2024 GCE Conference

Oregon State University
Corvallis, OR

August 8th – 10th

A message from the organizers

We welcome you to the 3rd Genetic Code Expansion Conference!

Every two years, this conference aims to bring together the thriving and diverse GCE community. After a COVID-induced hiatus, we are excited to have the community back together to celebrate recent advances in the GCE field.

The GCE Conference is meant to connect users of this technology, leading to a more rapid expansion of the use of noncanonical amino acids in biomedical research.

There has been remarkable progress in the cutting-edge technical capabilities associated with GCE. Additionally, the robustness of GCE tools has significantly improved and there is a surge of interest in adopting this technology in both academic and industrial settings.

The 3rd GCE Conference aims at bringing together diverse scientific disciplines that focus on developing and using GCE technology. Our aim is to catalyze cross-pollination of ideas and formation of new collaborative networks.

We're looking forward to spending the next few days together in science.

Cheers,

Abhishek Chatterjee,
Boston College

Kathrin Lang,
ETH Zurich

Ryan Mehl,
Oregon State University
GCE4All Center

Join the GCE4All Community

- Review our current projects
- Learn more about our current technologies & reagents
- Join our online discussion board (GCEbb)
- Register for our monthly International GCE Webinar
- Fill out our online form to let us know how GCE can support your research!

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Schedule of Events

THURSDAY, AUGUST 8TH

Arrival & Registration (5:00 –7:00 pm)
Dinner (7:00 - 8:00 pm)
Welcome Address (8:00 – 8:15 pm)
Keynote #1: Peter Schultz (8:15 – 9:15 pm) *An Expanding Genetic Code*

FRIDAY, AUGUST 9TH

8:00 am	**Hot breakfast available**
Session 1 (9:00 – 10:35 am) Chair: Carly Schissel	
9:00 – 9:25 am	**Satpal Virdee,** University of Dundee *Expanding the ubiquitin system with engineered protein sensors*
9:25 – 9:50 am	**Andrew Ellington**, University of Texas at Austin, USA *Engineering tRNA loading for genetic code modification*
9:50 – 10:15 am	**Sebastian Greiss**, University of Edinburgh *Genetic code expansion in C. elegans and its application for the development of in vivo tools*
10:15 – 10:35 am	**John Lueck**, University of Rochester, USA *Development of therapeutic suppressor tRNAs*
Group Photo & Coffee Break (10:35 – 11:00 am)	
Session 2 (11:00 am – 12:45 pm) Chair: Yarra Venkatesh	
11:00 – 11:25 am	**Sharona Gordon**, University of Washington, USA *Real-time traffic: new optical tools for measuring exocytosis and endocytosis applied to TRPV1 ion channels*
11:25 – 11:50 am	**Ivana Nikic Spiegel**, Tübingen University, Germany *Axonal injury: from advanced imaging to genetic code expansion-based tools for minimally invasive protein tagging*
11:50 am – 12:15 pm	**Tao Uttamapinant,** VISTEC, Thailand *Detection of proteoforms and cellular translation events with genetic code expansion*
12:15 – 12:35 pm	**Richard Obexer**, University of Manchester, UK *Enantioselective Photoenzymes with Genetically Programmed Triplet Sensitisers*
12:35 – 12:45 pm	**Akos Neyerges**, Harvard Medical School, USA *Synthetic Genomes and Genetic Codes*
Lunch (12:45 – 2:30 pm) **Tour of the Linus Pauling Nobel Prize Special Collections** – Meet out front at 1 pm to walk over *Must RSVP in advance, only 15 slots available*	

Schedule of Events

FRIDAY, AUGUST 9TH Cont.

Session 3 (2:30 – 4:15 pm) Chair: Antje Kruger	
2:30 – 2:40 pm	**Erkin Kuru,** Harvard Medical School, USA *Harnessing the power of synthetic chemical biology to illuminate (and cure) disease*
2:40 – 3:05 pm	**Marcello Marelli**, AstraZeneca *An unnatural partnership: Satisfying the functional needs and manufacturing wants of drug development*
3:05 – 3:30 pm	**Feng Tian**, Luxvitae Therapeutics *Journey Toward Its Commercialization of the Expanded Genetic Codon--an Ambrx Story*
3:30 – 4:15 pm	**Poster Flash Talks** (Evens) *If you were selected for a session talk, you won't give a flash talk
Coffee Break (4:15 – 4:30 pm)	
Poster Session (4:30 – 6:00 pm) *Drinks and appetizers beginning at 5:00 pm*	
Dinner (6:00 – 7:00 pm)	
Session 4 (7:10 – 8:45 pm) Chair: Sebasthian Santiago	
7:10 – 7:30 pm	**Dan Groff**, Sutro Biopharma *Production of advanced therapeutics using non-natural amino acids*
7:30 – 8:30 pm	**Keynote #2: Mike Jewett,** Stanford University *Transforming cell-free systems for synthetic biology*

SATURDAY, AUGUST 10TH

8:00 am	**Hot breakfast available**
Session 1 (9:00 – 10:35 am) Chair: Yunan Zheng	
9:00 – 9:25 am	**Tao Liu**, Peking University, China *A humanized genetic code expansion system for non-canonical amino acid controlled gene expression*
9:25 – 9:50 am	**Irene Coin**, Leipzig University, Germany *GCE reveals structural and dynamic details of GPCR function from the live cell*
9:50 – 10:15 am	**James Van Deventer**, Tufts University, USA *Pharmacophore-driven antibody discovery*
10:15 – 10:40 am	**Shixian Lin**, Zhejiang University, China *New genetic code expansion strategies for probing the biological functions of protein PTMs*

Schedule of Events

SATURDAY, AUGUST 10TH Cont.

Coffee Break (10:40 – 11:15 am)	
Session 2 (11:15 am – 12:45 pm) Chair: May Chakrabandhu	
11:15 – 11:40 am	**Farren Isaacs**, Yale School of Medicine, USA
11:40 am – 12:05 pm	**Michelle Chang,** UC Berkeley, USA
12:05 – 12:25 pm	**Rick Cooley**, Oregon State University, USA *Decoding the Dark Proteome: Innovations in Genetic Code Expansion for Phosphorylated Proteins*
12:25 – 12:45 pm	**Ross Thyer**, Rice University, USA *Emulsion-based directed evolution platforms to engineer non-canonical amino acid biosynthesis*
Lunch (12:45 – 2:00 pm) *GCE Conference business meeting during lunch*	
Session 3 (2:00 – 4:10 pm) Chair: Tarun Iype	
2:00 – 2:25 pm	**Ali Deliz Liang**, University of Zurich, Switzerland *Enzyme engineering with genetic code expansion*
2:25 – 2:50 pm	**Tina Boville**, Aralez Bio
2:50 – 3:10 pm	**Cory Dunn**, GRO Biosciences *Disruption Activated Reporter Transcription, a Method for Recovery of Aminoacyl-tRNA Synthetases That Promote Incorporation of NSAAs*
3:10 – 3:30 pm	**Christine Koehler**, Veraxa *Biochemical innovations for expanding the therapeutics window of ADCs*
3:30 – 4:10 pm	**Poster Flash Talks** (Odds) *If you were selected for a session talk, you won’t give a flash talk
Coffee Break (4:15 – 4:30 pm)	
Poster Session (4:30 – 6:00 pm) Drinks and appetizers beginning at 5:00 pm	
Dinner (6:00 – 7:00 pm)	

Schedule of Events

SATURDAY, AUGUST 10TH Cont.

Session 4 (7:10 – 8:40 pm) *Poster Prizes will be announces during the 1st 10 min* Chair: Alex Eddins	
7:10 – 7:20 pm	**Yuda Chen,** University of California, San Francisco (UCSF) *Unleashing the Potential of Noncanonical Amino Acid Biosynthesis to Create Cells with Precision Tyrosine Sulfation*
7:20 – 7:30 pm	**Anamika Singh**, GCE4All Research Center, Oregon State University, USA *Evaluating Ligase Feasibility for Targeted Protein Degradation in Living Systems with Genetic Code Expansion*
7:30 - 7:40 pm	**Meghan Breen**, Furman University, USA *Developing GCE tools for Candida glabrata*
7:45 – 8:45 pm	**Keynote #3: Alanna Schepartz,** UC Berkeley, USA *Broadening the concept of a genetically encoded material*
Conference Ending Festivities 9:00 pm *At Ryan Mehl's house – walking distance away*	

2024 GCE Conference Abstracts

*Even numbered posters will present on Friday, August 9th 4:00 – 6:30 pm

*Odd numbered posters will present on Saturday, August 10th 4:00 – 6:30 pm

1. **Divyansh,** Michael C. Allen, P. Andrew Karplus, Kayla A. Jara, Richard B. Cooley, and Ryan A. Mehl
GCE4All Research Center, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, USA, 97331.
Two New Resources Supporting the GCE Community: the GCE Bulletin Board (GCEbb) and the GCE4All KnowledgeBase (GCEkb)
Genetic Code Expansion (GCE) has huge potential for accelerating basic research and applied pursuits such as developing next-generation designer therapeutics. The NIH-funded GCE4ALL research center optimizes GCE tools and promotes their use. In addition to ensuring GCE tools are robust and available, we are building resources that help researchers identify and successfully implement GCE tools to advance their research. Two initiatives are: (1) providing an online forum for experienced and new GCE users to discuss GCE-related endeavors, including helping each other with designing and troubleshooting GCE experiments; and (2) providing a platform helping the community find GCE tools suitable for advancing their research. Regarding the first initiative, the center has launched the Genetic Code Expansion Bulletin Board (GCEbb), an email listserv with searchable archives that will promote conversations among newcomers and experts, nurturing a welcoming and helpful GCE community. Regarding the second initiative, the center is developing the GCE4All KnowledgeBase (GCEkb), a crowd-sourced, expert-curated searchable database that will eventually be a one-stop-shop for all things GCE. The GCEkb is in an early stage of development with a basic structure in place, but still limited in the information that has been entered. Its two main sections are organized around (i) the non-canonical amino acids (ncAAs) that have been used in GCE studies and (ii) the corresponding cognate amino-acyl tRNA synthetase/tRNA pair(s) for incorporating given ncAAs into proteins. Here, we will introduce the GCEbb and GCEkb, and invite feedback and participation in their use and development.

2. **Hung Manh Nguyen,** Richard B. Cooley, and Ryan A. Mehl
GCE4All Research Center, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon, United States.
Computational design of tRNA/synthetase pairs for Amino-Acridone.
Genetic code expansion, relies on rational design and directed evolution to engineer tRNA/ amino acyl synthetase pairs for efficient encoding of non-canonical amino acids into proteins. Rational design depends heavily on domain expert knowledge and engineering synthetase systems when structural and functional information is lacking is a major hurdle.

Directed evolution, though effective for some amino acids, is resource-intensive and limited by the number of amino acyl synthetase mutational sites that can cover in a DNA library. To address these challenges, we explore the potential of cutting-edge AI methods for designing amino acyl synthetase. This approach can cover more amino acid sites in the amino acyl synthetase accessing larger amino acids and shorten development timeline. In this poster, our focus is specifically on designing novel synthetases for the fluorescent amino acid Amino-Acridone. We present our progress to date and outline future directions for using computation in GCE research.

