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Defining serine tRNA knockout as a strategy for effective repression of gene expression in organisms with a recoded genome

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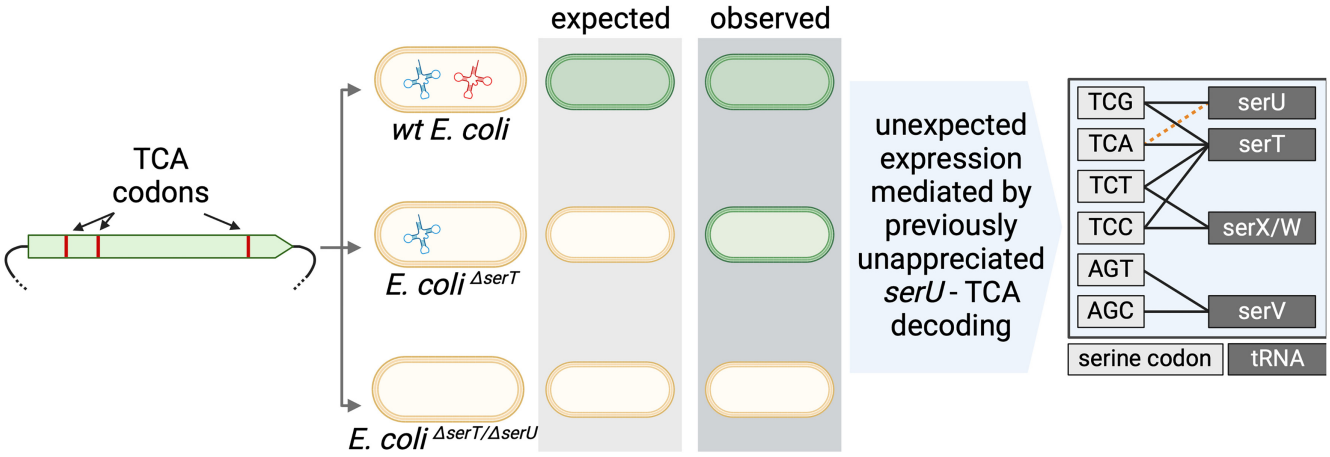
Abstract

Whole genome codon compression—the reassignment of all instances of a specific codon to synonymous codons—can generate organisms capable of tolerating knockout of otherwise essential transfer RNAs (tRNAs). As a result, such knockout strains enable numerous unique applications, such as high-efficiency production of DNA encoding extremely toxic genes or non-canonical proteins. However, achieving stringent control over protein expression in these organisms remains challenging, particularly with proteins where incomplete repression results in deleterious phenotypes. One platform enjoying increasing popularity utilizes serine TCA codon compression, relying on the prevailing understanding that deletion of $tRNA^{Ser(UGA)}$ (serT) would render the serine codon compressed organism incapable of translating any genes containing TCA codons. Here, we report evidence that $tRNA^{Ser(CGA)}$ (serU) can, surprisingly, also decode TCA, thereby precluding complete control over expression of TCA-containing genes in organisms with *serT* deletion. We then demonstrate the conditions necessary, including the precise modifications to the GRO and codon usage within the transgene, to overcome this interaction and achieve exceptionally stringent control over protein expression. Our findings provide critical insights and corresponding methods for guiding future use of serine codon compression for absolute control over protein expression, as well as a general strategy for optimizing repression via compression of other codons.

Graphical abstract



Introduction

The degeneracy of the standard genetic code, which describes how all 64 triplet codons encode the 20 standard amino acids, allows for multiple codons to encode the same amino acids (except tryptophan and methionine) (1). This feature has been exploited to create genomically recoded organisms (GROs) through a process termed whole genome codon compression, wherein all instances of selected codons are mutated to a synonymous codon (2,3). In turn, GROs are able to tolerate the knockout of the essential adapter molecules [i.e. transfer RNAs (tRNAs) or release factors] that decode the corresponding compressed codons (2,3). As these GROs cannot decode compressed codons (i.e. the codons that have been otherwise removed from the genome through synonymous mutations)

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in genes that contain them, it is possible to achieve exquisite control over expression of specific proteins by including these codons in the coding sequence (CDS) of the gene. Already, adapter molecule knockout in GROs has been adapted for synthesis of proteins containing non-standard amino acids (nsAAs) (3–7) as well as to provide a 'genetic firewall' that prevents horizontal gene transfer and enables pan-viral resistance (7). Since this strategy provides unparalleled repression of genes containing compressed codons, GROs may also serve as ideal biomanufacturing platforms for the production of toxic DNA and unstable gene circuits.

