Genetic Code Expansion

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MacMillan Research Group
Group Meeting
June 7th, 2022
The Central Dogma of Molecular Biology

“DNA makes RNA makes protein”
The Central Dogma of Molecular Biology

DNA makes RNA makes protein

The 20 canonical amino acids
Outside of few exceptions, proteins are made up of the 20 canonical amino acids
Expanding the Genetic Code

Synthetic organic chemistry allows us to make amino acids that are “noncanonical”

Proline analogue

Tyrosine analogue

EPR probe

Bioorthogonal click handle

Photoaffinity label
Expanding the Genetic Code

Synthetic organic chemistry allows us to make amino acids that are “noncanonical”

Proline analogue

Tyrosine analogue

EPR probe

Bioorthogonal click handle

Photoaffinity label

Is it possible to expand the genetic code to incorporate noncanonical amino acids into proteins?
Expanding the Genetic Code

- The traditional genetic code
- Expanding the genetic code
  - In vitro
  - In eukaryotes, prokaryotes, and mammalian cells
- Orthogonal ribosomes
- Genetically recoded organisms and synonymous codon compression
- Outlook
The Genetic Code

How does DNA / RNA sequence encode for protein sequence?
The Genetic Code

**Codon**: Sequence of three nucleotides

- 61 codons encode for amino acids
- 3 codons encode for stop codons

Codons specify which amino acid will be added during protein synthesis

Image source: Genomenon
The Genetic Code

Codons specify which amino acid will be added during protein synthesis

Image source: Genomenon
Translation of RNA to Protein via the Ribosome

Aminoacyl tRNA charged with an amino acid through its anticodon loop binds to a complementary codon

Image source: Biorender
Translation of RNA to Protein via the Ribosome

1. Initiation

2. Elongation

3. Termination

Released peptide

Image source: Biorender
The aminoacyl tRNA synthetase is responsible for “charging” the tRNA with amino acid to form an aminoacyl tRNA.
Production of Aminoacylated tRNAs

Production of Aminoacylated tRNAs

For each amino acid, there is at least one unique aminoacyl tRNA

In most organisms, for each amino acid, there is a unique aminoacyl tRNA synthetase.

The Adaptor Hypothesis

“Amino acid position within a protein is determined by the binding of mRNA with a tRNA carrying the amino acid”

Cysteine aminoacyl tRNA

mRNA

Cysteine is incorporated into the protein

Alanine aminoacyl tRNA

mRNA

Alanine is incorporated into the protein

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A General Method for the Synthesis of “Misacylated” tRNAs

**Problem:** tRNA charged with protecting group cannot be accepted by the ribosome

Protein Synthesis with Unprotected Aminoacyl tRNAs

Boc protection and deprotection enables synthesis of pCpA charged with unprotected amino acid

Protein Synthesis with Unprotected Aminoacyl tRNAs

pCpA charged with unprotected amino acid

T4 RNA ligase

pCpA charged with unprotected amino acid

tRNA\textsuperscript{Phe} charged with unprotected noncanonical amino acid can be synthesized

## Stop Codons

Of the three stop codons, the Amber stop codon is used the least in the genome.

<table>
<thead>
<tr>
<th>Codon</th>
<th>E. coli</th>
<th>Yeast</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amber</td>
<td>UAG</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Ochre</td>
<td>UAA</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Opal</td>
<td>UGA</td>
<td>1.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Codon frequency data:** https://www.genscript.com/tools/codon-frequency-table

**Amber Suppression with a Amber Suppressor tRNA Charged with Phe**

**Amber suppression:** Amber codon (UAG) has been reassigned from encoding “stop” to encoding an amino acid.

**RNA sequence:**

```
UUU AAC AAC CGG GCG
```

**Protein sequence:**

```
Phe Asn Asn Arg Ser Stop
```

**Amber Suppression**

```
Phe Asn Asn Arg Ser Phe Ser Asn Arg Stop
```

**Amber suppression:** 9 amino acid peptide

**Amber Suppression with a Amber Suppressor tRNA Charged with Phe**

**Step 1: Preparation of tRNA\textsuperscript{CUA} (amber suppressor tRNA) via anticodon loop replacement**

1) HCl-aniline
2) RNase A
3) Insert amber suppressor anticodon

Amber Suppression with a Amber Suppressor tRNA Charged with Phe

Step 2: Preparation of tRNA\textsuperscript{CUA} charged with phenylalanine

Phenylalanine tRNA synthetase accepts tRNA\textsuperscript{CUA}

Amber Suppression with an Amber Suppressor tRNA Charged with Phe

Amber Suppression with a Amber Suppressor tRNA Charged with Phe

Full length protein expression is dependent upon presence of tRNA charged with phenylalanine

