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Ribozyme-Based Transfer RNA Switches for Post-transcriptional Control of Amino Acid Identity in Protein Synthesis

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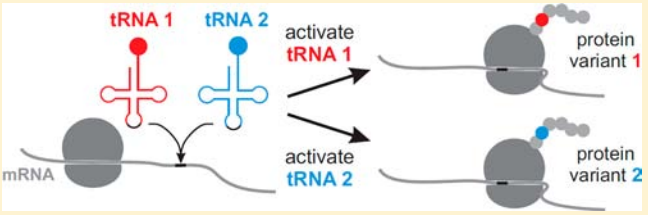
Supporting Information

ABSTRACT: Protein mutants are studied in a variety of contexts in the life sciences. However, individual mutations need to be generated in order to transcribe and translate the respective protein variants. Here, we introduce a novel strategy for controlling the incorporation of different amino acids in response to an amber stop codon by utilizing switchable designer transfer RNAs in *Escherichia coli*.



INTRODUCTION

In order to characterize proteins, often protein mutants are generated in transgenic cellular or organismal models to investigate the involvement of single amino acids with respect to the function of the protein. A given mutation needs to be generated and introduced into the respective model. However, transient studies with switching between mutant and wild-type phenotypes can hardly be realized utilizing conventional methods, making the study of essential or dominant negative mutations cumbersome. We have developed an RNA-based toolbox that allows for switching ribosomal amino acid incorporation from one amino acid to another by reading a given messenger RNA (mRNA) differently. Hence, the protein composition is controlled on a post-transcriptional level. The approach utilizes molecular RNA switches that allow for controlling the function of two competing transfer RNAs (tRNAs). The tRNAs both recognize and compete for suppression of an artificial amber codon introduced into the mRNA of interest, see Figure 1. Depending on the external addition of a small molecule trigger, a decision of utilizing tRNA 1 or tRNA 2 is reached. In order to do so, we had to develop several orthogonal tRNA control systems that were then combined in order to switch between incorporation of alanine, serine, or leucine. The introduced strategy needs only little sequence space and enables switching from one protein variant to another without further manipulating the genetic repertoire of a given cell or organism. The approach should also prove useful in order to fine-tune protein activity by adjusting the level of the functional variant in a dose-dependent manner.

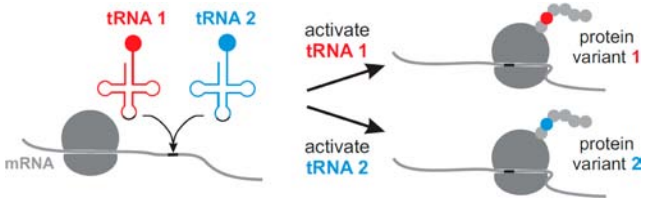


Figure 1. General principle of switching amino acid identity via small molecule-addressable tRNAs. Two different tRNAs (red and blue) both recognizing an amber stop codon (black) introduced at the site of interest in the target mRNA can be switched on or off individually. In the case of activating tRNA 1, amino acid 1 is incorporated into the protein of interest and vice versa.

Except for the report of a designed, RNA-guided deaminase for site-selective codon mutation,[1] to our knowledge, there is no method available for changing the identity of a specific amino acid post-transcriptionally. However, recent years have seen a series of discoveries related to RNA-based mechanisms that control gene expression. In bacteria, riboswitches are regulating gene expression by binding of small metabolites to mRNAs that encode metabolite biosynthesis and uptake.[2−5] In most cases, ligand binding to an aptamer domain residing in the 5′-untranslated region (UTR) triggers a conformational change of the so-called expression platform, thereby regulating transcription elongation or translation initiation of the respective mRNAs.[6,7] Interestingly, even before these natural mechanisms have been discovered, researchers have implemented similar but artificial RNA-based switches of gene expression by introducing aptamers into mRNAs.[8] Recent examples include the control of gene expression of viral, bacterial, as well as eukaryotic mRNAs by several mechanisms.[9−15] Although these examples allow the immediate control of expression without the need of additional protein factors, the approaches only allow control of whether a given message is translated or not. Here, we present a novel technology that allows reading of a given mRNA differently by implementing switchable designer tRNAs.

