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Expanding the Genetic Code of Yeast for Incorporation of Diverse Unnatural Amino Acids via a Pyrrolysyl-tRNA Synthetase/tRNA Pair

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Abstract: We report the discovery of a simple system through which variant pyrrolysyl-tRNA synthetase/$tRNA^{Pyl}_{CUA}$ pairs created in *Escherichia coli* can be used to expand the genetic code of *Saccharomyces cerevisiae*. In the process we have solved the key challenges of producing a functional $tRNA^{Pyl}_{CUA}$ in yeast and discovered a pyrrolysyl-tRNA synthetase/$tRNA^{Pyl}_{CUA}$ pair that is orthogonal in yeast. Using our approach we have incorporated an alkyne-containing amino acid for click chemistry, an important post-translationally modified amino acid and one of its analogs, a photocaged amino acid and a photo-cross-linking amino acid into proteins in yeast. Extensions of our approach will allow the growing list of useful amino acids that have been incorporated in *E. coli* with variant pyrrolysyl-tRNA synthetase/$tRNA^{Pyl}_{CUA}$ pairs to be site-specifically incorporated into proteins in yeast.

Introduction

The pyrrolysyl-tRNA synthetase/$tRNA^{Pyl}_{CUA}$ (PylRS/$tRNA^{Pyl}_{CUA}$) pairs from *Methanosarcina barkeri* (*Mb*) and *M. mazei* (*Mm*) are orthogonal in *Escherichia coli*.[1] These pairs have been evolved to direct the site-specific incorporation of a range of unnatural amino acids, including amino acids that are post-translationally modified, amino acids containing bio-orthogonal chemical handles, and amino acids protected with light- and acid-sensitive groups, into proteins in *E. coli* in response to the amber codon.[1−6] In contrast to other aminoacyl-tRNA synthetase/tRNA pairs for the incorporation of unnatural amino acids, which are orthogonal in either eukaryotic or prokaryotic hosts, the PylRS/$tRNA^{Pyl}_{CUA}$ pairs are orthogonal in both *E. coli* and mammalian cells.[2,6,7] Several unnatural amino acids have been site-specifically incorporated into proteins in mammalian cells by evolving the synthetase/tRNA pair in *E. coli* and subsequently transferring it to mammalian cells. This approach has the advantage of bypassing the requirement to evolve the amino acid specificity of the synthetase directly in a eukaryotic host.[8−10]

Many biological processes are more effectively addressed in the yeast *Saccharomyces cerevisiae* than in mammalian cells. Yeast has a rapid doubling time, bar-coded libraries of gene knockouts exist, protein interaction and transcriptome data is most complete, tap-tagged strains are readily available and powerful genetic approaches can be simply implemented. However, the requirement to evolve the current orthogonal pairs directly in yeast has limited the scope of unnatural amino acids that have been incorporated in yeast.

Preliminary work by Yokoyama and co-workers introduced a PylRS/$tRNA^{Pyl}_{CUA}$ pair into yeast and reported very weak phenotypes consistent with poor incorporation of N_ε-*tert*-butyl-oxycarbonyl-L-lysine,[7] but a properly characterized system for incorporating amino acids using PylRS/$tRNA^{Pyl}_{CUA}$ pairs has not been reported. Here we report the creation and characterization of a functional and orthogonal PylRS/$tRNA^{Pyl}_{CUA}$ pair in yeast