3. **Donovan J. McAfee,** Yogesh M. Gangarde, and Ryan A. Mehl*
GCE4All Research Center, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon, United States.
Triazine-Pyridyl Amino Acids for Bioorthogonal Click Chemistry and Metal Ion Binding
Expanding the toolkit of bioorthogonal handles enhances researchers' capabilities, as demonstrated by the significant impact of “clickable” amino acids in protein therapeutics and cellular imaging. Traditionally, bioorthogonal click reactions on proteins are accomplished by encoding amino acids into proteins containing azide, trans-cyclo-octene (TCO) or tetrazine-containing functional groups. Here, we describe development of synthetic routes for triazine-pyridyl amino acids. Triazine-pyridyl amino acids offer new capabilities as they can act as both bioorthogonal click chemistry handles, undergoing inverse electron demand Diels-Alder (IEDDA) reactions with trans-cyclooctene (TCO), as well as metal ion binders. Compared to tetrazine reaction handles, triazines are more stable and offer different selectivity toward TCO reagents compared to tetrazines, allowing for the simultaneous encoding and selective reactivity of triazine and tetrazine amino acids within the same biological system. After reacting with TCO groups, these triazine reagents bind metal ions with varying affinity by forming a 2,2′-bipyridine motif. Previously, no click-initiated reactions for loading metal ions onto proteins have been developed. Once encoded into proteins, this “turn on” metal binding capacity of triazines allows for controlled integration of metal binding sites on proteins, thus introducing a range of new functionalities and enabling the design of proteins with specific purposes based on the properties of the bound metal ions. Metal binding sites are crucial for various roles, including enzyme catalysis, signal transduction, and protein-protein interactions. Collectively, triazine-pyridyl amino acids provide a versatile new tool for bioorthogonal chemistry and metal ion binding, and their incorporation into proteins using genetic code expansion will greatly expand our ability to study and manipulate protein function.

4. **Ricardo Moreno Ballesteros**, Sunil Mathur, Adam Fletcher, Francisco Bustos, Carmen Espejo-Serrano
University of Dundee
Activity-based probes for RING E3 ligases
Investigation into the Ubiquitin Proteasome System (UPS), which is renowned for its regulatory function in protein degradation, is crucial for delineating fundamental biology and discovering new therapeutic targets. E3 ligases are essential players in the ubiquitin system as they recruit specific substrates and transfer ubiquitin to them. Central to understanding E3 ligase biology is establishing when and how they are activated but there is a paucity of tools for achieving this.
The Virdee lab has developed Activity-Based Probes (ABPs) for E3s with a catalytic cysteine residue, but most do not have this reactive feature. Approximately 300 single subunit RING E3 ligases catalyze substrate ubiquitination by allosterically activating the upstream E2~Ub donor. This activity can be regulated and involves a catalytic E2~Ub:RING intermediate with distinct geometry. By introducing a Bpa photocrosslinking amino acid into an experimentally determined position, we develop protein-based probes that undergo activity-dependent labelling of RING E3 ligase upon UV irradiation.
Photocrosslinking ABPs for RING E3s should be valuable tools for determining their regulatory mechanisms and their potential as therapeutic targets. We envision they will also have utility in identifying E3 ligases with desirable tissue/cell activity profiles that can be harnessed for improved targeted protein degradation strategies.

5. **Ibrahim Saleh**, Jennifer Ramirez, E. James Petersson, and Elizabeth Rhoades
University of Pennsylvania
Investigating the differential effects of authentic site-specific PTMs in tau
The neuronal microtubule cytoskeleton is regulated by microtubule-associated proteins (MAPs). Tau, the most abundant MAP in axons, controls motor motility, signaling, and microtubule dynamics. Tau dysfunction is linked to tauopathies such as Alzheimer's disease (AD). Tau is subject to a myriad of post-translational modifications (PTMs) such as phosphorylation and acetylation, yet our ability to understand how PTMs regulate tau functions in health and disease has been hindered by the absence of tools for generating site-specific PTMs on tau. Genetic code expansion (GCE) stands at the forefront of protein PTM research, enabling precise and customizable integration of modified amino acids into proteins expressed through recombinant methods. We used GCE to make acetylated and phosphorylated tau. We assessed the impact of these modifications on Tau’s binding to soluble tubulin and its ability to polymerize microtubules.

6. **Chintan Soni**, Noam Prywes, Matthew Hall, Malavika Nair, David Savage, Alanna Schepartz, and Abhishek Chatterjee
START: A translation-independent directed evolution platform for engineering aminoacyl-tRNA synthetases
Aminoacyl-tRNA synthetase (aaRS) engineering has enabled the co-translational incorporation of several hundreds of non-canonical -amino acids (ncAAs) into proteins in living cells. While majority of the current repertoire of ncAAs have a large side-chain diversity, they have very limited backbone diversity (mostly alpha-amines). There has been a significant effort in expanding the backbone diversity of proteins by facilitating incorporation of alpha-substituted monomers to generate sequence-defined biopolymers. However, one of the major bottlenecks is the scarcity of aaRS capable of acylating tRNAs with these alpha-substituted monomers.
Traditional approaches to evolve novel aaRS mutants typically rely on coupling the activity of aaRS to the expression of a reporter protein with a selectable phenotype (either antibiotic resistance or fluorescence). However, such translation-dependent evolution schemes are incompatible with monomers that are suboptimal substrates for the endogenous translational machinery (EF-Tu or ribosome). To enable the ribosomal incorporation of such exotic monomers, a two-step solution is needed: A) Engineering aaRS to acylate its cognate tRNA with these monomers, without relying on ribosomal translation as a readout, and B) Subsequent engineering of the translational machinery to accept the resulting acylated tRNA for translation. Here, we report a novel platform for aaRS evolution that directly selects for tRNA-acylation without ribosomal translation (START). In START, each distinct aaRS mutant is linked to a cognate tRNA containing a unique barcode sequence. Acylation by an active aaRS mutant protects the associated barcode-containing tRNAs from an oxidative treatment designed to damage the 3'-terminus of the non-acylated tRNAs. Sequencing of these surviving barcode-containing tRNAs is then used to reveal the identity of the said active aaRS mutant. The efficacy of START was demonstrated by identifying novel mutants of the M. alvus pyrrolysyl-tRNA synthetase from a naïve library that charge ncAAs.

7. **Moriah Mathis**[1], Phillip Zhu[1], Anamika Singh[1], Alex Eddins[1], Yunan Zheng[2], Kedar Puvar[2], Becca McCloud[2], Lina Blanco[2], Rick Cooley[1]*
[1]Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. [2]AbbVie, Inc., North Chicago, Illinois 60064
A novel, phosphorylation-dependent 14-3-3/cereblon protein complex may regulate protein degradation
Using genetic code expansion (GCE) methods, the Cooley lab has identified a novel, phosphorylation-dependent interaction between cereblon and the monomeric form of 14-3-3ζ, two human proteins of high interest. Cereblon (CRBN) is part of an E3 ligase complex and plays a critical role in recognizing target proteins for ubiquitination-directed protein degradation. It is currently the focus of intense study and drug development related to targeted protein degradation. 14-3-3ζ functions as an integral signaling hub that interacts

with hundreds of client proteins and is involved in critical cellular processes such as cell signaling, apoptosis, autophagy, and cell cycle regulation; it also contributes to many diseased states, including cancer and chemoresistance. The 14-3-3ζ/CRBN interaction is observed when the 14-3-3ζ dimer is phosphorylated at serine 58 and subsequently dissociates to its monomeric form; CRBN may also require phosphorylation to form the complex. GCE tools for encoding phosphoserine and non-hydrolyzable phosphoserine make it possible to synthesize 14-3-3ζ that is phosphorylated at residue 58 and constitutively monomeric, as well as site-specifically phosphorylated CRBN. Utilizing these phosphorylated proteins produced using GCE, we have begun characterizing this complex and probing its structural and functional details through the application of co-immunoprecipitation experiments, cross-linking assays, ubiquitination assays, mass photometry, and delivery of stably phosphorylated 14-3-3ζ by Chariot reagent into HEK293T cells. This work suggests that phosphorylated, monomeric 14-3-3ζ directs its clients to CRBN, where 14-3-3ζ, its client proteins, or both are ubiquitinated and targeted for proteasomal degradation. These experiments aim to elucidate a greater understanding of the process of protein degradation and explore how phosphorylation of 14-3-3ζ helps to rewire signaling systems during periods of stress and apoptosis.

8. **Ian Noonan**, Nathan Alexander, Richard Cooley, and Ryan Mehl.
GCE4All Center, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, USA, 97331.

Selecting Synthetases for the Genetic Encoding of Acetyllysine

Acetylation is a crucial post-translational modification that involves the addition of an acetyl group to lysine residues within proteins. This modification induces changes on protein stability, impacts DNA binding affinity and subsequently transcription factor activity. While dysregulation of acetylation has been linked to various diseases, studying such a modification is challenging due to low stoichiometry and rapid turnover. Current methods for site specific incorporation of acetyllysine into proteins using genetic code expansion systems are limited by the high amount of amino acid required, which hinders their effectiveness. To address these challenges, we are exploring the modification specificity of a PylRS/tRNA^{Pyl} pair from *Candidatus Methanomethylophilus alvus Mx1201 (Ma)* through directed selections. We aim to expand the scope of acetyllysine post-translational modification research by enhancing the efficiency and fidelity for the genetic encoding of acetyllysine. This holds potential to deepen our understanding of how site-specific acetylation influences biological systems.

9. **Sarah McGee**[1], Stanislau Stanisheuski[1], John Wang[2], Yiping Zhao[2], Richard B. Cooley[1], and Ryan A. Mehl[1].
[1]GCE4All Research Center, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, USA 97331. [2] Department of Genetics and Genome Sciences, School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4955.
Unveiling the Phosphatase and Denitrase Dual Functionality of Protein Tyrosine Phosphatase Receptor T's Tandem D1/D2 domains
Post-translational modifications (PTMs) are widespread across the human proteome. Among these PTMs, tyrosine nitration caused by oxidative stress is implicated in various diseases such as ALS and cancer. Traditionally deemed irreversible, our recent research indicates that the D2 "pseudo-phosphatase" cytosolic domain of Protein Tyrosine Phosphatase Receptor T (PTPRT), a trans-membrane receptor believed to be involved in tumor suppression, may reverse tyrosine nitration. Utilizing genetic code expansion (GCE), we are able to site-specifically encode nitrotyrosine into potential substrates of the D2 domain, resulting in pure nitrated protein that could not be achieved using non-specific methods of nitrating proteins such as exposure to peroxynitrite. Our study demonstrates the D2 domain's possible ability to remove nitrotyrosine from one of its potential substrates, ERK, as well as the capacity of the D1 domain of PTPRT to dephosphorylate ERK, showcasing PTPRT's dual functionality as a denitrase and phosphatase. Furthermore, we use GCE to incorporate phosphoserine into MEK – to create the active form of the kinase - and monitor its ability to phosphorylate different nitrated forms of ERK. We reveal that ERK nitration affects its MEK-mediated activation as well as its autophosphorylation kinetics, unveiling nitration-dependent phosphorylation previously unrecognized. This is also the first time a protein has been site-specifically both nitrated and phosphorylated, only made possible by the use of GCE. Altogether, this study highlights the interplay between nitration and phosphorylation and suggests a potential link in their removal mechanisms.