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RESULTS

It is possible to interfere with protein synthesis by controlling codon usage during translation.[16] We have recently presented specific designer amber stop codon recognizing tRNAs that are placed under control of a self-cleaving hammerhead ribozyme (HHR), see Figure 2A.[17] For this purpose, we connected the ribozyme such that the typical tRNA cloverleaf secondary structure is not able to fold in the case of an inactive ribozyme. Upon cleavage of the ribozyme, the two RNA fragments dissociate and enable the folding of the functional tRNA (see Figure 2A).[17] In addition, we have engineered the system in a way that it responds to externally added chemical triggers by attaching aptamers to stem III of the HHR and screening for optimized connection sequences. In a proof of concept study, a serine tRNA was activated upon addition of the small molecule theophylline to the growth medium.[17] Here, we present post-transcriptional control of amino acid identity in proteins by manipulating the activity of two orthogonally controlled tRNAs both decoding an artificial amber stop codon.

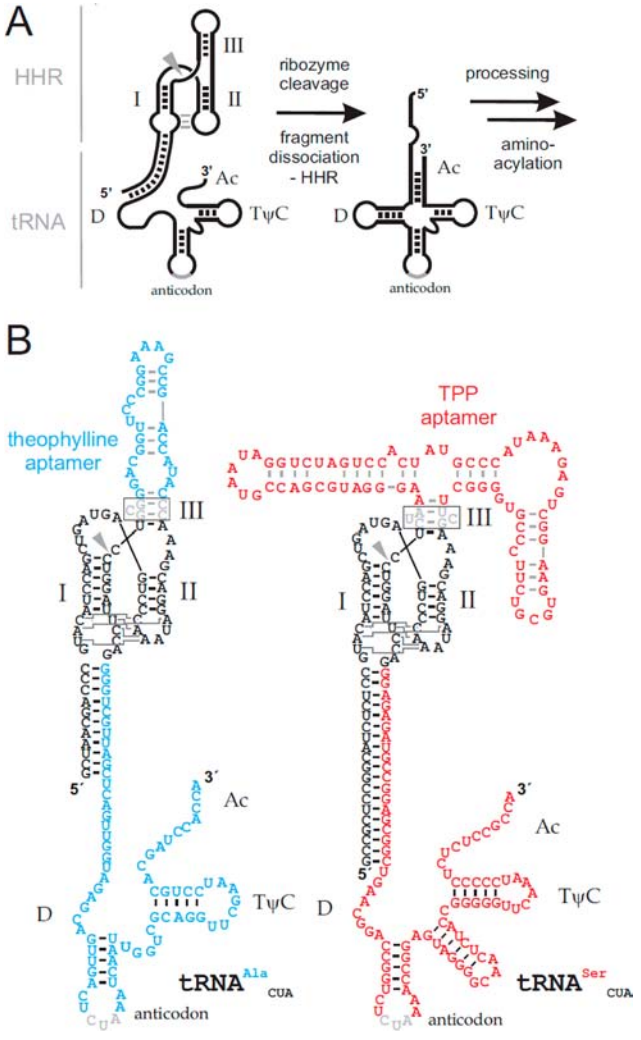

Figure 2. Aptazyme-mediated control of tRNAs in *Escherichia coli*. (A) General principle of ribozyme-mediated control of tRNA functionality. Connection of a HHR to the tRNA sequence disrupts the formation of acceptor (Ac) and D arm by a 5′-extension to the HHR. The cleavage site is marked by a gray arrowhead. Upon ribozyme cleavage, the tRNA is processed and serves as amber suppressor tRNA in translation. (B) Nucleotide sequence of the aptazyme−tRNA fusion constructs developed for controlling amino acid identity on a post-transcriptional level. Left: an alanine amber-suppressor tRNA under control of a theophylline-responsive ribozyme (theoON-tRNA$^{Ala}_{CUA}$); right: a serine amber-suppressor tRNA under control of a thiamine pyrophosphate (TPP)-responsive ribozyme (tppON-tRNA$^{Ser}_{CUA}$). Optimized communication module sequences connecting aptamer and ribozyme parts are boxed and shown in gray.