The Precedent for the Breakthrough

**Precedent 1:**
tRNAs can be charged with any amino acid using T4 ligase and pCpA-aa

The Precedent for the Breakthrough

Precedent 2: Phe can be incorporated at amber codon

Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schutz, P. G. Science 1989, 244, 182.
"aa" can be an unnatural amino acid

Precedent 2:
Phe can be incorporated at amber codon

The Breakthrough: Amber Suppression to Incorporate Noncanonical Amino Acids

Peter Schultz
Current Institution: Scripps Research

“aa” can be an unnatural amino acid

Can noncanonical amino acids be incorporated into proteins using this method?

Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schutz, P. G. Science 1989, 244, 182.
The Breakthrough: Amber Suppression to Incorporate Noncanonical Amino Acids

Preparation of pCpA charged with noncanonical amino acid

1) NPSCI, TEA
2) NPS-amino acid, CDI
3) Thiosulfate

14% overall yield

Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schutz, P. G. Science 1989, 244, 182.
The Breakthrough: Amber Suppression to Incorporate Noncanonical Amino Acids

pCpA charged with noncanonical amino acid

The Breakthrough: Amber Suppression to Incorporate Noncanonical Amino Acids

Method can be used to prepare \( \text{tRNA}^{\text{CUA}} \) with a variety of noncanonical amino acids

The Breakthrough: Amber Suppression to Incorporate Noncanonical Amino Acids

Previously prepared: tRNA$^{\text{CUA}}$ charged with Phe

Native β-lactamase can be expressed in E. coli lysate using amber suppression

Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schutz, P. G. Science 1989, 244, 182.
The Breakthrough: Amber Suppression to Incorporate Noncanonical Amino Acids

\[ \text{tRNA}^{\text{CUA}} \text{ charged with noncanonical amino acid} \]

Amber Suppression

Noncanonical amino acid

\[ \text{mRNA for } \beta\text{-lactamase} \rightarrow \text{Position 66 = noncanonical amino acid} \]

\[ \text{tRNA}^{\text{CUA}} \text{ charged with noncanonical amino acid incorporates the noncanonical amino acid at position 66 in the protein} \]

Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schutz, P. G. Science 1989, 244, 182.
The Breakthrough: Amber Suppression to Incorporate Noncanonical Amino Acids

\[ \text{tRNA}_{CUA} \text{ charged with noncanonical amino acid} \]

\[ \text{mRNA for } \beta\text{-lactamase} \]

\[ \text{UAG} \to \text{UAA} \]

\[ \text{Amber Suppression} \]

\[ \text{Noncanonical amino acid} \]

\[ \text{Position 66} = \text{noncanonical amino acid} \]

\[ \text{tRNA}_{CUA} \text{ charged with noncanonical amino acid incorporates the noncanonical amino acid at position 66 in the protein} \]

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The Breakthrough: Amber Suppression to Incorporate Noncanonical Amino Acids

The mRNA for β-lactamase is charged with a noncanonical amino acid at position 66. This results in the incorporation of the noncanonical amino acid at position 66 in the protein.

Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schutz, P. G. Science 1989, 244, 182.
**The Breakthrough: Amber Suppression to Incorporate Noncanonical Amino Acids**

Properties of β-lactamase mutants with noncanonical amino acids

<table>
<thead>
<tr>
<th>Amino acid at position 66 of β-lactamase</th>
<th>Km (substrate binding)</th>
<th>kcat (turnover #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type β-lactamase</td>
<td>55 ± 5</td>
<td>880 ± 10</td>
</tr>
<tr>
<td>β-lactamase prepared using tRNA^{CUA} with Phe or noncanonical amino acid</td>
<td>59 ± 6</td>
<td>870</td>
</tr>
<tr>
<td></td>
<td>59 ± 2</td>
<td>1120 ± 290</td>
</tr>
<tr>
<td></td>
<td>57 ± 4</td>
<td>370 ± 70</td>
</tr>
<tr>
<td></td>
<td>72 ± 14</td>
<td>150 ± 60</td>
</tr>
</tbody>
</table>

para-fluorophenylalanine increases enzyme turnover number

Expanding the Repertoire of Noncanonical Amino Acids

A diverse range of noncanonical amino acids can be incorporated via amber suppression.