10. **Sarah Louie**, Ryan A. Mehl, and Richard B. Cooley
GCE4All Research Center, Dept of Biochemistry and Biophysics, Oregon State University, Corvallis OR USA 97331
Differential roles of tyrosine nitration sites in ERK1 protein function and fate in HEK 293 cells
Protein tyrosine nitration is a marker of oxidative stress in disease states. Studying nitration effects is challenging because the levels of nitro-tyrosine in cells are relatively low compared to other post-translational modifications (PTMs). Additionally, generating site-specifically nitrated proteins with traditional methods of adding peroxynitrite is difficult as it leads to uncontrollable, off-target nitration. Historically, nitration has been considered an irreversible PTM. While several studies suggest the existence of "denitrase" enzymes responsible for declines in protein nitration, these enzymes have yet to be properly identified. Other studies suggest that nitration may serve as a marker for protein degradation. Notably, three nitration sites on ERK1 have been identified (Y156, Y210, and

Y333) whose biological roles are not well understood, but are hypothesized to affect ERK1 nuclear localization, phosphorylation, and folding. We optimized a Methanomethylophilus alvus GCE expression system for mammalian HEK293T cells and used it to encode nitro-tyrosine into ERK1. We explore the relationship between nitrated ERK1 and a putative denitrase, the D2 domain of receptor protein-tyrosine phosphatase T (PTPRT) by co-expressing the cytosolic D1/D2 domains with ERK1 nY156 or nY333 and measuring nitro-tyrosine levels with western blot analysis and mass spectrometry. We next explored the hypothesis that nitration of ERK1 at Y210 results in misfolding, which prevents nuclear localization, and leads to ubiquitination and proteasome degradation. These data aid our understanding of the different ways tyrosine nitration can alter protein function, serving as a cell signaling response to oxidative stress, and provide insight for development of novel therapeutics.

11. Cat Hoang Vesely[1], Shaima Nazaar[2], Patrick N Reardon[3], Blaine R Roberts[2], Ryan A Mehl[1], Richard B Cooley[1]

[1]GCE4All Research Center, Dept of Biochemistry and Biophysics, Oregon State University, Corvallis OR, [2]Department of Biochemistry, Emory University School of Medicine, Atlanta GA, [3]Oregon State University NMR Facility, Oregon State University, Corvallis OR

Unraveling The Structure, Interactions And Dynamics Of Phosphorylated Bcl-xL Using Genetic Code Expansion

Bcl-xL is an anti-apoptotic protein in the Bcl-2 family that interacts with pro-apoptotic proteins to regulate cytochrome-C release from mitochondria. Dysregulation of this interaction network protects cancer cells from death signals and cytotoxic agents thereby promoting chemoresistance, a hallmark of cancer. The intrinsically disordered region (IDR) of Bcl-xL contains several post-translational modifications (PTMs) including a principle regulatory phosphorylation site serine 62 (pSer62). Cell-based studies suggest phosphorylation of Ser62 initiates apoptosis, but the underlying biochemical and functional mechanisms are not clear, largely stemming from our inability to produce pure Bcl-xL with authentic phosphoserine at this site. In this work, we overcome this challenge by employing genetic code expansion (GCE) systems to produce Bcl-xL with genetically encoded pSer62 and its non-hydrolyzable analog (nhpSer) using PermaPhos, an efficient GCE platform for studying protein phosphorylation in cellular conditions. Moreover, we use our recently developed methodology to generate wild-type and phosphorylated Bcl-xL isotopically labeled with $^{15}N/^{13}C$ and collected 2D and 3D nuclear magnetic resonance (NMR) analyses. Through complementary binding assays with known partner proteins and characterizations of changes in Bcl-xL protein dynamics induced by phosphorylation, we gain important insights into the regulatory role(s) and the mechanism through which phosphorylation regulates Bcl-xL and apoptosis. Additionally, we site-specifically installed nhpSer at site 62 to analyze how phosphorylation influences Bcl-xL interaction networks using pull-down assays coupled with mass spectrometry. These insights will help development of therapeutic strategies that selectively target phosphorylated Bcl-family proteins.

12. **Cat Hoang Vesely**[1], Peter Chung[2], Ryan A Mehl[1], Richard B Cooley[1]
[1]GCE4All Research Center, Dept of Biochemistry and Biophysics, Oregon State University, Corvallis OR, [2]Department of Physics and Astronomy, University of Southern California, Los Angeles CA

Unlocking the chaos: A Versatile Approach to Producing Site-Specifically Phosphorylated Intrinsically Disordered Proteins

Over 30% of proteins in the higher eukaryotic proteome contain intrinsically disordered proteins (IDPs), characterized by their structural flexibility and lack of stable 3D structures. They have essential roles in cellular signaling and regulation, mediating interactions with hub proteins and serving as regulatory switches through reversible phosphorylation, influencing conformational dynamics, function, and allosteric interactions. However, producing phosphorylated IDPs faces two substantial challenges. First, IDPs themselves are often proteolyzed, unstable and aggregation prone, and sometimes toxic to the expression hosts. Second, there is the challenge of installing phosphoserine into IDPs at the targeted location and preventing it from being hydrolyzed during expression and purification. Here, we describe development of an innovative expression strategy using inclusion body tags that can be proteolytically removed at physiological pH to overcome these challenges and facilitate downstream IDP characterization. We showcase the utility of this strategy by expressing phosphorylated BAD (Bcl2-associated agonist of cell death), a proapoptotic IDP that in its unmodified form primes cells for apoptosis, but when phosphorylated at up to 3 sites is inactivated by sequestration with 14-3-3. It is also notorious for its instability when over-expressed in recombinant hosts and so for two decades it has eluded in vitro characterizations. To express BAD phosphorylated at Ser136 in E. coli, it was fused with a carrier protein to direct its expression into inclusion bodies to prevent degradation from cellular proteases. We then coupled this expression of BAD with genetic code expansion (GCE) systems to direct the site-specific translational installation of authentic phosphoserine (pSer) and non-hydrolyzable phosphoserine (nhpSer) at site 136 using PermaPhos, an efficient GCE platform where E. coli cells biosynthesize the nhpSer amino acid and encode it into proteins system. This method was extended to produce the longest human Tau isoform 2N4R and installed pSer and nhpSer at serine 404. Phosphorylation at this site has been observed in all neurodegenerative pathological stages, involving Tau seeding activity to aggregation in neurons. This methodology can be easily adapted for expression of many other phosphorylated IDPs ranging from short peptides to proteins with hundreds of amino acids, making them accessible for downstream in vitro characterization. Further, because the proteins are phosphorylated with a stable, functional mimic of pSer, transfections into mammalian cells to evaluate in vivo function of specific phosphorylated forms of IDPs are possible.

13. **Hannah Stuwe**[1], Patrick Reardon[2], Yu Zhen[1], Sahana Shah[1], Kaitlyn Hughes[1], Elisar Barbar[1].
[1]Dept of Biochemistry and Biophysics, Oregon State University, Corvallis OR, [2]Oregon State University NMR Facility, Oregon State University, Corvallis OR

Phosphorylation in the Ser/Arg-Rich Region of the Nucleocapsid of SARS-CoV-Regulates Phase Separation by Inhibiting Self-Association of a Distant Helix

The nucleocapsid protein (N) of SARS-CoV-2 is essential for virus replication, genome packaging, evading host immunity, and virus maturation. N is a multidomain protein composed of an independently folded monomeric N-terminal domain that is the primary site for RNA binding, and a dimeric C-terminal domain that is essential for efficient phase separation and condensate formation with RNA. The domains are separated by a disordered Ser/Arg-rich region preceding a self-associating Leu-rich helix. Phosphorylation in the Ser/Arg region in infected cells decreases the viscosity of N:RNA condensates promoting viral replication and host immune evasion. The molecular level effect of phosphorylation, however, has been missing from our current understanding. Using NMR spectroscopy and analytical ultracentrifugation we show that GCE-incorportated and native phosphorylation destabilizes the self-associating Leu-rich helix 30 amino acids distant from the phosphorylation site. NMR and gel shift assays demonstrate that RNA binding by the linker is dampened by phosphorylation, whereas RNA binding to the full-length protein is not significantly affected presumably due to retained strong interactions with the primary RNA binding domain. Introducing a switchable self-associating domain to replace the Leu-rich helix confirms the importance of linker self-association to droplet formation and suggests that phosphorylation not only increases solubility of the positively charged elongated Ser/Arg region as observed in other RNA binding proteins but can also inhibit self-association of the Leu-rich helix. These data highlight the effect of phosphorylation both at local sites and at a distant self-associating hydrophobic helix in regulating liquid-liquid phase separation of the entire protein.

14. **Patrick Allen**, Yogesh Gangarde, Nathan Alexander, Abigail Pung, Alex Eddins, Riley Bednar, Sarah McGee, Richard Cooley, and Ryan Mehl.
GCE4All Research Center, Dept of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331

Molecular basis for tetrazine-3-butyl M. Alvus tRNA synthetase binding: a case for the binding of both redox states

Genetic code expansion enables (GCE) the site-specific incorporation of fast, orthogonal ligation sites by way of tetrazine non canonical amino acids. While the speed of these ligations occurs on a time scale that allows live, in-cell labeling there has not been a tRNA synthetase (RS) that can encode tetrazines in both prokaryotic and eukaryotic systems. Selections of a methanomethlyophilus alvus RS 5-site library resulted in a universal tetrazine 3-butyl (T3B) encoding system. T3B, like prior tetrazine generations, can either be in the active state (oxidized) or inactive state (dihydro) which may decrease in vivo efficiency. To better understand potential molecular bindings of T3B redox, x-ray

crystallography revealed that both oxidized and dihydro forms bind to the active site. The two redox states can be distinguished by the unique orientations of conserved active site residue N166. A conserved water-mediated hydrogen bond network between the N166 side chain and A121/L122 backbones is absent when oxidized T3B is bound. N166 is instead hydrogen bonding with the T3B tetrazine ring. The disengagement of water molecule coordination allows simultaneous binding of T3B and malonate in the active site. However, when dihydro T3B is bound, N166 is perpendicular to the T3B tetrazine ring and the intrachain hydrogen bonding network is re-engaged. As a result, no malonate is detected in dihydro crystal structures. Consideration of these water or small molecule mediated contacts in the selection of a new active site library will ensure more efficient, and redox discriminating tetrazine tRNA synthetases

15. Pierce Eggan, Sharona Gordon, and William N. Zagotta
Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195
Ligand-Coupled Conformational Changes Revealed by Time-Resolved Transition Metal Ion FRET and the Unnatural Amino Acid Acridonylalanine
Cyclic nucleotide-binding domain (CNBD) ion channels play crucial roles in cellular-signaling and excitability and are regulated by the direct binding of cyclic adenosine- or guanosine-monophosphate (cAMP, cGMP). However, the precise allosteric mechanism governing channel activation upon ligand binding, particularly the energetic changes within domains, remains poorly understood. To better understand the conformational changes and energetics that describe them, we have developed an experimental system using a prokaryotic CNBD channel, SthK, and transition-metal ion fluorescence resonance energy transfer (tmFRET) with an unnatural amino acid fluorophore Acridonylalanine, Acd. We engineered donor-acceptor pairs at specific sites within SthK in *E.coli* for subsequent purification, electrophysiology analysis, and fluorescence measurements. Measuring tmFRET with fluorescence lifetimes of Acd, we determined intramolecular distance distributions in the absence and presence of cAMP or cGMP. Furthermore, we were able to use this system to assess the impact of protein oligomerization and ionic strength on the structure and energetics of the conformational states. This study demonstrates the effectiveness of our Acd-based time-resolved tmFRET technique in determining the conformational states and the ligand-dependent energetics in proteins.