In order to be able to implement two orthogonally controlled tRNAs, we developed a series of additional ligand-dependent ribozyme-regulated tRNAs. We have previously described an amber suppressor tRNA charged with serine that gets switched on in presence of theophylline, termed theoON-tRNA$^{Ser}_{CUA}$. We next constructed an amber suppressor tRNA for serine that switches on upon addition of thiamine (tppON-tRNA$^{Ser}_{CUA}$), a tRNA loaded with leucine switched on by theophylline (theoON-tRNA$^{Leu}_{CUA}$), and an alanine-incorporating tRNA, switched on by theophylline (theoON-tRNA$^{Ala}_{CUA}$), see the Supporting Information for details. We obtained these different ligand-regulated tRNA systems by connecting the respective tRNA to the corresponding aptazyme as shown in Figure 2. The ribozyme was positioned upstream of the respective tRNA. Next, a 5′-extension complementary to the 5′-end of the tRNA (the acceptor stem) was designed individually for each tRNA in order to achieve blocking of the formation of the typical cloverleaf secondary structure of the tRNA. The amber suppressor tRNA charged with alanine is derived from a lysine amber suppressor tRNA with two point mutations, and the amber suppressor tRNA charged with leucine originates from a leucine tRNA sequence.[18,19] The ribozymes contain an aptamer domain in stem III for regulation of cleavage activity upon external addition of a small molecule (theophylline or thiamine). In order to engineer ligand dependency of tRNA systems mentioned above, two key features were optimized in the individual designs: the length and composition of the 5′-sequence complementary to the 5′-end (acceptor stem) of the tRNA and the communication module (boxed and gray in Figure 2) connecting aptamer and ribozyme domains. For details of construction and characterization of the individual switches tppON-tRNA$^{Ser}_{CUA}$, theoON-tRNA$^{Ala}_{CUA}$, and theoON-tRNA$^{Leu}_{CUA}$, see the Supporting Information.

The aim of this study is to show that the developed tRNA switches are suited for controlling the identity of individual amino acids in protein synthesis post-transcriptionally. In order to do so, we first expressed the two competing amber suppressor tRNA systems tppON-tRNA$^{Ser}_{CUA}$ and theoON-tRNA$^{Ala}_{CUA}$ simultaneously. In order to characterize the performance of the system, enhanced green fluorescent protein (eGFP) was expressed containing an artificial amber stop codon at position 50. eGFP was purified via a His-tag from two *E. coli* cultures grown in presence of 2 mM theophylline and 0.1 mM thiamine, respectively. Protein masses were determined by electrospray ionization mass spectrometry (ESI-MS), see Table 1 and the Supporting Information. The obtained masses fit to the expected masses of the serine and alanine variants of the protein expressed under the respective conditions. Masses of proteins containing the nonanticipated amino acids were not

Table 1. ESI-MS of Purified Proteins

growth condition	calculated MW	measured MW
2 mM theophylline	29 294.8	29 293.9
0.1 mM thiamine	29 310.8	29 308.8

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found in the purified protein samples. Minor signals detected in some cases can be attributed *N*-formyl-methionine-carrying[20,21] protein variants, see the Supporting Information for details.

Although the whole-protein ESI-MS data demonstrate the validity of the approach, a quantitative analysis of the relative alanine and serine content in the expressed protein is not possible via this approach since the MS methodology is not sensitive enough with respect to the abundance of the obtained protein variants. For quantification purposes, we carried out a tryptic digest of eGFP samples expressed in presence of the different chemical triggers theophylline and thiamine and analyzed the resulting peptide fragments by high-performance liquid chromatography (HPLC). As shown in Figure 3B, the same peptide fragments were found in the digested protein samples. However, changed amounts of two peptides were found containing the amber stop codon site decoded with serine and alanine residues. The two peptides were verified by MS as FAVSGEGEGDATYGK (measured: 1488.3 Da; calcd: 1487.5 Da) and FSVSGEGEGDATYGK (measured 1504.4 Da; calcd: 1503.7 Da). Integration of the respective peaks in the HPLC elution profiles yielded quantitative data with respect to the performance of the switching process: the switching of amino acid identity was achieved to a ratio of 14/86 (Ser/Ala) in presence of 2 mM theophylline and 84/16 (Ser/Ala) in presence of 0.1 mM thiamine added to the growth medium. Hence, the ligand-dependent switching of tRNA systems results in some leakiness that is likely resulting from the imperfect switching performance of the individual switches (see the Supporting Information) and could possibly be optimized in future developments of this strategy.

