even when the A1 tag was present (Figure 3a). However, incubation of A1-tagged CPs with Cy5-CoA in the presence of 5- μ M AcpS rapidly yielded specific conjugation of Cy5 to a single subunit whose migration correlated closely with that expected for $\alpha 3$ or $\alpha 7$. No fluorescence was present in extracts lacking the A1 tag when AcpS was provided, indicating that labelling was highly subunit-specific and entirely dependent on the presence of the A1 tag. Similar results were obtained when CP containing $\alpha 7$ -S6 was incubated with Sfp and

Cy5-CoA (Figure S2). Importantly, labelling $\alpha 3$ and $\alpha 7$ with Cy5 did not obviously impact the structure (Figure 3b, Coomassie) or activity (Figure 3b, peptidase activity) of purified CP. The fluorescence intensity of CPs containing $\alpha 3$ -Cy5 were noticeably less bright than those containing $\alpha 7$ -Cy5 (Figure 3b, Cy5 fluorescence); the reason for this is unknown, but the $\alpha 3$ subunit can be replaced with a second copy of the proteasomal $\alpha 4$ subunit under some circumstances (Howell, Peterson, & Tomko, 2019; Kusmierczyk, Kunjappu, Funakoshi, & Hochstrasser, 2008; Padmanabhan, Vuong, & Hochstrasser, 2016; Velichutina, Connerly, Arendt, Li, & Hochstrasser, 2004), which may have contributed to the reduced fluorescence observed.

4 | CONCLUSION

In summary, we have established a new collection of template plasmids for PCR-based C-terminal tagging of yeast proteins with short peptide recognition sequences for enzymatic functionalization. Although a unique forward primer with homology to the desired peptide tag sequence is required for amplification of targeting constructs containing A1, S6 or SRS tags (Figure S1), the universal reverse primer described here is compatible with any of these plasmids, as well as essentially all previously described pFA6a-based plasmids (e.g., those in Funakoshi & Hochstrasser, 2009; Goldstein & McCusker, 1999; Wach et al., 1994). In addition, the *kanMX6*-, *hphMX4*- and *natMX4*-marked plasmids can be used for tagging in *Schizosaccharomyces pombe*, as each of these markers are functional in this organism as well (Bahler et al., 1998; Hentges, Van Driessche, Tafforeau, Vandehaute, & Carr, 2005).

Inclusion of a C-terminal FLAG tag will facilitate purification of proteins or complexes containing an intact peptide recognition sequence, ensuring maximal labelling. Notably, the inclusion of a C-terminal FLAG tag may be particularly useful in the case of protein modification using the SRS tag. The sequence C-terminal to the SRS threonine is removed and replaced with the donor N-terminal glycine-containing peptide during the transpeptidation reaction, and thus, the FLAG tag can in principle be utilized after functionalization to immunodeplete any unmodified target protein/complex. The 24 new plasmids and their sequences are available to the greater research community via the non-profit plasmid repository Addgene (<http://www.addgene.org>).

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CONFLICT OF INTEREST

The authors have no conflicts to declare.

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SUPPORTING INFORMATION

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