16. Linqi Cheng, Yu Hu, and Han Xiao
Department of Chemistry, Rice University, 6100 Main Street, Houston, Texas, 77005
Directed Evolution of a Cyclodipeptide Synthase to Biosynthesize Non-canonical Amino Acids-Containing Cyclodipeptides with Enhanced Efficiency and Specificity
The research on integrating non-canonical amino acids (ncAAs) into proteins and ribosomally synthesized and post-translationally modified peptides (RiPPs) through genetic code expansion (GCE) has been extensive. However, there is limited reporting on the

incorporation of ncAAs into other natural products, such as 2,5-diketopiperazines (DKPs), which are biosynthesized through the hijacking of aminoacyl-tRNAs (aa-tRNAs), using engineered aminoacyl-tRNA synthetases (aaRSs) evolved from yeast. In this study, we explore the synergistic utilization of evolved aaRSs and directed evolution of cyclodipeptide synthases (CDPSs) to generate a diverse range of cyclodipeptides incorporating 4-azido-L-penylalanine (AzF) with increased efficiency and specificity. Furthermore, we elucidate the catalytic mechanisms underlying the enhanced recognition of ncAA-tRNAs by these variants through molecular dynamics (MD) simulations and calculations of binding free energy. Expanding the substrate scope to incorporate ncAAs and extending this approach to encompass other CDPSs highlights their potential to produce diverse DKPs with unique structures and associated bioactivities. This potential is particularly evident when introducing additional tailoring enzymes for subsequent modifications.

17. **Andreas Torell**, Alfred N. Larsson, Luke Odell, and Daniel Fürth
SciLifeLab/Uppsala University
Detection of protein-protein interactions by bio-orthogonal fluorogenic proximity probes
Detecting protein-protein interactions within cells is challenging. Transgenic approaches risk altering protein function via fluorescent tagging, while in situ methods lack in vivo compatibility. Here, we introduce fluorogenic probes with dual-tetrazine pegylated branched arms linked to xanthene dye. Activation requires both tetrazine arms to interact simultaneously with target proteins, enabling dual-substrate recognition. We applied our method to detect protein-protein interactions in both fixed and living cells, utilizing antibody conjugation for fixed cells and genetic code expansion for real-time detection in living cells. Our strategy ensures versatile applicability and seamless transition between fixed and living systems.

18. **Gustavo Mendez**, Margaret Walker, Alex Ho, Ryan S Czarny, and Dr. P. Shing Ho
Colorado State University
Halogen Bonds as Enzymatic Catalysts
Halogen bonds can be utilized in protein design for structural and catalytic purposes. The Ho laboratory previously incorporated a halogen bond into an endonuclease active site by utilizing genetic code expansion. We demonstrated that the new enzyme is catalytic and activity is influenced differently by pH and magnesium levels compared to the metal-dependent wild type enzyme. We are now attempting to employ halogen bonds as catalysts in new systems to create new enzymatic catalytic centers.

19. **Jose Vazquez Rodriguez** and Sebastian Greiss
Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, United Kingdom
Genetic code expansion in *Caenorhabditis elegans*

Genetic code expansion (GCE) is at present mostly used in single celled systems such as bacteria or cultured eukaryotic cells. Many biological phenomena such as development, ageing or the functioning of nervous systems can however only be studied in a multicellular context. The nematode Caenorhabditis elegans is one of the most widely used multicellular model organisms. Despite its apparent simplicity (it consists of 959 somatic cells and has a nervous system made up of only 302 neurons), C. elegans can be used to study biological principles across most areas of the life sciences. Although a large number of genetic tools exist to manipulate and study the organism, GCE provides a new approach to overcome the limitations of existing techniques and allows for development of entirely new approaches.

Despite the inherent power of GCE, its adoption as a research approach has been held back, especially in complex organisms like animals. This is to a large part due to low efficiency and a lack of readily usable tools. We present our recent advances in i) developing GCE for multicellular organisms into a research technology that is robust, efficient, and ready for routine applications, and ii) we describe in vivo tools we have developed using non-canonical amino acids.

20. **Phuoc H Ngo**, Clark Edson, and Andrew Ellington

Modular Modification of One Pot Pure for Cell-Free Protein Synthesis Enabling Genetic Code Expansion and Unnatural Amino Acids Incorporation

The incorporation of unnatural amino acids are attractive methods for improving or bringing new and novel functions in peptides and proteins. Cell-free protein synthesis using the Protein Synthesis Using Recombinant Elements (PURE) system is an attractive platform for efficient unnatural amino acid incorporation. In this work, we further adapted and modified the One Pot PURE for a robust and modular system of enzymatic single site-specific incorporation of unnatural amino acid. With a single site-specific incorporation of unnatural amino acid, we demonstrated the flexibility of this system through the introduction of two orthogonal aminoacyl tRNA synthetase:tRNA (aaRS:tRNA) pairs to suppress two distinctive stop codons respectively within a mRNA template. Beyond this effort, we are investigating the possibility of utilizing this platform toward multiple enzymatic unnatural amino acid incorporation, prototyping engineered translational machinery, and introducing new to novel conjugation chemistry into protein.

21. **Kyle Shaffer**[1], Chloe M. Jones[1], Venkatesh Yarra[1], Vinayak V. Pagar[1], Richard B. Cooley[2], Ryan A. Mehl[2], and E. James Petersson[1]

[1]University of Pennsylvania, [2]GCE4All Research Center, Oregon State University

Development and use of fluorescent unnatural amino acids for biophysical studies

Fluorescent unnatural amino acids (Uaa’s) can be powerful biophysical tools, as they provide a site specific and minimally perturbing way to monitor protein folding and/or aggregation. The Petersson lab has previously shown that the Uaa acridonylalanine (Acd) can site specifically label proteins and be used as a Förster resonance energy transfer

(FRET) partner or a fluorescence polarization (FP) probe. Acd has impressive fluorescent properties such as 0.95 quantum yield (QY) in water, a fluorescence lifetime of 15 nanoseconds, and high photostability. Previously, Acd incorporation was limited to E. coli due to the limited orthogonality of it's synthetase. We have demonstrated successful incorporation of Acd into proteins in both HEK293 cells and E. coli via a newly evolved pyrrolysyl tRNA synthetase and demonstrated Acd's utility as a fluorescence lifetime probe. Currently, we are working towards the synthesis of red-shifted amino acids in order to produce a genetically incorporable FRET acceptor for Acd. We have also investigated Acd's effect on in vitro α-synuclein (αS) aggregation/disaggregation dynamics to validate its use for studying the effects of small molecules on αS, and we are working to express Acd-labeled αS in a neuronal cell type in order to carry out these studies in live cells

22. Paul Schnacke, Tiphaine Lainey, Shubhendu Palei, Maximilian Fottner, Kathrin Lang
Laboratory for Organic Chemistry (LOC), Department of Chemistry and Applied Biosciences (D-CHAB), ETH Zurich, Vladimir-Prelog-Weg 3, 8093 Zurich, Switzerland

Development of a modular toolbox for *in cellulo* ubiquitylation

The post-translational modifier ubiquitin (Ub) is a key regulator of protein fate. Substrates can be modified with single Ub monomers or oligomeric Ub chains that elicit distinct events depending on their topology. The resulting complex signaling network is inherently difficult to study in living cells. To address this challenge, we introduce a modular toolbox that enables *in cellulo* ubiquitylation in an orthogonal, and inducible fashion. For this, a previously reported system based on Sortase A and the use of genetic code expansion (GCE) has been improved by induced proximity to install these modifications in high yield. Furthermore, substrate scope and tunability have been expanded. These enhancements enable the installation of monoUb, ubiquitin-like modifiers (Ubls) as well as linear linked polyUb chains onto endogenous substrate-proteins within a cellular environment in a near-native-like manner. This should allow for the study of the thereby induced signaling events.

23. Maria Weyh, Marie-Lena Jokisch, Maximilian Fottner and Kathrin Lang
Laboratory for Organic Chemistry (LOC), Department of Chemistry and Applied Biosciences (D-CHAB), ETH Zurich, Vladimir-Prelog-Weg 3, 8093 Zurich, Switzerland

Deciphering functional roles of protein succinylation and glutarylation using genetic code expansion

Post-translational modifications (PTMs) dynamically regulate cellular processes. Lysine undergoes a range of acylations, including malonylation, succinylation (SucK) and glutarylation (GluK). These PTMs increase the size of the lysine side chain and reverse its charge from +1 to −1 under physiological conditions, likely impacting protein structure and function. To understand the functional roles of these PTMs, homogeneously modified proteins are required for biochemical studies. While the site-specific encoding of PTMs and their mimics via genetic code expansion has facilitated the characterization of the functional roles of many PTMs, negatively charged lysine acylations have defied this approach. We show the site-specific incorporation of SucK and GluK into proteins via temporarily masking their negative charge through thioester derivatives. We prepare succinylated and glutarylated bacterial and mammalian target proteins, including non-refoldable multidomain proteins. This allows us to study how succinylation and glutarylation impact enzymatic activity of metabolic enzymes and regulate protein-DNA and protein-protein interactions in biological processes from replication to ubiquitin signalling.

24. Filip Ilievski, Gerrit Branids, Ivan Volkov, Linnea Wikström, Magnus Johansson
Uppsala University

Fluorescence labelling of re-coded E.coli with non-canonical chemical entities

Single-molecule tracking (SMT) is a powerful method to study molecular binding kinetics in living cells. This is achieved by genetically fusing fluorescent proteins to the protein of interest (POI) or self-labeling tags that bind synthetic fluorophores. These genetically encoded labels can impair the protein function, due to their size and possible non-native interactions. Moreover, the location of the tag is limited either to the termini of the POI. Thus, the ideal labeling method must have minimal functional interference, flexibility in POIs location and provide environment as close to native physiological conditions. Genetic code expansion (GCE) is a powerful technology as a method for labeling POIs. It encompasses a variety of approaches of hijacking the host's transcriptional or translational machinery to insert new chemical entities. A series of genetically re-coded organisms (GROs) that originate from *E.coli* MG1655 phenotype were previously generated. All the in-frame amber codons (UAG) have been reassigned and release factor 1 (RF1) that terminates on UAG was removed. Moreover, series of click-chemistry based non-canonical amino acids (ncAAs) and orthogonal tRNA/aminoacyl RNA synthetase pairs (OTS) were engineered. Previous attempts to use GCE as labelling techniques have focused on outer membrane proteins and protein localization. This method has still not been applied for single-molecule tracking in characterizing dynamic binding cycles of inter-cellular proteins. In this work, we have developed a GCE-based *in vivo* site-specific protein labelling scheme with small organic dyes. We have restored GROs to a near-wild type phenotype[MJ1] , selected an efficient OTS and labelled and tracked outer membrane proteins, inner membrane proteins, as well as cytosoloic proteins, and characterized their diffusion

compared to previously published data. This allows us to expand our repertoire of dye-labeled components, for further single-molecule tracking based molecular binding kinetics studies in living cells.

25. **Phuong Ngoc Pham**[1], Jiří Zahradník[2,3], Lucie Kolářová[1], Bohdan Schneider[1] and Gustavo Fuertes[1]
[1]Institute of Biotechnology of the Czech Academy of Sciences, Vestec, Czechia, [2]First Faculty of Science, BIOCEV center, Charles University, Prague, Czechia, [3]Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot, Israel.
Photocontrolled interleukin/receptor interactions through genetic code engineering
Cytokines are key modulators of the immune and inflammatory responses. To date several cytokines have been approved for cancer immunotherapy in the clinic e.g. interleukin-2 (IL-2). However, the therapeutic application of cytokines is often hindered by toxicities and/or modest efficacies. Thus, the spatiotemporal control of interleukin-receptor interactions may help to further expand the utility of these biomolecules as protein-based therapeutics. Here, we report on a light-induced ON-switch IL-24/IL-20R2 heterodimer assembly based on genetically encoded photocaged tyrosines. Using a combination of biophysical, molecular biology and cell-based assays, we show that a single ortho-nitrobenzyl-tyrosine residue introduced at position 70 of recombinant IL-20R2 dramatically reduces the binding affinity towards IL-24. Mild irradiation with UV light removes the caging group thus enabling complex formation and activation of the JAK/STAT signaling cascade. Similarly, a photoswitchable non-canonical amino acid (azobenzene-phenylalanine) provides a certain degree of control over interleukin-receptor interactions. These results provide a proof-of-concept for the rational design of photoactivatable interleukin/receptor pairs directed against several conditions such as oncology, autoimmune disorders, and viral infections.

