An Expanded Genetic Code in Candida albicans To Study Protein-Protein Interactions In Vivo

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For novel insights into the pathogenicity of Candida albicans, studies on molecular interactions of central virulence factors are crucial. Since methods for the analysis of direct molecular interactions of proteins in vivo are scarce, we expanded the genetic code of C. albicans with the synthetic photo-cross-linking amino acid p-azido-1-phenylalanine (AzF). Interacting molecules in close proximity of this unnatural amino acid can be covalently linked by UV-induced photo-cross-link, which makes unknown interacting molecules available for downstream identification. Therefore, we applied an aminoacyl-tRNA synthetase and a suppressor tRNA pair (EcTyrRNA_CUA) derived from Escherichia coli, which was previously reported to be orthogonal in Saccharomyces cerevisiae. We further optimized the aminoacyl-tRNA synthetase for AzF (AzF-RS) and EcTyrRNA_CUA for C. albicans and identified one AzF-RS with highest charging efficiency. Accordingly, incorporation of AzF into selected model proteins such as Tsa1p or Tup1p could be considerably enhanced. Immunologic detection of C-terminally tagged Tsa1p and Tup1p upon UV irradiation in a strain background containing suppressor tRNA and optimized AzF-RS revealed not only the mutant monomeric forms of these proteins but also higher-molecular-weight complexes, strictly depending on the specific position of incorporated AzF and UV excitation. By Western blotting and tandem mass spectrometry, we could identify these higher-molecular-weight complexes as homodimers consisting of one mutant monomer and a differently tagged, wild-type version of Tsa1p or Tup1p, respectively, demonstrating that expanding the genetic code of C. albicans with the unnatural photo-cross-linker amino acid AzF and applying it for in vivo binary protein interaction analyses is feasible.

The application of unnatural amino acids has widened the scope of protein biochemistry dramatically (1), even more so, since they can be efficiently incorporated into proteins by using orthogonal tRNAs and corresponding aminoacyl-tRNA synthetases (aaRS) in model organisms—a method that has been reported as “expanded genetic code.” This approach has first been described for Escherichia coli (2–4), but 10 years later, the genetic codes of other prokaryotic and eukaryotic model organisms or cell lines, including Saccharomyces cerevisiae (5–7) and even multicellular organisms such as Caenorhabditis elegans (8), have been expanded with ~80 unnatural amino acids (9). Applications of these unnatural amino acids include among others a variety of protein manipulation techniques and allow for structure or function analyses or detection of protein localization (10). However, an expanded genetic code in human pathogenic fungi, including Candida albicans, is still missing. C. albicans is the major cause of fungal infections in humans (11, 12); however, a comprehensive picture of the pathogenesis-related mechanisms that differentiate this fungus from other closely related nonpathogenic yeast species is still emerging. With the advent of next-generation sequencing techniques, more and more data have become available on the genomic and transcriptomic level, whereas protein interaction data are massively underrepresented and yet essentially needed to complement studies on the posttranslational level.

Due to its aberrant CUG codon usage (13, 14) and lacking efficient plasmid systems, “tailor-made” techniques to analyze protein interactions in C. albicans are scarce. Recent contributions include the adaptation of yeast two-hybrid techniques (15) and tandem-affinity purification tag techniques (16, 17), as well as the establishment of a vesicle targeting method (18), for C. albicans. However, amino acids represent the smallest building blocks of a protein that can be potentially substituted by unnatural amino acids, which offer a wider scope to add new biochemical properties to a protein of interest or to improve or manipulate its function. We therefore intended to make this toolbox available for C. albicans, and expanded its genetic code with the unnatural amino acid p-azido-1-phenylalanine (AzF). AzF is one of several unnatural amino acids, which can be photoactivated by UV light to induce covalent bonds to molecules in close vicinity. Photo-cross-linker amino acids are consequently suitable to specifically preserve molecular interactions and capture even weak or transient interactions in a living cell. Due to covalent bond formation, interactions are stable for further characterization such as mass spectrometry or Western blotting. Utilization of photo-cross-linker amino acids in the analysis of virulence factors therefore represents a promising strategy in elucidating regulatory networks in pathogens, which has recently been exemplified by uncovering unknown interacting proteins for the acid chaperone HdeA in the pathogenic prokaryote Shigella flexneri (19).

In the present study, we demonstrate the addition of the unnatural photo-cross-linker amino acid AzF to the genetic code of the eukaryotic pathogen C. albicans, using an orthogonal system derived from E. coli and adapting it for the molecular characteristics of C. albicans. Accordingly, the optimized orthogonal tRNA
(EcTrytRNACUA) and tRNA synthetase for AzF (AzF-RS) were integrated into the genome of the clinical isolate SC5314 (20) and used to incorporate AzF site-specifically in response to an amber stop codon into two model proteins, Tsa1p (21) and Tup1p (22). We used Tsa1p, an abundant cytosolic protein, and Tup1p, a universal transcriptional repressor, as proof of concept to elucidate the feasibility of using AzF to site specifically introduce cross-links between interacting proteins in \textit{C. albicans}. We demonstrated that an identification of known or unknown interaction partners is accomplishable in a eukaryotic background by mass spectrometry, since these photo-cross-linked protein complexes are covalently bound and highly stable for purification. In this way, we provide a general system for the investigation of \textit{in vivo} binary interactions between \textit{C. albicans} proteins that might be helpful in elucidating assumed or unknown regulatory interactions.

\section*{MATERIALS AND METHODS}

\textbf{Media and growth conditions.} \textit{C. albicans} strains were routinely grown in YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) or SC medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% glucose, supplemented with amino acids). When AzF was added to the medium, a stock solution of 667 mM AzF (Bachem) was freshly prepared in 1 M NaOH and added to the culture to a final concentration of 1 mM AzF. Hyphal growth was induced by growth in YPD plus 10% fetal calf serum at 37°C. \textit{S. cerevisiae} strains were grown in -Leu/-Try SC medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% glucose, supplemented with amino acids, but without Leu and Trp). If AzF was added to the medium, a stock solution of 1 M AzF was freshly prepared in 1 M NaOH and added to the culture to a final concentration of 1 mM AzF.

\textbf{Plasmid and strain construction.} (i) pSFS2A ActPr AzF-RS 3 CTG opt His6 ActT 3 FR. All 29 CUG codons of the original AzF-RS 3 were replaced by UUG codons via seven rounds of site-directed mutagenesis with the QuikChange multi-site-directed mutagenesis kit (Stratagene) to obtain plasmid pAR3 CTG opt-PGK1 plus 3SUP4+tRNACUA. First, the original ScADH1 promoter was replaced. The CUG-optimized AzF-RS with the ScADH1 downstream sequence was amplified with the primers AzRS for and ADH1 Term rev NotI, using the previously created plasmid as a template. The 500-bp ACT1 upstream sequence was amplified with the primers ActPr500 for NotI and ActPr rev for genomic DNA using the primers ActPr500 for NotI and ActPr rev NotI. The resulting product was cloned into vector pSFS2A to produce the vector pSFS2A AzF-RS 3 CTG opt His6 ActT 3 FR.

