DECIPHERING CONTEXT-DEPENDENT AMBER SUPPRESSION EFFICIENCY IN MAMMALIAN CELLS WITH AN EXPANDED GENETIC CODE

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DECLARATION OF CONTRIBUTIONS

Identification of permissive amber suppression sites for efficient non-canonical amino acid incorporation in mammalian cells

Michael Bartoschek and Sebastian Bultmann designed and conceived the study. Sebastian Bultmann and Kathrin Lang supervised the study. Michael Bartoschek, Enes Ugur, Kathrin Lang and Sebastian Bultmann interpreted the data. Michael Bartoschek performed molecular and cellular experiments. Geraldine Rodschinka assisted with molecular cloning. Michael Bartoschek generated stable cell lines with help from Geraldine Rodschinka. Michael Bartoschek performed flow-cytometry and analysis. Michael Bartoschek and Enes Ugur conducted proteomic experiments. Enes Ugur performed mass spectrometry and analysis under the supervision of Michael Wierer. Sebastian Bultmann performed linear regression analysis. Tuan-Anh Nguyen performed chemical synthesis of DiazK and the Biotin-tetrazine conjugate. Michael Bartoschek and Sebastian Bultmann wrote the manuscript and prepared figures. All authors read, discussed, and approved the manuscript.

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Oliver Seitz conceived the study. Michael Bartoschek constructed the stable CHO cell lines and contributed Supplementary Figure 8-1. Michael Bartoschek discussed and proofread the manuscript.

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CONTENTS

ABBREVIATIONS

aaRS	Aminoacyl-tRNA synthetase
BcnK	Bicyclo[6.1.0]nonyne-L-lysine
BONCAT	Bioorthogonal non-canonical amino acid tagging
cAA	Canonical amino acid
CuAAC	Copper(I)-catalyzed azide-alkyne cycloaddition
DNA	Deoxyribonucleic acid
EF-Tu	Elongation factor thermo unstable
eRF1	Eukaryotic release factor 1
FACS	Fluorescence-activated cell sorting
FUNCAT	Fluorescent non-canonical amino acid tagging
G418	Geneticin
GOI*	Gene of interest harboring the (re-)assigned codon(s)
GTP	Guanosine-5′-triphosphate
HEK293T	Human embryonic kidney 293T
iEDDAC	Inverse-electron-demand Diels-Alder cycloaddition
iPASS	Identification of permissive amber sites for suppression (regression model)
KO	Knock-out
mESC	Mouse embryonic stem cell
mRNA	Messenger ribonucleic acid
ncAA	Non-canonical amino acid
ncRS	Non-canonical amino acid adapted, orthogonal aminoacyl-tRNA synthetase
NMD	Nonsense-mediated decay
o-aaRS	Orthogonal aminoacyl-tRNA synthetase
o-Ribo	Orthogonal ribosome
o-tRNA	Orthogonal tRNA
OTS	Orthogonal translation system
POI	Protein of interest
POI^{ncAA}	Protein of interest harboring a non-canonical amino acid at a specified incorporation site
PTC	Premature termination codon
PTM	Post-translational modification
Pyl	Pyrrolysine

PylRS	Pyrrolysyl-tRNA synthetase
PylT	Pyrrolysine-specific amber suppressor $tRNA^{Pyl}_{CUA}$
RF1	Release factor 1
rRNA	Ribosomal ribonucleic acid
RS	tRNA synthetase
SD	Shine-Dalgarno
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence
SORT-E	Stochastic orthogonal recoding of translation with enrichment
SORT-M	Stochastic orthogonal recoding of translation with chemoselective modification
SPAAC	Strain-promoted azide-alkyne cycloaddition
tRNA	Transfer ribonucleic acid
UAA	Ochre stop codon
UAG	Amber stop codon
UGA	Opal stop codon
UPF1	Up-frameshift protein 1
UPF2	Up-frameshift protein 2
UTR	Untranslated region
wt	Wild-type

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SUMMARY

The genetic code of organisms can be expanded by introducing orthogonal translation systems (OTSs). One of the most commonly applied OTSs in mammalian cells is the archaeal pyrrolysyl-tRNA synthetase/$tRNA^{Pyl}_{CUA}$ (PylRS/PylT) pair from *Methanosarcina* species. Thereby, usually in-frame amber stop codons (UAG) are suppressed to site-specifically incorporate non-canonical amino acids (ncAAs) into target proteins. These ncAAs can harbor unique chemical moieties, allowing to probe or engineer protein structure and function with high precision. To date, applicability of an expanded genetic code has been particularly advanced in bacteria by optimizing OTS components, modifying host translation, and developing mutually orthogonal translation systems. In mammalian cells, development of genetic code expansion tools has been largely focused on intrinsic properties of the OTS itself, for instance by engineering OTS components or tuning their expression levels. However, several-fold differences in ncAA incorporation efficiency are frequently observed between different amber stop codon positions within a target protein. These unpredictable variations in incorporation efficiencies substantially hamper the theoretical advantage of ncAAs to modify any user-defined site within a target protein. Here, applying a proteomics-based approach and fluorescent reporter system, we compute and validate a linear regression model that predicts ncAA incorporation efficiency in mammalian cells based on the nucleotide context. Thereby, we demonstrate that the immediate context directly modulates the competition between ncAA incorporation and termination at UAG. Moreover, our data support a molecular model in which the identity of up- and downstream nucleotides influences translational efficiency independent of amino acid and tRNA identity. Instead, base stacking of neighboring nucleotides might uniquely affect codon-anticodon base pairing during decoding of UAG. Additionally, context-specific ribosomal pausing and speed could contribute to varying ncAA incorporation efficiency. Furthermore, treatment with aminoglycosides and inhibition of nonsense mediated decay are proposed to improve yields of ncAA-modified proteins in mammalian cells. Taken together, our strategy not only facilitates the applicability of an expanded genetic code in mammalian cells, but should also prove useful in further deciphering the molecular mechanisms that govern context effects in translational efficiency. A better general understanding of context effects in translation would in turn benefit synthetic expansion of the genetic code.

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ZUSAMMENFASSUNG

Der genetische Code von Organismen kann durch die Einbringung orthogonaler Translationssysteme (OTSe) erweitert werden. Das Pyrrolysyl-tRNA Synthetase/$tRNA^{Pyl}_{CUA}$ (PylRS/PylT) Paar der Spezies *Methanosarcina* ist eines der am häufigsten angewendeten OTSe in Säugerzellen. Üblicherweise wird damit das amber Stoppcodon (UAG) innerhalb eines Leserasters supprimiert, um an spezifischen Stellen eines Zielproteins nicht-kanonische Aminosäuren (nkASn) einzubauen. Diese nkASn können einzigartige chemische Motive enthalten, die es ermöglichen die Struktur und Funktion von Proteinen mit hoher Präzision zu untersuchen und zu manipulieren. Bisher wurde insbesondere in Bakterien die Anwendbarkeit eines erweiterten genetischen Codes verbessert, indem OTS Komponenten optimiert, die Translation in Wirtsorganismen modifiziert und wechselseitig orthogonale Translationssysteme entwickelt wurden. Die Weiterentwicklung von Methoden, um den genetischen Code in Säugerzellen zu erweitern, fokussierte sich überwiegend auf intrinsische Eigenschaften der OTSe selbst, zum Beispiel der Modifizierung von OTS Komponenten oder der Anpassung ihrer Expressionslevel. Häufig unterscheiden sich jedoch verschiedene UAG Positionen in ihrer Effizienz eine nkAS einzubauen in mehrfacher Höhe. Diese unvorhersehbaren Schwankungen in der Einbaueffizienz schränken den Vorteil von nkASn erheblich ein, theoretisch jede benutzerdefinierte Position innerhalb eines Zielproteins modifizieren zu können. In dieser Publikation berechnen und validieren wir mit Hilfe einer proteomischen Methode und eines fluoreszierenden Reportersystems ein lineares Regressionsmodell, das anhand des Nukleotidkontextes die Effizienz des nkAS Einbaus in Säugerzellen vorhersagt. Wir zeigen dadurch, dass der unmittelbare Kontext direkt das Verhältnis zwischen nkAS Einbau und Termination an UAG moduliert. Unsere Daten unterstützen zudem ein molekulares Modell, in dem die Identität der vorherigen und nachfolgenden Nukleotide die Effizienz der Translation unabhängig von der Identität der Aminosäure und tRNA beeinflusst. Hingegen könnte sich ein Basen-Stacking über benachbarte Nukleotide in einzigartiger Weise auf die Codon-Anticodon Basenpaarung während der Dekodierung von UAG auswirken. Zusätzlich könnten ein Pausieren sowie die Geschwindigkeit des Ribosoms in Abhängigkeit vom Kontext zu der uneinheitlichen Effizienz des nkAS Einbaus beitragen. Des Weiteren werden ein Behandlungsverfahren mit Aminoglycosiden und eine Inhibierung des Nonsense-mediated Decay vorgeschlagen, um die Ausbeute an nkAS-modifizierten Proteinen zu verbessern. Zusammenfassend vereinfacht unsere Strategie nicht nur die Anwendbarkeit

eines erweiterten genetischen Codes in Säugerzellen, sondern sollte sich auch als nützlich erweisen, um die molekularen Mechanismen, über die der Kontext die Translationseffizienz beeinflusst, weiter zu entschlüsseln. Ein besseres allgemeines Verständnis der Kontexteffekte bei der Translation würde wiederum die synthetische Erweiterung des genetischen Codes fördern.

3 INTRODUCTION

3.1 THAWING THE FROZEN ACCIDENT

3.1.1 *A universal genetic code?*

EVOLUTION OF THE GENETIC CODE In 1968, Crick postulated in his 'Frozen Accident Theory' that the genetic code is immutable and hence universal since "any change would be lethal" (Crick, 1968). Accordingly, this standardization of the genetic code must have occurred when life reached a level of complexity where an alternative code would have had a catastrophic mutagenic impact on global protein function and hence viability. First microorganisms are estimated to have formed on Earth between 3.9 and 4.3 billion years ago (Dodd et al., 2017) with speculations that major building blocks such as nucleotides are of extraterrestrial origin (Rimmer et al., 2018). To this time point, standardization of the genetic code might have been driven by the evolution of primitive cells as a single unit and not as individual organisms (Vetsigian et al., 2006; Woese et al., 2000; Woese, 2002). In contrast to Darwinian evolution, which affects populations from one to the next generation and hence vertically, here the genetic code would be optimized by horizontal gene transfer between primitive cells (Aggarwal et al., 2016; Sengupta et al., 2014; Vetsigian et al., 2006; Woese et al., 2000; Woese, 2002). This dependence on exchanging genetic information to cooperatively share innovations within the community of primitive cells would again foster universality of the code itself (Aggarwal et al., 2016; Sengupta et al., 2014; Vetsigian et al., 2006). With increasing cellular complexity, information-processing mechanisms might then have been fixed, thereby becoming less susceptible to horizontal gene transfer (Woese et al., 2000; Woese, 2002). This gradual transition to strictly vertical or Darwinian evolution would be accompanied by the emergence of the universal genetic code and the concomitant maturation of the last universal common ancestor to give rise to modern cell designs (Vetsigian et al., 2006; Woese et al., 2000; Woese, 2002). At which level of code complexity this Darwinian transition started can only be speculated, but independent transitions within each of the three domains of life – bacteria, archaea, and eukaryotes (Woese et al., 1990) – have been suggested (Grosjean et al., 2010; Vetsigian et al., 2006). At this, the high diversity in transfer ribonucleic acid (tRNA) modifications between bacteria, archaea, and eukaryotes suggests that the primordial code of the last universal common ancestor encoded less than 20 amino acids and was progressively expanded

by convergent evolution of the genetic code within the three domains of life (Grosjean et al., 2010). Moreover, amino acid sequences and structure of release factors are distinct between pro- and eukaryotes (Frolova et al., 1994; Song et al., 2000; Zhouravleva et al., 1995), further indicating convergent evolution of the translational machinery. In summary, the genetic code's universality, as a prerequisite for horizontal gene transfer and hence communal innovation sharing, might have been rather imperative for organismal life to evolve than merely a generic consequence of Darwinian selection.

Despite being seemingly frozen at first, the dogma of a universal and hence immutable genetic code has been challenged over the past five decades. For instance, the diversity in tRNA modifications among bacteria, archaea and eukaryotes highlights the evolvability and flexibility of the genetic code (Grosjean et al., 2010). Furthermore, in *Mycoplasma* (Yamao et al., 1985), mitochondria (Anderson et al., 1981; Barrell et al., 1979; Bonitz et al., 1980), and ciliated protozoans (Caron and Meyer, 1985; Helftenbein, 1985; Horowitz and Gorovsky, 1985; Meyer et al., 1991; Preer et al., 1985; Sánchez-Silva et al., 2003) selected stop codons encode amino acids during translation, which is generally referred to as codon reassignment (Ling et al., 2015). Moreover, also sense codons can be reassigned as observed in mitochondria (Anderson et al., 1981; Barrell et al., 1979; Bonitz et al., 1980) and yeast (Kawaguchi et al., 1989; Mühlhausen et al., 2016; Ohama et al., 1993; Santos and Tuite, 1995; Sugiyama et al., 1995). Additionally, unassigned codons, which do not code for amino acids or serve as translational termination signals, have been found for instance in *Mycoplasma* (Andachi et al., 1989). Remarkably, bacteriophages and prokaryotic cells can even be evolved to tolerate a synthetic amino acid replacing a canonical amino acid (cAA) throughout their proteome as a new biochemical building block (Bacher and Ellington, 2001; Bacher et al., 2003; Hoesl et al., 2015). One of the probably most striking examples demonstrating evolvability of the genetic code are trypanosomatida (Záhonová et al., 2016) and marine ciliates (Heaphy et al., 2016; Swart et al., 2016), in which all 64 codons are read as sense codons. With the three stop codons being reassigned to code for amino acids, translational termination in these organisms seems to be triggered by the proximity of a stop codon to the 3' untranslated region (UTR) (Heaphy et al., 2016; Swart et al., 2016). Therefore, although the genetic code initially seemed to be unambiguous or frozen, it is not reluctant to change over time.

Two predominant models have been proposed that describe the molecular changes governing the reassignment of codons. The 'codon capture' model assumes that the codon to be redefined together with its cognate tRNA first has to disappear from the genetic code, probably driven by genomic GC or AT pressure (Osawa and Jukes, 1989, 1995). Subsequently, the codon reappears with its new meaning (Osawa and Jukes, 1989, 1995). As the codon is stochastically reassigned to a new

tRNA, its initial removal from the genetic code would prevent detrimental effects on cell viability due to ambiguous decoding (Osawa and Jukes, 1989, 1995). In contrast, the 'ambiguous intermediate' model assumes decoding of the codon by a new mutant tRNA while still retaining its original meaning (Schultz and Yarus, 1994, 1996). This competition is then gradually lost over time as the codon fully adapts its new identity (Schultz and Yarus, 1994, 1996). However, as indicated by the variety of alternative genetic codes observed to date, the molecular changes governing the reassignment of codons are complex and therefore cannot be generalized just by one of the two models (Knight et al., 2001; Mühlhausen et al., 2016; Santos and Tuite, 1995; Sengupta and Higgs, 2005).

THE 21ST AND 22ND PROTEINOGENIC AMINO ACIDS Coding for solely 20 standard proteinogenic amino acids, the universal genetic code is largely redundant (Crick et al., 1961). This codon degeneracy is surprising, especially given the presumable prebiotic synthesis and hence bioavailability of chemically diverse amino acids on primordial Earth (Kvenvolden et al., 1971; Weber and Miller, 1981) and their potential to provide proteins with new or enhanced functions (Agostini et al., 2017; Jackson et al., 2006; Lee and Schultz, 2008; Mayer, 2019; Ugwumba et al., 2011; Xiao et al., 2015). To date, two natural expansions of the genetic code beyond the 20 standard cAAs have been described.

The 21st amino acid discovered in addition to the known cAAs was selenocysteine (Sec), which is with the exception of some bacterial species (Mukai et al., 2016) encoded by the opal stop codon (UGA) in pro- (Zinoni et al., 1986, 1990) and eukaryotes (Chambers et al., 1986; Mullenbach et al., 1988; Reddy et al., 1988). At first, the Sec-specific opal suppressor $tRNA^{Sec}_{UCA}$ is aminoacylated with serine and the seryl moiety subsequently converted to the selenocysteinyl moiety (Carlson et al., 2004; Forchhammer and Böck, 1991; Kaiser et al., 2005; Leinfelder et al., 1990; Yuan et al., 2006). The following co-translational incorporation of Sec is then coordinated by the reassigned codon together with a hairpin structure within the transcript termed selenocysteine insertion sequence (SECIS). In prokaryotes, SECIS is localized immediately downstream of the incorporation site (Berg et al., 1991; Chen et al., 1993; Zinoni et al., 1990), whereas in eukaryotes SECIS is found within the 3' UTR (Berry et al., 1991, 1993; Shen et al., 1993; Walczak et al., 1996). By binding a SECIS-specific protein, the SECIS stem-loop is critical in pro- and eukaryotes for recruiting the Sec incorporation machinery and directing the aminoacylated Sec-tRNA to its incorporation site (Baron et al., 1993; Copeland and Driscoll, 1999; Copeland et al., 2000, 2001; Fletcher et al., 2001; Heider et al., 1992; Hubert et al., 1996; Leibundgut et al., 2005; Low et al., 2000; Shen et al., 1995).

After the initial discovery of an efficiently decoded in-frame amber stop codon (UAG) in methylamine methyltransferase genes of the methanogenic archaea *Methanosarcina barkeri* (Burke et al., 1998; James et al., 2001; Paul et al., 2000), pyrrolysine (Pyl) was identified as the 22nd proteinogenic amino acid to be incorporated at UAG (Hao et al., 2002, 2004). Despite the initial identification of a putative hairpin structure immediately downstream of the reassigned amber stop codon (Namy et al., 2004; Théobald-Dietrich et al., 2005), Pyl incorporation is independent of a *cis*-acting secondary structure like SECIS (Namy et al., 2007; Zhang et al., 2005). Accordingly, no Pyl-specific elongation factor is required for ribosomal decoding of UAG unlike Sec incorporation at UGA (Blight et al., 2004; Polycarpo et al., 2006; Théobald-Dietrich et al., 2004). Furthermore, the Pyl-specific amber suppressor $tRNA^{Pyl}_{CUA}$ (PylT, encoded by *PylT*) is directly aminoacylated with the free amino acid Pyl by the pyrrolysyl-tRNA synthetase (PylRS, encoded by *PylS*) and not synthesized on its cognate suppressor tRNA like Sec (Blight et al., 2004; Polycarpo et al., 2004; Srinivasan et al., 2002). To date, Pyl containing proteins have only been found in members of the archaeal family *Methanosarcinaceae* (Srinivasan et al., 2002) and a few bacterial species (Prat et al., 2012), either belonging to the class of *Deltaproteobacteria* (Zhang and Gladyshev, 2007) or *Clostridia* like *Desulfitobacterium hafniense* (Herring et al., 2007b; Srinivasan et al., 2002) and *Acetohalobium arabaticum* (Prat et al., 2012). Interestingly, PylRS seems to have evolved before the appearance of the last universal common ancestor to then persist throughout evolution only in organisms that metabolize methylamines (Kavran et al., 2007). Alternatively, expansion of the genetic code with Pyl might have been transferred from a methanogenic archaeal ancestor to bacteria via horizontal gene transfer (Borrel et al., 2014; Prat et al., 2012). In summary, the requirements for expanding the standard genetic code with Pyl are distinct from Sec in that Pyl incorporation is independent of defined secondary structures in the transcript as well as specialized elongation factors. Furthermore, a specific tRNA synthetase (RS) is only available for Pyl and not Sec as Sec is synthesized from serine on its suppressor $tRNA^{Sec}_{UCA}$.