Changes in amino acid substitution at Ala82 affect thermal stability of the protein.

Expanding the Repertoire of Noncanonical Amino Acids


Alternate Codons for UAA Incorporation: Quadruplet Codons

Stop Codons
- Amber: UAG
- Ochre: UAA
- Opal: UGA

Problem: limited range (3) of endogenous codons to suppress

Solution: quadruplet codons
- ACCU
- CCCU
- CUAU
- AGGU
- CUCU
- CCUA
- CGGU
- GGGU

Alanine is incorporated at UCCA codon

Alternate Codons for UAA Incorporation: Quadruplet Codons

Frameshift Codon Suppression

charged quadruplet tRNA

mRNA coding for streptavidin

When the frameshift does not happen, a termination codon (UAA) appears, forming the truncated protein

Expanding the Genetic Code

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**Incorporation of UAAs in Eukaryotic Systems**

**5-hydroxytryptamine type 3 receptor (5-HT₃)**

Serotonin binding leads to ion channel opening

**Proline 8**

*Hypothesis:* Proline 8 plays an important role linking serotonin binding to channel opening through proline cis-trans isomerization

**Question:** How does cis-trans isomerization of proline 8 influence ion channel opening?

Incorporation of UAAs in Eukaryotic Systems

Proline analogues favoring the cis conformer

Proline analogues favoring the trans conformer

Incorporation of UAAs in Eukaryotic Systems

- tRNA charged with proline analogue
- mRNA labeled with an amber codon
- Xenopus oocytes (Frog egg)
- 5-HT receptor

Overall layout of the 5-HT receptor shows two adjacent subunits from a homology model of the 5-HT receptor. The neurotransmitter binding site Trp is shown in blue, with Pro 8* in the M2–M3 loop and parts of M2 and M3, shown in purple; loop 7 is red; loop 2 is green; the conserved Leu thought to be critical to the switch that interconverts the open and closed states of the channel is shown in blue, with Pro 8* in the region corresponding to the peptide studied by NMR (Fig. 4), comprising 2–4–8 helix. This indicates that mutations at Pro 8* in channel gating can be explored. The neurotransmitter binding site in the Cys-loop superfamily of ion channels is located about 60 Å from the channel pore, presenting a conundrum as to the molecular events that link binding and activation. Our results thus confirm the structure of the M2–M3 loop and the critical role of Pro 8* in the 5-HT receptor. To explore the possible role of Pro 8* in channel gating, we used site-directed mutagenesis, a series of proline analogues with varying preference for isomerization at a proline to study the effect on the gating of the receptor.

Evidence implicates the M2–M3 loop, and in low-resolution structural studies this region interacts with loops 2 and 3. In the cation-selective nACh receptor, the apex of the M2 loop is trans and the nACh receptor has the appropriate proline analogue attached at the 3 helix. This proline is essential for receptor function in the 5-HT receptor. When incorporated into a protein, proline disrupts main-chain hydrogen bonding, because it lacks a backbone NH moiety, disrupting the hydrogen bonds of the main chain. The binding site Trp is shown in blue, with Pro 8* in the region corresponding to the peptide studied by NMR (Fig. 4), comprising 2–4–8 helix. This indicates that mutations at Pro 8* in channel gating can be explored. The neurotransmitter binding site in the Cys-loop superfamily of ion channels is located about 60 Å from the channel pore, presenting a conundrum as to the molecular events that link binding and activation. Our results thus confirm the structure of the M2–M3 loop and the critical role of Pro 8* in the 5-HT receptor. To explore the possible role of Pro 8* in channel gating, we used site-directed mutagenesis, a series of proline analogues with varying preference for isomerization at a proline to study the effect on the gating of the receptor.