(ii) pSFS2A ActPr AzF-RS 3 V5 ActT 3 FR. A 532-bp fragment immediately downstream of the TSA1 ORF was amplified from genomic DNA using the primers Tsa1 FR2 for NotI and Tsa1 ORF rev w/o TAA XhoI 3. The resulting product was cloned into vector pSFS2A using the restriction enzymes KpnI and XhoI. The final vector pSFS2A CT Tsa1 FR2 was digested with KpnI and XhoI, while the PCR product from the overlap PCR was digested with KpnI/Sall. Upon cloning of the insert into the vector, a new Xhol restriction site was introduced via the insert. Therefore, the insertion of the \textit{EcTrytRNACUA} under \textit{snr52} regulation could be repeated for additional three rounds, creating plasmid pSFS2A snr52 FR2 4 tRNAs. To create a larger region for genomic homologous recombination, a 217-bp region upstream of the previously utilized upstream region was amplified from genomic DNA using the primers snR52 Pr 220 for KpnI-XhoI and snr52 Pr 300 rev XhoI to create the vector pSFS2A ActPr AzF-RS 3 V5 ActT 3 FR.

(iii) pSFS2A ActPr AzF-RS 1 ActT 3 FR. In order to eliminate the previously utilized V5 tag of the AzF-RS, which might interfere with likewise V5-tagged cross-link products in the Western blot, we amplified a 1,779-bp product of the ACT1 downstream flanking region was amplified from genomic SC5314 DNA with the primers ActPr500 for NotI and ActPr rev NotI. The resulting product was cloned into vector pSFS2A to produce the vector pSFS2A ActPr AzF-RS 3 V5 ActT 3 FR.

(iv) pSFS2A snR52 4 tRNAs up FR. To express the heterologous \textit{EcTrytRNACUA} in \textit{C. albicans}, the 426-bp snr52 downstream flanking region was amplified from genomic DNA using the primers snR52 FR2 for NotI and snr52 FR2 rev SacII and cloned into plasmid pSFS2A. A 258-bp fragment with novel KpnI Xhol restriction sites and an \textit{ACT1} downstream flanking sequence was PCR amplified from genomic DNA (strain SC5314) with primers snR52 Pr 220 for KpnI-XhoI and snr52 Pr 300 rev XhoI to create vector pSFS2A. A second PCR was carried out, using plasmid pSFS2A and \textit{EcTrytRNACUA} TAE 3 FR as a template and the primers tRNA N for and tTE Term rev Sall to amplify a 193-bp DNA fragment. Both products were fused in an overlap PCR with the primers snR52 Pr 220 for KpnI-XhoI and tTE rev Sall. Vector pSFS2A snr52 FR2 was digested with KpnI/XhoI, while the PCR product from the overlap PCR was digested with KpnI/Sall. Upon cloning of the insert into the vector, a new Xhol restriction site was introduced via the insert. Therefore, the insertion of the \textit{EcTrytRNACUA} under \textit{snr52} regulation could be repeated for additional three rounds, creating plasmid pSFS2A snr52 FR2 4 tRNAs. To create a larger region for genomic homologous recombination, a 217-bp region upstream of the previously utilized upstream region was amplified from genomic DNA using the primers snR52 Pr 220 for KpnI-XhoI and snr52 Pr 300 rev XhoI to create the vector pSFS2A snr52 FR2 4 tRNAs.

(v) pSFS2A CT. Plasmid pSFS2A CT was created to clone genes of interest for expression with a fused C-terminal V5/His6 tag. Oligonucleotides C3-5 and C5-3 were phosphorylated and annealed according to standard protocols. The hereby created double-stranded DNA sequence with overhangs was dead-end cloned into Xhol-digested vector pSFS2A.

(vi) pSFS2A CT Tsa1. A 532-bp fragment immediately downstream of the \textit{TSA1} ORF was amplified from genomic DNA using the primers Tsa1 FR2 for NotI and Tsa1 ORF rev w/o AAT XhoI 3. The resulting product was cloned into vector pSFS2A using the restriction enzymes KpnI and XhoI. The final vector pSFS2A CT Tsa1 FR2 was used as a template in PCR-based mutagenesis to insert position-specific amber mutations using the multi-site-directed mutagenesis kit from Stratagene or a PCR-based protocol similar to the Phusion site-directed mutagenesis kit (New England BioLabs) and the primers listed in Table S3 in the supplemental material.
pSFS2A HA. We created pSFS2A HA to facilitate expression of a gene of interest with a C-terminally fused C. albicans codon-optimized HA tag. Oligonucleotides CT HA for and CT HA rev were phosphorylated and annealed according to standard protocols and the hereby created double-stranded DNA fragment with single-stranded overhangs was dead-end cloned into XhoI-digested pSFS2A.

pSFS2A HA Tsa1. The Tsa1 ORF with upstream flanking region but without stop codon was cut out of pSFS2A CT Tsa1 FR1 ORF FR2 with Kpn1/XhoI and cloned into the equally digested vector pSFS2A CT HA to yield vector pSFS2A CT HA Tsa1 FR1 ORF. TSA1 FR2 was cut out of the vector pSFS2A CT Tsa1 FR1 ORF FR2 with NotI/SacII and ligated into vector pSFS2A HA Tsa1 FR1 ORF to create the final vector pSFS2A CT HA Tsa1 FR1 ORF FR2.

pSFS2A CT Tup1. A 1,254-bp product immediately downstream of the TUP1 ORF was amplified from genomic DNA using the primers Tup1 FR2 Ntot for and Tup1 FR2 rev SacII and cloned into vector pSFS2A CT to create the vector pSFS2A CT Tup1 FR2. The TUP1 ORF with an upstream flanking region was amplified without the stop codon from genomic DNA using the primers Tup1 FR1 FRKpnl and Tup1 ORF w/o TAA rev XhoI. The resulting 2,080-bp fragment was cloned into vector pSFS2A Tup1 FR2 with Kpn1 and XhoI restriction sites. The final vector pSFS2A CT Tup1 FR1 ORF FR2 was used as a template in site-directed mutagenesis to insert position-specific amber mutations using a PCR-based protocol and the primers listed in Table S3 in the supplemental material.

Northern blotting. For total RNA isolation, C. albicans and S. cerevisiae strains were grown in the respective SC media. Fresh cultures were inoculated from over-night cultures to an optical density of 0.4. After 4 h of growth, the cells were harvested by centrifugation and snap-frozen. Homogenization was carried out with a Mixer Mill MM 200 (Retsch), and a 50,000-fold dilution of SYBR Gold [Invitrogen]. Transfer to a dried transfer unit (Hoefer, TE77X semidry transfer unit) in 0.5 M NaCl plus 0.015 M sodium citrate–0.1% SDS and one washing step with 0.1× SSC–1% SDS, the membrane was exposed to a phosphorimager plate (Fuji Photo Film) for 3 to 6 h, and signals were read using a FLA-5100 laser scanner (Fuji Photo Film).

