An expanded genetic code in *C. albicans* to study molecular interactions *in vivo*

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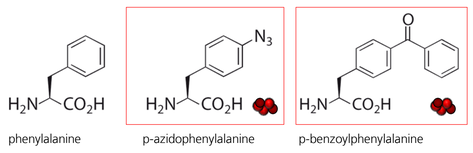
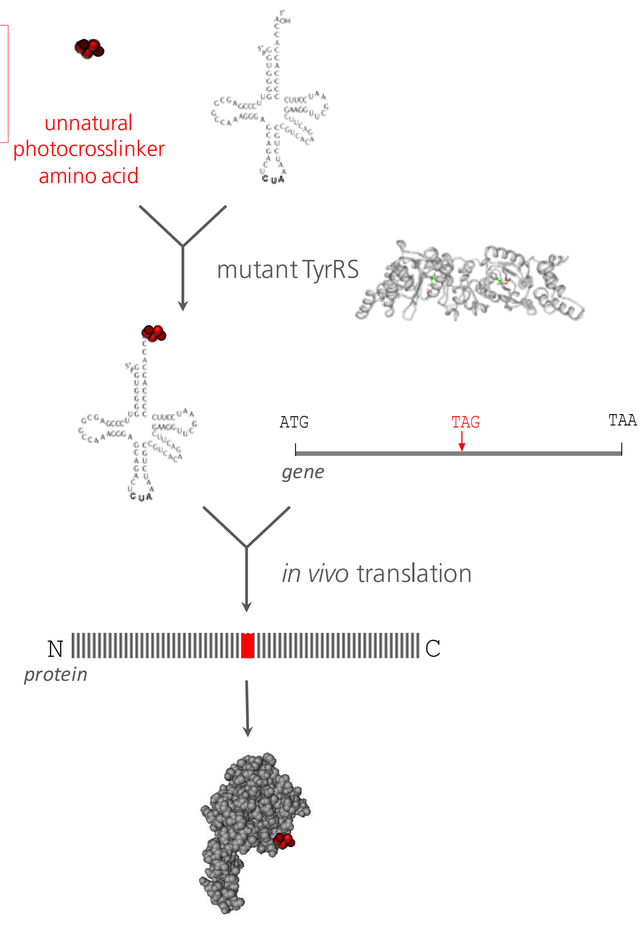
For novel insights into the pathogenicity of *C. albicans*, studies on molecular interactions of central virulence factors will prove themselves revealing. As methods for the analysis of direct interactions *in vivo* are scarce, we expanded the genetic code of *C. albicans* with the unnatural photocrosslinking amino acids p-azidophenylalanine and p-benzoylphenylalanine. Interacting molecules in close proximity of these unnatural amino acids can be linked by induction of a stable covalent bond via UV-photocrosslink, which makes unknown interacting molecules readily available for further downstream analyses and identification. For this purpose, we used aminoacyl-tRNA synthetase and suppressor tRNA pairs derived from *E. coli*, which were previously reported to be orthogonal in *S. cerevisiae.* Yet, after adaption and optimization of tRNA and aminoacyl-tRNA synthetase expression for *C. albicans*, the efficiency of the aminoacyl-tRNA synthetases in charging the heterologous tRNA with the respective unnatural amino acid was still limited. Therefore we analyzed additional mutagenized active-site variants and could identify one aminoacyl-tRNA synthetase for each unnatural amino acid with significantly improved charging efficiencies. As a result, translational suppression of different mutagenized reporter proteins could be considerably enhanced. As a model for protein-protein interaction we utilized C-terminally tagged Tsa1p variants with amber stop mutations at different positions in a strain background containing the suppressor tRNA and optimized azidophenylalanine tRNA synthetase. Immunologic detection of the tagged Tsa1p upon UV irradiation revealed not only the mutant monomeric form of Tsa1p but also a higher molecular weight complex, strictly depending on the position of the incorporated amino acid, the presence of p-azidophenylalanine and UV irradiation. By tagging and integrating a second, unmutagenized Tsa1p, we could identify the higher molecular weight complex as a homodimer consisting of a mutant Tsa1p and a wild-type, tagged Tsa1p, covalently bound via azidophenylalanine. In summary, we have established a novel tool in *C. albicans* with an expanded genetic code using unnatural photocrosslinker amino acids for *in vivo* binary protein interaction analyses.