However, recent investigations into these firewalls have revealed them to be more complex than first thought. Despite the removal of two tRNAs, a recoded *Escherichia coli* strain was found to be susceptible to horizontal gene transfer when a mobile genetic element carries with it tRNA genes that provide a complementary source of those removed from this GRO (8). Similarly, viruses that encode tRNAs capable of decoding the compressed codons have been shown to infect this same knockout GRO strain (9). As these firewalls rely on the interruption of codon decoding during translation, it is essential that the factors that dictate how this happens, as well as their limitations, are fully defined and understood in order to realize the full promise of the expanded functionality of GROs.

To that end, we examined the interactions between compressed sense codons and their cognate adapter molecules in the most extensively codon compressed GRO to date, Syn61. Syn61 is an *E. coli* strain in which the sense codons TCA (i.e. UCA in RNA form) and TCG (i.e. UCG in RNA form) (coding for serine), as well as the stop codon TAG, have been compressed. This allows Syn61 to tolerate the knockout of *serU* (encodes $tRNA^{Ser(CGA)}$, herein referred to as serU), *serT* (encodes $tRNA^{Ser(UGA)}$, herein referred to as serT) and *prfA* (encodes Release Factor 1) (2). Using this strain, we surprisingly discovered that serU is actually a weak decoder of TCA, and its presence is sufficient to disrupt the otherwise stringent repression of genes containing a limited number of TCA codons in a *serT* knockout variant of Syn61. By varying the number of compressed codons in Syn61 variants lacking *serT* and *serU*, we report here the specific transgene design elements and GRO modifications that enable exceptionally stringent control of gene and protein expression, further expanding the use of the Syn61 GRO.

Materials and methods

Experimental models

Escherichia coli strains: All Syn61 variants were cultured at 37°C with shaking in 2xYT media supplemented with streptomycin (100 μg/ml). All remaining strains were cultured at 37°C with shaking in LB media. Strains harboring pKD46 were cultured at 30°C with shaking until curing was required.

Method details

Plasmid cloning

Plasmids PV260, PV480 and PV476 were cloned through Gibson HiFi (New England Biolabs, cat. no. E2621L) assembly of synthesized DNA fragments (IDT) according to Supplementary Table S6. tRNA, nanoluciferase (NanoLuc) and Bxb1 recombinase expression plasmids were cloned via Golden Gate assembly (New England Biolabs, cat. no. E1601L) from synthesized DNA fragments (Twist and IDT) into PV480 or PV476 backbones according to Supplementary Table S7 (see Supplementary Table S8 for all synthesized DNA sequences).

Serine tRNA knockout

Recombineering was performed to knock out both *serU* and *serT* (10). Briefly, double-stranded DNA knockout templates consisting of 350–500 bp regions homologous to the Syn61 genome immediately up- and downstream of the *serT* and *serU* genes, flanking a chloramphenicol resistance cassette, were synthesized (IDT). Syn61 cells harboring pKD46 were grown at 30°C in 2xYT + 10 μg/ml L-arabinose to an OD_{600} of 0.5–0.6 and made electrocompetent by four washes with cold 10% glycerol and electroporated (1.8 kV, 200 Ω, 25 μF, 1 mm gap cuvette) with 1–1.5 μg of knockout template. Transformed cells were recovered in SOC at 37°C for 1 h, plated on 2xYT plates supplemented with chloramphenicol (5 μg/ml) and incubated at 37°C until colony growth was visible.

Colonies were isolated and cultured at 42°C, followed by gDNA purification (New England Biolabs, cat. no. T3010L). Roughly 1 μg of purified gDNA was sent for whole genome nanopore sequence by Plasmidsaurus to confirm knockout.

Kanamycin resistance transformation assays

All Syn61 strains were made electrocompetent as in the above section. For the serU rescue experiments, electrocompetent Syn61Δ3 was first transformed with the tRNA expression plasmids PV516 or PV518 and then made electrocompetent again. The cells were electroporated as above with 250 ng of either PV260 or PV480 plasmid DNA in three independent replicates and recovered for 1 h in SOC media. 1:5 serial dilutions were made of the recovered cells, which were then plated on 2xYT plates supplemented with kanamycin (50 μg/ml). Dilution points with 10–100 colonies were used to quantify the number of transformants/ml.