Acid urea-polyacrylamide gel electrophoresis (acid urea-PAGE). RNA isolation and analysis under acidic conditions was carried out according to the procedure described by Köhler and RajBhandary (26). For RNA isolation under acidic conditions, strains were grown under the same conditions as described for Northern blot. To analyze the acetylation of the Ec TyrRNA has in the presence of AzF, AzF was added to the medium to a final concentration of 1 mM as described above. After cell homogenization using the Mixer Mill MM 200 (Retsch), cell powder was resuspended in sodium acetate buffer (0.3 M sodium acetate, 10 mM EDTA [pH 5.0]). One volume of water-equilibrated phenol (pH 4.5) was added, and the samples were vortexed for 15 min in 10-s pulses. Between vortexing steps, the cell suspensions were kept on ice. After vortexing, the samples were centrifuged for 15 min at 16,000 × g, and the aqueous phase was transferred into a fresh reaction tube, while 250 μl of sodium acetate buffer was added to the phenol phase, and the vortexing steps and centrifugation were repeated. After both aqueous phases were pooled, 2.5 volumes of −20°C, 100% ethanol was added, and the nucleic acids were precipitated for at least 1 h at −20°C. The nucleic acids were pelleted by centrifugation for 30 min at 12,000 × g and 4°C. Pellets were washed twice with −20°C, 70% ethanol and allowed to air dry. Depending on the pellet size, it was resuspended in 80 to 120 μl of 10 mM sodium acetate (pH 5.0) or in the same volume of ice-cold water, if the sample was further subjected to deacetylation. For gel electrophoresis, a 6.5% polyacrylamide (19:1 acrylamide-bisacrylamide), 8 M urea, and 0.1 M sodium acetate (pH 5.0) gel of the dimensions 16 cm by 18 cm by 1 mm was cast. After 30 min of pre-electrophoresis in a running buffer of 0.1 M sodium acetate (pH 5.0), 20 μg of the acidic RNA sample was mixed with an equal volume of sample buffer (0.1 M sodium acetate [pH 5.0], 8 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol) and separated on the gel for 6 to 7 h at 160 V. For S. cerevisiae samples, only 1 μg of acidic RNA was loaded onto the gel. As a size standard, a low-range ssRNA ladder (New England BioLabs) was used according to the manufacturer’s protocols. After electrophoresis, the gel was stained in a 0.1 M sodium acetate (pH 5.0), 50,000-fold dilution of SYBR gold stain (Invitrogen). RNA was blotted onto a nylon membrane (Hybond-N; GE Healthcare) with a semidyed transfer apparatus (Hoefer, TE77X semidyed transfer unit) in transfer buffer (40 mM Tris–HCl [pH 8.0], 2 mM EDTA) for 45 min at 300 mA. After electrophoresis, RNA was cross-linked to the membrane using a UV cross-linker (Stratalinker; Stratagene). Labeling, hybridization, and exposure was carried out as described for the Northern blotting. The charging ratio of the AzF-RS variants was calculated from the exposed Northern blots of two independent experiments using AIDA image analyzer software (Raytest).

 Protein isolation and cross-link induction. Proteins were isolated from strains incubated in SC medium with or without 1 mM AzF. Depending on the analyzed protein, cultures were inoculated to a starting OD600 between 0.1 and 0.7 and incubated for 4 to 6 h. For cross-link induction, the respective samples were transferred into a petri dish or larger plastic container and irradiated with 365 nm for 30 min in a BioLink UV cross-linker (Peglab). The cells were either used directly for protein isolation or snap-frozen in liquid nitrogen. Cell lysis occurred by bead beating in PGSK buffer (Na2HPO4, 3.3 mM; NaH2PO4, 49.4 mM; NaCl, 48.4 mM; KCl, 4.9 mM; glucose, 61 mM) supplemented with 1× EDTA-free protease inhibitor cocktail (Complete; Roche) and phenylmethylsulfonyl fluoride (1 mM). After centrifugation, the crude cell lysates were precipitated according to Wessel and Fluegge (27), and protein pellets were resuspended in 1% SDS–25 mM NaOH.
Immunoprecipitation. To precipitate V5- or HA-tagged Tsa1p or Tup1p variants, strains were cultivated as described above for protein isolation. For each immunoprecipitation, 50 ml of culture was harvested by centrifugation, and the pellet was washed in immunoprecipitation buffer (IP buffer; 300 mM NaCl and 50 mM Tris HCl [pH 7.4]) supplemented with 1× protease inhibitor cocktail without EDTA [Complete] and 1 mM PMSE. Cells were lysed in IP buffer by vortexing with glass beads. After the lysates were cleared by centrifugation, Triton X-100 was added to a final concentration of 1%. Immunoprecipitation was carried out with the V5-tagged protein purification kit or the HA-tagged protein purification kit (MBL International) according to the manufacturer’s directions. Briefly, 500 μl of the prepared protein extracts was mixed with 20 μl of anti-V5-tag beads or anti-HA-tag beads and transferred to a column. Incubation for 1 h at 4°C rotating was followed by three washing steps with the supplied wash buffer. Elution was carried out in two steps with the supplied V5 peptide or HA peptide.

SDS-PAGE and Western blotting. To analyze translational suppression, 40 μg of total precipitated protein extract was separated on SDS–12% PAGE gels (Tsa1p) or SDS–8% PAGE gels (Tup1p). Up to 100 μg of precipitated protein extract was loaded onto the aforementioned SDS–PAGE gels, in order to analyze cross-link formation. As protein standards, the Spectra multicolor broad range protein ladder or the Spectra multicolor high range protein ladder (Fermentas) were used. Sample buffer, running buffer, and gels were prepared according to method of Laemmli (28). For immunodetection, proteins were transferred to a polyvinyldene difluoride membrane (Immobilon-P; Millipore) using a semidry transfer unit (Hoefer TE77X semidry transfer unit). Blocking of the membrane was carried out in phosphate-buffered saline (PBS; pH 7.4) with 5% skim milk for 1 h at room temperature or overnight at 4°C. After a washing step of 10 min in PBS (pH 7.4) with 0.05% Tween 20, membranes were incubated with mouse monoclonal anti-V5 antibody (1:5,000; Clone SV5-PK1 Acris) or rabbit polyclonal anti-HA antibody (1:5,000; Acris) in PBS (pH 7.4), 0.05% Tween 20, and 0.5% BSA. Subsequent detection occurred via peroxidase-coupled sheep anti-mouse antibody (1:1,500; GE Healthcare) or peroxidase-coupled goat anti-rabbit antibody (1:1,500; GE Healthcare) and the ECL Plus chemiluminescence substrate (Pierce) with a LAS-1000 CCD camera (Fuji Photo Film).