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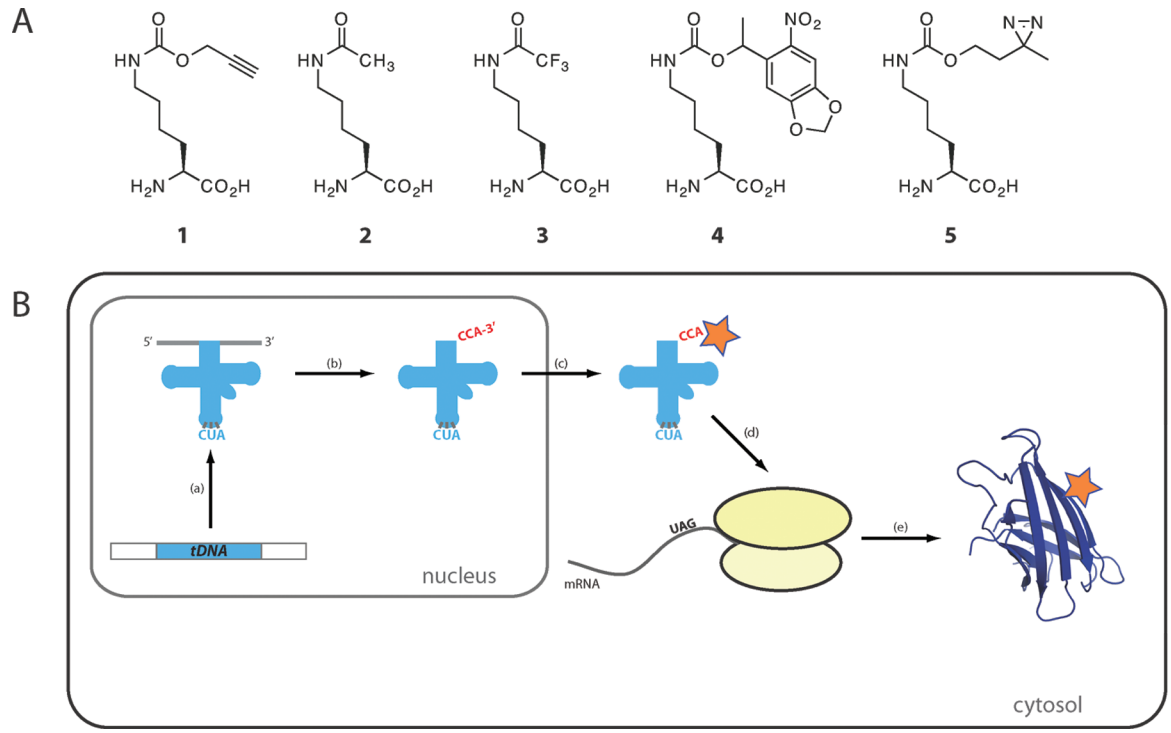

Figure 1. Genetically encoded incorporation of new unnatural amino acids in yeast. (A) Unnatural amino acids used in this study. (B) Amber suppression by foreign tRNAs in yeast. (a) The tRNA gene is transcribed by RNA polymerase III using A- and B-box promoter sequences internal to the structural gene. (b) Processing of tRNA precursor involving cleavage of 5′ and 3′ ends and addition of 3′-CCA. (c) Export to the cytoplasm for aminoacylation by aminoacyl-tRNA synthetases with an unnatural amino acid. (d) Ribosome-mediated incorporation of the unnatural amino acid in response to an amber codon on the mRNA. (e) Production of a full length protein containing an unnatural amino acid at the genetically defined site.

and demonstrate the incorporation of several useful unnatural amino acids using variants of this pair created in *E. coli* (Figure 1).

Results and Discussion

To investigate the amber suppressor activity and potential orthogonality of the *Mb*PylRS/tRNA$^{Pyl}_{CUA}$ pair in *S. cerevisiae* we used MaV203:pGADGAL4(2TAG) cells.[8,9] This yeast strain contains a *GAL4* transcriptional activator gene bearing amber codons, is auxotrophic for histidine, and contains *HIS3* and *LacZ* genes on GAL4-activated promoters. When a functional amber suppression system, such as the *Ec*TyrRS/tRNA$^{Tyr}_{CUA}$ pair,[8,9] is transformed into this strain, full length GAL4 is produced, leading to activation of *LacZ* and *HIS3* genes. Transcription of these genes allows cells to grow in the absence of histidine and turn blue in the presence of X-Gal.

We replaced the functional *Ec*TyrRS/tRNA$^{Tyr}_{CUA}$ pair with the *Mb*PylRS/*Mb*tRNA$^{Pyl}_{CUA}$ pair (Figure 2B construct 1) and supplemented with N_ε-[(2-propynyloxy)carbonyl]-L-lysine (**1**) (Figure 1A, a known substrate for *Mb*PylRS[5]) in MaV203:pGADGAL4(2TAG). These cells were unable to grow in media lacking histidine and did not turn blue in the presence of X-Gal, suggesting that this original construct is not functional (Figure 2D). We demonstrated by western blot that the yeast codon-optimized *Mb*PylRS was expressed in yeast cells (data not shown). However, analysis of northern blots indicated that *Mb*tRNA$^{Pyl}_{CUA}$ was not transcribed from our initial construct (Figure 2C). Since the *Ec*tRNA$^{Tyr}_{CUA}$ gene contains the consensus A- and B-box RNA polymerase III promoter sequences that direct its transcription in yeast,[11] but *Mb*tRNA$^{Pyl}_{CUA}$ does not (Figure 2A), it seemed likely that additional promoter elements would be required to direct the transcription of *Mb*tRNA$^{Pyl}_{CUA}$.