Collectively, the here described codon reassignments challenged the dogma of a frozen and hence universal genetic code. Moreover, these non-standard genetic codes found in nature prompted an intriguing application: The artificial expansion of the standard genetic code. As already noted by Crick (Crick, 1968), however, reassignment of canonical codons would impose detrimental effects on cellular function and viability. Hence, non-native molecular systems and their components must not interfere with their native counterparts. This essential prerequisite for expanding the genetic code in living organisms is referred to as (bio-)orthogonality. According to the central dogma of molecular biology, a fully orthogonal system would have to be isolated from the

host system at every level of the flow of genetic information: replication, transcription, and translation (Liu et al., 2018). Since such a system would be fully independent of the host, it would function in a predictable manner, buffering dynamically changing inputs like environmental factors (Liu et al., 2018). Hence, a fully orthogonal system would also be easily transferable between pro- and eukaryotes. Over the past decades, substantial progress has been made in establishing orthogonal translation as a means to (re-)assign codons. In particular, the straightforward molecular requirements for Pyl incorporation in comparison to Sec described above render the PylRS/PylT pair an attractive candidate to expand the genetic code of an organism. Soon after its discovery, the PylRS/PylT pair has been found to be orthogonal in many organisms including bacteria (Blight et al., 2004; Neumann et al., 2008; Yanagisawa et al., 2008b) and mammalian cells (Chen et al., 2009; Gautier et al., 2010; Mukai et al., 2008). In the main work presented in this thesis, we also apply the PylRS/PylT pair in further improving the efficiency of orthogonal translation in mammalian cells. In the following, a broad overview of orthogonal translation and the application of an expanded genetic code in general as well as the PylRS/PylT pair in particular will be given.

3.1.2 *Expanding the genetic code by orthogonal translation*

In synthetic biology, amino acids with chemically unique residues not found in cAAs are referred to as non-canonical amino acids (ncAAs). In the simplest approach expanding the standard amino acid repertoire, native aminoacyl-tRNA synthetase (aaRS)/tRNA pairs are leveraged for ncAA aminoacylation. Thereby, selected sense codons are globally reassigned to encode ncAAs. The finding that cAAs can be substituted with their non-canonical analogs dates back to the 1950s and 1960s (Richmond, 1962) and has since then been applied in protein chemistry to study protein synthesis, structure, function, and metabolism (Hortin and Boime, 1983; Wilson and Hatfield, 1984). This amino acid residue-specific recoding of the genetic code does not require prior genetic engineering of host translation systems and as such was the first strategy reported to simultaneously incorporate three distinct ncAAs in living cells (Lepthien et al., 2010). Mutating the editing or substrate-binding pocket of bacterial aaRSs to misacylate their cognate tRNAs with ncAAs offers an additional gateway to incorporate ncAAs (Döring et al., 2001; Kirshenbaum et al., 2002). However, replacement of a cAA with its non-canonical analog results in proteome-wide ncAA incorporation, negatively affecting cellular viability (Budisa et al., 1999).

In contrast to recoding of the standard genetic code using cAA analogs, the genetic code can be expanded and ncAAs site-specifically incorporated. Since nonsense codons do not encode any of the 20

standard cAAs, mainly these codons and in particular the amber stop codon, as the least frequently used termination codon in most bacteria (Korkmaz et al., 2014) and eukaryotes (Shabalina et al., 2004; Sun et al., 2005), have been reassigned (Torre and Chin, 2021). In response to an in-frame amber stop codon, an ncAA is then site-specifically incorporated. This genetic code expansion strategy is generally referred to as amber suppression. To co-translationally incorporate an ncAA at UAG, the amber suppressor tRNA has to be first aminoacylated by one of two approaches: in vitro by chemical or enzymatic conjugation or in vivo by an orthogonal translation system (OTS).

The first site-specific incorporations of ncAAs were accomplished in vitro by cell-free protein synthesis using chemically aminoacylated tRNAs suppressing an in-frame amber stop codon (Bain et al., 1989; Noren et al., 1989). In general, by adding the respective aminoacylated suppressor tRNAs to an in vitro translation system, also the opal or ochre (UAA) stop codon can be suppressed (Bain et al., 1991) and a wide variety of ncAAs incorporated (Ellman et al., 1992). Furthermore, acylated suppressor tRNAs can be injected into *Xenopus* oocytes, representing the first examples of site-specific ncAA incorporation in living cells (Nowak et al., 1995; Saks et al., 1996). In principle, these in vitro acylated suppressor tRNAs can also be delivered into cultured mammalian cells by electroporation (Monahan et al., 2003). However, application of chemically acylated suppressor tRNAs in in vitro translation systems or their delivery by microinjection or electroporation results in only low yields of ncAA-modified target proteins.

In contrast, genetically encoded and selective suppressor tRNA aminoacylation by orthogonal translation in vivo (Figure 1) enables the site-specific incorporation of ncAAs with greater efficiency (Furter, 1998). Thereby, modified protein levels are higher since after deacylation during translation, synthetic tRNAs can be reacylated with an ncAA by their cognate aaRS. To ensure orthogonality, the newly introduced aaRS/tRNA pair has to fulfill two essential prerequisites (Liu et al., 1997): First, the aaRS has to exclusively aminoacylate its cognate tRNA and no endogenous tRNA of the host organism. Second, the synthetic tRNA has to be exclusively aminoacylated by its respective aaRS and not by any endogenous aaRS (Figure 1). Any cross-reaction of the exogenous aaRS and/or tRNA with host aaRS/tRNA pairs will result in the misincorporation of ncAAs or cAAs. To date, a variety of aaRS/tRNA pairs from bacteria, archaea and also eukaryotes have been identified to be orthogonal in bacteria and/or eukaryotes.

ORTHOGONAL SYNTHETASE/TRANSFER RNA PAIRS In bacteria, the first orthogonal aaRS/tRNA pair to be identified originated from yeast (Liu and Schultz, 1999). To date, the *Saccharomyces cerevisiae* glutaminyl- (Liu et al., 1997; Liu and Schultz, 1999), tyrosyl- (Kowal et al., 2001; Ohno et al., 1998), aspartyl- (Pastrnak et al., 2000), and

tryptophanyl-RSs (Chatterjee et al., 2013d; Hughes and Ellington, 2010) in combination with selected amber suppressor tRNAs were demonstrated to be orthogonal in *Escherichia coli*. Additionally, derivatives of aaRS/tRNA pairs from archaea were identified as orthogonal in *E. coli*, like the leucyl-RS from *Methanobacterium thermoautotrophicum* (Anderson and Schultz, 2003) or the lysyl-RS from *Pyrococcus horikoshii* (Anderson et al., 2004). Recently, also some bacterial aaRS/tRNA pairs were described as orthogonal in *E. coli*, including the glutaminyl-RS from *Ilumatobacter nonamiensis* and the aspartyl-RS from *Sorangium cellulosum* (Cervettini et al., 2020). The most widely applied orthogonal aaRS/tRNA pair in *E. coli* is the tyrosyl-RS/$tRNA^{Tyr}_{CUA}$ pair from an archaeon, the thermophilic methanogen *Methanocaldococcus jannaschii* (Wang et al., 2000). This pair also constitutes the first OTS to incorporate an ncAA in vivo (Wang et al., 2001). In eukaryotes, the two mainly applied orthogonal aaRSs derived from *E. coli* are the tyrosyl- (Chin et al., 2003a; Edwards and Schimmel, 1990; Liu et al., 2007; Sakamoto et al., 2002; Young et al., 2009) and leucyl-RS (Wu et al., 2004; Young et al., 2009), although also the glutaminyl- (Drabkin et al., 1996; Kowal et al., 2001) and tryptophanyl-RS (Italia et al., 2017) are orthogonal. At this, the *E. coli* tyrosyl-RS has been the first orthogonal aaRS to incorporate an ncAA in mammalian cells (Sakamoto et al., 2002). However, neither are these bacterial aaRSs orthogonal in *E. coli* nor are the aforementioned yeast or archaeal aaRSs known to be orthogonal in eukaryotes.

In contrast, the archaeal PylRS from *Methanosarcina* species including *M. barkeri* and *Methanosarcina mazei* has been demonstrated to be orthogonal in both bacteria (Blight et al., 2004; Neumann et al., 2008; Polycarpo et al., 2006; Yanagisawa et al., 2008b) and also eukaryotes, ranging from yeast (Hancock et al., 2010) and mammalian cells (Chen et al., 2009; Gautier et al., 2010; Mukai et al., 2008) to plants (Li et al., 2013) and whole animals (Bianco et al., 2012; Chen et al., 2017; Greiss and Chin, 2011; Han et al., 2017; Liu et al., 2017). Importantly, this robust orthogonality allows to freely transfer the wild-type (wt) PylRS/PylT pair and its modified variants between pro- and eukaryotic organisms. Of note, PylRS/PylT pairs also from other methanogenic archaea have been recently identified as orthogonal in *E. coli* (Willis and Chin, 2018) and mammalian cells (Beránek et al., 2019; Meineke et al., 2018). Overall, OTSs derived from eukaryotes and some archaea are only orthogonal in bacteria, while the majority of bacterial OTSs discovered to date are only orthogonal in eukaryotes, with the archaeal PylRS/PylT OTS being a remarkable exception.

STRUCTURAL FEATURES CONFERRING ORTHOGONALITY Orthogonality of an aaRS/tRNA pair is based on structural features conferring unique tRNA as well as amino acid specificities. Hence, understanding these features is essential in successfully establishing and

adapting OTSs. At this, two features render the PylRS/PylT pair particularly suitable. First, the archaeal wt PylRS already tolerates a wide variety of ncAAs (Flügel et al., 2014; Kobayashi et al., 2009; Polycarpo et al., 2006) by binding its cognate amino acid Pyl via coordinated hydrogen bonding within an exceptionally deep hydrophobic pocket (Kavran et al., 2007; Yanagisawa et al., 2008a). Importantly, this catalytic pocket can also be further enlarged by engineering of only a small number of residues to accommodate a diverse set of even bulky ncAAs (Kavran et al., 2007; Schmidt et al., 2014b; Schneider et al., 2013; Takimoto et al., 2011; Yanagisawa et al., 2008b). Second, biochemical and structural analyses of the bacterial PylRS from *D. hafniense* revealed that the synthetase does not interact with its cognate tRNA acceptor stem or anticodon stem and loop, rendering the mechanism of PylRS/PylT recognition distinct from other aaRS/tRNA complexes (Herring et al., 2007b; Jiang and Krzycki, 2012; Nozawa et al., 2009). In contrast, the PylT variable loop, D stem, and T stem and loop were identified as tRNA identity elements (Jiang and Krzycki, 2012; Nozawa et al., 2009). This unique PylT binding interface including the minimal variable tRNA loop is essential for tight binding to the N-terminal PylRS domain (Herring et al., 2007a; Jiang and Krzycki, 2012; Nozawa et al., 2009; Suzuki et al., 2017). Thereby, the recognition of non-cognate host tRNAs is precluded, constituting the structural basis of PylRS/PylT's orthogonality (Herring et al., 2007a; Jiang and Krzycki, 2012; Nozawa et al., 2009; Suzuki et al., 2017). Accordingly, archaeal PylRSs from *Methanosarcina* species do not recognize the anticodon (Ambrogelly et al., 2007; Suzuki et al., 2017) with the two flanking nucleotides being PylT identity elements (Ambrogelly et al., 2007). Therefore, the PylT anticodon can be flexibly adapted to also decode other codons than the amber stop codon (Ambrogelly et al., 2007), although this change can also result in the loss of orthogonality of the PylT variant due to the recognition of the new anticodon by host aaRSs and hence mischarging with a cAA (Krishnakumar et al., 2013). In comparison, only aminoacylations by leucyl- and histidyl-RS in prokaryotes as well as seryl- and alanyl-RS in both pro- and eukaryotes are anticodon-independent, rendering the anticodon an essential identity element for the majority of pro- and eukaryotic tRNAs (Giegé et al., 1998). Of note, orthogonal PylRS/PylT pairs from methanogenic archaea other than *Methanosarcina* were recently identified that lack the N-terminal PylRS domain (Beránek et al., 2019; Meineke et al., 2018; Willis and Chin, 2018), suggesting additional modes of PylT binding that confer orthogonality. Taken together, the unique tRNA binding surface and deep catalytic pocket confer the orthogonality and amino acid polyspecificity of wt PylRS.

DIRECTED EVOLUTION OF ORTHOGONAL SYNTHETASE/TRANSFER RNA PAIRS To expand the genetic code of an organism with an

ncAA, also the substrate specificity of orthogonal aaRSs has to be engineered. By mutating orthogonal wt aaRSs, ncAA-adapted orthogonal aaRS (ncRS) variants can be established. In general, high binding affinity and elimination of non-cognate amino acids by proofreading activity enable aaRSs to charge their cognate amino acid with high fidelity to the cognate tRNA (Ling et al., 2009). To confer this specificity for a ncAA substrate, typically amino acid residues within the active site pocket, which must bind the ncAA, are randomly mutated (Cooley et al., 2014; Dumas et al., 2015; Nödling et al., 2019), but also mutagenesis of residues within non-catalytic aaRS domains can greatly improve catalytic activity and ncAA incorporation (Bryson et al., 2017; Cooley et al., 2014; Owens et al., 2017; Suzuki et al., 2017). Interestingly, the ability of an orthogonal aaRS to tolerate extensive engineering of its substrate specificity has been reported to depend on the aaRS's general structural robustness (Grasso et al., 2021). At this, loss of structural integrity and concomitant catalytic activity of an engineered ncRS is negatively correlated with its thermostability (Grasso et al., 2021). Hence, orthogonal aaRSs derived from thermophilic organisms might be especially suited for engineering ncAA substrate specificity. After synthesizing a mutant aaRS library, functional as well as orthogonal ncRSs are identified via directed evolution, a process which is based on iterative rounds of positive and negative selection in vivo (Liu and Schultz, 1999). During positive selection in the presence of an ncAA, usually expression of a viability-associated gene depends on efficient decoding of the ncAA-assigned codon (Chin et al., 2003a,b; Liu and Schultz, 1999; Wang et al., 2001). These genes can for instance complement an amino acid auxotrophy of the host organism (Chin et al., 2003b; Liu and Schultz, 1999) or confer antibiotic resistance (Liu and Schultz, 1999). Thereby, aaRS mutants are selected for acylating their cognate tRNA. During following negative selection in the absence of an ncAA, usually a lethality-associated gene must not be expressed, which depends on termination of translation at the ncAA-assigned codon (Chin et al., 2003a,b; Liu and Schultz, 1999; Wang et al., 2001). In bacteria, these cytotoxic effectors can be for instance the ribonuclease barnase (Liu and Schultz, 1999) or the CcdB toxin (Umehara et al., 2012). Thereby, aaRS mutants are selected for acylating their cognate tRNA exclusively with the ncAA and not with any cAA. Repetition of this positive and negative selection scheme over several rounds sequentially enriches for aaRS/tRNA pairs with a growth dynamic that is proportional to their ability to incorporate an ncAA and inversely proportional to their ability to incorporate a cAA instead (Liu and Schultz, 1999; Melançon and Schultz, 2009). Encoding both the positive and negative selection marker on a single vector further streamlines the identification of suitable ncRS mutants by omitting isolation and retransformation of plasmids encoding the aaRS library after each selection step (Melançon and Schultz, 2009;

Santoro et al., 2002). Additionally, colorimetric assays such as X-GAL blue-white screening can function as both positive and negative selection markers (Chin et al., 2003b; Owens et al., 2017). Expanding on this benefit of a single reporter gene, fluorescent proteins have been used in bacteria as readouts for positive and negative selection (Hohl et al., 2019a; Kuhn et al., 2010; Santoro et al., 2002). In combination with fluorescence-activated cell sorting (FACS), these fluorescent reporters not only allow for the directed evolution of functional and orthogonal aaRS in high-throughput, but also the quantification of ncAA incorporation efficiency based on fluorescence intensity (Hohl et al., 2019a; Kuhn et al., 2010; Santoro et al., 2002). However, due to the time effort of approximately one week for each cycle of positive and negative selection, directed evolution of ncRSs in bacteria is usually limited to only a few selection rounds (Bryson et al., 2017). This restriction can lead to suboptimal ncRSs mutants with greatly reduced ncAA affinity and as such catalytic activity (Umehara et al., 2012). In contrast, phage-assisted evolution of orthogonal ncRS variants allows for positive and negative selection over hundreds of generations (Bryson et al., 2017; Suzuki et al., 2017). At this, the selectivity and efficiency of ncAA charging by an orthogonal aaRS is coupled to phage propagation (Bryson et al., 2017; Suzuki et al., 2017). Remarkably, phage-assisted evolution of PylRS variants led to the identification of activity-enhancing mutations outside of the catalytic site and within the synthetase's N-terminal domain (Bryson et al., 2017; Suzuki et al., 2017), which binds PylT and is essential for PylRS activity (Herring et al., 2007a; Jiang and Krzycki, 2012). The discovery of these ncRSs highlights the substantial advantage of phage-assisted over directed evolution in bacteria in that identification of these novel ncRS mutations is independent of the prior selection and mutation of amino acid residues to prepare mutant aaRS libraries (Bryson et al., 2017; Suzuki et al., 2017). An additional new selection method leverages deep sequencing to identify ncRS variants obtained out of bacterial populations by parallel positive selection in the presence and absence of the ncAA (Zhang et al., 2017a). Since the method circumvents negative selection, selective ncRSs can not only be discovered more rapidly, but also identified for aaRSs that are poorly orthogonal in the absence of an ncAA (Zhang et al., 2017a). Notably, although an aaRS is usually evolved to accommodate a specific ncAA, some mutants can be permissive and acylate their cognate tRNA also with other ncAAs (Cooley et al., 2014; Guo et al., 2014; Miyake-Stoner et al., 2010). This ncRS polyspecificity can enable the incorporation of ncAAs for which *de novo* selection of aaRS mutants has failed (Cooley et al., 2014; Miyake-Stoner et al., 2010), but also interferes with the simultaneous and site-specific incorporation of structurally similar ncAAs (Guo et al., 2014; Kwok et al., 2019). To further decrease the promiscuity of an ncRS for other ncAAs and also cAAs, a selection scheme has

been developed that utilizes the N-end rule in bacteria (Kunjapur et al., 2018). At this, the stability of the suppressed target protein is regulated by the identity of its N-terminal amino acid residue. Hence, amber suppression of this N-terminal site can function as a readout to monitor selective incorporation of a specified ncAA versus cAAs (Kunjapur et al., 2018). Furthermore, parallel positive selection in combination with deep sequencing (Zhang et al., 2017a) or single fluorescent reporters in combination with flow-cytometry (Kwok et al., 2019) have been employed to identify ncRS variants with defined ncAA specificity. Moreover, also host aaRSs can be permissive for ncAAs (Fan et al., 2014; Hartman et al., 2006; Richmond, 1962), resulting in their proteome-wide incorporation. Hence, not only the ncRS/tRNA pair, but also the ncAA itself must be verified to be orthogonal within the host organism. In summary, a tailor-made orthogonal aaRS that incorporates a specified ncAA is typically evolved by iterative rounds of positive and negative selection, while the adaptation of new techniques such as deep sequencing or phage-assisted evolution further advances the identification of novel ncRS variants.

To establish ncRS variants, aaRSs that are orthogonal only in bacteria or eukaryotes also must be evolved in a selection host of either bacterial or eukaryotic origin, respectively. At this, OTSs for bacteria are usually evolved in *E. coli* (Liu and Schultz, 1999; Santoro et al., 2002; Wang et al., 2001), whereas OTSs for eukaryotes and as such mammalian cells are evolved in *S. cerevisiae* (Chin et al., 2003a,b; Liu et al., 2007; Wang et al., 2007b). However, evolving ncRS variants in yeast is technically more challenging and time-consuming than in fast-growing bacteria (Italia et al., 2017, 2018), which also can be used for phage-assisted evolution. Moreover, by applying distinct OTSs in bacteria and eukaryotes, genetically encoding a new ncAA requires the separate adaptation of each OTS, one for bacteria and one for eukaryotes. To this end, some bacterial aaRSs have been previously liberated in an engineered *E. coli* strain (Englert et al., 2017; Italia et al., 2017, 2018). Thereby, these bacterial aaRS/tRNA pairs, which are orthogonal in eukaryotes, can be evolved in bacteria instead of yeast, streamlining the identification of ncRS/tRNA pairs (Italia et al., 2017, 2018). However, the PylRS/PylT pair is of major advantage in terms of species orthogonality and hence ncRS evolution. Since wt PylRS is orthogonal in both bacteria and eukaryotes, its substrate specificity can be readily engineered in *E. coli* and directly transferred to eukaryotes (Bianco et al., 2012; Chen et al., 2009; Chen et al., 2017; Gautier et al., 2010; Greiss and Chin, 2011; Han et al., 2017; Hancock et al., 2010; Li et al., 2013; Liu et al., 2017; Mukai et al., 2008). Hence, in contrast to other OTSs, PylRS variants have to be evolved only once, rendering the PylRS/PylT pair a particularly useful OTS.