Fluorescence labeling of AzF containing Tsa1p. For covalent labeling of a mutant, AzF containing Tsa1p with an azide-reactive fluorescent dye, strains carrying the orthogonal pair and wild-type Tsa1p (SPC46) or N141X mutant Tsa1p (SPC55) were inoculated to an OD600 of 0.7 in SC medium with AzF and incubated for 4 h. Cells were harvested, washed twice in sterile PBS (pH 7.4), and frozen in liquid nitrogen. Per labeling reaction, 50 ml of culture was harvested. Cells were resuspended in PBS (pH 7.4), supplemented with 1× EDTA-free protease inhibitor cocktail (Complete; Roche) and lysed by bead beating. DyLight 550-phosphine (Pierce) was added to a final concentration of 1 mM to lysates, and the reaction was incubated for 3 h at 37°C. Triton X-100 was added to a final concentration of 1%, and the samples were immunoprecipitated with the V5-tagged protein purification kit (MBL International), as described and eluted in 40 μl. The sample was split into 10- and 30-μl portions and separated on a SDS–12% PAGE gel. The 10-μl sample was analyzed by Western blotting, and the 30-μl sample was visualized by fluorescence imaging of the SDS–PAGE gel using a FLA 5100 laser scanner (Fuji Photo Film) with a Cy3 filter and laser excitation at 532 nm.

Tup1p purification. For a mass spectrometric analysis of mutant Tup1p monomer and the higher molecular weight complexes, Tup1p was purified using a two-step protocol. The first purification step was carried out via the His6 tag; afterward, a purification using the V5 tag was conducted. An overnight culture of strain YBC6 carrying the orthogonal pair and Tup1 variant L63X was used to inoculate 1 liter of SC medium with 1 mM AzF to an OD600 of 0.5. The culture was incubated for 6 h at 30°C. Afterward, the culture was irradiated in the Bio-Link UV cross-linker (Peqlab) for 45 min at 365 nm. Cells were harvested by centrifugation and washed in native lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole [pH 8.0]), and the cell pellet was resuspended in a volume of ca. 10% of the pellet size and disrupted into liquid nitrogen. One liter of culture yielded a pellet of ~8 ml. The sample was stored at ~80°C until further processing. For cell lysis, the frozen cells were thawed on ice in 2 volumes of native lysis buffer supplemented with 1× EDTA-free protease inhibitor cocktail (Complete; Roche) and 1 mM PMSE. The sample was split and transferred into two 50-ml polypropylene reaction tubes, 4 ml of glass beads was added, and the cells were lysed by vortexing (four times, 5 min each time). Protein extracts were cleared by centrifugation for 20 min at 12,000 × g and 4°C. The cleared protein extract (10 ml) was applied to a gravity flow column, containing 1.5 ml of native lysis buffer equilibrated Ni-NTA agarose (Qiagen). All purification steps were carried out at 4°C. Each column was washed with 60 ml of native wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole [pH 8.0]) and elution was carried out in two 2-ml fractions of elution buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole [pH 8.0]). Elution fractions from the first elution step (E1) from two columns were pooled to obtain 4 ml of His6-tag-purified native eluate E1. Buffer exchange against IP buffer (300 mM NaCl, 50 mM Tris HCl [pH 7.4]) was carried out with Zeba spin desalting columns (Pierce) according to the manufacturer’s specifications. Triton X-100 was added to the buffer exchanged eluate to a final concentration of 1%, and the sample was split into seven 500-μl portions, which were processed with the V5-tagged protein purification kit (MBL International) as described above for immunoprecipitations. The seven 40-μl eluates after immunoprecipitation were pooled and concentrated using an Amicon Ultra-0.5 ml (MWCO, 10 kDa; Merck–Millipore) filter device. The retentate of ~25 μl was mixed with Laemmli sample buffer and loaded onto a SDS–8% PAGE gel (16 cm by 18 cm by 1 mm). Separation was carried out for 6 h at 130 V, and the gel was fixed in 40% ethanol–10% acetic acid for 1 h and washed twice for 10 min with water at room temperature. The gel was subsequently stained with colloidal Coomassie according to the Neuhoff protocol (29). A 0.1% Coomassie brilliant blue G250–2% ortho-phosphoric acid–10% ammonium sulfate stock solution was supplemented with methanol directly before use to a final concentration of 20% methanol. The gel was incubated in the staining solution for 24 h at room temperature and destained three times for 15 min in 1% acetic acid at room temperature. The protein bands of interest were cut out from the gel and stored at ~20°C until further processing.

Mass spectrometry. Tsa1p samples were analyzed with matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) at TopLab GmbH (Martinsried, Germany) according to standard protocols. Tup1p samples were analyzed by Nano-LC-ESI-MS/MS (nano-liquid chromatography-electrospray ionization–tandem mass spectrometry) by Hohenheim University. Proteins were in-gel digested using trypsin (Roche, Germany) according to the method of Shevchenko et al. (30). After digestion, the gel pieces were extracted with 50% acetonitrile (ACN) and 0.1% (vol/ vol) formic acid (FA) for 15 min. The supernatant was collected, and the gel pieces were covered with 5% FA for 15 min before the same volume of ACN was added. After incubation for 10 min, the supernatant was collected. The pooled supernatants were then dried in a vacuum centrifuge and stored at ~20°C. Dried samples were dissolved in 0.1% FA. Nano-LC-ESI-MS/MS experiments were performed on an Acquity Nano-UPLC system (Waters, USA) coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Germany). Tryptic digests were concentrated and desalted on a precolumn (2 cm by 180 μm, 5-μm particle size; Symmetry C18 [Waters]) and separated on a BEH 130 C18 reversed-phase column (25 cm by 75 μm, 1.7-μm particle size [Waters]). Gradient elution was performed from 1% ACN to 50% ACN in 0.1% FA within 90 min. The LTQ-Orbitrap was operated under the control of Xcalibur 2.0.7 software. Survey spectra (m/z = 250 to 2,000) were detected in the Orbitrap at a resolution of 60,000 at m/z = 400. Data-dependent tandem mass spectra were generated for the seven most abundant peptide precursors in the linear ion trap. For all measurements using the Orbitrap detector, internal calibration was performed using lock-mass ions from ambient air as described in Olsen et al. (31). Mascot 2.3 (Matrix Science, United
Kingdom) was used as the search engine for protein identification. Spectra were searched against the yeast subset of the NCBI protein sequence database downloaded as FASTA-formatted sequences from ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz. Search parameters specified trypsin as the cleaving enzyme, allowing two missed cleavages, a 5-ppm mass tolerance for peptide precursors, and a 0.6-Da tolerance for fragment ions. Carbamidomethylation of cysteine residues was set as a fixed modification. Methionine oxidation and serine/threonine/tyrosine phosphorylation were allowed as variable modifications. To ensure detection of the peptides derived from the V5/His tag, the resulting peptides were also matched to the protein sequence of the Tsa1p V5/His6 fusion protein. For the identification of p-amino-l-phenylalanine, p-amino-l-phenylalanine was added as a variable substitution of leucine 63 in CaTup1p or arginine 141 in CaTsa1p.