To address the challenge of creating new promoter elements to direct the transcription of *Mb*tRNA$^{Pyl}_{CUA}$, we investigated strategies to introduce A- and B-box sequences into our tRNA expression construct. We first mutated the sequence of the *Mb*tRNA$^{Pyl}_{CUA}$ gene to contain either near-consensus A box sequences (A11C/U15G/T24G, Figure 2B construct 2) or B box sequences (A56C, Figure 2B construct 3). Northern blot analysis demonstrated that the A56C mutation in the B box, led to very low but detectable levels of the mutant *Mb*tRNA$^{Pyl}_{CUA}$ (Supporting Information Figure 1), while expression of the (A11C/U15G/T24G) mutant tRNA was not detectable by northern blot. However, when the A56C mutant of *Mb*tRNA$^{Pyl}_{CUA}$ and *Mb*PylRS were transferred to MaV203:pGADGAL4(2TAG) in the presence of **1**, we did not observe phenotypes consistent with amber suppression (Figure 2D). This implies that either the tRNA is transcribed but not correctly folded or processed, or that the mutation abolishes synthetase recognition. Combining the A- and B-box mutations (Figure 2B construct 4) led to low levels of detectable tRNA production (Supporting Information Figure 1), but did not give phenotypes consistent with amber suppression (Figure 2D).

Since enhancing the transcription of *Mb*tRNA$^{Pyl}_{CUA}$ by mutation of the A- and B-box sequences within the structural gene did not produce a functional amber suppressor, we next investigated the potential of constructs that might augment the transcription of *Mb*tRNA$^{Pyl}_{CUA}$ using extragenic sequences. The 5′-leader sequence of the yeast *SNR52* primary transcript contains A- and B-box promoters that are post-transcriptionally removed to

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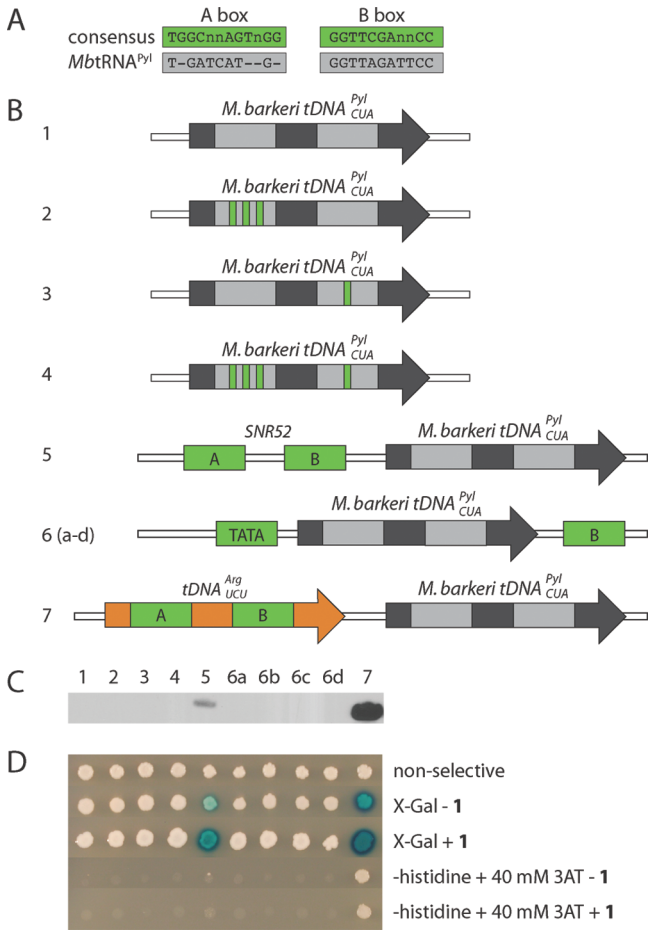

Figure 2. Creating a functional $tRNA^{Pyl}_{CUA}$ in yeast. (A) The consensus A- and B-box sequences and the A- and B-box sequences of *Mb*$tDNA^{Pyl}_{CUA}$. (B) The *Mb*$tDNA^{Pyl}_{CUA}$ expression constructs created and examined in this work. Constructs 6a−d were created using the 5′ and 3′ flanks from distinct tRNAs as described in the text. (C) Northern blots for *Mb*$tDNA^{Pyl}_{CUA}$ expression from various constructs. (D) Phenotyping constructs for amber suppression in MaV203:pGADGAL4(2TAG) cells, where 3AT is 3-aminotriazole and **1** was used at 2 mM. Cells contained *Mb*PylRS and the appropriate *Mb*$tDNA^{Pyl}_{CUA}$ expression construct.