In summary, this chapter illustrates how the genetic code can be further "thawed" to include ncAAs with new functionalities. Overall,

the process of expanding the genetic code of a living organism, pro- or eukaryote, can be subdivided into two essential steps: first, the identification of an aaRS/tRNA pair that is orthogonal in the host organism; and second, engineering of this pair to only accommodate and hence incorporate the selected ncAA. As outlined above, especially six features render the PylRS/PylT pair superior over other OTSs to expand the genetic code: (i) by nature, PylRS/PylT is orthogonal in bacteria as well as eukaryotes; (ii) in *E. coli* evolved PylRS variants can be readily applied in diverse eukaryotic hosts; (iii) wt PylRS is polyspecific and inherently accommodates ncAAs; (iv) the PylRS active site is permissive to directed evolution to accommodate even bulky ncAAs; (v) since PylRS does not bind the PylT anticodon loop, the anticodon can be flexibly exchanged; and (vi) unlike Sec, PylRS/PylT uses canonical co-factors for translation.

3.2 ADVANCING GENETIC CODE EXPANSION

3.2.1 *Optimizing key components of orthogonal translation*

To further develop OTSs as tools to efficiently and robustly expand the genetic code of an organism, each of its three key components has been modified: (i) the codon to be decoded with the orthogonal ncAA-tRNA; (ii) the orthogonal aaRS/tRNA pair and its expression as well as the transcript harboring the (re-)assigned codon(s); and (iii) the ncAA to be incorporated and its cellular bioavailability (Figure 1).

MODIFICATION OF CODONS The standard genetic code encompasses 64 triplet codons with 61 codons encoding amino acids (Crick, 1968; Crick et al., 1961). Only three codons that do not encode a cAA but instead terminate translation are available, consisting of the amber, ochre, and opal termination codons (Caskey et al., 1968). As outlined above, mostly the amber stop codon is reassigned to encode an ncAA by orthogonal translation in bacteria and eukaryotes. However, the availability of only a single codon, which also still functions as a signal for translational termination, inherently limits the efficiency and also the number of distinct ncAAs that can be site-specifically incorporated. Therefore, new free codons would allow expanding the genetic code even further. Here, the discovery that an extended tRNA anticodon loop of eight instead of seven nucleotides can form an additional Watson-Crick-Franklin base pair with the nucleotide following a triplet codon (Curran and Yarus, 1987; Gaber and Culbertson, 1984; Riddle and Carbon, 1973) lead to the design and subsequent identification of efficient four-base suppressor tRNAs that decode these quadruplet codons in vivo (Magliery et al., 2001). Upon decoding, the translational reading frame is then shifted by one base and the following triplet codon read out of phase, which hence is referred

to as frameshift suppression. The first site-specific incorporations of a cAA (Ma et al., 1993) or ncAA (Hohsaka et al., 1996) employing a four-base suppressor were conducted by in vitro translation. At this, the theoretical availability of 256 unique quadruplet codons allows for the simultaneous and site-specific incorporation of several distinct ncAAs into a single protein (Hohsaka et al., 2001; Hohsaka et al., 1999; Ohtsuki et al., 2005; Taki et al., 2002). After the identification of orthogonal four-base suppressor tRNAs in vivo (Anderson and Schultz, 2003), also the genetic code of *E. coli* could be expanded with an OTS that decodes a quadruplet codon with a ncAA (Anderson et al., 2004). Moreover, frameshift suppression is mutually orthogonal with amber suppression in vitro (Murakami et al., 2003) and in bacteria (Anderson et al., 2004), allowing the incorporation of two different ncAAs in parallel. However, decoding efficiency across different quadruplet codons varies in vitro (Hohsaka et al., 2001; Taira et al., 2005) and in vivo (Magliery et al., 2001) and efficient decoding in bacteria requires additional engineering of the ribosome (Neumann et al., 2010b). Orthogonal four-base suppressors have also been microinjected into *Xenopus* oocytes to incorporate ncAAs in living eukaryotic cells, although with reduced efficiency compared to amber suppression (Rodriguez et al., 2006). Additionally, frameshift suppression has been introduced in mammalian cells (Taki et al., 2006) and a single ncAA incorporated using a four-base PylT suppressor (Niu et al., 2013), yet awaiting further development and application.

Another intriguing possibility to design new free codons is the development of synthetic nucleoside bases that can form non-canonical codon-anticodon base pairs (Bain et al., 1992; Hirao et al., 2002). In vitro, this third base pair can be amplified (Li et al., 2014; Morris et al., 2017), transcribed, and translated (Bain et al., 1992; Hirao et al., 2002) to direct the site-specific incorporation of an ncAA. Moreover, a semi-synthetic *E. coli* strain now propagates, transcribes, and by applying the PylRS/PylT pair translates the artificial base pair to incorporate an ncAA in vivo (Malyshev et al., 2014; Zhang et al., 2017b,c). Furthermore, three orthogonal codon-anticodon pairs harboring the third artificial base pair have been identified to simultaneously and site-specifically decode up to three ncAAs in vivo (Fischer et al., 2020). Remarkably, the genetic alphabet has recently been expanded to an eight-letter code composed of the four standard letters as well as four synthetic letters that can be transcribed using an engineered RNA polymerase (Hoshika et al., 2019).

As a last possibility, compression of the genetic code to less than 64 codons liberates codons to also unambiguously encode ncAAs in vivo. At this, the amber stop codon has been completely removed in *E. coli* (Isaacs et al., 2011; Lajoie et al., 2013). Furthermore, by directly replacing genomic segments with synthetic deoxyribonucleic acid (DNA) sequences (Wang et al., 2016b), recently two serine codons in

addition to the amber stop codon have been replaced genome-wide with synonymous codons, generating a viable *E. coli* strain with a 61 codon genome (Fredens et al., 2019). Moreover, a synthetic 57 codon *E. coli* genome has been partly constructed replacing two arginine, serine, and leucine codons as well as the amber stop codon, which yet has to be fully assembled to generate a viable recoded organism (Ostrov et al., 2016). Of note, also the rare arginine codon AGG in *E. coli* has been reassigned genome-wide to encode an ncAA after synonymous exchange of the majority of AGG codons in essential genes (Mukai et al., 2015b). This strain can then be used to site-specifically incorporate an ncAA in response to the AGG codon (Ohtake et al., 2018). Taken together, the genetic code can be unambiguously expanded by quadruplet or even non-canonical codons as well as by fully reassigning sense and nonsense codons in genomically recoded organisms with a compressed standard genetic code.

MODIFICATION OF SYNTHETASE/TRANSFER RNA PAIRS AND TRANSCRIPTS Expression of the ncRS/tRNA pair in bacteria and concomitant incorporation of ncAAs can be significantly improved by encoding both the ncRS and tRNA on a single vector (Chatterjee et al., 2013b; Ryu and Schultz, 2006). Furthermore, inducible in parallel to constitutive ncRS expression in bacteria improves yields of a protein of interest (POI) harboring an ncAA at a specified incorporation site (POI^{ncAA}) (Young et al., 2010). In particular, ncRS expression levels have been identified as limiting factors in ncAA incorporation efficiency (Lammers et al., 2014; Young et al., 2010). In eukaryotes, high ncRS but especially tRNA expression levels are critical in improving POI^{ncAA} yields (Chen et al., 2007b; Garcia et al., 2019; Mukai et al., 2008; Parrish et al., 2012; Roy et al., 2020; Schmied et al., 2014; Uttamapinant et al., 2015; Wang et al., 2014b; Wang et al., 2007b; Zheng et al., 2017b). This dependence on high tRNA expression is in accordance with the positive correlation between stop codon suppression efficiency and aminoacylated suppressor tRNA levels (Janzen and Geballe, 2004). In difference to prokaryotes, however, eukaryotic tRNA transcription largely depends on promoter sequences within the tRNA coding region, called A- and B-box (Galli et al., 1981). Since A- and B-box promoter sequences are partially or even fully absent in the majority of orthogonal bacterial as well as archaeal tRNAs, efficient eukaryotic expression strategies for these foreign tRNAs had to be first engineered (Chen et al., 2007b; Hancock et al., 2010; Kowal et al., 2001; Sakamoto et al., 2002; Wang and Wang, 2008; Wang et al., 2007b; Zhang et al., 2004). However, modifying the orthogonal tRNA (o-tRNA) coding sequence to include A- and B-box sequences can disrupt its function (Hancock et al., 2010; Sakamoto et al., 2002). Here, the type-3 class of RNA polymerase III promoters has been found particularly useful in mammalian cells (Mukai et al., 2008; Schmied

et al., 2014; Wang et al., 2007b) with the H1 (Myslinski et al., 2001) or U6 small-nuclear RNA (Das et al., 1988; Kunkel and Pederson, 1989) type-3 promoters initiating transcription independent of downstream A- and B-box elements. To this end, PylT, which lacks mammalian A-and B-box sequences, is most efficiently expressed in mammalian cells from tandem promoter-tRNA copies of U6-PylT functional units (Mukai et al., 2008; Schmied et al., 2014). Moreover, in mammalian cells also the stoichiometry between ncRS and tRNA expression requires optimization as low suppressor tRNA levels in comparison to the ncRS have been found to limit ncAA incorporation efficiency (Schmied et al., 2014; Wang et al., 2007b; Zheng et al., 2017b). Since high intracellular ncRS and suppressor tRNA levels might adversely affect host translation, recently a doxycycline-inducible PylRS/PylT expression system has been developed, but not yet benchmarked against other PylRS/PylT expression systems in mammalian cells (Koehler et al., 2020). Additionally, a higher expression level of the gene of interest (GOI) harboring the (re-)assigned codon(s) (GOI*) in comparison to the ncRS has been reported to improve ncAA incorporation efficiency (Zheng et al., 2017b). However, since in amber suppression the GOI* transcript inherently harbors a premature termination codon (PTC), their expression might be additionally hampered by nonsense-mediated decay (NMD). Accordingly, inhibition of NMD has been reported to increase the efficiency of amber suppression and ncAA incorporation in yeast (Wang and Wang, 2008), *Caenorhabditis elegans* (Greiss and Chin, 2011; Parrish et al., 2012), and mammalian cells (Han et al., 2017). Furthermore, coding sequences of the orthogonal ncRS/tRNA pair and GOI* have to be efficiently delivered and contained in mammalian cells. At this, encoding the ncRS/tRNA pair as well as GOI* on a single vector greatly improves the efficiency of ncAA incorporation (Chatterjee et al., 2013c; Cohen and Arbely, 2016; Xiao et al., 2013; Zheng et al., 2017b). Additionally, PylRS together with PylT has been found to mainly localize to the nucleus, being spatially separated from cytoplasmic translation (Nikić et al., 2016). Preferentially sequestering PylRS into the cytoplasm by fusion to a nuclear export signal enhanced amber suppression efficiency up to 15-fold (Nikić et al., 2016). Moreover, combining phase separation and kinesin targeting, PylRS and the transcript harboring the reassigned stop codon were locally concentrated within eukaryotic cells, forming an artificial, membraneless organelle (Reinkemeier et al., 2019). Thereby, suppression of the in-frame nonsense codon by ncAA-PylT is spatially separated from the translation of host transcripts, increasing the specificity of ncAA incorporation (Reinkemeier et al., 2019). Furthermore, instead of transient transfections, baculovirus mediated delivery allows for the robust and homogenous expression of OTSs and GOI*s in diverse mammalian cell types (Chatterjee et al., 2013c; Zheng et al., 2017b) as well as the incorporation of multiple ncAAs

into a single target protein (Xiao et al., 2013). Moreover, the establishment of mammalian cells with a stably expanded genetic code further streamlined the long-term encoding and hence the applicability of ncAAs. To date, mammalian cell lines stably expressing the ncRS/tRNA and GOI* have been established by random genomic integration via selection pressure (Axup et al., 2012; Roy et al., 2020; Tian et al., 2014), pronuclear microinjection of fertilized eggs (Han et al., 2017), viral delivery (Ernst et al., 2016; Shen et al., 2011; Si et al., 2016), or transposases (Elsässer et al., 2016) as well as by self-replicating episomal vectors (Shao et al., 2020). Taken together, robust expression of the ncRS/tRNA pair and GOI* as well as fine-tuning of their stoichiometry is critical in expanding the genetic code especially of mammalian cells.

Besides OTS expression, the sequence or structure of the o-tRNA itself is a major factor to efficiently and robustly expand the genetic code of an organism. Analysis of ncAA-PylT kinetics in vitro revealed impaired binding of the prokaryotic elongation factor thermo unstable (EF-Tu) and delivery of the ncAA-PylT:EF-Tu:GTP ternary complex to the ribosome, resulting in up to 30-fold slower dipeptide formation compared to natural substrates (Wang et al., 2016a). To overcome this mistuning in the decoding capacity of an o-tRNA charged with an ncAA, especially in the bacterial expression context o-tRNAs have been extensively engineered to optimize ncAA incorporation efficiency (Uhlenbeck and Schrader, 2018) and tRNA orthogonality (Willis and Chin, 2018). In particular, tRNAs with a high affinity for EF-Tu have been demonstrated to substantially boost ncAA incorporation in vitro (Ieong et al., 2014; Katoh et al., 2017). Accordingly, o-tRNA acceptor stem and T-stem sequences, which are bound by EF-Tu, have been mutated to afford tighter EF-Tu binding and thereby increased amber suppression efficiency in bacteria, especially at multiple sites within a single GOI* (Chatterjee et al., 2012; Fan et al., 2015; Guo et al., 2009; Maranhao and Ellington, 2017; Young et al., 2010). Of note, likewise the identity of the ncAA bound to the evolved o-tRNA seems to influence the kinetics of EF-Tu binding and UAG decoding (Fan et al., 2015; Guo et al., 2009). Interestingly, modifications of the acceptor stem and T-stem also enabled binding of a $tRNA^{Sec}_{CUA}$ variant to EF-Tu and hence Sec incorporation in bacteria via canonical translation (Thyer et al., 2015). Moreover, not only EF-Tu binding, but also the anticodon sequence context and concomitant stability of codon-anticodon base pairing modulate the decoding efficiency of an o-tRNA (Uhlenbeck and Schrader, 2018). For instance, by directed evolution of the anticodon loop, o-tRNAs with enhanced amber suppression activity in *E. coli* were identified (Anderson and Schultz, 2003; Chatterjee et al., 2012; Maranhao and Ellington, 2017; Rogerson et al., 2015; Wang and Schultz, 2001). Additionally, ncAA incorporation by PylT can be further increased in bacteria (Chatterjee et al., 2013b) and mammalian

cells (Schmied et al., 2014) by substituting a wobble base pair in the anticodon stem, which has been originally reported to reduce suppression efficiency of a leucyl-tRNA (Anderson and Schultz, 2003). Especially efficient frameshift suppression in vitro and in bacteria by four-base suppressor tRNAs requires modification of the anticodon loop as well as other tRNA regions like the acceptor stem (Anderson and Schultz, 2003; Anderson et al., 2004; Magliery et al., 2001; Ohtsuki et al., 2005; Wang et al., 2014a). Interestingly, anticodon loop sequences vary between different quadruplet anticodons, indicating that the identities of the anticodon and neighboring nucleotides are interdependent (Magliery et al., 2001; Wang et al., 2014a). These evolved four-base suppressors can then also be applied in mammalian cells to decode quadruplet codons (Niu et al., 2013). Moreover, comparison of $tRNA^{Ser}_{CUA}$ isodecoders in human cells clearly demonstrated that the tRNA backbone sequence largely influences translational efficiency at the ribosome and as such amber suppression efficiency (Geslain and Pan, 2010). Furthermore, in mammalian cells intracellular PylT levels and concomitant POI^{ncAA} yields can be increased by rationally engineering the D-arm, T-loop, and anticodon stem (Serfling et al., 2018). Accordingly, poor efficiency of the amber suppressor tRNA in mammalian cells has been described as the most limiting factor in ncAA incorporation (Zheng et al., 2017b). Hence, improving o-tRNA quality by engineering its structural features instead of merely increasing the copy number of an o-tRNA can significantly improve decoding and thereby ncAA incorporation.

Moreover, in bacteria and eukaryotes 10% to 20% of tRNA residues are post-transcriptionally modified, maintaining their structural stability and ensuring for instance accurate tRNA aminoacylation or codon-anticodon base pairing (Lorenz et al., 2017; Suzuki, 2021). Here, a single post-transcriptional modification within the anticodon can determine whether a eukaryotic tRNA can act as a suppressor or not (Bienz and Kubli, 1981). Accordingly, post-transcriptional modifications of o-tRNAs and their effect on the capacity and fidelity of ncAA incorporation are emerging as a substantial factor in robustly expanding the genetic code of an organism (Baldridge et al., 2018; Biddle et al., 2016; Crnković et al., 2018; Serfling et al., 2018). For instance, post-transcriptional modification of the o-tRNA anticodon wobble position to inosine in *E. coli* resulted in off-target decoding of a synonymous codon by the affected o-tRNA (Biddle et al., 2016). Furthermore, overexpression or deletion of tRNA modifying enzymes in bacteria can substantially improve the activity of orthogonal suppressor tRNAs (Baldridge et al., 2018; Crnković et al., 2018). Likewise, post-transcriptional modification of the anticodon or adjacent nucleotides has been reported to modulate suppression activity of eukaryotic tRNAs in vitro (Zerfass and Beier, 1992) or in yeast (Beznosková et al., 2019; Blanchet et al., 2018; Klassen and Schaffrath, 2018). Interestingly,

an optimized PylT sequence with improved amber suppression efficiency in mammalian cells also gained a single post-transcriptional modification not present in the original PylT sequence, probably stabilizing the anticodon stem (Serfling et al., 2018). Therefore, activity and orthogonality of o-tRNAs can be considerably influenced by the post-transcriptional tRNA modification landscape of the host. However, directed evolution of o-tRNAs can diminish this interdependence between tRNA post-transcriptional modifications and their orthogonality within the bacterial selection host (Baldridge et al., 2018; Biddle et al., 2016). To which extent this interdependence can be modulated by evolved o-tRNAs in eukaryotes including mammalian cells remains unclear. In conclusion, both sequence and post-transcriptional modifications of o-tRNAs act together during decoding of their assigned codons and hence are important but yet emerging aspects in engineering OTSs.

MODIFICATION OF NON-CANONICAL AMINO ACIDS For cellular uptake, ncAAs are usually directly supplemented to the culture media. However, bioavailability especially of negatively charged and hence cell impermeable ncAAs can be increased by esterification of carboxyl groups for increased lipophilicity and hence membranous translocation (Takimoto et al., 2010) or by providing them as dipeptides for active cellular import via membrane transporters (Kang et al., 2013; Luo et al., 2017; Parrish et al., 2012). Furthermore, cellular uptake of ncAAs in bacteria has been enhanced by increasing expression levels of a natural amino acid transporter (Yu et al., 2014) or even development of a mutant membrane transporter (Ko et al., 2019). Additionally, engineering of metabolic pathways in *E. coli* can not only decrease intracellular ncAA turnover (Heinemann et al., 2012; Steinfeld et al., 2014), but also permit the biosynthesis of ncAAs from bioavailable carbon sources (Mehl et al., 2003; Zhang et al., 2017a). Of note, the latter represents the first self-sustaining and autonomous organism with orthogonal translation and as such a truly expanded genetic code (Mehl et al., 2003). Taken together, the intracellular bioavailability of the ncAA can be optimized by modifying the host organism or the ncAA itself.