NanoLuc assay

Two independent replicates of Syn61 variants harboring each of the NanoLuc expression plasmids were cultured for 16 h in 2xYT supplemented with kanamycin (50 μg/ml), after which OD_{600} was taken. Supernatant from the culture was then mixed with an equal volume of Nano-Glo reagent (Promega, cat. no. N1130) and luminescence was quantified via a Biotek Synergy H1 plate reader (gain: 100; integration time: 1 s). The following formula was used to calculate corrected luminescence values:

$$\text{Avg. luminescence} = \log_{10}\frac{\text{Lum}_1/\text{OD}_{600,1} \times \text{Lum}_2/\text{OD}_{600,2}}{2}.$$

Average luminescence values were then normalized on a 0–100 scale in GraphPad Prism.

Recombination assay

The same transformation procedure as for the kanamycin resistance (KanR) transformation assay was used to transform the Syn61 variants with each of the recombinase plasmids. After recovery, all cells were plated on 2xYT plates supplemented with kanamycin (50 μg/ml). Plates were incubated at 37°C until colonies were visible. One milliliter of LB was added to the plate and all colonies were resuspended by scraping. Plasmid DNA was then purified from the resuspended transformant population (Zymo Research, cat. no. D4036).

Nanopore sequencing was performed on the purified plasmid DNA by Plasmidsaurus.

A Python script (doi:10.5281/zenodo.10525174) was then used to determine the percent of reads that had undergone recombination. Briefly, this script filters reads by aligning them to the plasmid reference sequence; if the read is ≥70% the length of the reference and has ≥80% identity, the sequence flanked by the attP and attB sites and the attL sequence are both aligned to the read. If the flanked sequence is aligned with ≥75% identity and the attL sequence is not present, the read is considered to have not undergone recombination. If the flanked sequence is not present and the attL sequence is aligned with ≥99% identity, the read is considered to have undergone recombination.

Resources table

Reagent or resource	Source	Identifier
Bacterial and virus strains		
Syn61	Addgene	Cat#174513
Syn61Δ3	Addgene	Cat#174514
Syn61ΔT	This paper	
Syn61ΔU	This paper	
Chemicals, peptides and recombinant proteins		
Streptomycin sulfate	Sigma–Aldrich	Cat#S6501
Chloramphenicol	Sigma–Aldrich	Cat#R4408-10ML
L-(+)-Arabinose	Sigma–Aldrich	Cat#A3256-100G
Kanamycin sulfate	Sigma–Aldrich	Cat#60615-5G
Critical commercial assays		
NEBuilder HiFi DNA Assembly Master Mix	NEB	Cat#E2621L
NEBridge Golden Gate Assembly Kit (BsaI-HF v2)	NEB	Cat#E1601L
Monarch Genomic DNA Purification Kit	NEB	Cat#T3010L
Nano-Glo Luciferase Assay System	Promega	Cat#N1130
Zyppy Plasmid Miniprep	Zymo Research	Cat#D4036
Deposited data		
Raw whole plasmid sequencing reads	This paper	PRJNA1066256
Recombinant DNA		
pKD46	NovoPro Bioscience	V007013
See Supplementary Table S3 for plasmids generated in this study	This paper	
See Supplementary Table S4 for synthesized DNA sequences	This paper	
Software and algorithms		
Recombination Analyzer	This paper	doi:10.5281/zenodo.10525174

Results

serU (tRNA$^{Ser(CGA)}$) is a weak decoder of TCA codons

It is widely reported that serU (tRNA$^{Ser(CGA)}$) only decodes TCG and that TCA is only decoded by serT (tRNA$^{Ser(UGA)}$) (11–15). Given that genetic firewall effects in Syn61 are expected to be driven by these non-redundant interactions, we first generated Syn61 *serU* (Syn61ΔU) and *serT* (Syn61ΔT) single knockout strains (Figure 1B and C) and compared them against Syn61 and Syn61Δ3 (the *serU*, *serT* and *prfA* triple knockout strain) (Figure 1D). Based on the widely reported serine codon interaction map, only strains with a copy of *serT* should be susceptible to transformation by a plasmid encoding a KanR gene that contains 7 TCA codons out of 16 total serine codons (KanR^{TCA+}) (Figure 1E). As expected, no Syn61Δ3 transformants were observed on kanamycin plates. However, much to our surprise, we found that Syn61ΔT was actually susceptible to transformation by this plasmid, albeit with low efficiency (an ∼98% reduction in colonies relative to the same plasmid lacking TCA codons, KanR^{TCA-}) (Figure 1F and G, Supplementary Figure S1 and Supplementary Table S1).