produce mature *SNR52* snoRNA.[12] A previous report suggested that adding 5′-*SNR52* and 3′-*SUP4* flanking sequences to *Ec*$tDNA^{Tyr}_{CUA}$ and *Ec*$tDNA^{Leu}_{CUA}$ enhanced their amber suppression in yeast.[13] When *Mb*$tRNA^{Pyl}_{CUA}$ was cloned between 5′-*SNR52* and 3′-*SUP4* flanking sequences (Figure 2B construct 5), we could detect weak *Mb*$tRNA^{Pyl}_{CUA}$ transcription by northern blot (Figure 2C), and when the cassette was transformed into MaV203:pGADGAL4(2TAG) containing *Mb*PylRS and grown in the presence of **1**, we observed blue coloration on X-Gal plates but not growth in the absence of histidine in the presence of 40 mM 3-aminotriazole (3AT) (Figure 2D). These data suggest that addition of extragenic A- and B-box sequences via the 5′-*SNR52* and 3′-*SUP4* flanking sequences can partially compensate for the absence of consensus A- and B-box sequences in *Mb*$tRNA^{Pyl}_{CUA}$. However, since the *Ec*TyrRS/$tRNA^{Tyr}_{CUA}$ orthogonal pair supports growth on media lacking histidine and containing 40 mM 3AT[8,9] but this system does not, we decided that the system was suboptimal and opted to explore further extragenic sequences.

The yeast U6 (*SNR6*) gene assembles the same RNA polymerase III transcriptional machinery as tRNA genes but possesses an additional TATA-box promoter element 30 base pairs upstream of the transcription start site that binds TFIIIB.[14] The TATA-box enables TFIIIC-independent RNA polymerase III recruitment and is proposed to overcome the large separation (240 bp) of the A- and B-box promoter elements of this gene.[15] Several yeast tRNAs, some of which contain large introns between the A- and B-boxes, have TATA boxes that allow TFIIIC-independent RNA polymerase transcription.[15] We reasoned that by incorporating the flanking sequences of these genes into our tRNA cassettes it may be possible to compensate for the poor A- and B-box consensus of *Mb*$tRNA^{Pyl}_{CUA}$. We created constructs where the 5′-flanking region of *SNR6*, *Ile*{*TAT*}*LR1*, *Pro*{*TGG*}*FL*, and *Asp*{*GTC*}*KR* and the 3′-flanking region of *SNR6* sandwich *Mb*$tRNA^{Pyl}_{CUA}$ (Figure 2B constructs 6a−d). We also added a consensus sequence[16] found at the transcription start site of yeast tRNAs to the *SNR6* construct. Northern blots revealed low-level tRNA production from construct 6a (Supporting Information Figure 1). However, we did not observe phenotypes consistent with amber suppression when any of these constructs were transformed into MaV203:pGADGAL4(2TAG) containing *Mb*PylRS and grown in the presence of **1** (Figure 2D). These data suggested that, while these promoter elements may compensate for increases in the A- and B-box spacing, they cannot efficiently compensate for defects in the A- and B-box sequence in *Mb*$tRNA^{Pyl}_{CUA}$.

Yeast possess an unusual dicistronic $tDNA^{Arg}_{UCU}$−$tDNA^{Asp}_{GUC}$ gene in which the two mature tRNAs are generated from a single precursor RNA.[17] The A- and B-box promoter in the $tDNA^{Arg}_{UCU}$ gene directs the transcription of the precursor, and the transcription of $tDNA^{Asp}_{GUC}$ is entirely dependent on the promoter elements of $tDNA^{Arg}_{UCU}$. This suggests that $tDNA^{Arg}_{UCU}$ may provide the A- and B-box sequences required to transcribe tDNAs inserted in place of $tDNA^{Asp}_{GUC}$.[18] Indeed replacing $tDNA^{Asp}_{GUC}$ with human initiator tDNA or a transcriptionally inactive yeast tyrosine suppressor tDNA allows these tDNAs to be transcribed and processed to produce functional tRNAs in yeast.[19]

To test this system for the transcription of *Mb*$tRNA^{Pyl}_{CUA}$, we constructed a *Sc*$tDNA^{Arg}_{UCU}$−*Mb*$tDNA^{Pyl}_{CUA}$ cassette containing the natural 5′-, 3′-, and 10-base pair linker sequences (Figure 2B construct 7). Northern blot analysis revealed that *Mb*$tRNA^{Pyl}_{CUA}$ was transcribed from this construct much more efficiently than any other construct tested (Figure 2C). When transformed into MaV203:pGADGAL4(2TAG) in the presence of *Mb*PylRS and **1**, the *Sc*$tDNA^{Arg}_{UCU}$−*Mb*$tDNA^{Pyl}_{CUA}$ cassette conferred survival on media lacking histidine and containing 40 mM 3AT, and produced the strongest blue color of any construct tested when incubated with X-Gal (Figure 2D).