Strategy

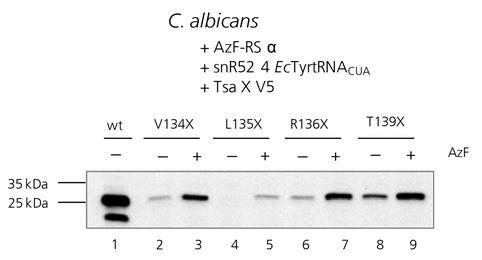


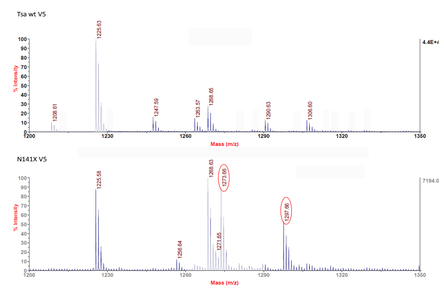


For the integration of unnatural photocrosslinker amino acids including p-azidophenylalanine (AzF) and p-benzoylphenylalanine (BzF) into proteins, the endogenous translational machinery is expanded with mutant, heterologous elements. A mutagenized, former tyrosyl tRNA and tRNA synthetases from *E. coli* are utilized to incorporate the unnatural amino acids site-specifically in response to an amber stop codon into a protein of interest.

Due to radical formation of the unnatural amino acids upon UV irradiation, interacting proteins in close proximity are linked by a stable covalent bond. This makes them readily available for further downstream processes like identification of interaction partners by mass spectrometry or western blotting.

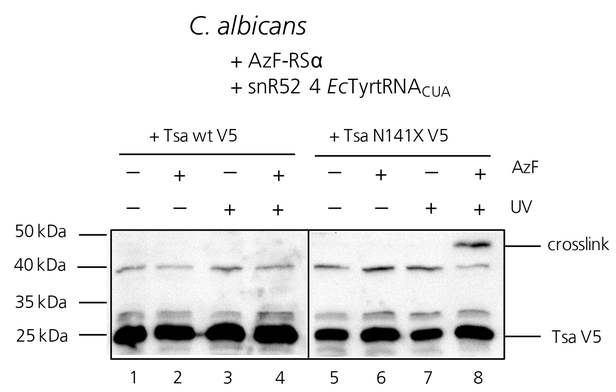
Incorporation of AzF into amber mutant Tsa1p

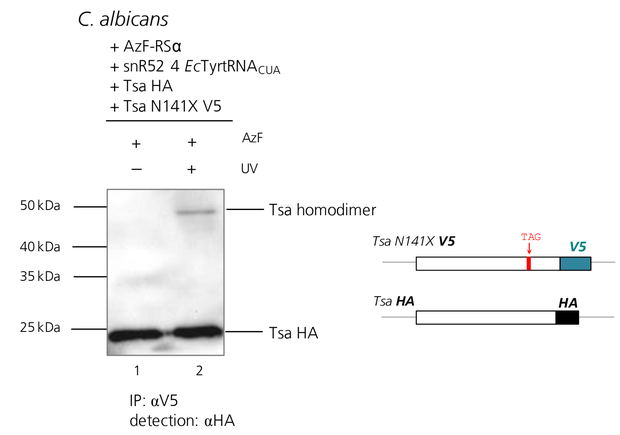




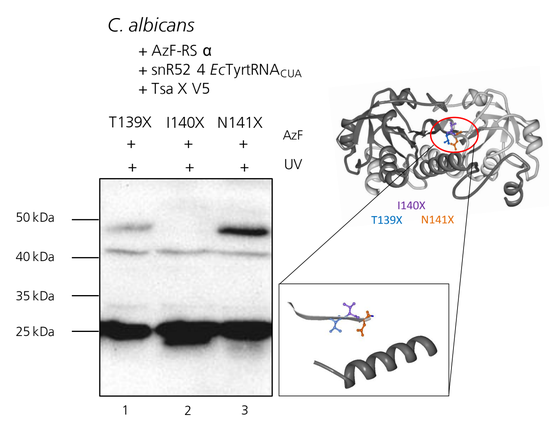
Stop codon readthrough with or without AzF is shown for different positions of the amber stop codon in Tsa1p. Depending on the position, the total amount of suppressed protein varies considerably. Incorporation of AzF into proteins could be demonstrated by MALDI-TOF analysis of Tsa1p variant N141X. Two additional peaks indicate integration of AzF and byproducts like aminophenylalanine.

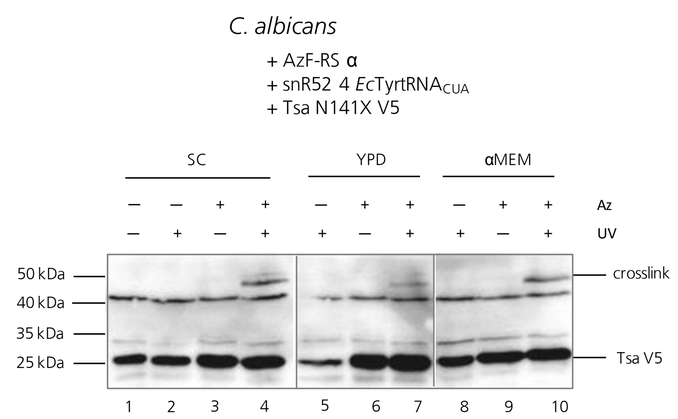
Crosslink induction with Tsa1p *in vivo*





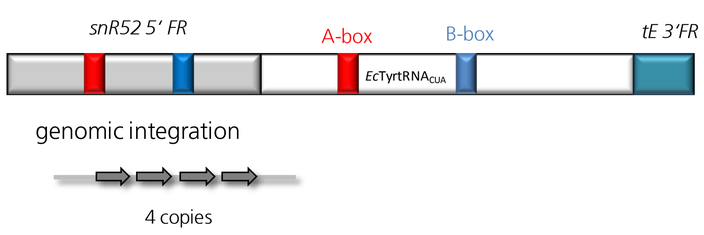
With Tsa1p as a model protein for di- and oligomerization, we could demonstrate that crosslink formation is strictly dependent on AzF and UV irradiation (left figure). The observed higher molecular weight complex could be immunologically identified by integration of a second, HA-tagged unmutagenized copy of Tsa1p as two interacting Tsa1p monomers (right figure).