To explore what allowed Syn61Δ3 to resist transformation compared to Syn61ΔT, we cloned a set of serU expression plasmids. Both plasmids encode the *E. coli serU* gene under control of the natural *serU* promoter, and differ only by their anticodon, where one possesses the wild-type *serU* anticodon (serU^{CGA}) and the other possesses the *serV* (present in both Syn61ΔT and Syn61Δ3) anticodon (serU^{GCU}) (Figure 1H). When Syn61Δ3 strains containing these plasmids were transformed with the same KanR plasmids, we found that the plasmid encoding wild-type *serU* (serU^{CGA}), but not the serU anticodon mutant plasmid (serU^{GCU}), rescued the transformation of Syn61Δ3 by KanR^{TCA+} (Figure 1I and Supplementary Table S2). Indeed, consistent with the low efficiency observed in Syn61ΔT, transformation by the KanR^{TCA+} plasmid, Syn61Δ3 containing serU^{CGA} was susceptible to low levels of transformation by KanR^{TCA+}, at ∼2% the efficiency of the KanR^{TCA-} plasmid. These results directly indicate that the tRNA encoded by *serU* in Syn61, tRNA$^{Ser(CGA)}$, is a decoder of TCA codons. Despite the seemingly poor efficiency of this interaction, the presence of *serU* was sufficient to circumvent the genetic firewall imposed by *serT* knockout in Syn61, allowing biologically relevant levels of expression of TCA-containing transgenes.

Compressed codon content in genes repressed by GROs

With this unexpected observation, we next examined the compressed codon thresholds that govern gene repression in tRNA knockout GROs. We first generated a set of nine plasmids containing the gene encoding NanoLuc, a 192 aa protein with six serine residues that we designed to be encoded by a differing number of TCA and/or TCG codons. When transformed into our tRNA knockout GRO panel (Syn61, Syn61ΔT, Syn61ΔU and Syn61Δ3), the presence of TCG codons in combination with *serT* and *serU* double knockout resulted in the tightest repression. With at least three TCG codons, we found that Syn61Δ3 exhibited the greatest repression, with over four orders of magnitude reduced NanoLuc expression. In contrast, luminescence from Syn61ΔT with NanoLuc containing as many as six TCA codons was only reduced by <2 orders of magnitude, consistent with the observations above, while there was no change in expression observed with any TCA or TCG combination in Syn61ΔU (Figure 2A and Supplementary Tables S3 and S4). We next investigated how these recoded codon thresholds extend beyond a small reporter gene.

Site-specific recombinases (SSRs) are commonly used elements in gene circuits, but their exceptionally high recombination efficiency means that even minimal leaky expression can profoundly impact gene circuit function (16,17). Thus, we again engineered a panel of plasmids containing (i) the Bxb1 SSR gene (500 aa with 25 serine residues) (18) with varying numbers of TCA and TCG codons, and (ii) attP and attB sites (the sequences the SSR acts upon) oriented in such a manner that their recombination by the SSR (which forms a distinct attL site) will excise ∼800 bp from the plasmid. We then evaluated this panel of Bxb1 SSR expression plasmids in the same tRNA knockout GRO panel by perform-