The tRNA constructs we discovered that are both transcribed (as judged by northern blot) and functional (as judged by phenotyping (constructs 5 and 7)) showed amber suppression

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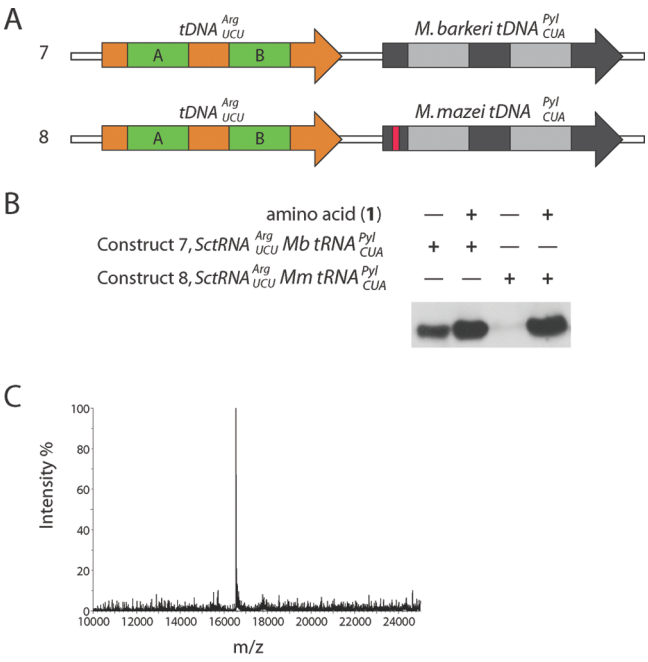

Figure 3. *Mm*tDNA$^{Pyl}_{CUA}$ is orthogonal in yeast, but *Mb*tDNA$^{Pyl}_{CUA}$ directs the incorporation of alanine and is not orthogonal in yeast. (A) Constructs used to compare orthogonality of tRNA$^{Pyl}_{CUA}$ in yeast. (B) Analysis of amber suppression by expression of hSOD33TAG-His$_6$ and detection by anti-His$_6$ western blot. Yeast cells containing the hSOD expression plasmid, *Mb*PylRS and the dicistronic *Sc*tDNA$^{Arg}_{UCU}$−tDNA$^{Pyl}_{CUA}$ construct were grown in the presence or absence of **1** (5 mM). (C) ESI-MS shows that alanine is incorporated into hSOD33TAG in cells producing amber suppressor *Mb*tDNA$^{Pyl}_{CUA}$ from construct 7 (Found 16553 ± 1.5 Da, expected 16553 Da), confirming that *Mb*tDNA$^{Pyl}_{CUA}$ is a substrate for yeast alanyl-tRNA synthetases.

phenotypes even in the absence of added amino acid **1**: construct 5 is blue on X-Gal in the presence and absence of **1,** and construct 7 is blue in the presence and absence of **1** and grows on media lacking histidine and containing 3AT in the presence and absence of **1**. These experiments revealed that *Mb*tRNA$^{Pyl}_{CUA}$ is not orthogonal in yeast.

To identify the molecular basis of the non-orthogonality of *Mb*tDNA$^{Pyl}_{CUA}$ we examined the sequence of *Mb*tRNA$^{Pyl}_{CUA}$ for nucleotides that match the positive identity elements within yeast tRNAs that are specifically recognized by yeast synthetases.[20] We realized that *Mb*tRNA$^{Pyl}_{CUA}$ contains an unusual G3·U70 base pair, which is a positive identity element by which yeast alanyl-tRNA synthetase recognizes *Sc*tRNA^{Ala}.[21] This suggested that *Mb*tRNA$^{Pyl}_{CUA}$ may be aminoacylated by alanyl-tRNA synthetase in yeast. To test this hypothesis we expressed human superoxide dismutase (hSOD) bearing an amber codon at position 33 (from pC1 hSOD33TAG-His$_6$ in the MJY125-derived strain SCY4[22]). Expression of hSOD was dependent on the presence of *Sc*tDNA$^{Arg}_{UCU}$−*Mb*tDNA$^{Pyl}_{CUA}$, but did not decrease substantially in the absence of **1** (Figure 3B), further confirming that the *Sc*tDNA$^{Arg}_{UCU}$−*Mb*tDNA$^{Pyl}_{CUA}$ cassette confers efficient amber suppression, which is not dependent on *Mb*PylRS. ESI-MS spectra of hSOD purified from expressions using *Mb*PylRS/*Mb*tRNA$^{Pyl}_{CUA}$ in the absence of unnatural amino acid were consistent with the incorporation of alanine in response to the amber codon in hSOD33TAG (Figure 3C), confirming our hypothesis on the molecular basis of *Mb*tDNA$^{Pyl}_{CUA}$ non-orthogonality.