RESULTS
Optimization and functionality of the orthogonal pair in C. albicans. Integration of unnatural amino acids into S. cerevisiae proteins is mediated by an amber codon-decoding suppressor tRNA7 from Escherichia coli (EcTyrRNA_CUA). The corresponding, former tyrosyl-tRNA synthetase from E. coli (TyrRS) has been extensively mutagenized in the active-site residues to ensure substrate affinity only for the unnatural amino acid AzF (6). Both tRNA and AzF-tRNA synthetase (AzF-RS) are termed "orthogonal pairs" since they function in parallel to other endogenous tRNAs and aaRS in the respective organism. Incorporation of the unnatural amino acid into a protein of interest is achieved by inserting an amber stop codon at the desired position of the gene of interest and encoding it together with the orthogonal pair in one strain (Fig. 1A). By the addition of AzF (Fig. 1B) to the medium, the AzF-RS specifically aminoacylates the EcTyrRNA_CUA with AzF, which is then efficiently incorporated in response to the amber codon during translation. To facilitate the introduction of the unnatural amino acid AzF into C. albicans proteins, we intended to utilize the orthogonal pair from S. cerevisiae (7). For heterologous expression of the AzF-RS, the E. coli gene had to be codon optimized for translation in C. albicans. Therefore, we replaced the 29 alternatively translated CUG codons with UUG codons to ensure leucine incorporation during protein synthesis (see Fig. S1 in the supplemental material). For strong constitutive expression, we placed the corresponding ORF between ACT1 regulatory sequences and integrated the construct into the ACT1 locus of the clinical isolate SC5314. Furthermore, constructs were created with a His6 tag or a V5/His6 tag fused to the synthetase for immunological detection of the protein. Although the AzF-RS gene was transcribed in C. albicans, no full-length protein could be detected by immunoblotting (data not shown). We hypothesized that the codon composition of AzF-RS hindered its efficient translation in C. albicans and completely optimized the gene according to the codon usage of C. albicans (see Fig. S1 in the supplemental material). Complete codon optimization yielded a full-length AzF-RS protein of the expected size of 48 kDa, which was detectable by immunoblotting (data not shown).

In eukaryotes, tRNAs are transcribed by RNA-polymerase III (Pol III), and transcription is regulated by internal sequences, the A- and B-boxes (32). Although the EcTyrRNA_CUA contained internal A- and B-boxes, comparison with yeast consensus sequences determined total conservation of the B-box, but only partial conservation of the A-box (33). Consequently, transcription from these regulating sequences alone could not be observed in C. albicans (Fig. 1C). We therefore fused the tRNA without its 3’ CCA repeat, which is added in eukaryotes enzymatically (34), to a SNR52 promoter and a glutamic acid tRNA (tECUA) terminator (see Fig. S2 in the supplemental material) that are Pol III-regulating sequences in C. albicans. The SNR52 gene encoding a small nucleolar RNA (snoRNA) is one of the few Pol III-regulated genes, with transcription driven from A- and B-boxes, which are external to the mature transcript, as opposed to tRNA genes with internal A- and B-boxes (35). The approach to fuse Pol III-regulating sequences with a heterologous tRNA has previously been described as successful in S. cerevisiae (36). We utilized the downstream region of the glutamic acid tRNA tECUA to the 5’ end of the EcTyrRNA_CUA since a 3'-rich stretch is sufficient for transcriptional termination (37). The SNR52-driven EcTyrRNA_CUA construct was subsequently integrated into the SNR52 locus. Using this construct, we could successfully determine the expression of the SNR52 regulated EcTyrRNA_CUA by detection with an EcTyrRNA_CUA-specific probe in total RNA of strain SPC30 (Fig. 1C).

To ensure highest charging efficiency of the EcTyrRNA_CUA, we analyzed six previously for S. cerevisiae described variants of AzF-RS (6) in C. albicans. Variants 1 to 6 were created based on the C. albicans codon usage-optimized AzF-RS variant 3 by site-directed mutagenesis of the active-site residues (see Table S4 in the supplemental material). In order to analyze the charging efficiency of the AzF-RS, an acid urea-PAGE/Northern blotting technique was applied to separate aminoacylated tRNAs from uncharged tRNAs (26) and to identify the AzF-RS variant with the highest aminoacylation rate for the EcTyrRNA_CUA. All except one variant were able to specifically charge the EcTyrRNA_CUA in the presence of AzF, although to widely varying degrees (data not shown). The codon-optimized variant 3 showed a charging efficiency in C. albicans (strain KC24, 49.5% aminoacylated tRNA), similar to that of the original AzF-RS 3 in S. cerevisiae (strain MBS1, 44.8% aminoacylated tRNA) (Fig. 2). However, the codon-optimized AzF-RS 1 (strain KC22) exhibited a significantly increased charging efficiency of 68.2% aminoacylated tRNA (Fig. 2). Interestingly, an unspecific background aminoacylation in the absence of AzF was detectable for the EcTyrRNA_CUA expressed in S. cerevisiae, as well as in C. albicans (Fig. 2). Since the charging ratio directly correlates to the yield of suppressed mutant protein, we further utilized AzF-RS 1 for efficient incorporation of AzF into proteins in subsequent analyses. These results also show that the unnatural amino acid AzF is efficiently taken up by the cells and transported into the cytosol, where it is charged to the EcTyrRNA_CUA by the AzF-RS.

Strong overexpression of suppressor stop tRNAs has previously been described as potentially toxic to cells (7, 38). We therefore analyzed strain SPC43 containing the orthogonal pair of SNR52-regulated EcTyrRNA_CUA in four copies and AzF-RS 1 for growth defects and morphology in standard media (see Fig. S3 in the supplemental material). No differences in growth rates or the induction of hyphal growth were discernible between the wild type (SC5314) and strain SPC43 under the tested conditions, including growth in SC, YPD, or YPD supplemented with serum at 37°C. Consequently, heterologous expression of the orthogonal pair has no adverse effects on C. albicans growth or morphogenesis. We could also observe no influence of the addition of the
unnatural amino acid AzF to the medium on growth or morphology of \textit{C. albicans}.

**Incorporation of AzF into model proteins Tsa1p and Tup1p.**

The \textit{C. albicans} Tsa1 protein (thiol-specific antioxidant-like protein) is a highly abundant, differentially localized peroxiredoxin, involved in protection against oxidative stress (21, 39). Distinct multimeric forms of the protein have previously been described for Tsa1p homologs in other species (40–42). Although oligomerization of \textit{Ca}Tsa1p has also been demonstrated, the function of these multimers still remains unclear in \textit{C. albicans} (39). Due to its di- and oligomerization, we chose Tsa1p as a model for potential AzF mediated cross-link induction between interacting monomers.