3.2.2 *Modifying host translation*

MODIFICATION OF TRANSLATIONAL TERMINATION Nonsense suppression of for instance the amber stop codon as the commonly reassigned codon in genetic code expansion (Torre and Chin, 2021) is in direct competition with translational termination (Figure 1). Since the termination efficiency of stop signals is enhanced by increasing levels of eu- or prokaryotic release factors (Cridge et al., 2006; Le Goff et al., 1997), engineering of release factors and release factor expression

has the potential to improve nonsense suppression mediated ncAA incorporation. In bacteria, release factor 1 (RF1) recognizes UAA and UAG, while UAA is also recognized by release factor 2 (Scolnick et al., 1968). Therefore, inactivation of RF1 should improve ncAA incorporation, particularly at amber stop codons, as first demonstrated in bacterial cell-free protein synthesis (Short et al., 1999). Following, after changing UAG stop codons in seven essential genes to UAA and in the presence of an amber suppressor tRNA, RF1 could be knocked-out in living bacteria, allowing the incorporation of an ncAA at five sequential amber stop codons via orthogonal translation (Mukai et al., 2010; Ohtake et al., 2012). Furthermore, unconditional knock-out (KO) of RF1 in *E. coli* with a reduced genome (Johnson et al., 2011) or even wt *E. coli* (Johnson et al., 2012) has been demonstrated to increase the efficiency of amber suppression and ncAA incorporation, especially at multiple sequential amber stop codons. Moreover, to overcome reduced cellular fitness of these strains (Johnson et al., 2012, 2011; Mukai et al., 2010), 95 UAGs in essential genes were replaced, mitigating the growth defect after RF1 KO (Mukai et al., 2015a). Additionally, applying dedicated genome engineering strategies (Wang et al., 2009; Wang et al., 2016b), synthetic *E. coli* strains were established where all amber stop codons have been removed, rendering RF1 functionally redundant (Fredens et al., 2019; Isaacs et al., 2011; Lajoie et al., 2013). Upon KO of RF1, these genomically recoded organisms retain their fitness (Fredens et al., 2019; Lajoie et al., 2013) and show increased suppression efficiency of GOI*s with multiple amber stop codons (Schwark et al., 2018; Zheng et al., 2016). Therefore, by deletion of RF1 in combination with genome engineering, UAG can be changed into a blank codon in bacteria to boost ncAA incorporation. However, translational termination in eukaryotes at any of the three termination codons is only promoted by the eukaryotic release factor 1 (eRF1) (Frolova et al., 1994; Konecki et al., 1977). Since its KO would be lethal, a dominant negative eRF1 has been engineered and co-expressed in mammalian cells, significantly improving ncAA incorporation at single and especially multiple UAGs using the PylRS/PylT pair (Schmied et al., 2014). Furthermore, inducible overexpression of a similar eRF1 mutant in a stable mammalian cell line selectively enhanced amber suppression up to two-fold at single sites without affecting cellular viability and termination at ochre and opal stop codons (Zhang et al., 2017d). Thus, although eRF1 cannot be knocked-out in eukaryotes, eRF1 mutants can be engineered and overexpressed to attenuate the competition of wt eRF1 with amber suppression.

MODIFICATION OF TRANSLATIONAL ELONGATION In bacteria, the efficiency of OTSs has not only be improved by addressing translational termination, but also by engineering translational elongation and as such elongation factors and the ribosome (Figure 1). All canon-

ical bacterial aminoacylated tRNAs bind EF-Tu and the ribosome with similar affinity, ensuring optimal translational kinetics (LaRiviere et al., 2001; Ledoux and Uhlenbeck, 2008; Louie et al., 1984; Schrader et al., 2011). Hence, for efficient decoding, ncAA-tRNAs would have to be recruited with comparable affinity. As outlined before, the o-tRNA can be modified to adjust its affinity for EF-Tu. However, the affinity of EF-Tu to the aminoacylated tRNA is not only determined by the tRNA itself, but also the aminoacly moiety (Fan et al., 2015; Guo et al., 2009; LaRiviere et al., 2001). At this, incorporation especially of bulky ncAAs has been improved in vitro by an EF-Tu mutant with an enlarged aminoacyl binding pocket (Doi et al., 2007; Ohtsuki et al., 2010). Furthermore, increasing the concentration of EF-Tu alone enhances ncAA incorporaion in vitro, indicating that the rate-limiting factor in POI^{ncAA} synthesis is rather ncAA-tRNA:EF-Tu:GTP ternary complex formation and efficient delivery of the ncAA-tRNA to the ribosome than peptide bond formation at the ribosome (Ieong et al., 2012). Moreover, in living bacteria EF-Tu variants have been evolved to increase incorporation efficiencies of ncAAs like O-phosphoserine (Park et al., 2011) or O-phosphotyrosine (Fan et al., 2016) as well as SECIS-independent Sec incorporation (Haruna et al., 2014). Additionally, tuning expression levels of an evolved EF-Tu variant in comparison to the OTS can substantially improve the simultaneous suppression of multiple amber stop codons in *E. coli* (Gan et al., 2017). Therefore, engineering of EF-Tu can benefit ncAA incorporation.

Specific ribosomal engineering that benefits ncAA incorporation is largely based on the development of an orthogonal ribosome (o-Ribo), which is specifically directed to an orthogonal transcript. After pioneering work on the development of specialized ribosomes for the translation of transcripts with a mutated Shine-Dalgarno (SD) sequence (Hui and Boer, 1987), o-Ribo/transcript pairs have been evolved in *E. coli* by altering the SD and anti-SD sequence (Rackham and Chin, 2005). Thereby, ribosome variants can be introduced that in combination with their dedicated transcripts constitute an independent translational unit next to their host counterparts (Rackham and Chin, 2005). As such, these ribosomes can then be adapted to optimize the efficiency and fidelity of ncAA incorporation specifically with GOI* transcripts. For instance, by mutating the ribosomal A-site, binding of RF1 to the o-Ribo has been decreased, thereby increasing the efficiency of amber suppression (Wang et al., 2007a). Furthermore, an o-Ribo has been evolved to efficiently decode quadruplet codons in *E. coli* (Neumann et al., 2010b). In combination with mutually orthogonal ncRS/tRNA pairs, this engineered ribosome facilitates the parallel incorporation of distinct ncAAs into a single POI^{ncAA} (Neumann et al., 2010b; Wang et al., 2014a). Additionally, an o-Ribo has been evolved to improve Sec incorporation at UGA in *E. coli* (Thyer et al., 2013). Remarkably, covalently linked ribosomal 16S and 23S RNA now allow

to specifically combine evolved ribosomal RNA subunits in a single o-Ribo, thereby fully separating orthogonal from native translation (Fried et al., 2015; Orelle et al., 2015). In summary, the efficiency and fidelity of OTSs can be optimized indirectly by engineering o-Ribos as well as release and elongation factors (Figure 1).

3.2.3 *Developing mutually orthogonal translation systems*

Parallel incorporation of distinct ncAAs into a single POI^{ncAA} in living cells requires ncRS/tRNA pairs that are not only orthogonal to the host aaRS/tRNA pairs but also to each other. Accordingly, three distinct crossreactivities between the ncRS/tRNA pairs have to be excluded (Zheng et al., 2017a): (i) an ncRS may aminoacylate a non-cognate suppressor tRNA; (ii) an ncRS may charge a non-cognate ncAA; and (iii) a suppressor tRNA may decode a non-cognate (re-)assigned codon. Pairs that preclude these three crossreactivities are defined as mutually orthogonal.

In bacteria, the *M. jannaschii* tyrosyl-RS/$tRNA^{Tyr}$ pair is orthogonal to the lysyl-RS/$tRNA^{Lys}$ pair of the archaeon *P. horikoshii* (Anderson et al., 2004) as well as the PylRS/PylT pair of *M. mazei* (Wan et al., 2010) or *M. barkeri* (Neumann et al., 2010b). Additionally, the PylRS/PylT pair of *M. mazei* has been identified to be orthogonal to the PylRS/PylT pair of methanogenic archaeon ISO4-G1 as well as an engineered PylRS/PylT variant of *Methanomethylophilus alvus*, which both lack the N-terminal PylRS domain (Willis and Chin, 2018). Moreover, up to three mutually orthogonal ncRS/tRNA pairs have been generated *de novo* by directed evolution of a single aaRS/tRNA pair like the *M. jannaschii* tyrosyl-RS/$tRNA^{Tyr}$ (Neumann et al., 2010a) or an archaeal prolyl-RS/$tRNA^{Pro}$ pair (Chatterjee et al., 2012). Additionally, the combination of parallel positive selection with deep sequencing allows to systematically identify ncRS/tRNA pairs with mutually orthogonal ncAA specificity (Zhang et al., 2017a). To date, application of mutually orthogonal ncRS/tRNA pairs in bacteria has been mainly restricted to the site-specific incorporation of two distinct ncAAs by decoding two nonsense codons (Chatterjee et al., 2013b; Venkat et al., 2018; Wan et al., 2010) or a nonsense and quadruplet codon (Anderson et al., 2004; Chatterjee et al., 2012; Neumann et al., 2010a,b; Wang et al., 2014a; Willis and Chin, 2018). Notably, suppression of all three termination codons by three OTSs in a modified *E. coli* strain recently enabled the targeted incorporation of three different ncAAs into a single POI^{ncAA} although with very low efficiency (Italia et al., 2019). Remarkably, using a computational scoring system to benchmark millions of foreign tRNAs for orthogonality, eight aaRS/tRNA pairs from diverse origins have been recently identified to be mutually orthogonal in *E. coli*, awaiting further application in genetic code expansion (Cervettini et al., 2020). In mammalian cells, the PylRS/PylT

pair from *Methanosarcina* species and the tyrosyl- or leucyl-RS from *E. coli* are mutually orthogonal (Xiao et al., 2013; Zheng et al., 2017a). Using these pairs, two distinct ncAAs have been first site-specifically incorporated in mammalian cells by suppressing the amber and ochre stop codon (Xiao et al., 2013). Furthermore, systematic evaluation of ncAA incorporation efficiency identified the combination of *E. coli* tyrosyl-RS with *M. barkeri* PylRS and of UAG with UGA as most favorable to mutually suppress nonsense codons in mammalian cells (Zheng et al., 2017a). Recently, also the N-terminally truncated and engineered PylRS/PylT variant from *M. alvus* has been demonstrated to be orthogonal to the *M. mazei* PylRS/PylT pair in mammalian cells, enabling site-specific incorporation of two distinct ncAA into a single POI^{ncAA} (Beránek et al., 2019; Meineke et al., 2018). Collectively, these developments and characterizations of mutually orthogonal ncRS/tRNA pairs further advance the applicability and utility of genetic code expansion to generate unique POI^{ncAA} in vivo.

3.3 APPLYING NON-CANONICAL AMINO ACIDS

3.3.1 *Modular functionalization via bioorthogonal labeling*

The goal of expanding the genetic code in vitro or in vivo is to robustly synthesize peptides with user-defined natural or unnatural properties. At this, ncAAs site-specifically install chemical moieties into a POI to rationally probe or engineer protein function and structure (Figure 2). Three main advantages render ncAAs superior over other genetically encoded tags: their comparably small size, their flexibility in tagging or modifying any protein site, and the diversity of functional groups that can be installed. However, to successfully add ncAAs to the genetic code of living cells, two key characteristics have to be fulfilled (Liu and Schultz, 1999): first, the ncAA must be able to enter the cell either by diffusion over the cell membrane or by active transport; second, the ncAA must not be a substrate of host aaRSs. Moreover, since the ncAA has to pass through the protein translation machinery, the size and chemical properties of ncAAs that can be accommodated without uncontrollable side reactions are inherently limited, which substantially constrains their design. Therefore, instead of directly incorporating the desired chemical moiety, modular approaches have been developed, where first a chemical handle is installed in the POI^{ncAA} that in a second step can be stably functionalized via covalent linkage with an external chemical probe (Figure 2). For in vivo application, these reactants and reactions have to be non-toxic, stable, and highly selective and preferentially react fast under physiological conditions, which is generally referred to as 'bioorthogonal' labeling (Lang and Chin, 2014). In genetic code expansion, three bioorthogonal labeling reactions are widely applied in living cells: Staudinger ligations based

on azide-phosphine reactions (Saxon and Bertozzi, 2000), cycloadditions based on azide-alkyne reactions (Huisgen, 1963), and Diels-Alder cycloadditions based on strained alkene/alkyne-tetrazine reactions (Blackman et al., 2008).

The Staudinger ligation of an azide and phosphine via an amide bond is bioorthogonal in living cells (Saxon and Bertozzi, 2000). To date, this conjugation approach has been applied in vitro and in vivo to covalently label biomolecules (Hang et al., 2007, 2003; Hangauer and Bertozzi, 2008; Luchansky et al., 2004; Ovaa et al., 2003; Tsao et al., 2005; Vocadlo et al., 2003; Yanagisawa et al., 2008b). However, compared to other chemoselective reactions the Staudinger ligation proceeds slowly with a low rate constant (Lin et al., 2005), which requires high concentrations of the respective reagent for labeling, increases the background signal, and hence limits application, particularly in vivo. Azides can also react with alkynes via an irreversible Huisgen cycloaddition, forming a covalent bond (Huisgen, 1963). This reaction is thermodynamically unfavorable at room temperature, but can be catalyzed by the addition of Copper(I) in vitro, which is referred to as Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (Rostovtsev et al., 2002; Tornøe et al., 2002; Wang et al., 2003). Since the addition of Copper(I) to living cells is toxic (Agard et al., 2006), strain-promoted azide-alkyne cycloaddition (SPAAC) is applied in vivo instead of CuAAC (Agard et al., 2004). In SPAAC an alkyne with a ring strain reacts with the azido group (Agard et al., 2004), which compared to the Staudinger ligation proceeds slightly faster (Agard et al., 2006; Gordon et al., 2012). Moreover, the reaction kinetics of strained alkyne-based probes in SPAAC can be increased multiple folds in vivo by introducing electron-withdrawing groups (Baskin et al., 2007; Codelli et al., 2008) as well as enhancing the ring strain (Gordon et al., 2012; Ning et al., 2008). However, for site-specific protein labeling via genetic code expansion, proteomic incorporation of alkynes and subsequent functionalization with azide-based probes is preferable and not vice versa, as azides once incorporated into proteins can be reduced in vivo, impeding subsequent functionalization with alkyne-based probes (Lang and Chin, 2014). At this, ncAAs with alkynyl groups have been site-specifically incorporated by amber suppression in bacteria and eukaryotes and subsequently labeled by CuAAC or SPAAC (Borrmann et al., 2012; Deiters et al., 2003; Deiters and Schultz, 2005; Fekner et al., 2009; Kaya et al., 2009; Nguyen et al., 2009b; Plass et al., 2011).

Lastly, the inverse-electron-demand Diels-Alder cycloaddition (iEDDAC) between an electron-rich dienophile and an electron-poor diene, in particular between a strained alkene/alkyne and a tetrazine (Balcar et al., 1983; Thalhammer et al., 1988), has been shown to proceed with extraordinarily fast kinetics under physiological conditions (Blackman et al., 2008). Hence, this strain-promoted iEDDAC reaction has found wide usage in bioorthogonal labeling strategies applying tetrazine-

based probes (Mayer and Lang, 2016). To this end, strained alkene functionalities have been encoded in bacteria and mammalian cells by expanding their genetic code with ncAAs bearing for instance *trans*-cyclooctenes (Lang et al., 2012b; Plass et al., 2012) or norbornenes (Kaya et al., 2012; Lang et al., 2012a; Plass et al., 2012). These ncAA-modified proteins can then be rapidly labeled with tetrazine-based probes in vitro and in vivo, with exceptionally high rate constants for *trans*-cyclooctene derivatives reaching the kinetics of enzymatic labeling approaches (Blackman et al., 2008; Lang and Chin, 2014; Lang et al., 2012b; Plass et al., 2012) and a more than 104 times slower reaction kinetic for norbornenes (Lang and Chin, 2014; Lang et al., 2012a). Furthermore, an ncAA with a strained alkyne, the cyclooctyne bearing ncAA bicyclo[6.1.0]nonyne-L-lysine (BcnK), has been genetically encoded via amber suppression and subsequently functionalized with tetrazine-based probes in bacteria and mammalian cells (Borrmann et al., 2012; Lang et al., 2012b). In strain-promoted iEDDAC labeling reactions with tetrazine conjugates, BcnK is approximately an order of magnitude less reactive than *trans*-cyclooctene derivatives (Lang and Chin, 2014; Lang et al., 2012b). However, contrary to *trans*-cyclooctene bearing ncAAs, excess intracellular BcnK can be better removed to reduce background labeling with tetrazine-based probes, rendering BcnK more suitable for intracellular bioorthogonal labeling approaches (Uttamapinant et al., 2015). Of note, CuAAC and SPAAC are mutually compatible bioorthogonal conjugation chemistries to iEDDAC, enabling site-specific dual-labeling of a single POI^{ncAA} harboring the respective two bioconjugation handles (Sachdeva et al., 2014; Zheng et al., 2017a). Additionally, mutually orthogonal strain-promoted iEDDAC reactions have been developed (Nikić et al., 2014; Wang et al., 2014a). Furthermore, photo-inducible versions of iEDDAC (Kumar et al., 2018, 2019; Mayer et al., 2019b; Zhang et al., 2016a) as well as SPAAC (Arumugam et al., 2013) have been reported, allowing to spatiotemporally control bioorthogonal labeling in living cells. Overall, in comparison to the Staudinger ligation and SPAAC, strain-promoted iEDDAC requires less labeling reagent in the micromolar range and results in less off-target labeling (Jakob et al., 2019). In conclusion, the iEDDAC reaction between a strained alkene or alkyne, in particular *trans*-cyclooctene derivatives or the cyclooctyne bearing ncAA BcnK, and a tetrazine conjugate is an ideal bioorthogonal labeling approach that proceeds exceptionally fast. This two-step bioconjugation approach, in which the POI^{ncAA} can be functionalized after biosynthesis with a compatible probe in vivo within seconds, circumvents the steric and bioreactive limitations imposed by the protein translation machinery on the non-canonical functionalities that can be genetically encoded.

3.3.2 *Probing protein structure and function*

To date, more than 200 ncAAs with diverse structures and functions have been encoded by genetic code expansion (Dumas et al., 2015; Vargas-Rodriguez et al., 2018), of which more than 100 can be currently incorporated in mammalian cells using OTSs (Nödling et al., 2019). The potential applications of these ncAAs can be broadly subdivided into two groups: to probe protein structure and function or to engineer protein structure and function. The first group includes functionalization either directly or indirectly via bioorthogonal labeling to incorporate fluorophores, spectroscopic probes, affinity tags, or photo-reactive or proximity-triggered crosslinkers (Figure 2).