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Incorporation of UAAs in Eukaryotic Systems

![Structures of amino acids: Pro, Pip, Aze, Tbp, Dmp](attachment:image.png)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Per cent cis*</th>
<th>EC_{50} (μM)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro</td>
<td>5</td>
<td>1.29 ± 0.07</td>
</tr>
<tr>
<td>Pip</td>
<td>12</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>Aze</td>
<td>18</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>Tbp</td>
<td>55</td>
<td>0.030 ± 0.024</td>
</tr>
<tr>
<td>Dmp</td>
<td>71</td>
<td>0.021 ± 0.009</td>
</tr>
</tbody>
</table>

Percent cis of proline isomer and EC_{50} for serotonin dependent ion channel opening are correlated.

Proline analogues favoring the cis isomer produce an ion channel highly dependent on serotonin binding.

Incorporation of UAAs in Eukaryotic Systems

Results suggest cis-trans isomerization of proline 8 interconverts the open and closed states of the ion channel.

Noncanonical Amino Acid Incorporation in Live Cells

All works mentioned thus far prepared tRNA_{CUA} charged with non canonical amino acid via T4 RNA ligase

**Question:** Can the tRNA_{CUA} charged with the non canonical amino acid be generated directly in cells?

Noncanonical Amino Acid Incorporation in Live Cells

Solution: Express enzymatic machinery to generate aminoacylated tRNA directly E. coli

The aminoacyl tRNA synthetase is responsible for “charging” the tRNA with amino acid to form an aminoacyl tRNA

For the 20 canonical amino acids, there are at least 20 different aminoacyl tRNAs charged by 20 different aminoacyl tRNA synthetases

Noncanonical Amino Acid Incorporation in Live Cells

The quest for an orthogonal tRNA / aminoacyl tRNA synthetase pair to be used in E. coli

Orthogonal aminoacyl tRNA synthetase

Cannot recognize endogenous tRNAs or amino acids

Orthogonal tRNA

Cannot be recognized by endogenous E. coli aminoacyl tRNA synthetases

Noncanonical amino acid

Cannot be recognized by endogenous aminoacyl tRNA synthetases

tRNA\textsuperscript{CUA} charged with noncanonical amino acid in live cells

Noncanonical Amino Acid Incorporation in Live Cells

The quest for an orthogonal tRNA / aminoacyl tRNA synthetase pair to be used in E. coli

Methanococcus jannaschii

Hyperthermophilic organism belonging to the kingdom Archaea

The tyrosyl tRNA / aminoacyl tRNA synthetase pair from M. jannaschii

- tRNA synthetase not recognize E. coli tRNAs
- Previously shown to charge tRNA^{CUA} with Tyr
- Can perform amber suppression in E. coli

Can the tyrosyl tRNA / aminoacyl tRNA synthetase pair from Methanococcus be evolved to be orthogonal?

Noncanonical Amino Acid Incorporation in Live Cells

Problem: orthogonal tRNA is still recognized by E. Coli aminoacyl tRNA synthetases

(t almost orthogonal, but not yet)

mutated tRNA_{CUA} library

endogenous from E. coli

mRNA for Barnase w/ amber codon

UAG

Barnase Ribonuclease (degrades RNA)

Problem: orthogonal tRNA is still recognized by E. Coli aminoacyl tRNA synthetases

Negative selection: If endogenous aminoacyl tRNA synthetase charges tRNA_{CUA}, barnase is produced, leading to cell death

Result: Orthogonal tRNA_{CUA} that cannot be recognized by E. coli aminoacyl tRNA synthetases

Noncanonical Amino Acid Incorporation in Live Cells

Goal: Evolve an orthogonal aminoacyl tRNA synthetase

氨酰化tRNA合成酶

氨酰化tRNA

从M. jannaschii

非典型氨基酸的tRNA

OH

AUC

**Noncanonical Amino Acid Incorporation in Live Cells**

**Goal:** Evolve an orthogonal aminoacyl tRNA synthetase

- Mutant aminoacyl tRNA synthetase library
- Orthogonal tRNA<sub>CUA</sub>
- Noncanonical amino acid
- mRNA for Chloramphenicol resistance w/ amber codon
- Chloramphenicol acetyltransferase
- Antibiotic resistance gene