In order to characterize the anticipated mechanism of ligand-induced tRNA activation, we analyzed ribozyme-tRNA levels using northern blots. Detection of the mature tRNA in the cleaved state is not possible since the natural, non-amber codon recognizing tRNA sequences are already expressed in *E. coli*. This is why we designed hybridization probes that target parts of the HHR sequence together with parts of the aptamer domain in order to discriminate the two expressed tRNA switches, see Figure 4. The probes were highly specific, detecting only the targeted RNA construct as evident by the absence of signals on the blot in samples that lack the respective tRNA switches. However, in presence of the tRNA switches, abundant fusion RNAs are only detected in absence of the respective ligands, see Figure 4. Hence, addition of thiamine or theophylline results in disappearance of the respective full length RNA construct in accordance with the anticipated mechanism of ligand-induced self-cleavage that yields products that are not recognized by the hybridization probes or degraded.

Although the RNA levels in vivo clearly respond to the externally added ligands, we next investigated whether the self-cleavage activities of the ribozyme−tRNA-fusion RNAs are indeed ligand-dependent. For this purpose, the full constructs tppON-tRNA$^{Ser}_{CUA}$ and theoON-tRNA$^{Ala}_{CUA}$ were in vitro transcribed. In order to prevent intramolecular cleavage during transcription, a blocking strand hybridizing to the catalytic core of the ribozyme was added in excess. After polyacrylamide gel electrophoresis purification, ribozyme reactions were initiated by addition of $MgCl_2$ to a final concentration of 2 mM in presence or absence of theophylline and TPP. The construct theoON-tRNA$^{Ala}_{CUA}$ exhibited a rate enhancement of almost 40-fold with a k_{obs} value of 0.1 min^{-1} in absence of theophylline and 3.89 min^{-1} in 2 mM theophylline, see Figure 5A. Upon