INCORPORATION OF FLUOROPHORES OR SPECTROSCOPIC PROBES
Compared to fluorescent proteins, organic fluorescent dyes are smaller, brighter, and more photostable, which are critical photophysical properties especially in single-molecule and super-resolution microscopy (Dempsey et al., 2011; Fernández-Suárez and Ting, 2008). Via bioorthogonal labeling, a POI^{ncAA} can be easily and within minutes equipped with these superior fluorophores under physiological conditions (Lang and Chin, 2014; Nikić et al., 2015), allowing to for instance perform super-resolution microscopy of intracellular proteins in mammalian cells (Uttamapinant et al., 2015). Since the ncAA incorporation site can be freely placed within the POI^{ncAA}, this minimally invasive labeling approach allows to track proteins that can neither be tagged on their N- or C-terminus with a fluorescent protein (König et al., 2020; Sakin et al., 2017). Additionally, coupling selected red or green fluorophores to tetrazines for subsequent bioorthogonal labeling quenches their fluorescence (Devaraj et al., 2010; Lang and Chin, 2014). Upon iEDDAC with the respective strained alkene or alkyne bearing ncAA, the fluorescence of these tetrazine-dye conjugates is then turned on, generating fluorogenic probes with a reduced background signal in microscopy (Devaraj et al., 2010; Lang and Chin, 2014). Furthermore, dual-color labeling of a single POI^{ncAA} with two fluorescent dyes enables single-molecule Förster resonance energy transfer measurements of protein targets previously inaccessible to this method (Gust et al., 2018; Milles et al., 2012). Besides fluorescent labeling of a POI^{ncAA} indirectly via conjugation of a dye probe, fluorescent ncAAs can be directly incorporated in bacteria and eukaryotes via genetic code expansion (Chatterjee et al., 2013a; Chen et al., 2019; Summerer et al., 2006; Wang et al., 2006). Thereby, even protein regions that are inaccessible for subsequent site-specific labeling at the ncAA incorporation site can be fluorescently labeled (Kalstrup and Blunck, 2013). These fluorescent ncAAs can not only be used to track target proteins, but also to monitor conformational changes (Kalstrup and Blunck, 2013; Shen et al., 2011) or protein-protein interactions (Park et al., 2019) in

living cells. Of note, in addition to their application in fluorescence microscopy, ncAAs have been functionalized for spectral measurements. These spectroscopic probes include iodinate ncAAs for protein crystallography (Sakamoto et al., 2009; Xie et al., 2004), the direct or indirect attachment of a spin-label for electron paramagnetic resonance (Fleissner et al., 2009; Kálai et al., 2011; Schmidt et al., 2014a), or the use of ncAAs as infrared labels (Schultz et al., 2006; Thielges et al., 2011; Völler et al., 2015) to elucidate POI^{ncAA} structure and dynamics. Taken together, the characteristics and diverse applications of fluorescent dyes directly or indirectly incorporated via genetic code expansion render this labeling approach superior over tagging with fluorescent proteins.

INCORPORATION OF AFFINITY TAGS Incorporation of ncAAs with bioorthogonal handles enables to selectively label ncAA-modified proteins with affinity tags for subsequent extraction and identification by mass spectrometry. To this end, bioorthogonal ncAA tagging (BONCAT) employs a non-canonical methionine derivative equipped with an azido group for labeling with an alkyne-biotin probe (Dieterich et al., 2007, 2006) or direct capture on a cyclooctyne resin by SPAAC (Nessen et al., 2009). In contrast to site-specific ncAA incorporation via amber suppression, the non-canonical methionine analog is a substrate for the endogenous translation machinery, resulting in its proteome-wide, stochastic incorporation in direct competition with methionine (Dieterich et al., 2007, 2006). Hence, after the addition of the azide-bearing methionine analog, the whole proteome is pulse labeled via BONCAT, permitting enrichment via the azide-handle and following identification of newly synthesized proteins by mass spectrometry (Bagert et al., 2016; Dieterich et al., 2006; Eichelbaum et al., 2012; Howden et al., 2013; Nessen et al., 2009). Additionally, this metabolic labeling approach can be paired with fluorescent instead of affinity tags, denoted fluorescent ncAA tagging (FUNCAT) to visualize newly synthesized proteins (Beatty et al., 2006; Beatty and Tirrell, 2008; Dieterich et al., 2010). Expanding on this residue-specific labeling approach, also codon-specific proteome labeling via orthogonal translation has been employed in bacteria, mammalian cells and whole animals (Elliott et al., 2014; Grammel et al., 2010; Ngo et al., 2012, 2009, 2013). For instance, stochastic orthogonal recoding of translation (SORT) employs the PylRS/PylT OTS to encode an ncAA at a selected set of sense codons to statistically label the whole proteome (Elliott et al., 2016, 2014). Following, the ncAA can be functionalized via CuAAC or iEDDAC to either visualize newly synthesized proteins by SORT with chemoselective modification (SORT-M) using fluorescent probes (Elliott et al., 2014), or to capture newly synthesized proteins by SORT with enrichment (SORT-E) using biotin probes (Elliott et al., 2016; Krogager et al., 2018). Moreover, PylRS/PylT or in general OTS expression

can be restricted to a specific cell type or tissue to exclusively label its proteome with the ncAA (Elliott et al., 2014; Grammel et al., 2010; Ngo et al., 2012, 2009). Thereby, the proteome of only OTS expressing cells and cell types can be exclusively labeled and selectively isolated even out of complex organs such as the mouse brain (Krogager et al., 2018), which is a major advantage over the OTS-independent approaches BONCAT and FUNCAT. Notably, also direct incorporation of biotinylated ncAAs using an evolved PylRS/PylT pair in bacteria and mammalian cells and streptavidin-mediated pulldown of these ncAA-modified proteins has been recently reported (Hohl et al., 2019b). In summary, ncAA pulse labeling and subsequent enrichment of newly synthesized proteins allows to monitor proteomic changes as well as in addition with orthogonal translation to selectively capture the proteome of defined cellular populations.

INCORPORATION OF CROSSLINKERS Expansion of the genetic code with ncAAs harboring crosslinking moieties allows to covalently capture dynamic and transient protein interactors with high spatiotemporal resolution as well as their interaction interface within their native context (Coin, 2018; Nguyen et al., 2018). To this end, ncAAs with photo-reactive side chains for protein-protein crosslinking, which are typically activated upon short-wavelength light exposure, have been developed, such as benzophenones (Chin et al., 2002a; Hino et al., 2005), diazirines (Hancock et al., 2010; Tippmann et al., 2007; Yanagisawa et al., 2012; Zhang et al., 2011), and aryl azides (Chin et al., 2002b; Lin et al., 2011). Using genetic code expansion, these ncAAs can be site-specifically incorporated in bacteria (Ai et al., 2011; Chin et al., 2002a,b; Chin and Schultz, 2002; Chou et al., 2011; Lacey et al., 2013; Tippmann et al., 2007; Yanagisawa et al., 2012), yeast (Chin et al., 2003a; Hancock et al., 2010), and mammalian cells (Ai et al., 2011; Chou et al., 2011; Hino et al., 2005; Lacey et al., 2013; Liu et al., 2007; Yanagisawa et al., 2012) to crosslink up to a radius of 15 Å (Yanagisawa et al., 2012). Over the past two decades, this approach has found wide application to capture and map protein interactions in vitro (Braig et al., 2009; Chen et al., 2007a; Dziuba et al., 2020; Liu et al., 2010; Simms et al., 2018; Weibezahn et al., 2004) and with high spatiotemporal resolution in vivo (Guan et al., 2018; Miyazaki et al., 2020; Mori and Ito, 2006; Okuda and Tokuda, 2009; Wang et al., 2016c; Wilkins et al., 2014; Zhang et al., 2016b), including receptor-ligand binding (Coin et al., 2013; Grunbeck et al., 2012, 2011; Hino et al., 2005, 2011; Kusano et al., 2012; Rannversson et al., 2016; Seidel et al., 2017; Wang et al., 2014b) and chromatin interactors (Kleiner et al., 2018; Xie et al., 2017; Yang et al., 2016a) in living mammalian cells. In combination with mass spectrometry, the ncAA-mediated crosslinking strategy represents a powerful means to profile interactomes in a context-dependent manner in living cells (Kleiner et al., 2018; Yang

et al., 2017). Moreover, multifunctional ncAAs harboring a crosslinking moiety as well as a bioconjugation handle, which after crosslinking can be functionalized with an affinity tag for pulldown, facilitate the specific enrichment and subsequent characterization of crosslinked complexes (He et al., 2017; Hoffmann et al., 2018; Joiner et al., 2017). Furthermore, ncAAs with cleavable and modifiable linkers allow to separate the bait and crosslinked prey proteins, which after cleavage retain a mass spectrometry identifiable label and affinity handle to better eliminate nonspecific interactors (He et al., 2017; Lin et al., 2014; Yang et al., 2016b). In addition to photo-inducible crosslinkers, also proximity-triggered chemical crosslinking has been developed. At this, an under physiological conditions chemically inert ncAA can only crosslink with an adjacent residue upon incorporation into a target protein (Nguyen et al., 2018). For instance, ncAAs bearing a reactive halide feature this proximity-enhanced crosslinking activity particularly for cysteine residues and have been successfully applied as crosslinkers to study protein interactions in vitro and in vivo (Chen et al., 2014; Cigler et al., 2017; Coin et al., 2013; Kobayashi et al., 2016; Xiang et al., 2014, 2013). Thereby, transient protein interactions can be stabilized, also aiding their co-crystallization and following structural elucidation (Cigler et al., 2017). In summary, incorporation of ncAAs harboring fluorescent dyes, spectroscopic probes, affinity tags, or photo-reactive or proximity-triggered crosslinkers is an elegant approach to probe protein structure and function.

3.3.3 *Engineering protein structure and function*

Incorporation of ncAAs into a target protein can also be applied to engineer protein structure and function, including their stability, catalytic activity, post-translational modifications (PTMs), or even biocontainment of whole recombinant organisms (Figure 2). As for probing protein structure and function, these applications can be conferred either directly by incorporating the ncAA harboring the respective functional chemical moiety or indirectly via bioorthogonal labeling. For instance, the latter has been exploited to create defined antibody-drug conjugates with improved stability, efficacy, and hence safety as site-specific incorporation of ncAAs with unique conjugation handles allow precise control over the conjugation site and drug load (Hallam et al., 2015). Applying genetic code expansion, ncAA-functionalized antibodies have been produced in cell-free expression systems, bacteria, and transiently transfected or stably expressing mammalian cell lines (Axup et al., 2012; Roy et al., 2020; Tian et al., 2014; VanBrunt et al., 2015; Zimmerman et al., 2014). Besides their usefulness in conjugation, ncAAs find wide usage in other protein engineering strategies as outlined in the following.

ENGINEERING OF PROTEIN STABILITY AND CATALYTIC ACTIVITY

The structural stability of a POI^{ncAA} especially in the presence of thermal or chemical denaturation can be reinforced by introducing additional non-covalent or covalent intramolecular interactions. For the latter, incorporation of ncAAs with proximity-triggered crosslinking activity has proven particularly useful. To this end, ncAAs with reactive halides or other electrophilic moieties have been encoded in bacteria and mammalian cells to crosslink with proximal residues or between homodimers and to staple secondary structures (Chen et al., 2014; Kobayashi et al., 2016; Li et al., 2018, 2019; Liu et al., 2016; Xiang et al., 2014, 2013; Xuan et al., 2016, 2017a). Thereby, the thermal stability of the modified POI^{ncAA} has been dramatically increased (Liu et al., 2016; Xiang et al., 2014, 2013; Xuan et al., 2016), for instance by more than 40% for the homoserine o-succinyltransferase harboring only a single ncAA incorporation site (Li et al., 2018, 2019). Additionally, the stability of proteins can be enhanced without the introduction of intramolecular crosslinks, for instance by the incorporation of superhydrophobic halogenated cAA analogs into the hydrophobic core of a coiled-coil protein (Bilgiçer et al., 2001; Tang and Tirrell, 2001; Tang et al., 2001). At this, genetic code expansion has been employed to substitute multiple cAAs with halogenated ncAAs, increasing protein half-life up to 5-fold, which is most likely mediated by stabilizing interactions of the bulky halogen moieties within the protein core (Ohtake et al., 2018, 2015). Besides structural stability, site-specific incorporation of ncAAs with diverse chemical moieties can also greatly enhance the catalytic activity of a target protein, which would be impossible to achieve by cAA mutagenesis only (Green et al., 2016; Jackson et al., 2006; Kolev et al., 2014; Ugwumba et al., 2011; Xiang et al., 2013; Xiao et al., 2015). Moreover, designer enzymes with novel catalytic activity have been generated, where the ncAA residue confers catalytic activity (Drienovská et al., 2018; Mayer et al., 2019a). Furthermore, the development of metal-chelating ncAAs and their site-specific incorporation via amber suppression (Lee et al., 2009; Liu et al., 2012, 2013; Xie et al., 2007) now allows to manipulate coordination of metal ions within enzymatic sites and thereby engineering of artificial metalloenzymes (Bersellini and Roelfes, 2017; Drienovská et al., 2015). For instance, the metal-chelating ability of an ncAA has been exploited to equip a DNA binding protein with sequence-specific endonuclease activity (Lee and Schultz, 2008). Additionally, fluorescent proteins have been engineered with metal-chelating and other ncAAs to function as fluorescent biosensors for the detection of small molecules and ions in living cells, such as Copper(II), hydrogen peroxide, or peroxynitrite (Ayyadurai et al., 2011; Chen et al., 2012; Chen et al., 2013; Liu et al., 2014; Wang et al., 2012). In conclusion, expansion of the genetic code bears the potential to evolve proteins with improved stability as well as enhanced or even new catalytic activity and functions.

ENGINEERING OF POST-TRANSLATIONAL MODIFICATIONS Targeted manipulation of PTMs as key regulators of biological processes would allow to better understand and also leverage their protein regulatory and cellular functions. At this, genetic code expansion offers an attractive route to site-specifically install PTMs into a POI^{ncAA} in vitro and in vivo, including phosphorylation of serine (Lee et al., 2013; Park et al., 2011; Rogerson et al., 2015), tyrosine (Fan et al., 2016; Luo et al., 2017), and threonine (Zhang et al., 2017a), as well as lysine acetylation (Mukai et al., 2008; Neumann et al., 2008; Umehara et al., 2012) and methylation (Nguyen et al., 2009a). Since ncAAs encoding these PTMs can be directly incorporated into the target site, also residues otherwise inaccessible by for instance chemical ligation can be modified, such as the structured core of histones (Neumann et al., 2009). Moreover, leveraging mutually orthogonal ncRS/tRNA pairs, the simultaneous impact of distinct PTMs on protein activity can be investigated (Venkat et al., 2018). Additionally, posttranslationally modified ncAAs can also be applied to probe their turnover like deacetylation by sirtuins (Xuan et al., 2017b). Furthermore, proteins can be site-specifically and stably ubiquitylated in vitro by applying amber suppression and subsequent native chemical ligation (Virdee et al., 2011) as well as in living cells by combining genetic code expansion and sortase-mediated transpeptidation (Fottner et al., 2019). Therefore, even proteins with sterically demanding PTMs can now be synthesized in vivo in a user-defined, controllable manner.

CONTROLLING ENZYMATIC ACTIVITY The genetic code can be also expanded with photocaged ncAAs to spatiotemporally control protein function. To date, in pro- and eukaryotes including mammalian cells a big variety of photocaged cAA analogs have been encoded via amber suppression such as lysine (Chen et al., 2009; Gautier et al., 2010; Luo et al., 2014), tyrosine (Arbely et al., 2012; Deiters et al., 2006), serine (Lemke et al., 2007), or cysteine (Kang et al., 2013; Nguyen et al., 2014; Uprety et al., 2014; Wu et al., 2004). Site-specific incorporation of these ncAAs has been applied to spatiotemporally control enzymatic activity (Deiters et al., 2006; Endo et al., 2004; Luo et al., 2014; Nguyen et al., 2014; Palei et al., 2020; Uprety et al., 2014; Walker et al., 2016; Wang et al., 2019; Wolffgramm et al., 2021; Wu et al., 2004), signaling activity (Arbely et al., 2012; Gautier et al., 2011; Kang et al., 2013; Liu et al., 2017; Tsai et al., 2015; Wang et al., 2019), cellular trafficking (Gautier et al., 2010; Lemke et al., 2007), as well as genomic (Brown et al., 2018; Hemphill et al., 2015) and synthetic protein engineering (Böcker et al., 2015; Ren et al., 2015). For instance, photoactivation of epigenetic regulators has been exploited to investigate DNA methylation dynamics with high temporal resolution (Palei et al., 2020; Walker et al., 2016; Wolffgramm et al., 2021). Recently, computational modeling has been successfully implemented to streamline the identification of proximal

active-site residues for photocaging with an ncAA (Wang et al., 2019). This strategy might greatly facilitate the design and application of photoactivatable proteins.

BIOCONTAINMENT OF RECOMBINANT ORGANISMS Lastly, expansion of the genetic code with an ncAA can not only be applied to engineer a single protein, but also to control the growth and propagation of whole recombinant organisms. In an early proof-of-principle study, propagation of a poliovirus amber mutant could be linked to the inducible expression of an amber suppressor tRNA in mammalian cells (Sedivy et al., 1987). Expanding on this biocontainment strategy, Zhou and coworkers applied transgenic cell lines stably expressing the PylRS/PylT pair to produce fully infectious viruses harboring multiple in-frame amber stop codons (Si et al., 2016). Importantly, in cells lacking amber suppression, these viruses are then avirulent, rendering them highly effective vaccines (Si et al., 2016). Furthermore, genomically recoded organisms with an expanded genetic code have been established, in which expression of multiple essential genes depends on the availability of an ncAA (Mandell et al., 2015; Rovner et al., 2015). Since ncAAs as non-natural compounds are environmentally unavailable, this 'genetic firewall' functions as a biocontainment strategy, preventing the proliferation of genetically modified organisms in natural ecosystems with very low to undetectable escape frequencies (Mandell et al., 2015; Rovner et al., 2015). Furthermore, effective biocontainment of bacteria has been demonstrated by substituting cAAs with ncAAs in only two (Xuan and Schultz, 2017) or even a single essential protein (Gan et al., 2018; Koh et al., 2017, 2019). Taken together, expanding the genetic code with ncAAs permits engineering the stability, function, catalytic activity, and PTM status of single proteins up to controlling the propagation of whole organisms (Figure 2).

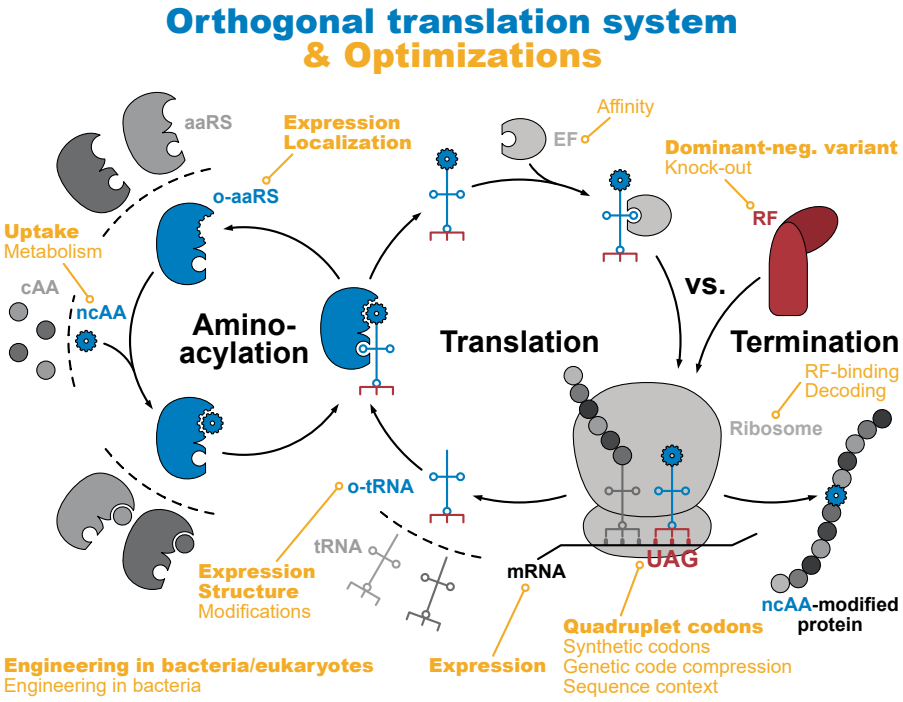

Figure 1: Key components of an orthogonal translation system (blue) next to host translation components (grey) and their optimizations (yellow) to expand the genetic code of living cells. During aminoacylation, a non-canonical amino acid (ncAA) is specifically recognized by an orthogonal aminoacyl-tRNA synthetase (o-aaRS) to be attached to an orthogonal tRNA (o-tRNA). These two key components, orthogonal aaRS/tRNA pair and ncAA, must not crossreact with endogenous aaRS/tRNA pairs and canonical amino acids (cAA). To synthesize a protein that is site-specifically modified with an ncAA, the aminoacylated o-tRNA must be bound by the elongation factor (EF) and then decoded by the ribosome in response to its assigned codon located within the respective transcript. At this, the amber stop codon (UAG) is commonly suppressed, which is in direct competition with translational termination by the release factor (RF). Orthogonal translation as a means to expand the genetic code has been optimized by engineering aminoacylation, translation, and in the case of amber suppression termination in pro- and/or eukaryotes.