Noncanonical Amino Acid Incorporation in Live Cells

**Goal:** Evolve an orthogonal aminoacyl tRNA synthetase

Mutant aminoacyl tRNA synthetase library

Noncanonical amino acid

**Positive selection:** If the mutant aminoacyl tRNA synthetase can charge tRNA\(^{\text{CUA}}\) with any amino acid, the cells are antibiotic resistant

Chloramphenicol acetyltransferase
Antibiotic resistance gene

Noncanonical Amino Acid Incorporation in Live Cells

**Goal:** Evolve an orthogonal aminoacyl tRNA synthetase

Positive selection: If the mutant aminoacyl tRNA synthetase can charge tRNA\textsuperscript{CUA} with any amino acid, the cells are antibiotic resistant

Noncanonical Amino Acid Incorporation in Live Cells

**Goal:** Evolve an orthogonal aminoacyl tRNA synthetase

**Problem:** The mutant aminoacyl tRNA synthetase can charge tRNA\(^{\text{CUA}}\) with an endogenous amino acid and still survive

Noncanonical Amino Acid Incorporation in Live Cells

**Goal:** Evolve an orthogonal aminoacyl tRNA synthetase

Successful aminoacyl tRNA synthetases from round 1

Orthogonal tRNA

mRNA for Chloramphenicol resistance with amber codon

Isolate the cells that died from an identical plate supplemented with the unnatural amino acid

**Life:** antibiotic resistant

Antibiotic-resistant aminoacyl tRNA synthetase incorporates endogenous amino acid

**Death** via Chloramphenicol

Antibiotic-resistant aminoacyl tRNA synthetase incorporates noncanonical amino acid

Orthogonal tRNA synthetase / tRNA pair incorporates O-Me tyrosine into DHFR

All components required for expression of DHFR with noncanonical amino acid

Noncanonical Amino Acid Incorporation in Yeast

**Goal:** Evolve an orthogonal aminoacyl tRNA synthetase for use in yeast

*The tyrosyl tRNA / aminoacyl tRNA synthetase pair from E. coli*

**Noncanonical Amino Acid Incorporation in Yeast**

**Goal:** Evolve an orthogonal aminoacyl tRNA synthetase for use in yeast

**Mutant aminoacyl tRNA synthetase library**

**Orthogonal tRNA\textsuperscript{CUA}**

**Noncanonical amino acid**

**mRNA for GAL4 transcriptional activator w/ amber codon**

**HIS3** **URA3**

**Reporter genes**

**Positive selection:** If the mutant aminoacyl tRNA synthetase can charge tRNA\textsuperscript{CUA} with any amino acid, the cells produce histidine and uracil and live.

Noncanonical Amino Acid Incorporation in Yeast

**Goal:** Evolve an orthogonal aminoacyl tRNA synthetase for use in yeast

**Mutant aminoacyl tRNA synthetase library**

**Orthogonal tRNA\textsuperscript{CUA}**

**mRNA for GAL4 transcriptional activator w/ amber codon**

**UAG**

**Reporter genes**

HIS3  URA3

*required genes for making histidine and uracil*

**Life**

**Positive selection:** If the mutant aminoacyl tRNA synthetase can charge tRNA\textsuperscript{CUA} with any amino acid, the cells produce histidine and uracil and live

Endogenous amino acid

\[
\text{HO} \quad \text{NH}_2 \quad \text{CO} \quad \text{NH} \quad \text{OH}
\]

grow cells with media lacking histidine and uracil

Noncanonical Amino Acid Incorporation in Yeast

**Goal:** Evolve an orthogonal aminoacyl tRNA synthetase for use in yeast

- **Aminoacyl tRNA synthetases from Round 1**
- **Orthogonal tRNA**
- **5-fluoroorotic acid**
- **mRNA for GAL4 transcriptional activator w/ amber codon**
- **Reporter genes HIS3 URA3**
- **Death**

**Negative selection:** If the mutant aminoacyl tRNA synthetase can charge tRNA with an endogenous amino acid, URA3 will be expressed, and the cell will die via 5-fluoroorotic acid.