According to the crystal structure of human erythrocyte thioredoxin peroxidase b (41) (Protein Data Bank accession number 1QMV), which shares 57.6% protein sequence identity with \textit{Ca}Tsa1p, we selected five amino acids along the proposed monomer-monomer interface for substitution with AzF by replacing the corresponding codons with amber codons. To discriminate mutant Tsa1p from wild-type Tsa1p, we fused a V5/His6 tag to the C terminus of the mutant Tsa1p. The mutant, tagged Tsa1p variants were subsequently inserted into one allele of the endogenous \textit{TSA1} gene locus of strain SPC43. Mutant, suppressed Tsa1p was detectable in protein lysates of the corresponding strains (Fig. 3A). For every substituted position, the translational suppression of the amber codon was stronger in the presence of AzF; however, a variable amount of suppressed Tsa1p was observed without addition of AzF to the media (Fig. 3A). This unspecific background
suppression might be due to less stringent orthogonality of the components in *C. albicans*, indicating a cross-reaction with endogenous factors, which is in agreement with the tRNA aminoaacylation results (Fig. 2). Nevertheless, the stronger amber suppression in the presence of AzF indicates a preferred incorporation of AzF in response to a stop codon.

As a second example for protein-protein interactions, we selected a transcription factor which is not as abundant as Tsa1p, to investigate the dynamic range of the method considering incorporation of AzF and cross-link formation. Tup1p is a central repression of reporter genes, while tetramerization remained un-detectable without the support of structure data, we chose the affected (47). Accordingly, we mutagenized position L63 in the C-terminal domain of Tup1p, only structural data for the C-terminal domain of S. cerevisiae Tup1p (ScTup1p) forms homotetramers via its N-terminal domain (43) and interacts with the transcriptional cofactor Ssn6p (44, 45). The C-terminal domain forms a seven-bladed propeller consisting of WD40 repeats that is thought to act as a general interface for protein-protein interactions. When we started our experiments with AzF but absent from Tsa1p wt (Fig. 3C). Accordingly, the labeling reaction specifically bound the fluorescent dye to the AzF containing protein. Tsa1p wild type or of two Tsa1p N141X molecules. Notably, not all positions were equally suited for cross-link induction. In alpha helices, the orientation of every amino acid residue depends on the turn of the helix. Since close proximity (3 to 4 Å) of the azido group with an interacting molecule is a prerequisite for cross-link product may consist of a mutant Tsa1p N141X variant and Tsa1p wild type or of two Tsa1p N141X molecules. Notably, not all positions were equally suited for cross-link induction. In alpha helices, the orientation of every amino acid residue depends on the turn of the helix. Since close proximity (3 to 4 Å) of the azido group with an interacting molecule is a prerequisite for cross-link induction, even neighboring residues can vary greatly with respect to cross-link efficiency, depending on their orientation toward the interacting molecule. In the case of Tsa1p, higher-molecular-mass complexes were observed for mutant Tsa1p variants T139X and N141X but not for Tsa1p variant I140X (Fig. 4B). When comparing the corresponding residues in the crystal structure of human D62X and V64X, no full-length protein was detected (data not shown). Interestingly, whereas for Tsa1p a significant amount of unspecific background suppression of the amber codon was observed, full-length translation of mutant Tup1p variants without the addition of AzF was hardly detectable.

Biochemical detection of an azide-containing protein is feasible by labeling with a triarylphosphine fluorescent dye, which is covalently bound to the azido-group in a Staudinger Ligation (48, 49). To determine whether AzF is intact in vivo and available for covalent cross-link formation, we labeled cell lysates from Tsa1p wt (SPC46) or Tsa1p N141X (SPC55) by Staudinger reaction using a fluorescently labeled phosphine. After the labeling reaction, wild-type Tsa1p and mutant Tsa1p were purified via the V5 tag, and immunoprecipitates were analyzed by SDS-PAGE. A specific fluorescence signal was only evident for Tsa1p N141X, incubated with AzF but absent from Tsa1p wt (Fig. 3C). Accordingly, the labeling reaction specifically bound the fluorescent dye to the AzF containing protein.

To further verify site-specific incorporation of AzF into the respective proteins, we analyzed mutant proteins Tsa1p L63X and Tsa1p N141X by mass spectrometry. Due to the highly reactive nature of the azide-group, p-azido-l-phenylalanine is known to be reduced to p-amino-l-phenylalanine during sample preparation (6, 7). The expected p-amino-l-phenylalanine peak was detected for Tsa1p N141X by MALDI-TOF measurement (Fig. 3D). Peptides resulting from substitution of leucine 63 with p-amino-l-phenylalanine (relative mass calculated, 1,392.67 Da) in mutant Tsa1p L63X were also detected by Nano-LC-ESI-MS/MS (data not shown). Taken together, we conclude that (i) AzF is site specifically incorporated in response to an amber codon into proteins, (ii) it is not degraded in vivo either before or after incorporation into the mutant protein, and (iii) it is thus available for cross-link formation in *C. albicans*.

**Induction of cross-links with mutant proteins Tsa1p and Tup1p.** With the mutant Tsa1p and Tup1p variants, we next sought to analyze amino acid positions involved in protein-protein interaction. Strains containing the orthogonal pair and mutant Tsa1p or Tup1p constructs were grown in AzF supplemented media and subjected to UV exposure at 365 nm for 30 min. For Tsa1p, position N141X yielded the strongest cross-link signal in response to incubation with AzF and UV irradiation as indicated by the appearance of a higher-molecular-mass band at 48 kDa (Fig. 4A). Cross-link formation was strictly dependent on UV excitation and addition of AzF to the medium, whereas no cross-link signal was found for Tsa1p wt. Due to the apparent molecular mass of the cross-link of ~48 kDa and the known homo-oligomerization of Tsa1p, it seemed likely that the observed cross-link product may consist of a mutant Tsa1p N141X variant and Tsa1p wild type or of two Tsa1p N141X molecules. Notably, not all positions were equally suited for cross-link induction. In alpha helices, the orientation of every amino acid residue depends on the turn of the helix. Since close proximity (3 to 4 Å) of the azido group with an interacting molecule is a prerequisite for cross-link induction, even neighboring residues can vary greatly with respect to cross-link efficiency, depending on their orientation toward the interacting molecule. In the case of Tsa1p, higher-molecular-mass complexes were observed for mutant Tsa1p variants T139X and N141X but not for Tsa1p variant I140X (Fig. 4B). When comparing the corresponding residues in the crystal structure of human

**FIG 2** Functionality of the orthogonal pair in *C. albicans*. (A) Aminoacylation efficiency and thus functionality of the heterologous AzF-RS variants was analyzed by acid urea-PAGE. Uncharged EcTyrRNA<sub>C</sub> (tRNA) and aminoacylated EcTyrRNA<sub>C</sub> (aa-tRNA) were detected by hybridization with an EcTyrRNA<sub>C</sub>-specific probe. Strains were incubated in SC medium with (lanes 2, 4, and 6) and without (lanes 1, 3, and 5) AzF. (B) Quantification of bands by densitometry.
erythrocyte peroxidase b (Fig. 4C), isoleucine 140 is apparently
directed toward the inside of the monomer, whereas positions 139
and 141 are directed toward the interacting Tsa1p monomer;
therefore, a cross-link induction with AzF at position 140 is un-
likely to produce a covalent bond to an interacting protein.