To create an *Mb*tDNA$^{Pyl}_{CUA}$ construct that is orthogonal in yeast we converted the G3·U70 base pair in *Mb*tRNA$^{Pyl}_{CUA}$ to A3-U70. This changes *Mb*tRNA$^{Pyl}_{CUA}$ to *Mm*tRNA$^{Pyl}_{CUA}$ (Figure 3A construct 8). Yeast containing *Mb*PylRS/*Sc*tDNA$^{Arg}_{UCU}$−*Mm*tDNA$^{Pyl}_{CUA}$ produced full-length hSOD-His$_6$ from pC1SOD33TAG only in the presence of **1** (Figure 3B). These experiments establish that functional *Mm*tRNA$^{Pyl}_{CUA}$ is produced from the dicistronic construct and is orthogonal in yeast.

To begin to demonstrate the range of amino acids that can be incorporated in yeast using our approach, we incorporated the important post-translational modification N_ε-acetyl-L-lysine (**2**) and its analog N_ε-trifluoroacetyl-L-lysine (**3**), a photocaged lysine derivative N_ε-[(1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethoxy)-carbonyl]-L-lysine (**4**), and photo-cross-linker N_ε-[(2-(3-methyl-3*H*-diazirin-3-yl)ethoxy)carbonyl]-L-lysine (**5**) into hSOD-His$_6$ produced in *S. cerevisiae* (Figure 4A) using *Mb*PylRS and variants of *Mb*PylRS we have previously evolved in *E. coli*.[1−3] While we have not specifically evolved a synthetase for N_ε-trifluoroacetyl-L-lysine, we have found that AcKRS2,[1] previously evolved for incorporating N_ε-acetyl-L-lysine, efficiently incorporates this amino acid. We demonstrated the incorporation of each amino acid by western blot (Figure 4A). We carried out large-scale expression and purification of hSOD in the presence of **1**, **2**, and **3** (Figure 4B), which unlike **4** and **5** are not photosensitive and are available in gram quantities, to further confirm the site and identity of amino acid incorporation by ESI-MS and MS/MS sequencing (Figure 4C−H). We have demonstrated the specific incorporation of an amino acid into SOD in the presence of **4** and **5.** In addition we have reported MS and MS/MS data for the incorporation of amino acids **4** and **5** into proteins in other organisms.[2,23] However, we have not yet obtained MS data directly in yeast and cannot rule out the possibility that an aspect of yeast metabolism—that is not conserved in either other eukaryotes or bacteria—leads to the selective post-translational modification of these amino acids *in vivo*. Purified hSOD yields were 30−100 μg/L of yeast culture which is similar to the 50 μg/L yield reported for incorporating *p*-acetyl-L-phenylalanine into hSOD using the *Ec*TyrRS/tRNA$^{Tyr}_{CUA}$ pair in yeast.[8]

Conclusions

In summary, we have solved the key challenges of producing a functional and orthogonal tRNA$^{Pyl}_{CUA}$ in yeast. We have discovered an *Mb*PylRS/tRNA$^{Pyl}_{CUA}$ pair that is orthogonal in yeast, and described a simple system through which variant *Mb*PylRS/tRNA$^{Pyl}_{CUA}$ pairs created in *E. coli* can be transplanted to expand the genetic code of yeast for a wide range of unnatural amino acids. Using our approach we have incorporated the alkyne-containing amino acid N_ε-[(2-propynyloxy)carbonyl]-L-lysine (**1**), an important post-translationally modified amino acid N_ε-acetyl-L-lysine (**2**), and an analog of N_ε-acetyl-L-lysine, trifluoroacetyl-L-lysine (**3**), a photocaged lysine derivative N_ε-[(1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethoxy)carbonyl]-L-lysine (**4**), and a photo-cross-linker N_ε-[(2-(3-methyl-3*H*-diazirin-3-yl)ethoxy)carbonyl]-L-lysine (**5**) into proteins in yeast. Amino acid **1** may be used for bio-orthogonal [3 + 2] cycloadditions