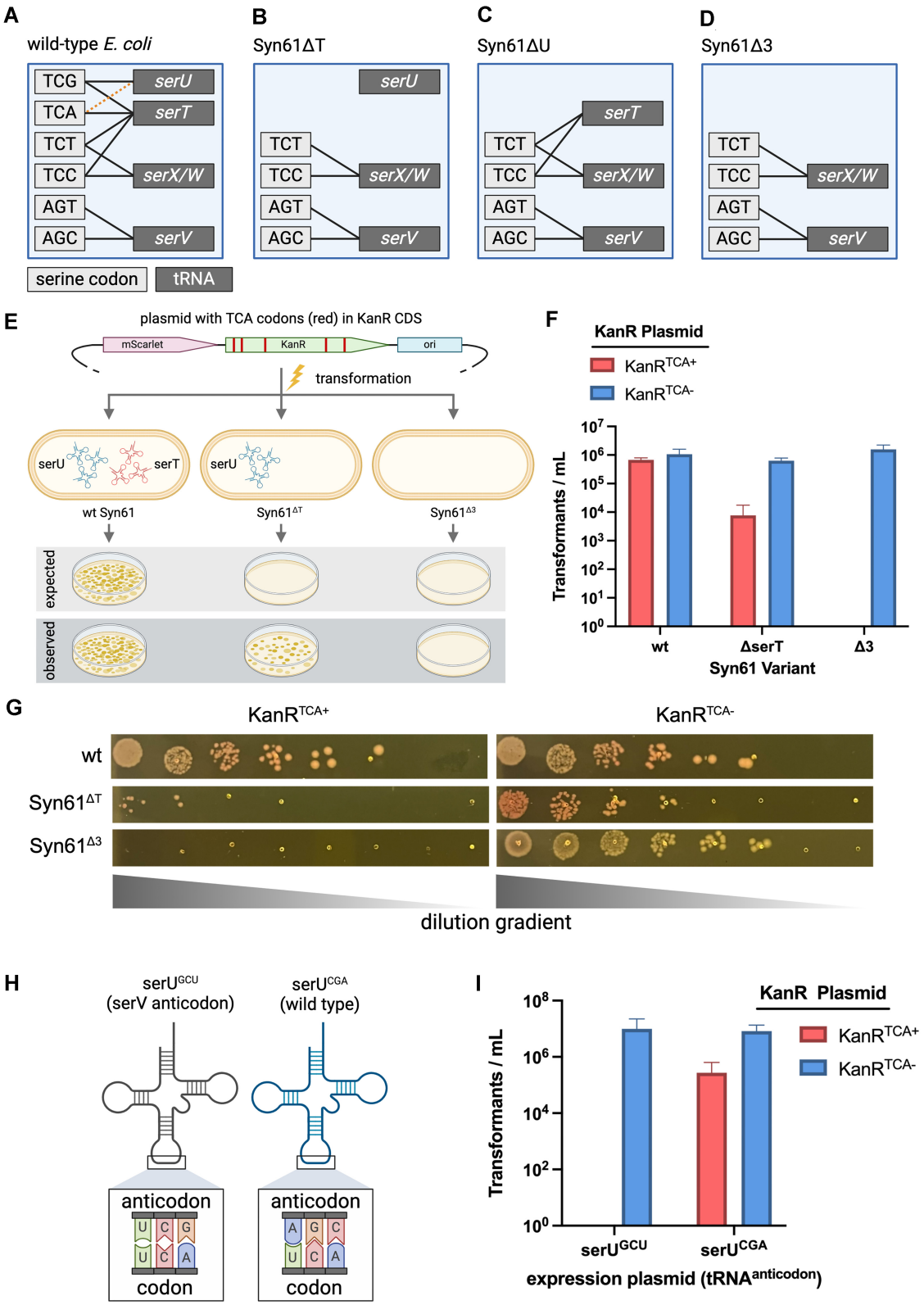

Figure 1. serU (tRNA$^{Ser(CGA)}$) decodes TCA codons. The tRNA–codon interaction map of (**A**) wild-type *E. coli* (dashed orange line represents the proposed serU decoding of TCA described here), (**B**) Syn61$^{\Delta T}$, with *serT* (encodes tRNA$^{Ser(UGA)}$) knocked out, (**C**) Syn61$^{\Delta U}$, with *serU* (encodes tRNA$^{Ser(CGA)}$) knocked out, and (**D**) Syn61$^{\Delta 3}$, with both *serT* and *serU* knocked out. In all Syn61 strains, every TCA and TCG codon has been removed from the genome via synonymous mutations, indicated by the lack of codon boxes. (**E**) A plasmid encoding a KanR gene is transformed into different Syn61 tRNA knockout variants (vertical lines represent the presence of TCA codons in the KanR CDS). Given the widely reported interaction map in panel (A), transformation of this plasmid into Syn61ΔT and Syn61Δ3 is expected to not yield transformants. (**F**) Mean + standard deviation of colony forming units (transformants) resulting from this transformation reaction ($n = 3$). (**G**) Representative images of the transformant plates quantified in panel (F). Syn61Δ3 containing (**H**) helper plasmids that encode wild-type serU (with anticodon CGA) or a form of serU where the anticodon has been mutated to that of serV (GCU) were transformed with the same KanR plasmids as in panels (F)–(G). (**I**) Mean + standard deviation of transformants resulting from this transformation reaction ($n = 3$). Created in BioRender. Voorhees, P. (2025) https://BioRender.com/v18p889.