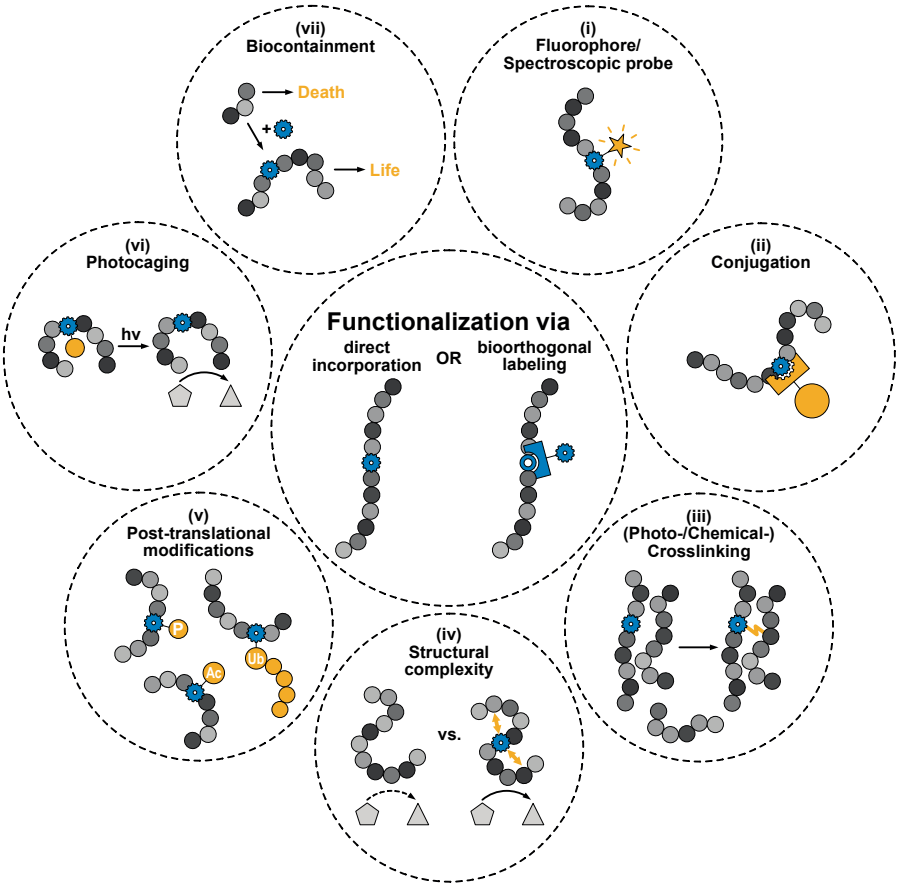

Figure 2: Applications of non-canonical amino acids (blue) to probe and engineer protein structure and function. In all examples, proteins can be site-specifically functionalized either directly by incorporating the respective functional group during translation or indirectly via bioorthogonal labeling with the respective probe. Seven potential functionalizations are indicated (clockwise): (i) fluorophores and spectroscopic probes to probe protein structure and function; (ii) bioorthogonal handles for protein conjugation in general to for instance selectively label proteins with affinity tags or produce defined antibody-drug conjugates; (iii) photo- or chemical-crosslinking to probe interactors or reinforce structural stability; (iv) engineering the structural complexity of proteins by introducing new chemical moieties to improve protein stability and/or catalytic activity or even confer new catalytic activity; (v) post-translational modifications like acetylation (Ac), phosphorylation (P), or ubiquitylation (Ub) to engineer proteins; (vi) photocaging to spatiotemporally control protein activity including enzymatic function; and lastly (vii) intrinsic biocontainment of recombinant organisms addicted to the bioavailability of a non-canonical amino acid.

4 DISCUSSION

4.1 IDENTIFICATION OF PERMISSIVE AMBER SUPPRESSION SITES

Incorporation of ncAAs in bacteria has been substantially improved by engineering OTS components as well as host organisms as outlined before. In comparison, however, optimization of genetic code expansion in eukaryotes lags behind (Figure 1). Therefore, in particular in mammalian cells application of ncAAs to probe and engineer protein structure and function is considerably more difficult. The main objective of this thesis was to develop new strategies that can be readily and easily applied in mammalian cells to optimize ncAA incorporation efficiency and applicability.

We focused our efforts on amber suppression by the PylRS/PylT OTS to incorporate ncAAs as this strategy has been progressively optimized over the last decade and is to date one of the most commonly used genetic code expansion tools in mammalian cells. In a first step, we engineered two mammalian cell lines to stably integrate PylRS/PylT and expand their genetic code (Bartoschek et al., 2021). Theoretically, in these stable cell lines ncAAs can then be freely placed within a POI by including an in-frame UAG within the respective transcript. However, in the following we detected large variations in amber suppression efficiency between different GOI* sites (Bartoschek et al., 2021), a phenomenon which has also been observed by others in bacteria and mammalian cells (Chin, 2017; Liu et al., 2009; Liu and Schultz, 2010; Pott et al., 2014; Sakin et al., 2017; Schvartz et al., 2017; Schwark et al., 2018; Xu et al., 2016; Young et al., 2010). This unpredictable variability in ncAA incorporation efficiency depending on the UAG position within a GOI* considerably complicates the application of ncAAs. Furthermore, the flexibility to incorporate an ncAA at any user-defined site within a POI is substantially compromised. We mitigate these limitations by developing two complementary tools: on one hand we establish a regression model, which we termed iPASS (Identification of Permissive Amber Sites for Suppression), to predict context-specific amber suppression and concomitant ncAA incorporation efficiency in silico; on the other hand we establish a fluorescent reporter system to rapidly and reproducibly quantify and validate these predicted ncAA incorporation efficiencies in vivo (Bartoschek et al., 2021). Thereby, we simplify the identification of UAG sites that are permissive for suppression by PylT and generally advance the applicability of ncAAs in mammalian cells (Bartoschek et al., 2021).

Importantly, these results also contribute to our mechanistic understanding of the interdependence between codon context and amber suppression efficiency in particular as well as translational efficiency in general. In the following, this interdependence will be discussed in detail. Additionally, experimental setups will be suggested to further decipher context effects and optimize ncAA incorporation in mammalian cells. Prior, two remarks: First, decoding of the stop codon by a near-cognate tRNA will be referred to as 'readthrough' of the stop codon. During readthrough the tRNA anticodon pairs with the stop codon by non-cognate base pairing. Decoding of the stop codon by a suppressor tRNA will be referred to as 'suppression' of the stop codon. In contrast to near-cognate tRNAs, the suppressor tRNA decodes the codon via cognate codon-anticodon base pairing. However, both suppression and readthrough of stop codons are in direct competition with termination. Second, amber suppression efficiency is governed by factors that are dependent or independent of the nucleotide context. For instance, tRNA identity is an essential factor in amber suppression efficiency as introduced before. However, engineering the suppressor tRNA usually improves amber suppression at all sites and is therefore context-independent. This stimulatory effect has to be delineated from context-dependent effects and molecular mechanisms, which will be discussed hereafter. To this end, we focus on the close-by nucleotide context (-6 to +9, with UAG at +1, +2, +3) and its contribution to the observed several-fold differences in ncAA incorporation efficiencies (Bartoschek et al., 2021).

4.2 MECHANISTIC PERSPECTIVES

4.2.1 *Contribution of context-dependent termination efficiency*

AMBER SUPPRESSION EFFICIENCY IN BACTERIA DOES NOT EXCLUSIVELY DEPEND ON TERMINATION EFFICIENCY The identity of the +4 nucleotide following the amber stop codon was recognized first to influence the efficiency of translational termination in bacteria (Bossi and Roth, 1980). Following, formation of a tetranucleotide rather than trinucleotide termination signal has been proposed (Brown et al., 1990b). Since then this context effect of the +4 nucleotide has been extensively studied and described as a major factor in the efficiency of translational termination, and in this respect stop codon readthrough and suppression. Termination codons in *E. coli* are strongly biased for +4 U (Brown et al., 1990b; Cridge et al., 2006; Poole et al., 1995; Tate et al., 1996). Corresponding to their frequency, the hierarchy of release factor selection rates at UAGN tetranucleotides in bacteria has been reported as +4 U>G>C>A (Pedersen and Curran, 1991) or +4 G>U=A>C (Poole et al., 1995). Furthermore, the efficiency of nonsense suppression by natural suppressor tRNAs (stop codon suppressing

derivatives of natural tRNAs) has been demonstrated to depend on the surrounding sequence context (Feinstein and Altman, 1977; Garen, 1968; Yahata et al., 1970). In particular, UAG contexts with +4 U and +4 C were found to be poorly suppressed, whereas +4 A and +4 G enhance suppression efficiency (Bossi, 1983; Bossi and Roth, 1980; Miller and Albertini, 1983; Pedersen and Curran, 1991; Phillips-Jones et al., 1993; Stormo et al., 1986). Additionally, in bacteria with an expanded genetic code especially +4 A following the amber stop codon has been found to boost ncAA incorporation (Pott et al., 2014; Schinn et al., 2017; Schwark et al., 2018; Xu et al., 2016). This preference for +4 A is in sharp contrast to its depletion within the iPASS motif (Bartoschek et al., 2021), confirming initial observations that UAG contexts differ in their suppression efficiency between bacteria and mammalian cells (Phillips-Jones et al., 1995, 1993). As such, iPASS is a eukaryotic-specific model that cannot be applied to predict ncAA incorporation efficiency in bacteria. Of note, also lack of reliable factors in predicting amber suppression efficiency in *E. coli* was previously reported (Hostetler et al., 2018). Overall, termination at UAGN tetranucleotides in bacteria seems to be less stringent with +4 A, which in turn enhances their suppression. This consistent effect of +4 A indicates that context-dependent variations in release factor selection directly modulate ncAA incorporation efficiencies. However, the aforementioned stimulatory effect of +4 G on amber suppression despite being an efficient termination signal suggests contribution of additional factors. Moreover, KO of RF1 in bacteria generally improves multi-site but not single-site ncAA incorporation (Schwark et al., 2018; Zheng et al., 2016). Hence, the efficiency of release factor selection, which strongly depends on the identity of the +4 nucleotide, is not the only factor governing context-dependent amber suppression efficiency in bacteria. However, whether similar molecular features of permissive amber suppression sites can also be found in eukaryotes and in particular in mammalian cells was largely unclear.

INCORPORATION OF NON-CANONICAL AMINO ACIDS IN MAMMALIAN CELLS PROFITS FROM WEAK TERMINATION CONTEXTS

In contrast to bacteria, the +4 nucleotide following the stop codon in eukaryotes is biased for purines, especially +4 G (Brown et al., 1990a; Cavener and Ray, 1991; Cridge et al., 2018, 2006; McCaughan et al., 1995; Shabalina et al., 2004). Despite genome-wide frequencies of termination signals in eukaryotes are overall uncorrelated with their termination efficiency (Cridge et al., 2006; Martin, 1994; Sun et al., 2005; Williams et al., 2004), this preference is especially evident at highly expressed genes (Brown et al., 1990a; Cridge et al., 2006; Kochetov et al., 1998; McCaughan et al., 1995; Trotta, 2013, 2016). Hence, also in eukaryotes formation of a tetranucleotide rather than triplet termination signal has been proposed, at which termination efficiency

correlates with the identity of the +4 nucleotide (Brown et al., 1990a; Cridge et al., 2006; Kochetov et al., 1998; McCaughan et al., 1995; Sun et al., 2005; Trotta, 2013, 2016). The iPASS motif indicates the UAGC tetranucleotide to be most favorable for amber suppression (Bartoschek et al., 2021). The few existing studies on eukaryotic context rules in amber suppression efficiency confirm this stimulatory effect of +4 C (Cridge et al., 2018; Phillips-Jones et al., 1995, 1993). Interestingly, +4 C has been identified as the most important sequence feature of eukaryotic readthrough motifs (Anzalone et al., 2019; Mangkalaphiban et al., 2021). Additionally, +4 C has been consistently demonstrated to promote stop codon readthrough in living mammalian cells (Cridge et al., 2018; Harrell et al., 2002; Loughran et al., 2014; McCaughan et al., 1995; Pacho et al., 2011; Schueren et al., 2014; Wangen and Green, 2020), suggesting that compromised release-factor binding and termination at least partially contribute to the stimulatory effect of +4 C in amber suppression. Notably, increased readthrough particularly in the context of +4 C has been attributed to compromised eRF1 recruitment, whereas readthrough of +4 A, G, and U stop codon contexts seems to be predominately dependent on the availability of suitable near-cognate tRNAs (Beznosková et al., 2016; Cridge et al., 2018). Furthermore, crystal structures of mammalian ribosome complexes during termination confirmed decoding of stop codons by formation of a compacted tetranucleotide, which is stabilized by stacking of +4 purines with 18S ribosomal ribonucleic acid (rRNA) (Brown et al., 2015; Matheisl et al., 2015; Shao et al., 2016). To this end, we found depletion of +4 purines and in particular +4 A via iPASS (Bartoschek et al., 2021), pointing towards destabilization of the termination complex in favor of suppression by PylT. Accordingly, +4 purines were also demonstrated to increase termination efficiency in mammalian cells (Cridge et al., 2018; Harrell et al., 2002; Loughran et al., 2014; McCaughan et al., 1995; Pacho et al., 2011; Schueren et al., 2014; Wangen and Green, 2020). Additionally, the efficiency of amber suppression by PylT was linearly correlated with readthrough by near-cognate tRNAs (Bartoschek et al., 2021). At this, the concentration and also identity of the recruited tRNA differ between suppression and readthrough, whereas the cellular concentrations of release factors and GOI* transcript should be similar. Therefore, this linear correlation further supports a context-dependent decrease in release factor recruitment that generally boosts both ncAA incorporation during amber suppression and cAA incorporation during readthrough. In general, this model of compromised release factor selection in favor of PylT recruitment is in accordance with increased ncAA incorporation efficiency in mammalian cells that co-express dominant negative eRF1 mutants (Schmied et al., 2014; Zhang et al., 2017d). However, these correlation values between suppression and readthrough were lower compared to the correlation values between ncAA incorporation

efficiency and iPASS score (Bartoschek et al., 2021), indicating that the efficiency of amber suppression is influence by additional context-specific factors, which are at least partially covered by iPASS too. Thus, not only the identity of the +4 nucleotide and its influence on release factor recruitment contribute to the context-dependent increase in ncAA incorporation efficiency in mammalian cells.

ADDITIONAL FACTORS BEYOND RELEASE FACTOR RECRUITMENT AND TERMINATION EFFICIENCY MUST BE INVOLVED In addition to the +4 nucleotide, we analyzed the sequence context up to 6 base pairs up- and downstream of the amber stop codon. We detected at each position depletion or enrichment of nucleotides (Bartoschek et al., 2021), confirming that the context effect extends beyond the +4 position and its well characterized influence on release factor selection. Accordingly, defined motifs comprising the nucleotides up to six base pairs up- and downstream of UAG have been identified that boost ncAA incorporation efficiency in bacteria with an expanded genetic code (Pott et al., 2014; Xu et al., 2016). Furthermore, in living eukaryotic cells up- as well as downstream sequences have been demonstrated to substantially contribute to the efficiency of stop codon readthrough (Beznosková et al., 2016, 2019; Bonetti et al., 1995; Cassan and Rousset, 2001; Cridge et al., 2018; Harrell et al., 2002; Jungreis et al., 2011; Loughran et al., 2014; Mangkalaphiban et al., 2021; McCaughan et al., 1995; Mottagui-Tabar et al., 1998; Namy et al., 2001; Pacho et al., 2011; Schueren et al., 2014; Skuzeski et al., 1991; Stahl et al., 1995; Tork et al., 2004; Wangen and Green, 2020; Williams et al., 2004). Moreover, stop codon readthrough was found to be synergistically influenced by sequences flanking the stop codon (Bonetti et al., 1995; Williams et al., 2004). Using the dual-fluorescence reporter, we could also demonstrate this synergistic effect of up- and downstream sequences in modulating the efficiency of ncAA incorporation. In particular, we found that in mESCs the combined context effect was greater than the sum of ncAA incorporation efficiency caused by either the optimized up- or downstream context alone (Bartoschek et al., 2021). At this, iPASS revealed the context -6 UUC AAU UAG AAA GAU +9 to be worst and the context -6 CAU GGA UAG CUC AUG +9 to be most favorable for amber suppression (Bartoschek et al., 2021). Interestingly, the latter context is largely distinct from stop codon contexts found in eukaryotic viruses like the tobacco mosaic virus (Goelet et al., 1982) that are known to permit high readthrough in mammalian cells (Harrell et al., 2002; Stahl et al., 1995). Furthermore, the iPASS motif is distinct from motif analyses of stop codon readthrough in human cells that also apply a linear regression model (Schueren et al., 2014; Wangen and Green, 2020). The regression models are similar to iPASS but have been computed using context-specific readthrough and not suppression efficiencies. We used one of the previously published linear regression models (Schueren et

al., 2014) to score the readthrough propensity of each context analyzed with the fluorescent reporter system. In comparison to iPASS scores, readthrough scores of each context were correlated to a lesser extent with their ncAA incorporation efficiencies (unpublished results). This result supports that optimal readthough and suppression contexts differ in their nucleotide preferences. Importantly, the readthrough analyses by Schueren et al. (2014) and Wangen and Green (2020) were performed in bulk over all three termination codons with UGA being positively correlated. This correlation with UGA might mask context effects that are specific for UAG. In fact, unique context effects extending +4 C were previously found to govern the readthrough propensity of UAG, UGA, and UAA in mammalian cells (Cridge et al., 2018). In agreement with our analysis, the study identified the +4 CUC motif to permit some of the highest readthrough at the amber stop codon in mammalian cells (Cridge et al., 2018). However, the underlying molecular mechanisms by which contexts extending the +4 nucleotide modulate eukaryotic stop codon readthrough and suppression are still poorly understood.

To this end, direct interactions between the ribosome and messenger ribonucleic acid (mRNA) nucleotides could contribute to the efficiency of amber suppression. While the mRNA traverses the mRNA channel, it interacts not only with tRNAs but also with the ribosome (Yusupova et al., 2001). During translational elongation, eukaryotic ribosomes occupy approximately 27 nucleotides (-15 to +12) (Ingolia et al., 2009; Wolin and Walter, 1988; Wu et al., 2019). However, binding of the pre-termination complex is accompanied by pulling the mRNA by two 3' nucleotides into the ribosomal mRNA channel to form the tetranucleotide termination signal within the A-site (Alkalaeva et al., 2006; Brown et al., 2015; Ingolia et al., 2011; Kryuchkova et al., 2013; Matheisl et al., 2015; Shirokikh et al., 2010; Wangen and Green, 2020). The nucleotide sequence downstream of the stop codon could modulate formation of this compacted mRNA conformation and thereby termination efficiency. For instance, motifs that are complementary to the 40S ribosomal subunit might restrict flexibility of the mRNA within the mRNA channel, inhibiting termination (Anzalone et al., 2019; Namy et al., 2001). As such, these motifs should also generally boost readthrough as well as suppression of nonsense codons. However, the previously described discrepancy between 3' readthrough motifs and iPASS only suggests involvement but no dominant role of these mRNA-rRNA interactions in context-dependent amber suppression efficiency.