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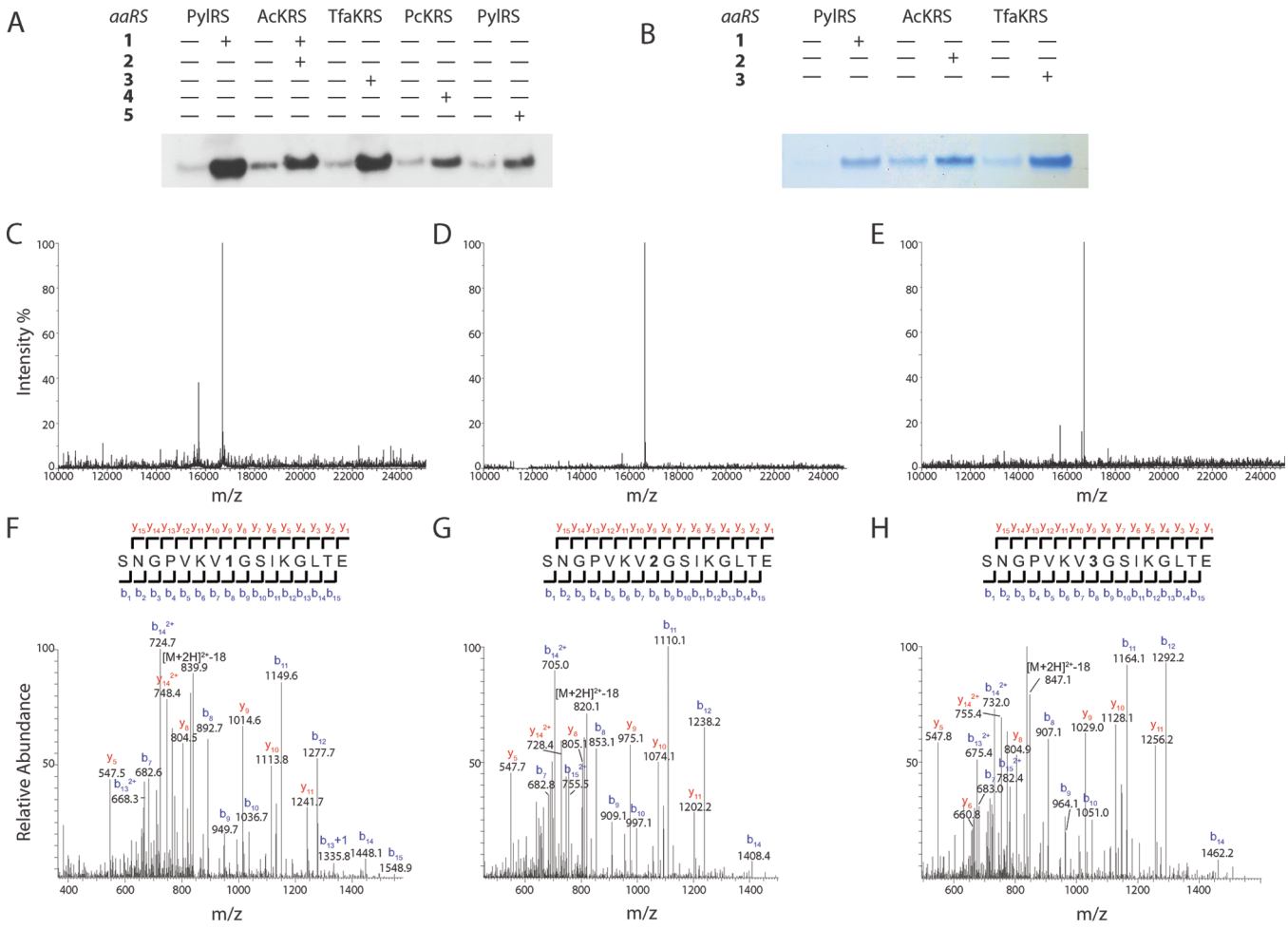

Figure 4. Characterization of unnatural amino acid incorporation in yeast with the orthogonal *Mb*PylRS/*Mm*tDNA$^{Pyl}_{CUA}$ pair. (A) Amber suppression efficiency of hSOD33TAG-His$_6$ in yeast in the presence or absence of **1** (5 mM), **2** (10 mM), **3** (10 mM), **4** (2 mM), or **5** (1.3 mM) by anti-His$_6$ western blot. Yeast cells containing the hSOD expression construct were transformed with the dicistronic *Sc*tDNA$^{Arg}_{UCU}$−*Mm*tDNA$^{Pyl}_{CUA}$ construct for expressing the orthogonal *Mm*tDNA$^{Pyl}_{CUA}$ in yeast and the appropriate aminoacyl-tRNA synthetase (aaRS). PylRS (wild-type *Mb*PylRS), AcKRS (a variant of *Mb*PylRS that has been evolved to use **2**[3]), TfaKRS (a variant of *Mb*PylRS that can use **3**, see text), PcKRS (a variant of *Mb*PylRS that has been evolved to use **4**[2]). (B) Coomassie SDS-PAGE analysis of purified hSOD from expressions in the presence and absence of **1**, **2**, or **3**. Full protein MS (C−E) and Glu-C MS/MS (F−H) confirm the incorporation of unnatural amino acids **1** (C/F found 16691 ± 1.5 Da, expected 16691 Da), **2** (D/G found 16651 ± 1.5 Da, expected 16651) and **3** (E/H found 16705 ± 1.5 Da, expected 16705) at the genetically encoded site. hSOD is copurified as a heterodimer with yeast SOD (minor additional peak in spectra at 15722 Da; identity was confirmed by Glu-C MS/MS). For full gels and western blots and larger versions of MS and MS/MS data see Supporting Information Figure 2.