For the mutant Tup1p variants, cross-links were generated
for positions L63X and A66X (Fig. 4D). Cross-link formation
again was dependent on AzF supplementation and UV irradi-
ation (see Fig. S4 in the supplemental material). Strikingly, the
higher-molecular-mass products differed between the two po-
sitions. Mutant Tup1p L63X exhibited two higher-molecular-
mass products upon UV exposure, with apparent molecular
masses of approximately 125 and 165 kDa. UV light irradiation
of mutant Tup1p variant A66X led to the cross-link formation
of a single 165-kDa product. Regarding the apparent molecular
mass and the overall role of the N-terminal domain in te-
tramerization of Tup1p, we hypothesized that the lower cross-
link might be a homodimer.

Identification of interacting proteins. In a subsequent step,
we sought to identify the interacting proteins of the mutant Tsa1p
and Tup1p variants. Since in both cases a homodimerization was
highly likely, we pursued two different approaches. The first ap-
proach comprised the direct detection of the homodimer by tag-
ging a second copy of Tsa1p or Tup1p with an HA tag and by
subsequent immunoprecipitation and immunoblotting of the
higher-molecular-weight complexes with both anti-V5 antibody
and anti-HA antibody. As expected, the higher-molecular-mass
complex formed by mutant Tsa1p could be identified as a combi-
nation of mutant, V5-tagged Tsa1p N141X and an HA-tagged
wild-type molecule of Tsa1p by immunoblotting (Fig. 5A). For
Tup1p, both higher-molecular-mass complexes of mutant Tup1p
variants L63X and A66X were detected with the anti-HA antibody
as binary interactions between the mutant Tup and the wild-type
HA-tagged Tup1 (Fig. 5B). Remarkably, although no lower cross-
link band was observed in protein lysates for Tup1p A66X (Fig.
4D), immunoprecipitation produced also a faint band at
125 kDa (Fig. 5B). In a complementary approach, we intended to
identify the unknown interacting proteins by an open and unbi-
ased method. For this purpose, we purified the higher-molecular-
weight complexes of Tup1p (see Fig. S5 in the supplemental ma-
terial) for mass spectrometric analysis. Nano-LC-ESI-MS/MS of
highly purified mutant Tup1p L63X upper and lower cross-linked
complexes confirmed the findings of the immunoblots. In the
upper cross-link band, Tup1p was identified with a sequence cov-
erage of 70% (see Fig. S5 in the supplemental material). The lower
cross-link band also contained Tup1p with a sequence coverage of
66% (see Fig. S5 in the supplemental material). In both samples,
peptides were detected that unambiguously identified mutant
Tup1p via the V5 tag, as well as wild-type Tup1p, containing the

FIG 3  In vivo incorporation of AzF into proteins in C. albicans. (A) Incorporation
of AzF into C-terminally tagged Tsa1p (24.6 kDa) in response to an amber codon
at position T139, I140, or N141 in the presence (lanes 3, 5, and 7) and absence
(lanes 2, 4, and 6) of AzF. For comparison, C-terminally tagged Tsa1p without a
stop mutation (lane 1, strain SPC46) was utilized. (B) Incorporation of AzF into
C-terminally tagged Tup1p (60.6 kDa) in response to an amber codon inserted at
position L63, V65, or A66 in presence (lanes 3, 5, and 7) and absence of AzF (lanes
2, 4, and 6). For comparison, C-terminally tagged Tup1p without a stop mutation

(lane 1, strain YBC1) was also analyzed. (C) Detection of reactive $p$-azido-L-
phenylalanine by Staudinger ligation using a fluorescent phosphine (DyLight
phosphine). Tsa1p wt (lane 1, strain SPC46) and Tsa1p N141X (lane 2, strain
SPC46) were subjected to the labeling reaction and subsequent SDS-PAGE
separation. Fluorescent signals were measured with a laser scanner (upper
lane) and Tsa1p amounts were determined by Western blotting (lower lane).
(D) Mass spectra of tryptic peptides from purified Tsa1p wt and Tsa1p N141X.
Detection of $p$-amino-L-phenylalanine, the decomposition product of
$p$-azido-L-phenylalanine, in peptide QITIN*DLPVGR (calculated mass,
m/z 1,273.77; observed mass, m/z 1,273.66, marked with a black circle).
original leucine at position 63. A peptide covering p-azido-l-phenylalanine/p-amino-l-phenylalanine at position 63 in the mutant Tup1p was obviously not found in the cross-linked complexes, since all available mutant Tup1p was covalently bound at this position.

DISCUSSION

In our efforts to add an unnatural amino acid to the genetic code of C. albicans, we accomplished the heterologous expression of an orthogonal pair of tRNA and aaRS from E. coli following extensive optimization. No discernible effects of the orthogonal pair on C. albicans growth or morphology could be determined. Furthermore, the results of global transcriptional analyses of SPC43 show no significantly differentially regulated genes compared to the wild-type strain (data not shown). We therefore would expect this expanded genetic code to be not only suitable for the analysis of binary interactions between C. albicans proteins but also for more complex assays, including in vitro or in vivo infection models. Studies of host-pathogen interactions could benefit considerably from this method, since it is generally not restricted to certain media or growth conditions (data not shown).

For both model proteins, Tsa1p and Tup1p, suppressed mutant protein yields only a fraction of wild-type protein levels. This is not surprising, as translational stop suppression is always competing with translational termination (50, 51). Furthermore, nonsense-mediated decay of mRNA can also affect transcript abundances, as premature translation-termination codon containing transcripts are selectively

![Cross-link induction with mutant proteins](image-url)
bound and degraded (52). Utilization of NMD-deficient S. cerevisiae strains yielded increased levels of mutant proteins (36), but, generally, knockout of components of the NMD pathway is not necessary to obtain mutant protein by stop codon suppression.