Noncanonical Amino Acid Incorporation in Yeast

Noncanonical amino acids are successfully incorporated into human superoxide dismutase (hSOD) in yeast

Noncanonical Amino Acid Incorporation in Mammalian Cells

Noncanonical amino acid incorporation in CHO and 293T cells

A unique aminoacyl tRNA synthetase was evolved for each amino acid

Noncanonical Amino Acid Incorporation in Mammalian Cells for Imaging

Imaging cell surface proteins via bioorthogonal click chemistry

EGFR-GFP with amber codon at position 128

EGFR-GFP labeled with TAMRA fluorophore

Noncanonical Amino Acid Incorporation in Mammalian Cells for Imaging

Imaging cell surface proteins via bioorthogonal click chemistry

eGFP and TAMRA signal colocalize

Genetic Encoding of Traceless Post-Translational Modifications

Phosphorylation of a protein can lead to activation, deactivation, degradation, or membrane transport

Phosphorylated serine and tyrosine can be installed at specific positions via noncanonical amino acid incorporation


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Orthogonal Ribosomes

**Problem:** Release factors compete with tRNA\(^{CUA}\), diminishing amber suppression efficiency

Decreased interaction of release factor 1 would lead to increased efficiency of amber suppression

**Orthogonal Ribosomes**

**Problem:** Release factors compete with tRNA\(^{CUA}\), diminishing amber suppression efficiency

Can an orthogonal ribosome be engineered to specifically translate an orthogonal mRNA and increase amber suppression?

Orthogonal Ribosomes

16S rRNA of 30s ribosome recognizes the Shine-Dalgarno (SD) site upstream of the start codon

Solution an orthogonal ribosome: Alternate Shine-Dalgarno site and anti-SD sequence

Image source: Researchgate

Orthogonal Ribosomes

Ribo-X improves noncanonical amino acid incorporation and is hypothesized to decrease ribosomal interaction with Release Factor 1

Orthogonal Ribosomes

The orthogonal ribosome Ribo-Q1 efficiently decodes quadruplet codons and amber codons.

Orthogonal Ribosomes

The orthogonal ribosome Ribo-Q1 efficiently decodes quadruplet codons and amber codons

Can the 23s rRNA of the 50s ribosomal subunit be engineered as well to improve noncanonical amino acid incorporation?

Previous work: Engineering of 16s rRNA of the 30s ribosomal subunit

Problem: Engineered rRNA of the 50s / 30s ribosomal subunits can interact with endogenous 30s / 50s subunits

Orthogonal Ribosomes

**Solution:** ‘Stapled ribosomes’ link 16S and 23S rRNA together

**This work:** The first fully orthogonal ribosome (both subunits)

**Problem:** Low activity

Orthogonal Ribosomes

Orthogonal translation pathways

Cross-assembly pathways

‘Stapled ribosomes’ can still interact with endogenous 50s and 30s subunits

Orthogonal Ribosomes

This work: Engineer a ‘stapled ribosome’ that does not associate with endogenous subunits

Orthogonal ‘stapled’ ribosomes have activities comparable to that of the parent orthogonal ribosome

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Genomically Recoded Organisms

Problems with amber suppression

Release factors compete with tRNA\textsubscript{CUA}, diminishing amber suppression efficiency

Charged tRNA\textsubscript{CUA} suppress endogenous UAG stop codons

Solution: Recode endogenous UAG (amber codon) to UAA and delete Release Factor 1

**Stop Codons**

- **Amber**  UAG
- **Ochre**  UAA
- **Opal**  UGA

**Release factor 1**

Recognizes amber codon to release new peptide from the ribosome

**Problems with amber suppression**

Release factors compete with \( \text{tRNA}^{\text{CUA}} \), diminishing amber suppression efficiency

Charged \( \text{tRNA}^{\text{CUA}} \) suppress endogenous UAG stop codons

**Solution:** Recode endogenous UAG (amber codon) to UAA and delete Release Factor 1
**Genomically Recoded Organisms**

**Stop Codons**
- Amber: UAG
- Ochre: UAA
- Opal: UGA

**Problems with amber suppression**
Release factors compete with \( \text{tRNA}^{\text{CUA}} \), diminishing amber suppression efficiency