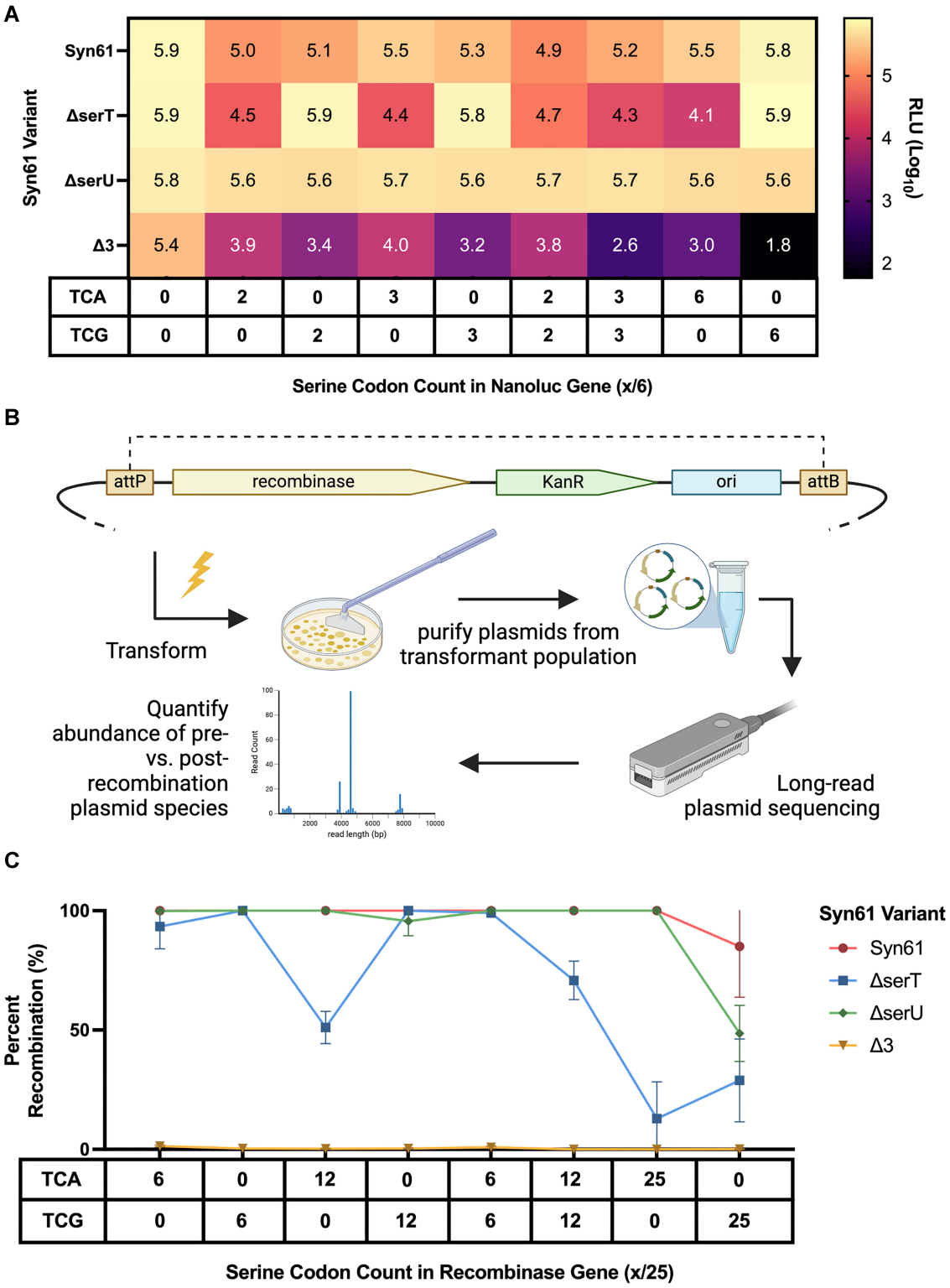

Figure 2. Compressed codons mediate repression in select GROs only if sufficient codons are present. (**A**) Mean $\log_{10}$ luminescence intensity across a panel of Syn61 tRNA knockout variants (*y*-axis) transformed with a NanoLuc gene containing varying numbers of TCA and TCG codons in the coding sequence (*x*-axis) ($n = 2$). (**B**) Overview of the recombination assay. A panel of plasmids encoding (i) the Bxb1 site-specific recombinase with varying numbers of TCA and TCG codons in the coding sequence and (ii) the Bxb1 attP and attB sites, which are oriented such that their recombination by the SSR (which forms an attL site) excises ∼800 bp from the plasmid, is transformed into Syn61 tRNA knockout variants. Plasmid DNA is purified from the entire population of transformants and sequenced by long-read nanopore sequencing. The reads are then analyzed for signatures of recombination and the percent of sequenced plasmid molecules that have undergone recombination is calculated. (**C**) Mean + standard deviation of percent recombination from the protocol described in panel (B) ($n = 2$). Created in BioRender. Voorhees, P. (2025) https://BioRender.com/v26h108.

ing long-read nanopore sequencing of plasmid DNA purified from the whole transformant population to quantify the number of plasmid molecules that had undergone recombination (Figure 2B). As expected, 100% of the sequenced plasmid molecules were recombined in Syn61 (*serU* and *serT* competent). Consistent with the NanoLuc results, we saw no recombination with plasmids containing 6–25 TCG codons in Syn61Δ3, suggesting complete repression with as few as 6 TCG codons. In Syn61ΔT, plasmids with 6–12 TCA codons in the SSR gene exhibited low to moderate levels of repression, while the plasmid with 25 TCA codons in the SSR gene yielded full repression. These results suggest that a much larger TCA codon threshold is needed to effectively shut off expression in GROs lacking *serT*. Interestingly, <100% recombination was observed in Syn61ΔT when all 25 serines in the SSR gene were encoded by TCG codons. In Syn61ΔU, little to no recombination was observed with the presence of 6–25 TCA codons and 6–12 TCG codons. However, similar to Syn61ΔT, less than half the sequenced plasmid molecules were recombined with 25 TCG codons present (Figure 2C and Supplementary Table S5).