26. **Ruixi Wan**[1,2], Yulin Chen[1,2], Lihui Lao[1], Yingying Zheng[1], Ling Tang[1], Zhongyu Zou[3,4], Chuan He[3,4],Guojian Shao[5], Yun Ge[5], Ying Yuan[6] and Shixian Lin[1,2,6,7]

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O-GlcNAcylation determines the translational regulation and phase separation of YTHDF proteins

N^6-methyladenosine (m^6A) is the most abundant internal mRNA nucleotide modification in mammals, regulating critical aspects of cell physiology and differentiation. The YTHDF proteins are the primary readers of m6A modifications and exert physiological functions of m6A in the cytosol. Elucidating the regulatory mechanisms of YTHDF proteins is critical to understanding m^6A biology. Here we report a mechanism that protein post-translational modifications control the biological functions of the YTHDF proteins. We find that YTHDF1 and YTHDF3, but not YTHDF2, carry high levels of nutrient-sensing O-GlcNAc modifications. O-GlcNAcylation attenuates the translation-promoting function of YTHDF1 and YTHDF3 by blocking their interactions with proteins associated with mRNA translation. We further demonstrate that O-GlcNAc modifications on YTHDF1 and YTHDF3 regulate the assembly, stability and disassembly of stress granules to enable better recovery from stress. Therefore, our results discover an important regulatory pathway of YTHDF functions, adding an additional layer of complexity to the post-transcriptional regulation function of mRNA m^6A .

27. Sandhya Jaiswal, Surender Reddy Jakka and Govindasamy Mugesh
Indian Institute of Science (IISc)

In Vivo Biosynthesis of Chalcogen-Containing Unnatural Amino Acids and their Incorporation into GFP using GCE

The genetic code expansion (GCE) of an organism provides an interesting strategy to access novel variety of physiochemical landscapes of proteins. Unnatural amino acids (UAAs) that are fluorescent, photo-responsive, bio-orthogonally reactive, and mimic various protein modifications have been successfully incorporated into proteins for studying their roles in labeling, biocatalytic activities, protein photochemistry, spectroscopic studies, and more. Although, the GCE enables the expansion of protein chemistry through the co-translational incorporation of a wide variety of functional groups in the form of UAAs, the source of UAAs is mainly by means of laboratory chemical synthesis. Furthermore, the chemical synthesis of UAAs often involves numerous steps. And the solubility of chemically synthesized UAAs is contingent upon the functional groups attached to them, which can show challenges during the stock preparation. There are numerous UAAs soluble in acidic or basic conditions, often requiring the addition of a small amount of organic solvents. These limitations underscore the necessity of exploring biosynthetic pathways for the synthesis of UAAs within organisms to overcome such barriers and obtain proteins with novel UAAs. Therefore, we are working on a strategy in which an organism biosynthesizes UAAs. We focused on biosynthesizing tyrosine derivatives using an enzyme called Tyrosine Phenol Lyase (TPL) and incorporates them into proteins simultaneously using orthogonal translational machinery. In this discussion, I will cover: 1) the biosynthesis of tyrosine derivatives and their incorporation into proteins, 2) novel chemistry, and 3) the structural aspects of modified proteins (GFP). Further future directions involve utilizing redox-sensitive UAAs to develop protein-based antioxidant systems.

28. **Ling Tang**, Hongxia Zhao, Ling Tang, Chao Liu, Yu Fang, and Shixian Lin
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Manipulating Cation−π Interactions of Reader Proteins with Genetic Code Expansion
Cation-π interactions are the major noncovalent interactions for molecular recognition and play a central role in a broad area of chemistry and biology. Despite tremendous success in understanding the chemical nature and the importance of cation−π interactions in a range of biological processes, particularly in epigenetic regulation, the design and synthesis of stronger cation-π interactions remain elusive. Here, we report a chemical approach to engineer cation−π interactions in the histone methylation reader domains via the site-specific replacement of key Trp residues in the reader domains with electron-rich Trp derivatives in vitro and in mammalian cells. We show that this site-specific Trp replacement strategy is generally applicable for the engineering of high-affinity reader domains for the major histone H3 trimethylation marks, such as H3K4me3, H3K9me3, H3K27me3, and H3K36me3, with high specificity. Furthermore, we demonstrate that engineered reader domains can serve as powerful tools for various in vitro and in vivo biological applications, including detection, enrichment, imaging of histone methylation, and capture of the protein interactome at chromatin marks. Therefore, our study paves the way for the design of enhanced cation−π interactions in reader proteins for a variety of biological applications.

29. **Alex Eddins**, Yogesh M. Gangarde, Anamika Singh, Subhashis Jana, and Ryan A. Mehl
Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR USA.
Highly efficient protein labeling in living cells by controlling the redox state of encoded tetrazines
The site-specific attachment of fluorophores, probes or drugs to proteins in living systems is critical advancing our understanding of biology and drug development. However, bioorthogonal labeling of proteins is limited by slow reaction kinetics, unstable bioorthogonal handles, and low cell permeability of reagents. Current bioorthogonal labeling methods work well on extracellular proteins however labeling of intracellular proteins requires excess labeling reagents with extended labeling times, resulting in incomplete protein labeling and substantial background signal. To combat these limitations, and ideal system would display rapid reaction rates, site-specificity and afford quantitative labeling. We've developed genetic code expansion (GCE) systems to site-specifically incorporate tetrazine amino acids (Tet) into proteins in mammalian cells, which will rapidly and quantitatively react with trans-cyclo-octene (TCO) labels. We have recently learned that once Tet is encoded into proteins inside living cells, it is in equilibrium between an oxidized (reactive) and reduced (non-reactive) state. In its oxidized state the encoded Tet reacts rapidly (~30 min) and at low label concentrations (~1 uM) inside cells, but there exists a subpopulation (up to 60%) of reduced Tet-protein that remains unlabeled, depending on conditions. Upon lysis, Tet-protein expressed in mammalian cells completely reacts,

indicating that the unreactive fraction is due to intracellular reduction of Tet on proteins. To convert reduced, non-reactive Tet-protein to the oxidized reactive state, we identified a strategy to irradiate cells with gentle, low-energy light in the presence of a photocatalyst, leading to the 'photo-oxidization' of intracellular reduced encoded Tet. These treatments increased in-cell reactivity of Tet-protein from approximately 50% to over 90% and did so for a variety of structurally diverse Tet ncAAs. Furthermore, by rationally tuning the structure and reactivity properties of our encoded tetrazine ncAAs, we were able to circumvent this photooxidation process by encoding Tet ncAAs that remain oxidized under biological conditions. These data represent the first instances of stoichiometric protein labeling within cells, enabling researchers to more effectively study biological events using a variety of small, unobtrusive labels with desired biophysical properties, such as different fluorescent wavelengths and spin labels.

30. Satoshi Ishida, Andrew Ellington
University of Texas at Austin

Directed Evolution of a SelB Variant That Does Not Require a SECIS Element for Function

The introduction of non-canonical amino acids (ncAA) into proteins has enabled researchers to modify physicochemical and functional properties of proteins. However, some type of ncAAs such as beta-amino acid or D-amino acids still struggle for its low incorpoation efficiency. This is partially due to the weakened binding affinity of tRNA charged with ncAAs to EF-Tu; an elongation factor that recruits all natural aminoacyl-tRNA to the ribosome. Researchers have therefore attempted to engineer the amino acid binding pocket of EF-Tu to restore its weak affinity to ncAAs and increase its incorpoation efficiency. However, mutating EF-Tu could potentially alter its recruitment ability of tRNA charged with natural amino acids as well. Thus, a separate elongation factor that functions in paralle to EF-Tu could be a promising engineering platform for ncAA incorporation. To achieve this, we focused on a homologue of EF-Tu called SelB. SelB is an elongation factor that is specific for selenocysteineyl-tRNASec (Sec-tRNASec). SelB specifically interact with selenocysteineyl-tRNASec and not with other nautural tRNAs which makes SelB a perfect candidate of ncAA specific elongation factor. However, in order for SelB to recruit Sec-tRNA to ribosome, it needs a specific RNA sequence, the SECIS (selenocystein insertion sequence) element, on the mRNA which is an undesired function for engineering purpose. Therefore, development of SECIS independent SelB was attempts with the use of directed evolution.

31. George Augustin, Alex J Eddins, Dan Liefwalker, Richard Cooley, Ryan A Mehl
Department of Biochemistry and Biophysics, 2011 Agricultural and Life Sciences, Oregon State University, Corvallis, OR 97331, USA

Virus-mediated delivery of orthogonal facile-labeling of human cells through genetic code expansion

Expanding the genetic code enables the site-specific incorporation of "designer" non-canonical amino acids into proteins, facilitating the development of new approaches for in situ labeling of proteins across the cellular milieu. Current methods for cell labeling using genetic code expansion (GCE) methods are restricted to a few immortalized cell lines due to the challenges associated with low transfection efficiency and poor transgene expression. To overcome these limitations, we have developed a modular, non-cytotoxic, non-integrating, baculovirus-based delivery system for transducing genetic code expansion (GCE) machinery components into mammalian cells with greater efficiency in order to introduce amino acids with unique functional moieties into proteins with residue-specific precision in living cells. Here, we develop a novel baculovirus transfer vector system that harbors the Candidatus Methanomethylophilus alvus Mx1201 (Ma) pyrrolysine tRNA (PyltRNA)/pyrrolysyl-tRNA synthetase (PylRS-B5) pair to encode Tetrazine 3.0 butyl amino acid into proteins and label them with strained trans-Cyclooctene (sTCO) probes in mammalian cells. We demonstrate successful viral-mediated delivery of this GCE system into HEK293 cells and confirm successful encoding of Tet-3.0-butyl and labeling of the model reporter super-folder GFP protein with sTCO-conjugated fluorophores and polymers. Due to its versatility and compatibility with a wide range of cell types, we anticipate this virus-based GCE delivery approach will enable facile click-labeling of many proteins and open new avenues of study in cell biology. In particular, future work in our lab will be aimed at monitoring transcription factor localization and mechanism of regulations in live cells.

32. **Subhashis Jana**, George Augustin, P. Andrew Karplus, Richard B. Cooley, Ryan A. Mehl*
Department of Biochemistry and Biophysics at Oregon State University, Corvallis, Oregon, USA-97331

Studying the dynamics of transcription factor Sox2 – DNA binding interaction using site-specifically installed DNA-Intercalating fluorogenic probe

The transcription factor (TF) Sox2 plays a central role in embryonic stem cell maintenance. Its interaction with DNA serves as a cornerstone in regulating gene expression, and preserving stem cell pluripotency. Exploring the dynamics of Sox2 binding to DNA with single-molecule (SM) fluorescence is one of the most effective techniques for studying the Sox2-DNA binding event at the molecular level. This technique provides insights into the dynamics of Sox2 binding to DNA, such as the speed at which Sox2 can bind and release from its target sites, which is essential for its regulatory role in gene expression. SM fluorescence also allows visualization of the movement and binding patterns of Sox2 to specific DNA motifs and promoters. Fluorescent labeling of Sox2 is essential for these studies. However, current fluorescent labeling methods, like genetic fusions with fluorescent proteins or larger-sized halo-tags, disrupt protein function. Additionally, the low abundance of transcription factors within cells requires rapid and precise labeling methods to ensure complete labeling without compromising cellular integrity. To address these

limitations, we employed a site-specific labeling strategy using genetic code expansion (GCE) in which tetrazine-containing amino acids were encoded at various positions, including six distinct TAG sites of which one was opposite to the protein-DNA contacts within the Sox2-HMG. Leveraging the fast reaction rates between tetrazines and strained trans-cyclco-octene (sTCO) groups, we were able to observe the dynamics of Sox2 DNA binding with high precision and efficiency by first synthesizing the DNA-responsive fluorogenic probe, sTCO-Yo-Pro, as a Tet-protein label. The DNA binding Protein (DxP) labels become fluorescent only when the protein is bound to DNA, allowing for functional readout of proteins like transcription factors in vivo. After conjugation of the tetrazine-encoded Sox-HMG variants with sTCO-YoPro labels, we found that DxP labels positioned at sites of TF-DNA interaction exhibited high turn-on fluorescence upon contact with DNA, while those situated opposite to the interaction surface remained non-fluorescent. Encouraged by these proof-of-concept outcomes, ongoing research is aimed at exploring Sox2-DNA binding events within live cells.