**Charged \( \text{tRNA}^{\text{CUA}} \) suppress endogenous UAG stop codons**

**Solution:** Recode endogenous UAG (amber codon) to UAA and delete Release Factor 1

---

Genomically Recoded Organisms

Project resulted in the E. coli species C321ΔA

Phages rely on host to express proteins necessary for propagation

T7 virus fitness is reduced when release factor 1 is removed

Total Synthesis of E. coli with a Recoded Genome

Total synthesis of *Escherichia coli* with a recoded genome

Julius Fredens¹,⁴, Kaihang Wang¹,²,⁴, Daniel de la Torre¹,⁴, Louise F. H. Funke¹,⁴, Wesley E. Robertson¹,⁴, Yonka Christova¹, Tiongsun Chia¹, Wolfgang H. Schmied¹, Daniel L. Dunkelmann¹, Václav Beránek¹, Chayasith Uttamapinant¹,³, Andres Gonzalez Llamazares¹, Thomas S. Elliott¹ & Jason W. Chin¹*

Total Synthesis of E. coli with a Recoded Genome

Serine: TCA = AGT and TCG = AGC

Stop codon: TAG = TAA

**Total Synthesis of E. coli with a Recoded Genome**

E. coli genome was divided into 8 fragments which were created using REXER

Project resulted in the successful creation of the *E. coli* species **Syn61**

Total Synthesis of E. coli with a Recoded Genome

**Syn61Δ3**

**Serine**

<table>
<thead>
<tr>
<th>Codon</th>
<th>tRNA Anticodon</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCG</td>
<td>CGAserU</td>
</tr>
<tr>
<td>TCA</td>
<td>UGAserT</td>
</tr>
<tr>
<td>TCT</td>
<td>TCT</td>
</tr>
<tr>
<td>TCC</td>
<td>GGAserWX</td>
</tr>
<tr>
<td>AGT</td>
<td>GCUserV</td>
</tr>
</tbody>
</table>

**E. coli**

**STOP**

<table>
<thead>
<tr>
<th>Codon</th>
<th>Release Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGA</td>
<td>RF2prfB</td>
</tr>
<tr>
<td>TAA</td>
<td>RF1prfA</td>
</tr>
<tr>
<td>TAG</td>
<td>RF1prfA</td>
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</tbody>
</table>

**Syn61**

**Serine**

<table>
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<tr>
<td>TCT</td>
<td>GGAserW,X</td>
</tr>
<tr>
<td>TCC</td>
<td>GGAserW,X</td>
</tr>
<tr>
<td>AGT</td>
<td>GCUserV</td>
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</table>

**STOP**

<table>
<thead>
<tr>
<th>Codon</th>
<th>Release Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGA</td>
<td>RF2prfB</td>
</tr>
<tr>
<td>TAA</td>
<td>RF1prfA</td>
</tr>
</tbody>
</table>

2 rounds of parallel mutagenesis & dynamic selection to create Syn61(ev2)

3 rounds of parallel mutagenesis & dynamic selection

deletion of serT, serU, prfA to create Syn61Δ3

**Syn61 can be evolved to remove serine tRNAs and release factor 1**

Total Synthesis of E. coli with a Recoded Genome

Phages use endogenous translation machinery to reproduce

What happens when phages try to infect Syn61Δ3?

**Total Synthesis of E. coli with a Recoded Genome**

**Syn61Δ3**

\[ \lambda^{+}P1\text{vir}^{+}T4^{+}T6^{+}T7 \]

<table>
<thead>
<tr>
<th>Phage</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syn61</td>
<td>ev2</td>
<td>ΔRF1</td>
<td>Δ3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0 h

4 h

*Syn61Δ3* is more resistant to phage infection

Total Synthesis of E. coli with a Recoded Genome

The codons TCG, TCA, and TAG are no longer present in the genome

TCG, TCA, and TAG can be recoded to incorporate three different noncanonical amino acids

Syn61Δ3 can be used to synthesize noncanonical heteropolymers and macrocycles

New Conceptual Applications and the Future of Genetic Code Expansion

Non-natural nucleic acid base pairs


Genomically recoded mammalian cells?

Additional mechanistic exploration to improve noncanonical amino acid incorporation

Questions?