Furthermore, a position effect in stop codon suppression for both proteins Tsa1p and Tup1p was visible, although this effect was more pronounced in Tsa1p. It therefore seems likely that efficient amino acid incorporation is not only depending on the identity of the protein but also on the substituted amino acid position. This might be due to codon context effects that influence translational efficiency (53, 54), especially in combination with stop codon readthrough. For some positions (Tup1p D62X and V64X) no translational suppression at all was detectable (data not shown), which might equally be explainable with the respective codon context. A protein and position effect was also observed for the unspecific background integration of one or more natural amino acids in amber codons of Tsa1p constructs in the absence of AzF in the medium. This background suppression was also detectable, when only the N-terminal aaRS with natural amino acids. However, cross-reaction of the antisera with HA-tagged Tsa1p constructs in the absence of AzF and UV excitation. We could identify the cross-linked complexes as dimers consisting of mutant V5-tagged Tsa1p (L63X or A66X) and Tup1p wt. Interestingly, for Tup1p L63X both the upper and the lower cross-linked band turned out to be a homodimer with wild-type Tup1p.

For Tsa1p, we could see a good correlation between the structural data and cross-link formation for the selected residues. Recently, the crystal structure of S. cerevisiae Tsa1p C47S has been deposited at the Protein Data Bank (Protein Data Bank accession number 3SBC). Since it was not available at the time we conducted our experiments, all structural data applied in the present study is based on the crystal structure of the N-terminal domain that has been published recently (59). Of the selected residues, solely substitution of L63 and A66 with AzF produced higher-molecular-weight bands in the Western blot after UV light exposure. We could identify the cross-linked complexes as dimers consisting of mutant V5-tagged Tsa1p (L63X or A66X) and Tup1p wt. Interestingly, for Tup1p L63X both the upper and the lower cross-linked band turned out to be a homodimer with wild-type Tup1p. For Tup1p A66X, after immunoprecipitation, the lower cross-link band was also detectable with anti-V5 antibody and anti-HA antibody, although with much weaker intensity. The apparent molecular mass of the upper detected dimers is ~165 kDa and thus considerably higher than the expected size for two Tup1p monomers (computational calculation of the molecular mass: Tup1p wt, 57.8 kDa; Tup1p wt with C-terminal V5/His, tag, 60.6 kDa). This aberrant migration behavior in a reducing SDS-PAGE might be due to the intermolecular covalent bond that prevents complete unfolding and uniform SDS binding which in turn leads to a reduced electrophoretic mobility, analogous to intramolecular disulfide bonds (60, 61). However, depending on the position of the intermolecular bond and the protein, the contrary effect is also possible (62). We reason that these effects might apply to the two observed higher-molecular-mass cross-link bands for Tup1p L63X. It is conceivable that AzF at position L63X and to a lesser extend also at position A66X exhibits a certain steric flexibility and could react with two different positions or maybe even two different monomers of an interacting Tup1p wild-type molecule and thus produces two diversely migrating homodimers. However, molecular-weight determination by mass spectrometry might elucidate whether the mass of the observed dimers is indeed identical or if modifications might be the cause of the aberrant migration pattern.

We were not surprised to find no interaction between Tup1p L63X and CaSn6p, as a mutation of the corresponding L62 residue in ScTup1p abolishes this interaction (47). However, that no interaction occurred between the neighboring residues and...
CaSn6p was unexpected. Kaneko et al. could demonstrate the interaction between CaTup1p and CaSn6p by tandem affinity purification and furthermore found another interacting protein, CaTcc1p (63). Since the growth conditions were similar (blastospore morphology), a reason for the missed interaction might be the identity of the substituted amino acids. The specificity of the photo-cross-linker-mediated bond depends on the exact localization and orientation of the unnatural amino acid in relation to the interacting protein. With the limited set of positions we substituted, we simply might have missed the exact position necessary for a cross-link to CaSn6p. Another reason might be an overall structural change due to incorporation of the photo-cross-linker amino acid. Matsumura et al. not only solved the crystal structure of the quaternary N-terminal domain of ScTup1p (NTD Tup1p) but also of the L62R mutant N-terminal ScTup1p domain (NTD Tup1p-L62R) (59). Interestingly, NTD Tup1p-L62R revealed an altered structure, which is no longer associated via four antiparallel helices but in parallel helices. We cannot rule out that incorporation of AzF might also affect the structure of mutant Tup1p and therefore possibly influences the interaction with CaSn6p. However, for the following reasons, we hypothesize that this might not apply here. (i) The observed interaction between mutant Tup1p L63X occurs not with another mutant Tup1p L63X but with wild-type Tup1p. Consequently, we hypothesize that the overall structure of the mutant Tup1p with three additional wild-type Tup1p would not be as strongly affected compared to the homotetramers of ScTup1p-L62R. (ii) Replacement of leucine 63 in CaTup1p was carried out with the equally hydrophobic amino acid AzF instead of a positively charged arginine. Strikingly, replacement of L62 in ScTup1p with the equally hydrophobic valine resulted in a moderate effect on the repression of target genes and thus interaction with ScSn6p (59). Investigation of the target gene repression in the mutant Tup1p L63X C. albicans strains in a Δtup1 background might determine the effect of AzF incorporation on the structure and association with CaSn6p.

Regarding the physiological effects of the incorporation of unnatural amino acids in proteins, only limited data are available. Most proteins analyzed thus far were heterologous (e.g., superoxide dismutase [SOD] [7], glutathione S-transferase [GST] [64], green fluorescent protein [GFP] [36], and myoglobin [38]) since a complete dismutase [SOD] [7], glutathione S-transferase [GST] [64], green fluorescent protein [GFP] [36], and myoglobin [38]) since the CUG codon is decoded in vivo as serine and not leucine in C. albicans. Nucleic Acids Res. 23:1481–1486. 11. White TC, Andrews LE, Maltby D, Agbáían N. 1995. The “universal” leucine codon CTG in the secreted aspartyl proteinase 1 (SAP1) gene of Candida albicans encodes a serine in vivo. J. Bacteriol. 177:2933–2935. 12. Stynen B, Van Dijck P, Tournu H. 2010. A CUG codon adapted two-hybrid system for the pathogenic fungus Candida albicans. Nucleic Acids Res. 38:e184. 13. Blackwell C, Brown JD. 2009. The application of tandem-affinity purification to Candida albicans. Methods Mol. Biol. 499:133–148. 14. Kaneko A, Umezuma T, Hanaoka N, Monk BC, Uehara Y, Niimi M. 2004. Tandem affinity purification of the Candida albicans septin protein complex. Yeast 21:1025–1033. 15. Boysen JH, Fanning S, Newberg J, Murphy RF, Mitchell AP. 2009. Detection of protein-protein interactions through vesicle targeting. Genetics 182:33–39. 16. Liu S, Zhang Z, Xu H, Li L, Chen S, Li J, Hao Z, Chen PR. 2011. Site-specific incorporation of photo-cross-linker and bio-orthogonal amino acids into enteric bacterial pathogens. J. Am. Chem. Soc. 133:20581–20587. 17. Gillum AM, Tsay EY, Kirsch DR. 1984. Isolation of the Candida albicans gene for orotidine-5’-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations. Mol. Gen. Genet. 198:179–182.
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