33. **Yogesh M. Gangarde**, Patrick Allen, Alex J. Eddins, Abigail Pung, Riley Bednar, Nathan Alexander, Richard B. Cooley, P. Andrew Karplus and Ryan A. Mehl*
GCE4All Research Center, Department of Biochemistry & Biophysics, Oregon State University, Corvallis OR 97331

A universal GCE system for efficient tetrazine amino acid encoding and protein labeling

The development of genetic code expansion (GCE) tools has enabled the site-specific incorporation of tetrazine-bearing non-canonical amino acids (Tet-ncAAs) into proteins, facilitating diverse biochemical applications. Recently, a pyrrolysyl-tRNA synthetase (PylRS) tRNA pair (PylRS/$tRNA^{Pyl}$) pair derived from *Methanomethylophilus alvus* (Ma) was reported to encode a tetrazine-bearing ncAA in proteins. The evolved *Ma* system was associated with a few limitations such as low tRNA suppression efficiency, high concentration of Tet ncAA required for encoding on protein that attributed to the increased cost of ncAA and the toxicity exerted by ncAA on the expression host. Creating an improved GCE system to encode Tet-ncAAs is essential to overcome these limitations. The aminoacyl tRNA synthetase (aaRS) is a central component of the bioorthogonal translation machinery and it largely influences the efficiency of the GCE system and so here we sought to identify novel Ma PylRS variants able to encode improved Tet-ncAAs. We report the development of a Ma PylRS/tRNA-based GCE platform that efficiently encodes a rapidly reactive and biologically stable tetrazine-bearing ncAA, termed “Tet 3.0 butyl,” in proteins that overcomes limitations of the prior systems. We performed multiple rounds of double-sieved life/death selection on the Ma PylRS active site randomized library to generate this efficient RS that encodes Tet 3.0 butyl. The selected RS demonstrated high fidelity and efficiency (UP50 ~10 μM) for Tet 3.0 butyl encoding in proteins, comparable to wild-type RS in *E. coli*. Crystal structures of this new Ma PylRS variant with Tet3-butyl in its active site provides the structural basis for the improved aaRS activity. We successfully utilized the aaRS/tRNA pair to encode Tet 3.0 butyl

in *E. coli* for the expression of nanobodies, ScFv's, Fabs, and other proteins. Additionally, we showed efficient encoding and labeling of Tet 3.0 butyl in proteins in eukaryotic cells (HEK293T) for protein labeling applications and in yeast (*Saccharomyces cerevisiae*). The efficient encoding of Tet 3.0 butyl into proteins using this selected MaRS platform requires only small quantities of amino acid and can be used across multiple model expression hosts and platforms, underlying its enhanced and broad utility.

34. Riley M. Bednar, Richard B. Cooley, and Ryan A. Mehl.
GCE4All Research Center, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, 97331.

Towards an Untargeted, Continuous Evolution Platform for the Optimization of Genetic Code Expansion Machinery

A significant challenge in genetic code expansion technologies is the low catalytic efficiency of encoding machinery, often three orders of magnitude less efficient than ancestral systems. Current selection approaches, relying on saturation mutagenesis of only a few residues, limit the breadth and depth of mutational space that can be explored. An untargeted selection approach unrestricted by combinatorial library sizes could access novel evolutionary trajectories. We bridge this gap by developing a Continuous Adaptive Laboratory Evolution platform for Genetic code expansion Optimization (CALEGO). CALEGO exploits the noncanonical amino acid (ncAA)-dependent production of a single metabolic enzyme that serves as both a positive and negative selection marker, depending on the context. Under positive conditions, ncAA addition enables production of TrpS, an enzyme that converts indole to tryptophan in an auxotrophic strain. Under negative conditions, the withholding of ncAA leads to TrpS truncation, preventing the enzyme from converting fluoro-indoles into toxic fluoro-tryptophans. By utilizing a single selection marker for both positive and negative selections, only mutants that faithfully produce TrpS in an ncAA-dependent manner should survive, eliminating the most common escape mutants encountered in traditional selection approaches, and thereby rendering the platform compatible with a continuous evolution format. By increasing the amount of TAG sites in TrpS (or additional enzymes), the selective pressure can be ratcheted up over time, allowing for untargeted selection of encoding machineries with activities that exceed those currently attainable. Improving the efficiency of genetic code expansion machinery promises access to new materials, such as encodable noncanonical polymers, enhances industrial potential, and improves the applicability and utility of genetic code expansion tools for a broader research community.

35. Anamika Singh[1], Zoey Niu[2], Violeta Marin[2], Jonathan Young[2], Paul Richardson[2], Marcus L. Hemshorn[1], Richard B. Cooley[1], P. Andrew Karplus[1], Justin M. Reitsma[2*], Yunan Zheng[2*], Ryan A. Mehl[1*]

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Targeted Protein Degradation: From PROTACs to Genetic Code Expansion (GCE)

In maintaining cellular health, the relative abundance of proteins must be carefully controlled and indeed dysregulation of protein homeostasis is linked to numerous diseases. As part of these processes, the ubiquitin-proteasome system serves to control proper protein levels by marking specific proteins with ubiquitin molecules and orchestrating protein breakdown through the enzymes E1 (Ub activating), E2 (Ub conjugating), and E3 (Ub protein ligases). Targeted protein degradation (TPD) has recently emerged as a promising strategy to treat such imbalances in protein homeostasis. Among such TPD therapeutics are so-called proteolysis-targeting chimeras (PROTACs) that non-covalently tether disease-associated proteins to specific E3 ligases, enabling selective degradation. While this strategy has worked with a few E3 ligases including Cereblon (CRBN) and Von Hippel-Lindau (VHL), there exist dozens of other E3 ligases for which no small molecule binders exist to tether substrates. Tools to validate and study other E3 ligases as suitable conduits for targeted protein degradation are not yet available, though critically needed to expanded TPD therapies. Here, we used Genetic Code Expansion (GCE) to encode tetrazine-containing non-canonical amino acids (Tet-ncAAs) surrounding the well-studied immunomodulatory drugs (IMiD) binding pockets of the CRBN E3 ligase. These tetrazines can be efficiently conjugated in living cells to target-protein binding ligands that are functionalized with strained trans-cyclooctene (sTCO) and in doing so, target proteins can be recruited to the CRBN E3 ligase and targeted for degradation. To test this hypothesis, we encoded clickable Tet-ncAAs into the CRBN E3 ligase and covalently tethered it to BRD2/4-binding molecules that recruited BRD2/4 for CRBN mediated degradation, thus bypassing the need for CRBN specific binding molecules. BRD2/4 degradation was dependent on location of the Tet-ncAA encoding on CRBN and the linker length connecting the sTCO and BRD2/4 binding molecule. This approach has the advantages of not only maintaining the native state of E3 ligase, but also allowing the interrogation of E3 ligases and target protein partners under intracellular conditions and can be universally applied to other unliganded known E3 ligases as well.

36. **F. Aaron Cruz Navarrete**[1,2,#], Wezley C. Griffin[1,2,#], Yuk-Cheung Chan[3,#], Maxwell I. Martin[1,2], Jose L. Alejo[1,2], Ryan A. Brady[1,2], S. Kundhavai Natchiar[1,2], Isaac J. Knudson[4], Roger B. Altman[1,2], Alanna Schepartz[4,5,6,7,8], Scott J. Miller[3,*], and Scott C. Blanchard[1,2,*]

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β-amino acids reduce ternary complex stability and alter the translation elongation mechanism

Templated synthesis of proteins containing non-natural amino acids (nnAAs) promises to vastly expand the chemical space available to biological therapeutics and materials. However, existing technologies still limit the identity and number of nnAAs than can be incorporated into a given protein. Addressing these bottlenecks requires deeper understanding of the mechanism of messenger RNA (mRNA) templated protein synthesis by the ribosome and how this mechanism is perturbed by the inclusion of nnAAs. Here, we examine the impact of nnAA analogues of L-α-Phe: *p*-azido-Phe, D-α-Phe and the four β-Phe stereoisomers, on the formation and ribosome-utilization of a central protein synthesis substate: the ternary complex of native, aminoacylated transfer RNA (aa-tRNA), thermally unstable elongation factor (EF-Tu) and GTP. By performing ensemble and single-molecule fluorescence resonance energy transfer measurements, we show that, compared to L-a-Phe, *p*-azido-Phe-tRNA shows similar levels of ternary complex formation, while D-α-Phe- and (*R*) and (*S*)-β^2-Phe-tRNA disrupt ternary complex formation to levels below *in vitro* detection limits, and (*R*)- and (*S*)-β^3-Phe-tRNA reduce ternary complex stability by one order of magnitude. Consistent with these findings, D-α-Phe- and (*R*)- and (*S*)-β^2-Phe-charged tRNAs were not utilized by the ribosome, while (*R*)- and (*S*)-β^3-Phe were utilized inefficiently. (*R*)-β^3-Phe but not (*S*)-β^3-Phe also exhibited order of magnitude defects in the rate of translocation after mRNA decoding. We conclude from these findings that non-natural amino acids can negatively impact the translation mechanism on multiple fronts and that the bottlenecks for improvement must include consideration of the efficiency and stability of ternary complex formation.

37. Nikolaj G. Koch, Jaime Fernández De Santaella, Michael Nash
University of Basel / ETH Zurich, Switzerland

High-throughput epitope mapping of human arginase-1 using amber suppression scanning and bioorthogonal chemistry

Understanding antibody-mediated immune response to foreign proteins at high resolution is pivotal for a broad range of biomedical applications, from vaccine development to antibody-based therapies. Here we present the use of genetic code expansion (GCE) in combination with an amber scanning library and bacterial display to identify reactive epitopes of the therapeutic enzyme human arginase1 (hArg1). hArg1 is a promising enzyme for metabolic therapy of various cancer types including hepatocellular carcinoma and melanoma but immunogenicity is a concern.[1]Epitope mapping methods, like alanine[2] or cysteine[3] scanning, have helped to engineer such enzymes to reduce immune responses.[4,5] However, these epitope mapping strategies have inherent limitations. Alanine scanning may yield false negatives since alanine mutations can produce weak disturbances at the protein-antibody-interface which may not prevent antibody binding.[6] Cysteine scanning is restricted to cysteine-free proteins to prevent false positives resulting from cysteine bioconjugation strategy. Our approach enhances these epitope mapping techniques by facilitating the introduction of more potent perturbations while avoiding side reactions with cysteines through the use of bioorthogonal chemistry. We encoded S-allyl-cysteine (Sac) at amber stop codons, and specifically addressed the bioorthogonal allyl group of Sac for bioconjugation, serving as the epitope disruptor. This is particularly relevant since hArg1 has three cysteine residues. Bioconjugation was carried out with the state-of-the-art bioorthogonal inverse electron demand Diels–Alder reaction.[7] This strategy is generalizable for all displayable proteins. Also, given that Sac is one if the smallest non-

canonical amino acids (ncAAs) encodable with GCE, the conformational disturbance of the target protein is minimized,[8] which reduces the chance of false positives based on target protein unfolding. Unlike alanine and cysteine scanning, which use hard coded libraries that contain one mutation per residue, the amber scanning library can be repurposed for a few hundred ncAAs depending on the GCE system. We are the first to combine GCE[9], bacterial display[1], and the concept of an amber scanning library for epitope mapping. In our approach the efficient psychrophilic Pyrrolysyl-tRNA synthetase (PylRS) of *Methanococcoides burtonii* ensures higher display percentages than with commonly used PylRS. Also, in contrast to phage display, the bacterial workflow is able to detect conformational epitopes while also being less labor intensive. Both the use of a variety of ncAAs and biorthogonal conjugations, provide a controllable and versatile platform, where the resilience of protein-protein interactions to different size perturbations can be studied in detail. We envision these implementations would help further improve the robustness of the method and establish it as the epitope mapping of choice in the future.