In fact, several findings by us and others indicate that not only variations in termination efficiency govern the context effect in amber suppression efficiency. First, in agreement with studies on readthrough in mammalian cells (Cassan and Rousset, 2001; Cridge et al., 2018; Loughran et al., 2014; Pacho et al., 2011; Schueren et al., 2014; Wan-

gen and Green, 2020), we not only found down- but also upstream nucleotides to impact amber suppression efficiency (Bartoschek et al., 2021). Second, in line with other studies in mammalian cells (Cassan and Rousset, 2001; Cridge et al., 2018; Phillips-Jones et al., 1995, 1993), we found the efficiencies of readthrough and suppression at selected stop codon contexts to be similar but not identical (Bartoschek et al., 2021). Third, the iPASS motif and established readthrough motifs differ in their sequence composition (Bartoschek et al., 2021). And lastly, compromised termination by eRF1 has been previously found to predominantly occur in the weak termination context of +4 C in yeast and mammalian cells, whereas readthrough at +4 G, A, and U contexts was suggested to mainly dependent on the availability of suitable near-cognate tRNAs (Beznosková et al., 2016; Cridge et al., 2018). It is therefore unlikely that the flanking nucleotides solely modulate amber suppression efficiency by directly affecting release factor recruitment. In fact, observations that in pro-and eukaryotes codon pairs are biased and that this bias modulates translational efficiency (Ahmed et al., 2020; Buchan et al., 2006; Chevance and Hughes, 2017; Chevance et al., 2014; Coleman et al., 2008; Gamble et al., 2016; Gobet et al., 2020; Gutman and Hatfield, 1989; Irwin et al., 1995; Letzring et al., 2010; Moura et al., 2011) further support a model in which unique synergies between the tRNAs bound to the ribosomal P- and A-site contribute to context-dependent suppression efficiencies. These synergies could occur on two distinct levels that will be discussed in the following: the amino acid charged to the tRNA or the identity of the tRNA itself.

4.2.2 *Contribution of context-dependent translational efficiency*

THE NUCLEOTIDE CONTEXT MEDIATES ITS EFFECT INDEPENDENT OF AMINO ACID IDENTITY The effect of the UAG context could be mediated indirectly by the encoded amino acids and not only the nucleotide sequence itself. While PylT is bound within the ribosomal A-site to decode the amber stop codon, the preceding codons have already been translated into amino acids or are bound by a peptidyl-tRNA within the P-site. Hence, in particular the identity of amino acids upstream of UAG might impact the efficiency of amber suppression. To this end, previous analyses of readthrough in eukaryotic cells found no correlation between the identity of surrounding amino acids and readthrough propensity (Cassan and Rousset, 2001; Harrell et al., 2002; Mangkalaphiban et al., 2021; Namy et al., 2001; Tork et al., 2004; Williams et al., 2004), although also contradicting results have been reported in yeast for the penultimate amino acid before the termination codon (Mottagui-Tabar et al., 1998). Analyses whether the identity of flanking amino acids is also predictive in stop codon suppression are only available for bacteria. At this, biochemical

properties of the two 5′ amino acid residues have been previously reported to influence UGA or UAG suppression (Björnsson et al., 1996; Mottagui-Tabar et al., 1994; Zhang et al., 1996). In contrast, in bacteria with an expanded genetic code, no influence of the ultimate amino acid on ncAA incorporation efficiency could be detected (Xu et al., 2016), although specific amino acid motifs surrounding the amber stop codon were described (Pott et al., 2014). As such, this amino acid preference might be the direct consequence of the observed nucleotide preference around UAG. To further elucidate this causality in mammalian cells, we first attempted to compute the amino acid motif flanking UAG, but the number of contexts identified by SORT-E was too limited to perform a statistically sound linear regression analysis (unpublished results). We then asked whether synonymous exchange of the two codons up- and downstream of UAG would also affect ncAA incorporation efficiency. If the identity of the flanking amino acids is the main determinant, only marginal variations in ncAA incorporation efficiency between isocodons would be expected. We detected significantly improved ncAA incorporation efficiencies for the majority of contexts (85%) after synonymous codon exchange (Bartoschek et al., 2021), suggesting the presence of mechanisms that are independent of the immediate amino acid context. In agreement with this finding, also synonymous mutations of codon pairs have been found to affect translational efficiency and to be under selective pressure (Chevance and Hughes, 2017; Chevance et al., 2014; Coleman et al., 2008; Gamble et al., 2016; Moura et al., 2011). Of note, we silently mutated all four neighboring codons in parallel, which masks the individual influence of single codons. Therefore, we cannot determine the extent to which the identity of a single neighboring amino acid like the ultimate cAA impacts ncAA incorporation in comparison to the nucleotide sequence. In this regard, identity of amino acids within the ribosomal A- and P-site has been recently reported to contribute to translational efficiency in yeast (Ahmed et al., 2020). However, our results demonstrate that the efficiency of mammalian amber suppression also depends on the nucleotide context itself. Additionally, we showed that the UAG context influences ncAA incorporation in mammalian cells independent of the ncAA identity (Bartoschek et al., 2021), which is in agreement with data on amber suppression efficiency in *E. coli* (Kipper et al., 2017; Pott et al., 2014; Young et al., 2010). Hence, other than peptide-related molecular mechanisms contribute to the observed context effect in amber suppression efficiency.

THE NUCLEOTIDE CONTEXT MEDIATES ITS EFFECT INDEPENDENT OF TRANSFER RNA IDENTITY The second possibility would be that context-dependent efficiency of amber suppression is also mediated by the identity of recruited tRNAs. At this, the identity of the tRNA bound in the ribosomal P-site could influence recruitment of

the suppressor tRNA. Once the suppressor tRNA relocated to the ribosomal P-site, likewise decoding of the following codon could be affected. Conflicting results have been reported in eukaryotic stop codon readthrough with two studies confirming (Mottagui-Tabar et al., 1998; Williams et al., 2004) and one study questioning (Tork et al., 2004) an effect of the P-site tRNA on readthrough propensity. For nonsense suppression in eukaryotes, data on the relationship between nucleotide context and tRNA identities are sparse. In a previous study three different ncRS/tRNA pairs were comparable in their suppression efficiency in mammalian cells but only a single defined context was tested (Zheng et al., 2017a). Furthermore, two PylT variants were tested for their suppression efficiency at two distinct UAG contexts in mammalian cells (Serfling et al., 2018). The published quantification of suppression efficiencies by western blot (Serfling et al., 2018) suggests that relative ncAA incorporation efficiencies of these PylT variants are generally independent of the nucleotide context. Lastly, relative amber suppression efficiency of three $tRNA^{Ser}_{CUA}$ isodecoders was reported to be highly similar between two distinct contexts in human cells (Geslain and Pan, 2010). Accordingly, relative efficiency of nonsense suppression across different contexts in bacteria has been described to be largely independent of the used suppressor tRNA (Bossi, 1983; Miller and Albertini, 1983; Stormo et al., 1986), but also few context-specific differences were detected (Bossi, 1983; Kleina et al., 1990; Miller and Albertini, 1983). In this regard, two distinct ncRS/tRNA pairs were found to most efficiently suppress slightly different albeit largely related UAG contexts in bacteria (Pott et al., 2014). Mechanistically, compatibility of the anticodon loops of adjacent P- and A-site tRNAs has been suggested to influence tRNA decoding at the ribosomal A-site in bacteria (Smith and Yarus, 1989). Of note, this loop has not been varied in the study by Geslain and Pan (2010) comparing $tRNA^{Ser}_{CUA}$ isodecoders in their amber suppression efficiency in human cells. Taken together, the structure of decoding tRNAs might only marginally if at all contribute to the context effect in amber suppression efficiency.

This conclusion is supported by our finding that the relative efficiency of ncAA incorporation is independent of cell line identity. In mammals, albeit the 61 sense codons are decoded by 47 (mouse) or 48 (human) anticodons, more than 400 tRNA genes are present to express well over 200 tRNA isodecoder families (Chan and Lowe, 2016; Goodenbour and Pan, 2006). Importantly, in mammals expression of these tRNA isodecoders varies between cell types (Behrens et al., 2021; Ishimura et al., 2014; Kutter et al., 2011; Pinkard et al., 2020). Therefore, although data on isodecoder sequence conservation or direct comparison of isodecoder levels between different species are still unavailable, it is likely that the composition of isodecoder pools also varies between mouse and human cell lines. Despite these suggested

variations, context-dependent ncAA incorporation efficiencies were highly correlated between mESCs and human embryonic kidney 293T (HEK293T) cells (Bartoschek et al., 2021). Thus, the tRNA structure might mediate its effect on amber suppression and in general translational efficiency independent of the codon context and neighboring tRNAs by interacting with main components of the translational machinery like the ribosome. Whether the influence of the tRNA structure and codon context on amber suppression efficiency are truly uncoupled could be easily tested by comparing the context preference of ncRS/tRNA pairs other than PylRS/PylT using our SORT-E approach as well as fluorescent reporter assay. If relative suppression efficiencies of the identical contexts are correlated between structurally different suppressor tRNAs, a dominant role of tRNA-tRNA interactions in context-dependent suppression efficiency would be unlikely. In this regard, also analysis of SORT-E data and context-specific suppression efficiencies at the codon level would be more informative than at the level of single nucleotides.

THE NUCLEOTIDE CONTEXT MIGHT UNIQUELY AFFECT CODON-ANTICODON BASE PAIRING VIA BASE STACKING How else could the close-by nucleotide context then mechanistically influence the efficiency of amber suppression in addition to modulating termination efficiency? According to the evidence presented before, this influence would have to be mediated largely independent of amino acid and tRNA identity. One potential factor in suppression efficiency not discussed yet is how efficiently the suppressor tRNA anticodon can base pair with the nonsense codon in the ribosomal A-site. Changing the anticodon of orthogonal tRNAs to suppress the ochre or opal instead of amber stop codon in mammalian cells both reduce their suppression efficiency by more than fourfold with the respective nonsense codons being in the same context (Xiao et al., 2013; Zheng et al., 2017a). However, the general hierarchy of nonsense codons from highest to lowest termination efficiency in eukaryotes has been consistently reported as UAA>UAG>UGA (Cridge et al., 2018; Howard et al., 2000; Jungreis et al., 2011; Keeling and Bedwell, 2002; Loughran et al., 2014; Mangkalaphiban et al., 2021; Manuvakhova et al., 2000; Stiebler et al., 2014; Wangen and Green, 2020). On one hand, this discrepancy between suppression efficiency and overall termination hierarchy additionally supports the presence of release factor independent context effects. On the other hand, these variations in suppression efficiency upon solely changing the anticodon implicate a mechanism by which the same tRNA structure reads different cognate codons with different efficiencies. In fact, several additional lines of evidence indicate that the close-by nucleotide context directly influences codon-anticodon base pairing within the ribosomal A-site. For instance, the identity of the tetranucleotide termination codon and near-cognate tRNAs

permitting their readthrough have been demonstrated to be interdependent in eukaryotes (Beier et al., 1984; Beznosková et al., 2016, 2019; Blanchet et al., 2014; Chittum et al., 1998; Feng et al., 1990; Kuchino et al., 1987; Roy et al., 2015; Valle et al., 1987). Additionally, unique context effects extending +4 C were reported for the readthrough of UAG, UGA, and UAA in mammalian cells (Cridge et al., 2018). Furthermore, mammalian amber, ochre, and opal suppressor tRNAs differed in their codon preference 3′ of each nonsense codon with the exception of +4 C that resulted in overall above average suppression efficiency (Cridge et al., 2018). Importantly, these three suppressor tRNAs shared the same backbone sequence (Cridge et al., 2018), which excludes involvement of the overall tRNA structure in these stop codon-specific context effects. Taken together, codon-anticodon base pairing might be influenced by the nucleotide context. This direct effect could explain our observation that silently mutating up- or downstream nucleotides significantly affects ncAA incorporation efficiency independent of cell line identity (Bartoschek et al., 2021) and as such tRNA isodecoder pools.

How could this direct effect of the flanking nucleotides on codon-anticodon base pairing be mechanistically explained? An abrupt turn in the tRNA sugar-phosphate backbone separates the 5′ nucleotide from the anticodon, rendering it unavailable for base pairing with the +4 nucleotide of the codon (Holbrook et al., 1978; Quigley and Rich, 1976). Hence, an influence of the +4 nucleotide on amber suppression efficiency via base pairing with the suppressor tRNA can be excluded, which also has been confirmed in living cells (Ayer and Yarus, 1986). Alternatively, contribution of the adjacent nucleotides to the stacking energy of the codon-anticodon interaction and thereby decoding efficiency has been proposed (Ayer and Yarus, 1986; Bossi and Roth, 1980; Grosjean et al., 1976; Pedersen and Curran, 1991). To this end, unpaired terminal nucleotides are known to contribute via base stacking to the stability of RNA duplexes in a sequence-dependent manner (Freier et al., 1986; Martin et al., 1971; O'Toole et al., 2006; Ohmichi et al., 2002; Sugimoto et al., 1987; Tateishi-Karimata et al., 2014). Furthermore, during translation the codon-anticodon pair located at the ribosomal P-site could also uniquely affect stacking and consequently base paring at the A-site. Accordingly, wobble base pairing of the tRNA at the ribosomal P-site has been implicated in the efficiency of decoding at the A-site (Björnsson et al., 1996; Curran, 1995; Gamble et al., 2016). To date, the potential contribution of nucleotide stacking across the codon context to translational efficiency has not been systematically investigated. This stacking model is supported by observations that simply swapping two consecutive codons can already significantly affect translational speed in vivo (Chevance et al., 2014; Gamble et al., 2016). Furthermore, this base stacking effect has been proposed to extent over up to two codons up- and downstream (Chevance and

Hughes, 2017). Of note, in yeast the effect of codon pair bias on translational efficiency recently has been mainly attributed to the identity of amino acids at the A- and P-site (Ahmed et al., 2020). However, this finding is in contrast to our results in mammalian cells, demonstrating that changing the nucleotide and not amino acid sequence already significantly affects ncAA incorporation efficiency (Bartoschek et al., 2021). Collectively, these reports and our results suggest that the close-by amber stop codon context can influence suppression efficiency via base stacking to stabilize codon-anticodon base pairing at the ribosomal A-site, an effect that seems to be unique for each codon-anticodon pair. Importantly, this stacking and codon-anticodon base pairing model can also explain the aforementioned codon pair bias, suggesting synergies between the ribosomal P- and A-site. This potential direct effect of the close-by nucleotides is still underappreciated in explaining variations in translational efficiency. Moreover, this stacking effect might be of particular importance during nonsense suppression at which efficient decoding by the suppressor tRNA at the ribosomal A-site could better out-compete release factor recruitment.

TRANSLATIONAL SPEED AND PAUSING MIGHT CONTRIBUTE TO NON-CANONICAL AMINO ACID INCORPORATION EFFICIENCY We demonstrate iPASS to predict approximately 50% of the context-dependent variation in ncAA incorporation efficiency (Bartoschek et al., 2021), indicating the presence of additional features next to the identity of close-by single nucleotides. To this end, ribosome profiling (Ingolia et al., 2009) revealed that the density of ribosomes and hence translational speed is not uniform across transcripts (Ingolia et al., 2011). In particular at termination codons ribosomes are stalled, which reflects the slower dynamics of termination compared to elongation (Ingolia et al., 2011; Schuller et al., 2017; Wangen and Green, 2020; Wolin and Walter, 1988). Whether and how translational speed and amber suppression are related can only be speculated. For instance, two distinct dynamics could both favor ncAA incorporation: on one hand, reduced translational speed around the incorporation site could reinforce translational fidelity, facilitate suppressor tRNA and ncAA accommodation within the ribosome, and ultimately enhance amber suppression efficiency; on the other hand, high translational speed of the surrounding codons could disfavor recruitment of the termination complex to the advantage of decoding by the amber suppressor tRNA. Moreover, simple rules governing the distribution of ribosomal footprints are yet missing (Collart and Weiss, 2020; Hanson and Coller, 2018), constraining interpretation of the iPASS context regarding translational speed. One feature that correlates with ribosomal pausing sites in eukaryotes is the usage of non-optimal codons that are decoded by low abundant tRNAs (Hussmann et al., 2015; Weinberg et al., 2016; Wu et al., 2019). To this end, comparing published tRNA levels of HEK293T

cells (Behrens et al., 2021) with the iPASS codon context revealed no clear correlation (unpublished data). Interestingly, despite heterogeneity of isodecoder pools, the abundance of tRNA anticodon pools and as such isoacceptor levels were reported to be highly similar between different cell types (Kutter et al., 2011; Pinkard et al., 2020; Schmitt et al., 2014). However, recent work applying state-of-the-art tRNA profiling techniques revealed small yet significant differences in tRNA anticodon pools between cell types (Behrens et al., 2021). Whether these subtle variations lead to differences in translational speed between cell lines remains unclear. We found context-dependent ncAA incorporation efficiencies to be highly correlated between mESCs and HEK293T cells (Bartoschek et al., 2021). Assuming that these small differences in tRNA anticodon pools lead to variations in translational speed between cell lines, our data would indicate that the close-by nucleotide context affects translational efficiency independent of speed. Of note, the regulation of translational speed is not only simply correlated with tRNA abundance, but also other factors such as varying aminoacylation levels of tRNA isoacceptors seem to be involved (Darnell et al., 2018; Li et al., 2012; Subramaniam et al., 2013). Recently, also a computational model to infer codon-specific elongation rates has been developed and demonstrated to reliably predict overall translational efficiency of defined transcripts (Trösemeier et al., 2019). This in silico simulation of ribosome dynamics along a defined transcript could be helpful in further deducing the relationship between amber suppression efficiency and translational speed.

Ribosome profiling data in yeast (Pop et al., 2014) and mammalian cells (Gobet et al., 2020) indicate that the mechanisms regulating translational elongation are more complex than simply a correlation with tRNA abundance or codon usage. For instance, a defined ribosome interface has been recently reported to interact with the codon 3′ of the ribosomal A-site in yeast through hydrogen bonding (Scopino et al., 2020). The strength of this ribosome-mRNA interaction was found to depend on the mRNA sequence and posttranslational modification status of the interacting ribosomal protein, suggesting an additional layer of complexity in the regulation of translational efficiency (Barr et al., 2020; Scopino et al., 2021). Furthermore, sequences that hybridize with the anti-SD sequence in 16S rRNA were described to cause translational pausing in bacteria (Chevance et al., 2014; Li et al., 2012). However, in following reports not the SD motif (Borg and Ehrenberg, 2015; Mohammad et al., 2016) but proline, asparagine, and glycine codons within the three active sites of the ribosome were reported to primarily cause pausing (Mohammad et al., 2019), indicating an mRNA independent mechanism. Ribosomal pausing at one or combinations of these three amino acids has also been observed in yeast (Gutierrez et al., 2013; Schuller et al., 2017) and mammalian cells (Gobet et al., 2020; Ingolia et al., 2011), suggesting that rates of peptidyl

transfer are universally slow for these amino acid motifs. Interestingly, the iPASS motif (Bartoschek et al., 2021) includes proline (codon: CCN) and glycine (codon: GGN) as the penultimate and ultimate amino acid, respectively. This preference indicates that increased ribosomal pausing around UAG benefits amber suppression. To further investigate this hypothesis, previously reported ribosomal pause sites (Ingolia et al., 2011; Schuller et al., 2017) could be rapidly evaluated with the dual-fluorescence reporter assay. Overall, peptide-related translational speed might contribute to the remaining 50% of context-dependent variation not covered by iPASS, underlining the importance of evaluating motifs not only at the level of single nucleotides but also codons and hence amino acids.