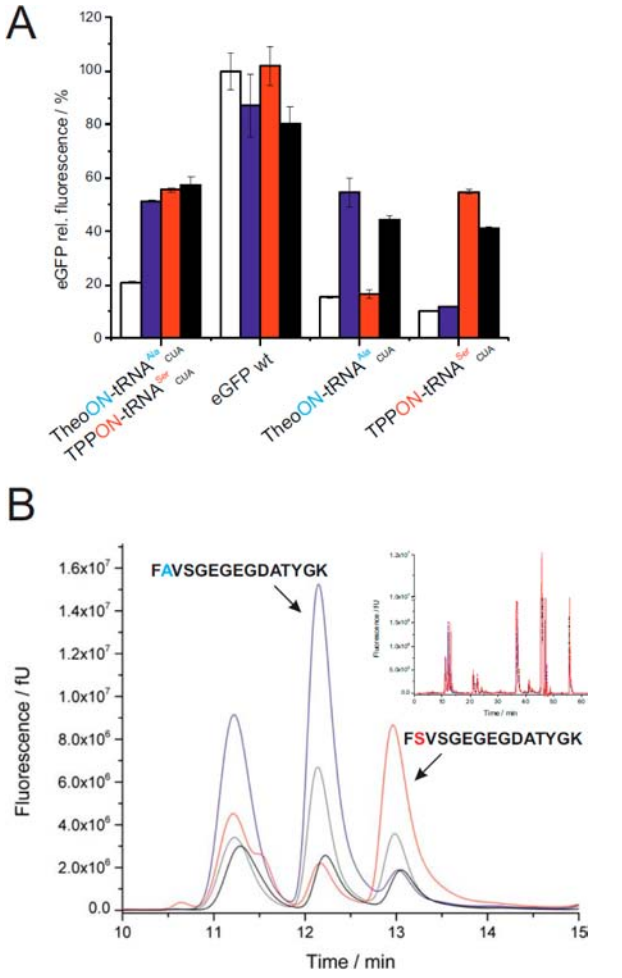

Figure 3. Analysis of amino acid incorporation into eGFP. (A) Fluorescence analysis of eGFP expression in *E. coli* using the following constructs: an alanine amber-suppressor tRNA under control of a theophylline-responsive ribozyme and simultaneously a serine amber-suppressor tRNA under control of a thiamine-responsive ribozyme (theoON-tRNA$^{Ala}_{CUA}$-tppON-tRNA$^{Ser}_{CUA}$); a control clone with a wild-type eGFP gene lacking the amber stop codon (eGFP wt); the individual alanine amber-suppressor tRNA under control of a theophylline-responsive ribozyme (theoON-tRNA$^{Ala}_{CUA}$); and the individual serine amber-suppressor tRNA under control of a thiamine-responsive ribozyme (tppON-tRNA$^{Ser}_{CUA}$). All eGFP mRNA constructs except eGFP wt contain an amber stop codon at amino acid position 50 of eGFP. White bars: no compound added; blue: 2 mM theophylline; red: 0.1 mM thiamine; black: both compounds added. (B) LC profile of the tryptically digested, His-tag purified protein expressed in absence (black line), presence of theophylline (blue), presence of thiamine (red), or both ligands simultaneously (gray) with combined theoON-tRNA$^{Ala}_{CUA}$-tppON-tRNA$^{Ser}_{CUA}$ systems. Insert shows whole LC elution profile, and the two peptides FAVSGEGEGDATYGK and FSVSGEGEGDATYGK are highlighted.

addition of TPP to the tppON-tRNA$^{Ser}_{CUA}$ construct, cleavage activity increased 43-fold from 0.0134 ± 0.0002 to 0.579 ± 0.030 min^{-1}, see Figure 5B. Addition of TPP to the theophylline-responsive construct and vice versa showed no significant influence on cleavage activity (data not shown). These results demonstrate that the designed tRNA switches are indeed responsive to the ligands theophylline and TPP, adding further evidence to the anticipated mechanism of regulating

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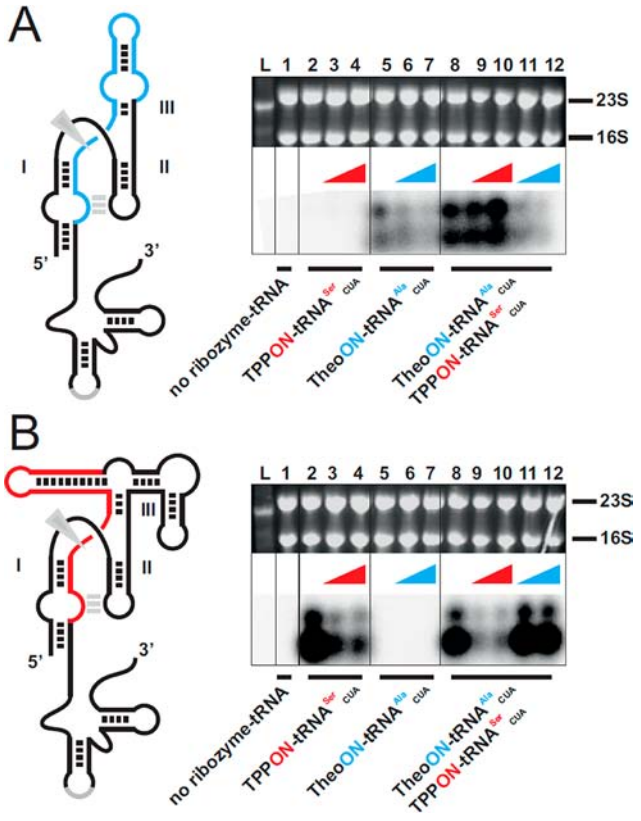

Figure 4. Northern blots of ligand-dependent HHR–tRNA fusion constructs. (A) Northern blot detecting the theophylline-induced system theoON-tRNA$^{Ala}_{CUA}$ using a hybridization probe complementary to the blue region of the RNA construct. (B) Detection of tppON-tRNA$^{Ser}_{CUA}$ construct using a probe directed against the RNA region shown in red. Cells expressing the indicated RNA systems were grown as follows: lane 1: no ligand added; 2: no ligand added; 3: 0.01 mM thiamine; 4: 0.1 mM thiamine; 5: no ligand added; 6: 0.2 mM theophylline; 7: 2 mM theophylline; 8: no ligand added; 9 and 10: 0.01 mM and 0.1 mM thiamine; 11 and 12: 0.2 mM and 2 mM theophylline.