38. Yuda Chen, Han Xiao, and Abhishek Chatterjee
University of California, San Francisco (UCSF)

Unleashing the Potential of Noncanonical Amino Acid biosynthesis and Genetic Incorporation to Engineer cells with Diverse Functions

Despite the great promise of genetic code expansion technology to modulate structures and functions of proteins, external addition of ncAAs is required in most cases and it often limits the utility of genetic code expansion technology, especially to noncanonical amino acids (ncAAs) with poor membrane internalization. During my PhD study, we have reported biosynthetic pathways for several genetic encodable ncAAs including sulfotyrosine, 5-hydroxyltryptophan, 3,4-dihydroxyl-L-phenylalanine and *p*-amino phenylalanine, through exploration of metabolic engineering and bioinformatics. With both the biosynthetic pathway from simple carbon source and genetic incorporation machinery, the engineered cells exhibit unique functions including manufacture of enahnced therapeutic proteins with site-specific post-translational modification, detection of oxidative stress, preparation of proteins with site-specific modifications and generation of sticky cells for material application.

39. Sebasthian Santiago Gonzalez, Riley Bednar, Kishor Mutyala, Richard Cooley, Ryan Mehl, Sarah Maddux, Aiko Umeda, Weiwen Deng, Yuan Cheng, and Melissa Thomas.
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Harnessing Genetic Code Expansion for the Development of Multispecifics

Site-specific incorporation of unnatural amino acids (uAAs) into proteins via genetic code expansion (GCE) provides a powerful protein engineering tool for the development of next-generation biopharmaceuticals. Here, we aim to use GCE to incoporate a tetrazine amino acid and harness its exceptional bioorthogonal reactivity to generate antibody-cytokine conjugates. Different protein sites will be evaluated in terms of uAA incorporation and conjugation efficiency, and resulting conjugates will be evaluated against their fusion counterparts in vivo to determine optimal conjugation sites and linkages

40. Christopher G. Thomson, and Amanda G. Jarvis

University of Edinburgh

Genetic Code Expansion with Novel Metal-Binding Unnatural Amino Acids for the Development of Artificial Metalloenzymes

Artificial metalloenzymes (ArMs) have the potential to provide highly-selective, new-to-nature, chemical synthesis pathways for challenging transformations in aqueous environments.[1] To date, ArMs development has largely relied on bioconjugation strategies to install pre-formed transition metal complex catalysts within a protein scaffold.[2] However, the incorporation of metal-binding unnatural amino acids directly within a protein through genetic code expansion (GCE) presents several significant advantages, such as specific control over the incorporation site with complete selectivity, and enabling directed evolution. This poster will present our groups recent work in this area, covering the synthesis of unnatural amino acids with metal-binding functionalities, such as bipyridylalanine, and their subsequent incorporation within the human steroid carrier protein (SCP-2L) using GCE. The resulting artificial apoproteins are capable of selectively binding abiotic metal co-factors for a diverse range of applications, including imaging agents and enantioselective Lewis-acid catalysis.[3] Introducing the same active site to the protein *via* traditional methods, such as cysteine bioconjugation, was investigated and produced surprising differences in selectivity.

41. Beyer JN, Serebrenik Y, Shalem O, and Burslem GM

University of Pennsylvania

Developing a method for protein editing in mammalian cells

The ability to tag proteins has been transformative for the study of proteins, and has enabled researchers to track a protein of interest's location, functions, and interactions. However, adding a tag to a protein comes with its own set of problems, particularly in mammalian cells. All tags, whether a 30 kDa GFP or a short epitope tag, are adding additional residues and bulk to the tagged protein, and this addition can impact key characteristics of the protein of interest, such as protein localization, half-life, or interactions. For example, some proteins cannot tolerate a tag at their N or C termini, requiring their native sequence to carry out specific functions. In a similar vein, many proteins lack specific antibodies, rendering them challenging to study. From these scenarios, it is clear that there is a need for a less disruptive tagging strategy that still enables informative experiments. To fill this gap in the field, we have developed a method for protein editing that enables the rapid installation of unnatural amino acids into proteins in mammalian cells. In this way, we can introduce useful functional groups with temporal resolution, in an effort to carry out classic cell biology experiments with significantly less disruption to the protein of interest. In order to do this, our technology leverages split intein-mediated protein trans-splicing, recombinant protein expression, and genetic code expansion to "edit" an unnatural amino acid, such as a click chemistry handle, photo-crosslinker, or authentic PTM, into a user-defined site into proteins inside mammalian cells. To demonstrate the utility of this technology, we have installed the unnatural amino acid p-azido-phenylalanine (pAzF), with TAMRA and biotin conjugated by click chemistry, into proteins in mammalian cells, including calnexin, β-actin, and c-Myc. Using these proteins as model systems, we have been able to validate our protein editing method by microscopy, mass spectrometry, and immunoblotting. We demonstrate that our protein

editing method is rapid, nearly traceless, has a low background, and can be easily multiplexed to incorporate a variety of useful labels.

42. Emerson M. Carmona, William N. Zagotta, and Sharona E. Gordon
Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195

Exploring the conformational dynamics of the human voltage-gated proton channel by incorporating acridonylalanine with genetic code expansion in *E. coli*

The human voltage-gated proton channel (hH_v1) is a membrane protein that contains a selective permeation pathway for protons. Different stimuli, including membrane potential, pH, and mechanical forces, regulate the open probability of this permeation pathway. Although structural models of hH_v1 have been reported, the molecular mechanisms explaining the hH_v1 function are still poorly understood. We hypothesize that studying the conformational dynamics of hH_v1 through transition metal ion FRET (tmFRET) will provide insight into the structural basis of its function. We incorporated the fluorescent noncanonical amino acid acridon-2-yl-alanine (Acd) in the H_v1 sequence using genetic code expansion (GCE) in *E. coli* as a tmFRET donor. We studied the expression, stability, function, and fluorescence of the Cysless version of hH_v1 with Acd incorporated at 14 positions along the protein sequence. The level of expression and the presence of truncated products varied across positions. Moreover, differences in the fluorescence-detection size exclusion chromatography (FSEC) peaks were observed between constructs. We successfully purified functional hH_v1-Acd for 12 of these constructs. Changes in the fluorescence spectrum and lifetime of hH_v1 with Acd incorporated in certain positions were observed when the protein was transferred from a detergent micelle to a lipid bilayer. The next step in this project is to insert cysteine residues in these hH_v1-Acd constructs to measure distance changes when the protein is labeled with a transition metal as a tmFRET acceptor.

43. Carly Schissel, Alanna Schepartz, and Matthew Francis
UC Berkeley

Post-translational acyl rearrangement in ribosomal products

The ribosome catalyzes amide bond formation between L-alpha-amino acids at an astonishing ~300 bonds per second. Increasingly we discover that not only can this translation machine catalyze bond formation between noncanonical residues, but it can also form non-amide bonds, including esters and thioesters. Chemical acylation of tRNA combined with cell-free protein translation permits exploration of unnatural peptide and non-peptide products formed by the ribosome. Here, we combine non-amide bond formation in the ribosome with post-translational chemistry to form unique non-peptide products. We've developed a novel non-amino acid that when installed within a peptide, chemically or ribosomally, isomerizes at neutral pH to form a reactive species that can be diversified into a variety of motifs common in FDA therapeutics. This described method may provide opportunities to genetically encode materials that begin to look more like natural products than peptides.

44. **Akos Nyerges**[1], Bogdan Budnik[2], Regan Flynn[1], Siân V. Owen[3], Eleanor A. Rand[3], Michael Baym[3], Maximilien Baas-Thomas[1], Svenja Vinke[1], Owen Spencer[1], Venkat Ayalavarapu[1], Shirui Yan[1], Min Liu[4], Kangming Chen[4], Qingmei Zheng[4] , Catherine Zhen[4], George M. Church[1,2]

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Synthetic genomes with radically modified genetic codes

Engineering the genetic code of an organism provides the basis for (i) making any organism safely resistant to natural viruses and (ii) preventing genetic information flow into and out of genetically modified organisms while (iii) allowing the biosynthesis of genetically encoded unnatural polymers. Driven by these three goals, we are constructing a computationally redesigned 57-codon *Escherichia coli* genome, in which seven codons have been replaced with synonymous alternatives in annotated protein-coding genes. I will describe how we synthesized this 3.97 Mbp genome, the challenges we faced during genome construction, and a novel technology that expedites the construction and troubleshooting of genomes with radically altered genetic codes (Nyerges, A. et al., *unpublished*). Furthermore, to achieve our goals in a single living cell, we developed a novel type of recoded organism bearing an amino-acid-swapped genetic code that—using repurposed viral tRNAs—reassigns two of the six serine codons to leucine during translation and combines this technology with nsAA-based biocontainment. This amino-acid-swapped genetic code renders cells completely resistant to viral infections by mistranslating viral proteomes and prevents the escape of synthetic genetic information by engineered reliance on serine codons to produce leucine-requiring proteins (Nyerges, A. et al., *Nature*, 2023). This work underscores the feasibility of bottom-up genome construction—through computational design, multi-omics analyses, genome synthesis, engineering, and genetic code expansion—toward biological systems with functions unavailable in extant organisms.

45. **Birthe Meineke**, Johannes Heimgärtner, Rozina Caridha, Michael Landreh and Simon J Elsässer

Karolinska Institute

Dual stop codon suppression in mammalian cells with genomically integrated genetic code expansion machinery

Pyrrolysyine-tRNA (tRNAPyl)/PylRS pairs from methanogenic archaea, as well as engineered tRNA/aaRS pairs derived from bacteria, are used for genetic code expansion in mammalian cells. Amber suppression is routinely achieved by transient introduction of the components leading to short-term and heterogeneous expression. Here, we demonstrate that stable integration of tRNA/aaRS genes allows for efficient, genetically encoded ncAA incorporation in diverse mammalian cell lines. We extend a general plasmid design and PiggyBac (PB) integration strategy developed for the Methanosarcina mazei tRNAPyl/PylRS pair to genomic integration of two tRNA/aaRS pairs of bacterial origin. We further explore suppression of ochre and opal stop codons and parallel incorporation of two distinct ncAAs, both accessible for click chemistry, by dual suppression in stable cell lines. Clonal selection allows for isolation of cells with high dual suppression efficiency and dual site-specific

fluorescent labeling of a cell surface receptor using bioorthogonal click chemistries on live mammalian cells.