Discussion

The recently developed GRO, Syn61, is built in part on the long-standing assumption that serU ($tRNA^{Ser(CGA)}$) decodes only TCG, and TCA is only decoded by serT ($tRNA^{Ser(UGA)}$) (Figure 1A). Thus, serine codon compression that eliminates all TCA and TCG codons would allow for removal of both *serU* and *serT*. In this context, knocking out *serT* alone from Syn61 should also prevent the decoding and expression of proteins encoded by genes containing any number of TCA codons. Here, we show that, contrary to this prevailing understanding (11–15), *serU* can actually decode TCA. Our findings here appear to be consistent with two recent studies on GROs (8,9), which have shown that Syn61Δ3 is susceptible to viruses and mobile genetic elements when they carry with them a copy of either *serU* or a viral $tRNA^{Ser(CGA)}$. Consequently, this incomplete firewall allows for expression of genes with up to 12 TCA codons present at biologically relevant levels.

While translation of genes containing TCA codons in Syn61ΔT was detectable in all assays used here (including the serU rescue assay in Syn61Δ3; Figure 1I), indicating decoding of TCA by serU, it should be noted that the translation efficiency with this underappreciated tRNA interaction appears relatively low. In the antibiotic resistance assay, there was a >98% reduction in the number of Syn61ΔT colonies that formed when the KanR CDS contained TCA. In the NanoLuc assay, there was a >95% reduction in the amount of luminescence the Syn61ΔT sample produced compared to the Syn61 sample when the NanoLuc CDS contained six TCA codons. Finally, in the recombination assay, there was a >87% reduction in the amount of recombination observed in Syn61ΔT when the recombinase CDS contained 25 TCA codons. These results clearly underscore that *serT* deletion can offer meaningful reductions in protein expression. It is, thus, only when exceptionally stringent repression is needed that *serT* deletion alone is inadequate. This also likely explains why the decoding of TCA by serU was not previously discovered.