General benefits of the method are demonstrated by agreement of crosslink formation with the crystal structure, which is shown for residues T139X, I140X and N141X and the corresponding residues in the crystal structure of the human erythrocyte peroxidase (left figure). Furthermore, the method is applicable to different media and morphologies, as shown for yeast and filamentous growth, making it robust and universal (right figure).

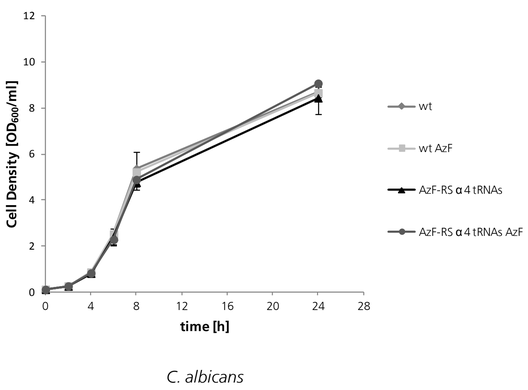
Expression of the molecular components



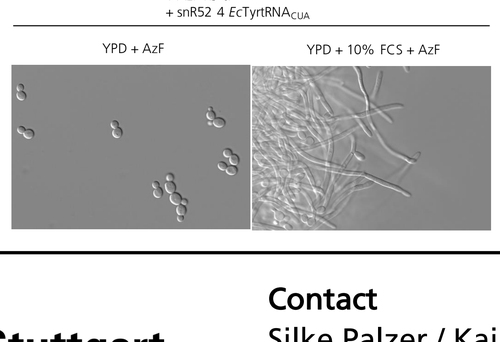
For heterologous expression in *C. albicans*, the *Ec*TyrtRNA$_{CUA}$ was fused with the A- and B-box containing snR52 promoter. The construct was integrated into the genome in 4 copies.

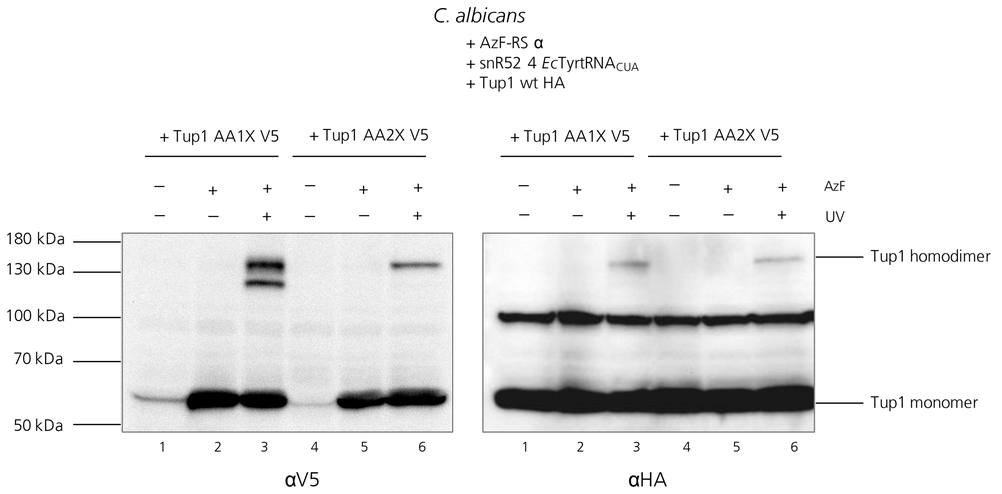
Crosslink induction with transcription factor Tup1p

Growth of strains containing the 4 tRNAs under snR52 regulation and the AzF-RS α in presence and absence of AzF are identical to growth of wild type strain SC5314. This indicates no toxic effects of *Ec*TyrtRNA$_{CUA}$ overexpression in *C. albicans*.



Additionally, no effects on cell morphology could be observed in yeast and hyphal growth conditions.





The applicability of this method could also be demonstrated for other proteins, here illustrated for the transcription factor Tup1p. UV irradiation of mutant Tup1p resulted in the formation of one or two crosslink products, depending on the position of incorporated AzF. By integration of a HA-tagged unmutagenized copy of Tup1 into the strains, the identity of the upper band could be determined as a Tup1 homodimer. All interactions are currently investigated by MS analysis.

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