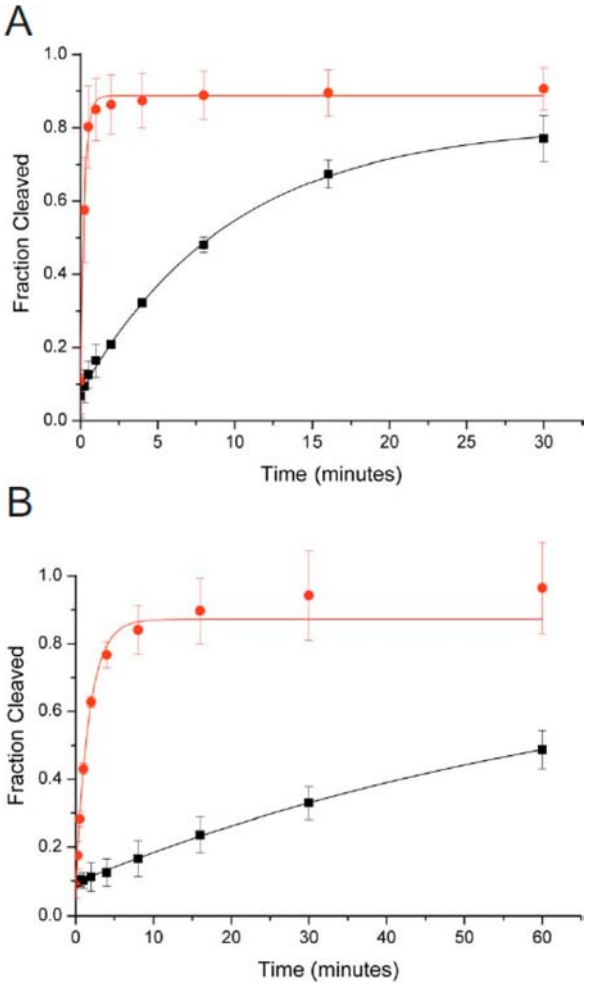

Figure 5. In vitro cleavage kinetics using purified RNA constructs. Ribozyme reactions were initiated by addition of $MgCl_2$ to a final concentration of 2 mM. (A) The self-cleavage of theoON-tRNA$^{Ala}_{CUA}$ proceeds with rates of k_{obs} = 0.10 ± 0.01 min^{-1} in absence and 3.89 ± 0.30 min^{-1} in presence of 2 mM theophylline, respectively, representing a 38.9-fold increase in activity. (B) The cleavage reaction of the RNA construct tppON-tRNA$^{Ser}_{CUA}$ is accelerated 43-fold from 0.0134 ± 0.0002 min^{-1} in absence of TPP to 0.579 ± 0.030 min^{-1} in presence of 0.1 mM TPP.

tRNA utilization in vivo via a ligand-dependent cleavage of the ribozyme–tRNA constructs.

Having characterized the anticipated regulatory mechanism, we next aimed at further increasing the applicability of the strategy by controlling the incorporation of an additional amino acid. In order to do so, we constructed a ribozyme-controlled amber tRNA system that is charged with leucine, see the Supporting Information for details. We then combined switchable tRNAs charged with serine and leucine by coexpressing two constructs tppON-tRNA$^{Ser}_{CUA}$ and theoON-tRNA$^{Leu}_{CUA}$ allowing for switching between these two amino acids in protein synthesis, see the Supporting Information for details. In conclusion, utilizing these two ribozyme-controlled tRNAs switching between serine and leucine is facilitated with incorporation ratios of 10/90 (Ser/Leu) in presence of 2 mM theophylline and 79/21 (Ser/Leu) in presence of 0.1 mM thiamine. Hence, the efficiency of switching amino acid identity is comparable to the serine/alanine system described in detail above. This result demonstrates the high modularity of the switchable tRNA systems.