Recent developments in our understanding of decoding indicate that the ribosome restricts the geometry of the first and second base pairs during codon–anticodon pairing in the decoding center of the ribosome, such that they must be canonical Watson–Crick base pairs and maintain a Watson–Crick-like helix (19). The geometry of the third pair is not constrained, however. Thus, the data put forth here suggest that, while not a canonical Watson–Crick pair, the A·C wobble pair—which can occur through *trans* Watson–Crick/Watson–Crick, *trans* Watson–Crick/Hoogsteen and *trans* Watson–Crick/sugar-edge base pairing (20)—observed at the third position of the TCA–CGA interaction when serU decodes TCA is likely tolerated and sufficient to allow decoding. Despite this, perfect Watson–Crick pairing of the first two bases of the TCA codon only has moderate thermodynamic favorability (−2.2 kcal/mol versus −3.1 kcal/mol when the first two bases contain only G/C) (21). By further decreasing favorability through an A·C wobble pair, TCA–CGA pairing may not be kinetically favored. While this may help explain both why it is so often misreported that TCA codons cannot be decoded by serU and why we see dramatically reduced expression in our data when TCA codons are present in a Syn61ΔT-translated transcript, further experiments are needed to mechanistically validate the interactions between serU and TCA.

Beyond the unexpected serU and TCA interactions, our data demonstrate the parameters by which complete repression of gene expression can be achieved in Syn61. Specifically, within the sensitivity of our assays, we found that knocking out both *serU* and *serT* affords complete repression of proteins encoded with as few as six TCG codons in its gene. In contrast, when only *serU* is knocked out, expression levels do not change, as serT can adequately decode TCA and TCG codons. More importantly, when only *serT* is knocked out, expression is only markedly reduced (though still not completely prevented) when as many as 25 TCA codons are present in a gene. This observed reduction in expression is likely attributed to multiple factors. In addition to the potential contribution of the unfavorable kinetics of the TCA–serU interaction during decoding, the more dramatic reduction in expression we observed in our recombination assay when the gene contained 25 TCA codons may also be due, in part, to a bottleneck caused by the size of the serU tRNA pool. SerU is the lowest abundance serine tRNA, with only ∼25% the amount of serT molecules (15), and it is possible that such a high number of TCA codons in a constitutively expressed transcript driven by a strong promoter may over burden this relatively small resource pool. Importantly, our data also indicate that TCG codons yield superior levels of repression in Syn61Δ3, compared to the same number of TCA codons (by roughly an order of magnitude, on average). Together, these results provide a clear blueprint for a method of blocking transgene expression with exquisite control.

With the promise to enhance cellular properties and expand their functions, genome recoding and GROs stand at the forefront of synthetic biology and bioengineering (22). It is, therefore, essential to develop a complete understanding of the codon–tRNA interactions that mediate these functions. Our work here suggests that further investigations of our current understanding of tRNA–codon interactions in GROs are likely needed to achieve complete control over the functions of these organisms. A better understanding of these interac-

tions will be critical not only in achieving full control of gene expression in GROs, but also in preventing circumvention of genetic firewalls by viruses and mobile genetic elements, producing safer biocontainment systems, and in realizing purer, more reproducible production of proteins containing nsAAs.

Data availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact: Samuel K. Lai (lai@unc.edu).

Materials' availability

Plasmids generated in this study are available from the lead contact with a completed material transfer agreement. Syn61 and Syn61Δ3 are available through Addgene (#174513 and #174514, respectively). There are restrictions to the availability of Syn61ΔT and Syn61ΔU due to a material transfer agreement, but these strains may be requested from Jason W. Chin.

Data and code availability

Whole genome sequencing data for Syn61 single knockout strains have been deposited to NCBI (BioProject: PRJNA1065929). Whole plasmid sequencing data have been deposited to NCBI (BioProject: PRJNA1066256). Code has been deposited to https://zenodo.org/doi/10.5281/zenodo.10525173.

Supplementary data

Supplementary Data are available at NAR Online.

Acknowledgements

Syn61 (Addgene plasmid #174513) and Syn61Δ3 (Addgene plasmid #174514) were a gift from Jason W. Chin. Figures were created, in part, with BioRender.com.

Author contributions: Conceptualization, P.J.V. and S.K.L.; methodology, P.J.V.; software, P.J.V.; investigation, P.J.V. and X.C.; formal analysis, P.J.V.; writing—original draft, P.J.V.; writing—review & editing, P.J.V. and S.K.L.; funding acquisition, S.K.L.

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Conflict of interest statement

P.J.V. and S.K.L. are inventors of a provisional patent application covering the use of genetically recoded organisms to produce specific viral vectors. The terms of these arrangements are managed by UNC-CH in accordance with its conflict-of-interest policies.

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