in yeast proteins.[24] Amino acid **2** may be used for producing acetylated proteins directly in yeast and synthetically controlling processes normally regulated by acetylation in yeast. Amino acid **3** is a very poor substrate for sirtuins, but not for HDACs,[25] and should allow us to install irreversible acetylation at sites directly regulated by sirtuins *in vivo*. It should allow us to probe the deacetylases that act on a given site in a protein. Amino acid **4** is a photocaged lysine with demonstrated utility for controlling protein function in eukaryotic cells,[2] and we anticipate that genetically encoded photocontrol of proteins in yeast will be a powerful approach for gaining a temporal and spatial understanding of cellular processes. Amino acid **5** is a photo-cross-linking amino acid with demonstrated utility for mapping protein interactions in *E. coli*,[23] and we believe that this will find wide utility in mapping protein−protein interactions in yeast. Given the growing list of amino acids that can be incorporated using *Mb*PylRS and its variants,[1−6] we anticipate that our approach will allow the introduction of a wide range of chemical functional groups into yeast. Finally, the strategies we have explored for creating and expressing heterologous, orthogonal tRNAs in yeast may be useful for improving other orthogonal aminoacyl-tRNA synthetase/tRNA$_{CUA}$ pairs.[8−10]

Experimental Section

General Methods. N_ε-[(2-Propynyloxy)carbonyl]-L-lysine,[5] N_ε-[(1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethoxy)carbonyl]-L-lysine[2] and N_ε-[(2-(3-methyl-3*H*-diazirin-3-yl)ethoxy)carbonyl]-L-lysine[23] were synthesized as previously reported. N_ε-Acetyl-L-lysine and N_ε-trifluoroacetyl-L-lysine were purchased from Bachem.

Northern Blot Analysis. Total RNA was purified from yeast cells using TRI reagent (Sigma) and ethanol precipitated. The RNA was denatured, separated on a 6% Novex TBE-urea gel (Invitrogen), blotted onto Biodyne B modified nylon membrane (Thermo Scientific), and cross-linked by UV fixation. The membrane was hybridized overnight at 55°C with a biotinylated probe 5′-

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GGAAACCCCGGGAATCTAACCCGGCTGAACGGATTTAG-AG, which is specific for *Mb*tDNA$^{Pyl}_{CUA}$. The hybridized probe was detected with North2South chemiluminescent hybridization and detection kit (Pierce). The number of cells was used to control the total amount of RNA loaded.

Phenotyping Yeast Cells. Phenotyping was performed as described in Chin et al..[8] Briefly, *S. cerevisiae* MaV203 (Invitrogen) was transformed by the lithium acetate method with the pGADGAL4-(2TAG) reporter, p*Mb*PylRS and tDNA$^{Pyl}_{CUA}$ constructs. Overnight cultures were serially diluted and replica plated onto selective media in the presence or absence of 2 mM N_ε-[(2-propynyloxy)carbonyl]-L-lysine (**1**). X-Gal assays were performed using the agarose overlay method.

Protein Expression, Purification, western blot Analysis, and Mass Spectrometry. Appropriate selective medium ± unnatural amino acid was inoculated with a stationary phase culture to give an $OD_{600} \approx 0.2$. Cultures were grown at 30°C for 24−48 h. Proteins were extracted from yeast cells using Y-PER reagent (Thermo Scientific) containing complete, EDTA-free inhibitor cocktail (Roche). Clarified supernatants were separated by SDS-PAGE, and western blots were performed using anti-His_6 (Qiagen). Human superoxide dismutase was purified using Ni^{2+}-NTA resin (Qiagen) as previously described.[26] For expressions with N_ε-acetyl-L-lysine (**2**), 20 mM nicotinamide was added to the cultures and to lysis buffers; for expressions with N_ε-trifluoroacetyl-L-lysine (**3**), 10 mM sodium butyrate was added to the cultures and to lysis buffers. Protein concentration was determined using the Biorad Protein Assay in comparison to IgG standard. Total mass analysis was performed on a LCT time-of-flight mass spectrometer with electrospray ionization (Micromass) with protein solutions in 20 mM ammonium bicarbonate and mixed 1:1 with 1% formic acid in 50% MeOH. Samples were injected at 10 $\mu L \cdot min^{-1}$, and calibration was performed in positive ion mode using horse heart myoglobin. MS/MS analysis was performed on a LTQ-Orbitrap mass spectrometer on protein samples that were in-gel digested with Glu-C (Roche).

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Supporting Information Available: Supplementary figures, details of plasmid construction, and construct sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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