In addition, we combined two other tRNA switches, again enabling switching between serine and leucine: the combination of a serine tRNA switched on by theophylline with a tRNA coding for leucine switched off by the same ligand allows for regulation of amino acid identity with only one chemical trigger needed (theoON-tRNA$^{Ser}_{CUA}$ and theoOFF-tRNA$^{Leu}_{CUA}$); in absence of theophylline, leucine is incorporated during translation, and upon addition of theophylline, serine is utilized, see the Supporting Information. The incorporation ratios are 18/82 (Ser/Leu) in absence of the ligand and 71/29 (Ser/Leu) in presence of 2 mM theophylline. In comparison to the two systems described above, the theoON-tRNASerCUA/theoOFF-tRNALeuCUA combination switches less efficiently but has the advantage that it can be used in complex media such as LB broth, since we use minimal, thiamine-depleted media for the thiamine-dependent RNA switches. However, the use of two ligands for controlling tRNA functionality has the advantage that, in total, four protein expression states can be addressed individually. For example, in the case of utilizing two ON-switches, when no compound is added, the protein is not expressed (or just in a truncated version); addition of one or the other chemical allows for expression of the desired protein

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variant; when both stimuli are added a mixture of proteins is obtained.

We have shown that the incorporation of amino acids serine, alanine, and leucine can be controlled by ligand-activated switching of the respective competing amber suppressor tRNAs. However, all experiments shown so far have been carried out with an amber stop codon at position 50 of eGFP. In order to demonstrate that the strategy is independent of the respective position, we alternatively inserted the amber codon at positions 108 and 169. At both sites, control of amino acid identity was achieved with comparable efficiencies utilizing the two switches tppON-tRNA$^{Ser}_{CUA}$ and theoON-tRNA$^{Ala}_{CUA}$, see Figure S11 of the Supporting Information for details.

CONCLUSIONS

In conclusion, we have demonstrated that amino acid identity can be regulated in vivo on a post-transcriptional level by switching designer tRNAs via ligand-dependent control of ribozyme activity. Without further manipulating the genotype of proteins, phenotypic properties such as enzyme activity, binding affinity, and so forth could now be controlled utilizing the introduced strategy. In principle, the system should even enable "pulse-chase"-like experiments with protein mutants and modifications. Since the protein of interest is expressed in all states of the experiment and only the residue of interest is changed, the study of proteins with multiple functions or essential and potentially dominant negative activities should be possible. In addition, the strategy should improve ways to study aspects of protein turnover and time-resolved experiments. Furthermore, the technique profits from but is also expected to contribute to the vibrant field of incorporating genetically encoded, unnatural amino acids in proteins.[22−24] Since the majority of such approaches available today rely on the use of amber suppressor tRNAs, the introduced system could be adapted easily to switching, for example, a natural amino acid to an unnatural, modified amino acid derivative. In general, since the ribozyme-based switching mechanism relies on controlling the formation of the cloverleaf tRNA secondary structure and the latter seems to be a universal prerequisite for tRNA processing and functionality in all kingdoms of life,[25,26] the introduced strategy should also be transferable to higher organisms.

ASSOCIATED CONTENT

Supporting Information

Details about the construction and characterization of the individual switches tppON-tRNA$^{Ser}_{CUA}$, theoON-tRNA$^{Ala}_{CUA}$, and theoON-tRNA$^{Leu}_{CUA}$, the dual systems tppON-tRNA$^{Ser}_{CUA}$-theoON-tRNA$^{Leu}_{CUA}$, and theoON-tRNA$^{Ser}_{CUA}$-theoOFF-tRNA$^{Leu}_{CUA}$, and additional ESI-MS data of tppON-tRNA$^{Ser}_{CUA}$-theoON-tRNA$^{Ala}_{CUA}$ and tppON-tRNA$^{Ser}_{CUA}$-theoON-tRNA$^{Leu}_{CUA}$, incorporation of amber codon at two alternative positions, and details about LC-MS analysis are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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