STRUCTURAL ELEMENTS IN MESSENGER RNA MIGHT CONTRIBUTE TO NON-CANONICAL AMINO ACID INCORPORATION EFFICIENCY

Additionally, mRNA sequences or secondary structures further than six nucleotides downstream of the stop codon have been found to modulate readthrough propensity in eukaryotes. For instance, in mammalian cells nucleotides up to +15 have been reported to regulate stop codon readthrough (Loughran et al., 2014). Furthermore, programmed stop codon readthrough in several eukaryotic viruses is strictly dependent on the presence of defined 3′ mRNA pseudoknot or stem-loop structures (Alam et al., 1999; Brown et al., 1996; Feng et al., 1992; Firth et al., 2011; Honigman et al., 1991; Houck-Loomis et al., 2011; Kuhlmann et al., 2016; Napthine et al., 2012; Wills et al., 1991, 1994). Moreover, pairing probability of a stem-loop as well as its distance from the stop codon were recently identified as the most important features for eukaryotic amber stop codon readthrough after identity of the +4 nucleotide and GC content (Anzalone et al., 2019). How these structural elements can mechanistically modulate readthrough propensity remains unclear (Jaafar and Kieft, 2019). Two possibilities would be that the secondary structure acts either indirectly by recruitment of a trans-acting factor or directly by interacting with the ribosome. These modes of action might then interfere with release factor recruitment and/or boost accommodation of a near-cognate tRNA. Notably, no ribosomal pausing could be detected at a single viral readthrough promoting stem-loop structure investigated to date (Napthine et al., 2012), questioning the relevance of translational speed also in amber suppression efficiency. Intriguingly, however, the spacing between stop codon and the readthrough-promoting mRNA secondary structure has been reported to be generally between 8 and 15 nucleotides (Anzalone et al., 2019; Feng et al., 1992; Firth et al., 2011; Honigman et al., 1991; Napthine et al., 2012; Wills et al., 1991). This defined spacing places the secondary structure immediately in front of the ribosome, suggesting a direct effect of the secondary structure on ribosomal function. To this end, formation of a stem-loop structure in front of the ribosome

might interfere with the aforementioned compaction of mRNA within the ribosomal channel during termination. Thereby, formation of the tetranucleotide termination signal might be slowed impeding release factor binding in favor of tRNA recruitment. Hence, the robustness of iPASS in predicting suppression efficiencies could be further improved by evaluating the presence of RNA structural elements downstream of UAG. In this respect, engineering stem-loops 3′ of the stop codon might further improve ncAA incorporation efficiency.

In conclusion, the nucleotide context could mediate amber suppression efficiency by modulating both translational termination and elongation. Since our analysis and optimization by iPASS focuses on the immediate nucleotide context (-6 to +9), regulatory mechanisms must be present that influence translational efficiency independent of the extended sequence context and as such overall translational speed as well as mRNA secondary structures. To this end, ncAA incorporation at amber stop codons seems to be in general efficient for 3′ contexts where termination is poor and vice versa. However, the iPASS motif differs from established readthrough motifs in mammalian cells and we detect a clear influence of the 5′ nucleotides on ncAA incorporation efficiency (Bartoschek et al., 2021). Additionally, experimentally measured amber suppression efficiency and readthrough propensity are correlated to a lesser extent than amber suppression efficiency and iPASS score (Bartoschek et al., 2021). These results suggest that the close-by nucleotide context not only affects release factor recruitment but also translational elongation. At this, several steps of the translation elongation cycle could be modulated by the codon context like tRNA accommodation, hybrid state formation, translocation, and exit. We demonstrate that the sequence context can influence amber suppression independent of the nascent peptide (Bartoschek et al., 2021) and hence peptide bond formation. Furthermore, we show that the context affects ncAA incorporation efficiency independent of cell line identity (Bartoschek et al., 2021), which likely differ in their tRNA isodecoder pools available for decoding. Thus, interactions between tRNA backbone sequences at the ribosomal P- and A-site might only marginally contribute to translational efficiency. Instead, our data together with the limited data published on context-effects in translational efficiency suggest that the codon context uniquely affects codon-anticodon base pairing via base stacking. Overall, our results are in support of a model where the effect of an individual codon on translational efficiency depends on its context. To date, only very few studies have explored this interdependence, which might be a key factor in regulating translational elongation. Importantly, by further characterizing context-effects in nonsense suppression, a deeper mechanistic insight into the relationship between immediate codon context and translational efficiency in general would be gained, advancing our understanding of how a functional proteome is produced in vivo.

4.3 FUTURE PERSPECTIVES

4.3.1 *Deciphering context effects in amber suppression and translational efficiency*

Our data provide insights into the interdependence between codon context and translational efficiency and termination. As such, nonsense suppression by OTSs could be used as a tool to systematically characterize the contribution of different factors on translational efficiency: the charged amino acid, the tRNA backbone, the anticodon, the decoded codon, the amino acid context, and the nucleotide context. Our SORT-E and dual-fluorescence reporter approach should prove useful in further delineating the relationship between codon context and nonsense suppression efficiency as well as translational efficiency.

APPLICATION OF THE SORT-E AND FLUORESCENT REPORTER STRATEGY TO BETTER UNDERSTAND CONTEXT EFFECTS The proposed stacking model implicates that the optimal context is different for each codon-anticodon pair. In fact, as discussed before, unique sequence features seem to be present that depend on the codon-anticodon interaction either of a near-cognate or suppressor tRNA. Whether our characterization of permissive amber suppression sites is generalizable to ochre and opal suppression is unclear. Modifying only the anticodon of designated tRNA backbones like PylT to suppress UGA or UAA and performing our SORT-E and fluorescent reporter strategy would allow to further dissect the interdependence between nonsense codon identity and context, suppressor tRNA identity, and suppression efficiency. However, the direct contribution of nonsense codon-anticodon base pairing to suppression efficiency would be confounded with varying termination efficiencies at UGA and UAA. To better separate the influence of the stop codon context on nonsense codon-anticodon base pairing from release factor recruitment, readthrough could be compared with suppression efficiency and as such non-canonical with canonical codon-anticodon base pairing. If codon-anticodon base pairing is uniquely affected by the context, relative efficiencies between readthrough and suppression should not only vary at defined amber (Bartoschek et al., 2021), but also at ochre and opal stop codon contexts. Furthermore, evaluating also other suppressor tRNA isodecoders would be effective in delineating the effect of the tRNA structure on nonsense suppression efficiency. To this end, our SORT-E and dual-fluorescence reporter strategy could be easily performed with other OTSs. If unique tRNA-tRNA interactions are present, also context preferences should vary between suppressor tRNA isodecoders. Overall, these experiments would contribute to our understanding of context effects in translational efficiency.

COLLECTION OF UNBIASED DATASETS TO OVERCOME LIMITATIONS OF IPASS The iPASS motif reflects in combination with the overall GC content the influence of individual nucleotide positions on amber suppression efficiency. Despite the identity of single nucleotides (Anzalone et al., 2019; Cassan and Rousset, 2001; Loughran et al., 2014; Pacho et al., 2011; Skuzeski et al., 1991; Zerfass and Beier, 1992), also the combination of surrounding nucleotides seems to synergistically affect readthrough propensity (Bartoschek et al., 2021; Bonetti et al., 1995; Cridge et al., 2018; Harrell et al., 2002; Namy et al., 2001; Williams et al., 2004). In this respect, identity of the codon (and not single nucleotides) at the ribosomal P-site has been recently identified as an informative predictor of readthrough efficiency in yeast (Mangkalaphiban et al., 2021). This non-additive, combinatorial effect of nucleotides is not covered by our linear regression analysis of SORT-E data, focusing on each nucleotide position individually. Furthermore, our SORT-E approach in stable cell lines with an expanded genetic code is limited to endogenous UAG contexts, which are biased as outlined before. Additionally, these contexts have to be sufficiently expressed in the respective cell line to be detected by mass spectrometry, further reducing the number of contexts that can be probed. In this respect, we considered endogenous contexts to be suppressed as soon as the respective protein was enriched in the streptavidin pulldown. A more rigorous approach would be to only consider 'readthrough peptides', which are comprised of amino acids up- and downstream of the suppressed amber stop codon. The respective proteomic workflow to identify these peptides has been already established for the *E. coli* proteome (Aerni et al., 2015). We established a similar workflow but detected too few readthrough peptides in mammalian cells to perform a linear regression analysis (unpublished data). Despite these limitations, we demonstrate iPASS to accurately predict approximately 50% of the context-dependent variation in amber suppression efficiency (Bartoschek et al., 2021).

However, a statistically sound analysis at the codon (61 possibilities per position) instead of single nucleotide (4 possibilities per position) level would be critical to further delineate synergies between individual nucleotide positions. Additionally, the contribution of amino acid or tRNA identity to context-specific suppression efficiency could be further deciphered. Compared to iPASS, this analysis would require the collection of a much larger dataset that vastly oversamples the sequence space of potential contexts. To this end, by applying the dual-fluorescence reporter as a readout of suppression efficiency, an unbiased suppression assay could be established. After cloning a degenerate library of stop codon contexts into the reporter, efficiently suppressed contexts could be enriched by FACS and subsequently identified by sequencing. As an alternative to the expression of a fluorescent protein, expression of an antibiotic resistance gene could be

linked to stop codon suppression and used for selection of permissive amber suppression sites. Given the identification of an adequate number of distinct contexts, a machine learning algorithm similar to that used for UAG readthrough (Anzalone et al., 2019) could be trained that then aids the screening and design of permissive suppression sites. Thereby, accurate prediction of context-dependent amber suppression efficiency could be improved.

4.3.2 *Complementary strategies to further improve ncAA incorporation*

TREATMENT WITH AMINOGLYCOSIDES TO COUNTERACT TERMINATION AT PREMATURE TERMINATION CODONS Treatment of eukaryotic cells with aminoglycosides has been long known to induce stop codon readthrough in a context-dependent manner (Barton-Davis et al., 1999; Bedwell et al., 1997; Beznosková et al., 2019; Bidou et al., 2004; Burke and Mogg, 1985; Chowdhury et al., 2018; Howard et al., 2000; Howard et al., 1996; Malik et al., 2010; Martin et al., 1989; Palmer et al., 1979; Singh et al., 1979; Wangen and Green, 2020; Wilschanski et al., 2003). However, the precise molecular mechanisms of readthrough induced by different aminoglycosides are not yet fully understood. Aminoglycosides such as geneticin (G418) or paromomycin bind in the ribosomal A-site and stabilize the decoding center in the tRNA-bound conformation (Loubresse et al., 2014; Ogle et al., 2001; Ogle et al., 2002; Prokhorova et al., 2017). This conformational change has been suggested to promote selection of near-cognate tRNAs by increasing the flexibility for non-cognate codon-anticodon base pairing (Loubresse et al., 2014; Ogle et al., 2001; Ogle et al., 2002; Prokhorova et al., 2017). Interestingly, promoting readthrough by G418 treatment in human cells markedly renders the identity of the stop codon and +4 nucleotide more important relative to the remaining surrounding nucleotides (Wangen and Green, 2020). This result indicates that while G418 relaxes codon-anticodon base pairing, release factor recruitment becomes the dominant competitor of readthrough. Accordingly, treatment with aminoglycosides might diminish the importance of other nucleotide positions in nonsense suppression efficiency, which would have the advantage that selected sites could be easier adapted to confer high ncAA incorporation rates. However, to which extent relaxed codon-anticodon base pairing caused by aminoglycosides like G418 would stimulate decoding by suppressor tRNAs that already bind the stop codon via cognate codon-anticodon base pairing remains unclear.

Additionally, release factor binding might be sterically disfavored (Shalev and Baasov, 2014; Youngman et al., 2007), in particular by aminoglycosides containing a 6′-NH_2 constituent in ring I like gentamicin (Prokhorova et al., 2017). Our results indicate that competition with termination is one of the main factors counteracting amber suppression efficiency. Hence, treatment with some selected amino-

glycosides might benefit stop codon suppression by directly inhibiting release factor recruitment. In particular, the small molecule, non-antibiotic aminoglycoside analog ELX-02 (also referred to as NB-124) (Kandasamy et al., 2012) would be an interesting candidate to boost ncAA incorporation. Similar to gentamicin, the ring I of ELX-02 contains a 6'-NH_2 constituent. Furthermore, upon ELX-02 treatment of mammalian cells, readthrough of PTCs is almost 3x greater than with gentamicin while demonstrating improved tolerability (Bidou et al., 2017; Brasell et al., 2019; Kandasamy et al., 2012; Xue et al., 2014). Moreover, ELX-02 has been reported to specifically promote readthrough of PTCs and not native stop codons (Crawford et al., 2020), reducing the risk of adverse effects on the global proteome and cell viability. Notably, also gentamicin X2, a gentamicin congener, has been recently found to induce readthrough comparable to ELX-02 while being even better tolerated (Friesen et al., 2018). However, while improving nonsense suppression, aminoglycosides could boost readthrough by near-cognate tRNAs to a similar extent, resulting in the misincorporation of cAAs instead of the ncAA. Additionally, aminoglycoside treatment can result in the misincorporation of cAAs at sense codons (Fan-Minogue and Bedwell, 2008; Prokhorova et al., 2017). These trade-offs should be carefully monitored. To promote ncAA incorporation, we also treated mammalian cells with Chardonnay. Since the results were inconclusive, a bottle of the respective wine can be requested from the author for follow-up assays. We additionally assessed potency of the non-aminoglycoside compound Ataluren (also referred to as PTC-124), a fluorinated oxadiazole, in enhancing amber suppression efficiency. Ataluren was previously reported to efficiently promote PTC readthrough in mammalian cells (Roy et al., 2016; Welch et al., 2007). However, in line with a previous study (Lancia et al., 2014), we detected no increase in POI^{ncAA} yield in the presence of Ataluren (unpublished data). Interestingly, Ataluren has been proposed to exert its readthrough stimulating function most efficiently at UGA through direct interactions with the opal stop codon and flanking nucleotides (Lentini et al., 2014; Tutone et al., 2019). This sequence-specific mode of action would explain the observed lack of activity at amber stop codon contexts. Taken together, treatment of mammalian cells with selected aminoglycosides might substantially enhance site-specific ncAA incorporation.

INHIBITION OF NONSENSE MEDIATED DECAY TO STABILIZE TRANSCRIPTS WITH LONG 3' UNTRANSLATED REGIONS Our data of the dual-fluorescence reporter also indicate that its transcript levels are susceptible to NMD (Bartoschek et al., 2021). In mammalian cells, extension of the 3' UTR can trigger NMD independent of introns and therefore exon-junction complexes (Bühler et al., 2006; Eberle et al., 2008; Singh et al., 2008; Yepiskoposyan et al., 2011). Despite the obser-

vation that readthrough of the PTC (and in this sense also suppression of the amber stop codon) stabilizes transcripts by counteracting NMD (Baker and Hogg, 2017; Hogg and Goff, 2010; Kurosaki and Maquat, 2013; Zünd et al., 2013), inhibition of NMD has been reported to increase POI^{ncAA} yields in mammalian cells (Han et al., 2017). Hence, although our data confirm that suppression of the in-frame UAG mitigates NMD (Bartoschek et al., 2021), transcript levels of the dual-fluorescence reporter might be still susceptible to NMD upon ncAA addition. Moreover, the potential 3′ UTR of the dual-fluorescence reporter transcript, which could trigger NMD, is particularly long since the GOI* is followed by the open reading frame of mNeonGreen. However, 3′ UTR length does not correlate per se with NMD susceptibility in mammalian cells (Hurt et al., 2013; Tani et al., 2012) but rather depends on the recruitment of trans-acting factors (Eberle et al., 2008; Fatscher et al., 2014; Hogg and Goff, 2010; Joncourt et al., 2014; Peixeiro et al., 2012; Silva et al., 2008; Singh et al., 2008). At this, binding of up-frameshift protein 1 (UPF1) to the 3′ UTR and its activation by phosphorylation is a key factor in triggering NMD (Eberle et al., 2008; Hogg and Goff, 2010; Kurosaki et al., 2014; Kurosaki and Maquat, 2013; Lee et al., 2015; Zünd et al., 2013). Interestingly, UPF1 has been found to preferentially bind to G- and GC-rich sequences in 3′ UTRs, causing stalling of UPF1 translocation, phosphorylation, and consequently NMD (Hurt et al., 2013; Imamachi et al., 2017). Therefore, deletion of these motifs downstream of the suppressed amber stop codon might boost POI^{ncAA} yields. This sequence optimization strategy could be readily implemented into the iPASS algorithm. Furthermore, 3′ UTR-mediated NMD is selectively inhibited by reduced levels of up-frameshift protein 2 (UPF2), which interacts with UPF1 and stimulates its activity in NMD (Boehm et al., 2014; Metze et al., 2013). Hence, knock-down of *Upf2* might be also beneficial, which has already been demonstrated for mouse embryonic fibroblasts with an expanded genetic code (Han et al., 2017). Taken together, counteracting 3′ UTR-mediated NMD might improve transcript levels especially of the dual-fluorescence reporter and therefore POI^{ncAA} yields.

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ANNEX

A.1 PUBLICATIONS

A.1.1 *Identification of permissive amber suppression sites for efficient non-canonical amino acid incorporation in mammalian cells*

Bartoschek, M. D., E. Ugur, T.-A. Nguyen, G. Rodschinka, M. Wierer, K. Lang, and S. Bultmann (June 2021). *Nucleic Acids Res.* 49, e62.

DOI: 10.1093/nar/gkab132

A.1.2 *Live cell PNA labelling enables erasable fluorescence imaging of membrane proteins*

Gavins, G. C., K. Gröger, M. D. Bartoschek, P. Wolf, A. G. Beck-Sickinger, S. Bultmann, and O. Seitz (Jan. 2021). *Nat. Chem.* 13, pp. 15–23.

DOI: 10.1038/s41557-020-00584-z

A.2 CURRICULUM VITAE

MB CV 1/2

Michael David Bartoschek

INDUSTRIAL AND LAB-BASED WORK EXPERIENCE

06/2015 – 12/2020 **Doctoral candidate**
Ludwig-Maximilians-Universität München, Germany
Department of Human Biology and BioImaging, Dr. Sebastian Bultmann
Interdisciplinary project with TU Munich, Synthetic Biochemistry, Prof. Dr. Kathrin Lang

04/2014 – 08/2014 **Intern**
Baxter Healthcare International, Unterschleißheim, Germany
Business unit Hemophilia

05/2011 – 03/2014 **Research assistant**
Ludwig-Maximilians-Universität München, Germany
Department of Genetics, Prof. Dr. Martin Parniske

UNIVERSITY EDUCATION

06/2015 – 09/2021 **Doctorate (Dr. rer. nat.) in biology**
Ludwig-Maximilians-Universität München, Germany
Graduate program: International Max Planck Research School for Molecular Life Sciences, Martinsried, Germany

10/2012 – 03/2015 **Master of Science (M.Sc.) in biology**
Ludwig-Maximilians-Universität München, Germany

10/2009 – 09/2012 **Bachelor of Science (B.Sc.) in biology**
Ludwig-Maximilians-Universität München, Germany

PUBLICATIONS

Michael David Bartoschek

Gavins GC, Gröger K, Reimann M, Bartoschek MD, Bultmann S, Seitz O. Orthogonal coiled coils enable rapid covalent labelling of two distinct membrane proteins with peptide nucleic acid barcodes. *RSC Chemical Biology* (2021). DOI: 10.1039/D1CB00126D

Kempf JM, Weser S, Bartoschek MD, Metzeler KH, Vick B, Herold T, Völse K, Mattes R, Scholz M, Wange LE, Festini M, Ugur E, Roas M, Weigert O, Bultmann S, Leonhardt H, Schotta G, Hiddemann W, Jeremias I, Spiekermann K. Loss-of-function mutations in the histone methyltransferase EZH2 promote chemotherapy resistance in AML. *Scientific Reports* (2021). DOI: 10.1038/s41598-021-84708-6

Bartoschek MD, Ugur E, Nguyen TA, Rodschinka G, Wierer M, Lang K, Bultmann S. Identification of permissive amber suppression sites for efficient non-canonical amino acid incorporation in mammalian cells. *Nucleic Acids Research* (2021). DOI: 10.1093/nar/gkab132

Gavins GC, Gröger K, Bartoschek MD, Wolf P, Beck-Sickinger AG, Bultmann S, Seitz O. Live cell PNA labelling enables erasable fluorescence imaging of membrane proteins. *Nature Chemistry* (2020). DOI: 10.1038/s41557-020-00584-z

Mulholland CB, Nishiyama A, Ryan J, Nakamura R, Yiğit M, Glück IM, Trummer C, Qin W, Bartoschek MD, Traube FR, Parsa E, Ugur E, Modic M, Acharya A, Stolz P, Ziegenhain C, Wierer M, Enard W, Carell T, Lamb DC, Takeda H, Nakanishi M, Bultmann S, Leonhardt H. Recent evolution of a TET-controlled and DPPA3/STELLA-driven pathway of passive DNA demethylation in mammals. *Nature Communications* (2020). DOI: 10.1038/s41467-020-19603-1

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