46. Yarra Venkatesh,[1] Priyanda Giri,[1] Moriah Mathis,[2] Christina Hurley,[1] Richard Cooley,[2] Rahul Kohli,[1] Ryan Mehl,[2] E. James Petersson*[1]
[1]University of Pennsylvania, Philadelphia, PA, United States. [2]Oregon State University, Corvallis, OR, United States

Genetic Code Expansion for Site-Specific Dual Encoding of Fluorophore-Quencher Pairs

The ability to precisely modify proteins at multiple locations in their natural environment represents an unprecedented opportunity for answering biological questions at the molecular and cellular levels. In this work, we introduce a versatile dual incorporation approach, which involves the site-specific incorporation of two distinct noncanonical amino acids with bioorthogonal properties into proteins *in vitro*. This innovative strategy has demonstrated remarkable efficacy in incorporating acridonylalanine (Acd) and *m*-4-methyltetrazinyl phenylalanine (Tet) bearing amino acids at respective TAG and TAA codons in a single protein using mutually orthogonal tRNA/synthetase sets. As proof-of-concept, we successfully applied the dual encoding approach to study two different proteins of interest: 1) α-synuclein, a protein that aggregates to form amyloid fibrils which play an important role in Parkinson's disease, 2) the LexA protein, a key regulator of the SOS response that enables acquired antibiotic resistance in bacteria. In this design, Tet can serve as an intrinsic quencher of Acd fluorescence or a biorthogonal reactive handle for attachment of FRET acceptors such as BODIPY through strained *trans*-cyclooctene (sTCO) labeling. Strategically introducing Acd/Tet modifications at specific positions in the α-synuclein or LexA protein enables 1) helps probe α-synuclein structural changes, amyloid fibril kinetics, and molecular interactions, yielding insights into disease mechanisms and 2) real-time monitoring of pH mediated or RecA*-dependent LexA cleavage kinetics through fluorescence activation and screening small-molecule inhibitors for their effects on both RecA*-stimulated cleavage and LexA autoproteolysis. These studies highlight the potential for Acd/Tet double labeling to be used in mechanistic analysis and drug discovery in a complex biochemical pathway with important implications for suppressing antibiotic resistance in pathogenic bacteria.

47. Kishor Kumar Reddy Mutyala, Nathan Alexander, Richard B. Cooley, and Ryan A. Mehl
Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR USA.

A Genetic code expansion tool kit for metal binding amino acids

One third of all proteins contain metal ions which play a significant role in maintaining structural and functions roles. Metals can function as cofactors in complex protein catalytic and electron-transfer processes, and their unique spectroscopic and fluorescence properties of protein-bound metals can be exploited to interrogate detailed atomic level

mechanisms to better understand how they mediate higher-level architecture of cellular regulatory and signaling networks. Thus, the ability to introduce new metal binding sites into proteins offers a powerful approach to both study native proteins as well as create proteins with new functions. Here, we use genetic code expansion (GCE) to engineer rationally designed metallo-proteins by translationally encoding metal binding amino acids at specific positions of a protein of interest. In the GCE4All center we have benchmarked the currently available encoding systems Methanocaldococcus jannaschii amber codon suppressor tyrosyl-tRNA (MjtRNATyr CUA)/tyrosyl-tRNA synthetase (MjTyrRS) pair for bipyridyl alanine (Bpy-Ala), We also explore new GCE encoding systems that are compatible with eukaryotic cells and the synthesis and encoding of metal binding amino acids with diverse affinities for metal ligands.

48. Wenlong Ding, Hongxia Zhao, and Shixian Lin
Zhejiang Univ

Rare codon recoding for efficient noncanonical amino acid incorporation in mammalian cells

The ability to genetically encode noncanonical amino acids (ncAAs) has empowered proteins with improved or previously unknown properties. However, existing strategies in mammalian cells rely on the introduction of a blank codon to incorporate ncAAs, which is inefficient and limits their widespread applications. In this study, we developed a rare codon recoding strategy that takes advantage of the relative rarity of the TCG codon to achieve highly selective and efficient ncAA incorporation through

systematic engineering and big data-model predictions. We highlight the broad utility of this strategy for the incorporation of dozens of ncAAs into various functional proteins at the wild-type protein expression levels, as well as the synthesis of proteins with up to six-site ncAAs or four distinct ncAAs in mammalian cells for downstream applications.

49. Abigail Pung, Michael Allen, Richard Cooley, and Ryan Mehl
GCE4All Research Center, Department of Biochemistry & Biophysics, Oregon State University, Corvallis OR 97331

Advancing Expression Conditions for Fully Reactive Tetrazine-Encoded Antibody Fragments

Antibodies and antibody fragments are imperative to the advancement of science and medicine as protein therapeutics, diagnostics, and research tools. Due to their versatility of action and application, optimization of both antibody expression and functionality is of constant interest to researchers and is necessary for the development of new therapeutics. One way to expand the current functionality of antibodies is by site-specifically encoding noncanonical amino acids (ncAAs) through Genetic Code Expansion (GCE). This then

eliminates the need to conjugate fluorescent dyes, labels, and drugs to cysteines and unlocks many more sites of incorporation. Here, we wanted to pair the encoding of the rapidly reacting bioorthogonal tetrazine ncAAs with E.coli protein expression methods to create homogenous antibody fragments ready for drug- or probe- conjugation. Tetrazine ncAAs serve as an excellent reactive group because they are highly stable, highly selective for their strained-transcycloctene reaction partner, and most importantly display rapid kinetics enabling complete antibody-conjugation at stoichiometric levels in a manner of minutes. Producing conjugation-ready antibodies in E. coli is desirable to lower expression costs, increase the scale of antibody production and hasten production time, which would in turn expand their capacity to be used as medical and scientific tools. This is hindered by the difficulty of overexpressing disulfide bond-containing proteins in the cytoplasm of E. coli, due to an overwhelmingly reducing environment. We evaluated the use of solubility tag fusion proteins, various expression conditions and a recently published E. coli chaperone and disulfide isomerase system to create soluble antibody fragments, specifically scFV and Fab fragment proteins. We then synthesized this with an improved tetrazine ncAA encoding system to produce tetrazine Fabs and homogenous Fab conjugates. Our research greatly improves the means and accessibility of producing ncAA-containing antibody fragments and enables rapid and complete conjugation of probes to a given antibody site.

50. Richard B. Cooley*, Cat Hoang Vesley and Ryan A. Mehl.
GCE4All Research Center, Department of Biochemistry & Biophysics, Oregon State University, Corvallis OR 97331

Shedding new light on the "dark proteome": enhancing Genetic Code Expansion for biologically relevant phosphorylated proteins

Genetic code expansion (GCE) technologies have the potential to transform science, medicine and drug discovery. However, their widespread impact has been limited because often first-generation GCE systems are not efficient enough to generate biologically relevant proteins with non-canonical amino acids (ncAAs). Additionally, these systems are not easily adapted by non-experts into standard laboratory protein expression workflows and so many spectroscopists, structural biologists, cell biologists, and biophysicists are unable to harness GCE tools effectively. At the GCE4All Center, our mission is to optimize and adapt transformative GCE technologies to make the production of biologically relevant proteins efficient and approachable for a variety of applications. A primary focus here includes enhancing access to custom site-specifically phosphorylated proteins. Key advancements include (i) the generation of a healthy "truncation-free" *E. coli* expression host and compatible target protein vectors for multi-protein phospho-dependent complexes and intrinsically disordered proteins, which are typically prone to proteolysis and hydrolysis, (ii) improving target protein folding and solubility through co-expression of chaperones and tuning of expression levels, (iii) creating methods to generate biotinylated phospho-proteins for avidin immobilization or screening assays, (iv) producing isotopically (15N/13C) labeled proteins for NMR studies and mass spectrometry standards, (v) engineering dual ncAA

encoding for the generation of phospho-proteins with tetrazine "click" handles for rapid attachment of fluorophores, biophysical probes, and polymers and (vi) engineering the *E. coli* "PermaPhos" system for fully-autonomous, efficient and scalable expression of proteins with non-hydrolyzable pSer, making them resistant to phosphatase activity in eukaryotic environments. These efforts will expand opportunities in our pursuit to reveal the so-called "dark proteome" that is the countless number of phosphorylated protein variants found in biology.

51. **Nathan D. Alexander**[1], David Sokolov[1], Christopher A. Ahern[2], Rick B Cooley[1], Ryan A Mehl[1]
[1]GCE4All Research Center, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, USA 97331. [2]Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA 52242.

Forging a toolset for site specific 19F NMR and precise control of electrostatic p-p interactions using variably fluorinated amino acids

With Genetic Code Expansion (GCE), researchers possess a versatile toolset to investigate and modify function, structure, and interactions of proteins with atomic-level resolution. This technique relies on engineered tRNA/tRNA synthetase (tRNA/RS) pairs to orthogonally and faithfully inject location-specific non-canonical amino acids (ncAAs) into polypeptides ranging from medically important proteins to industrially relevant enzymes. Fluorinated aromatic ncAAs are highly useful as spectroscopic probes (e.g. 19F NMR, 18F PET), for tuning protein-substrate binding and exploring p-p and cation-p interactions. The similar size and shape of fluorinated ncAAs compared to their natural counterparts make them ideal probes since they minimally perturb native protein structure, but this also makes them very difficult to selectively encode into proteins using GCE. We addressed this challenge at the GCE4All Center by engineering and characterizing a family of Methanomethlyophilus alvus (Ma) Pyrrolysyl tRNA synthetase (PylRS)/tRNA pairs able to incorporate 7 different fluorinated phenylalanine and 2 fluorinated tryptophan derivatives with high efficiency and fidelity, which can be used in both prokaryotic and eukaryotic expression hosts. We began with a multimillion phenotype library of the Ma PylRS enzyme,and subjected library members to life and death selections that favor the survivability of mutants that only aminoacylate the cognate tRNA with the choice fluorinated ncAAs and not natural amino acids. Fluorescence based screens were then used to refine surviving RS pools for their ability to encode differentially fluorinated Phe and Trp isomers, revealing a suite of RSs that selectively incorporate mono, di, tri, tetra and penta fluoro-Phe in multiple configurations or diversely fluorinated tryptophans. Plasmid architecture and expression conditions were optimized to ensure high fidelity of encoding and compatibility with common culturing media. These engineered systems are now being adopted to interrogate biologically relevant p-p and cation-p interactions both in vitro and in vivo. These studies serve as demonstration of the relevance and applicability of GCE to study and modify diverse proteins of interest.

52. Wooree Ko[1,*], Joseph J. Porter[1,*], Sacha Spelier[2,3], Isabelle van der Windt[2,3], Priyanka Bhatt[4], Tyler Couch[1], Kevin Coote[4], Martin Mense[4], Jeffrey M. Beekman[2,3,5] and **John D. Lueck**[1,6,7,*]

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AntiCodon Engineered (ACE) tRNAs are a platform therapeutic for suppression of nonsense mutations.

Nonsense mutations arise from single nucleotide substitutions that result in premature termination codons (PTCs). PTCs result in little to no full-length protein production and loss of mRNA expression through the nonsense-mediated mRNA decay (NMD) pathway. We demonstrate that anticodon engineered (ACE-) tRNAs efficiently suppress the most prevalent cystic fibrosis (CF) causing PTCs, promoting significant rescue of endogenous cystic fibrosis transmembrane conductance regulator (CFTR) transcript abundance and channel function in different model systems. We demonstrate that our best-performing ACE-tRNA, that decodes all UGA PTCs to a leucine amino acid, markedly rescues CFTR channel function from the most prevalent CF causing PTCs that arise from non-leucine encoding codons. Using this single ACE-tRNA variant, we demonstrate significant rescue of CFTR channel function in an immortalized airway cell line and two different primary CF patient-derived intestinal cell models with CFTR nonsense mutations. Thus, ACE-tRNAs have promise as a platform therapeutic for CF and other nonsense-associated diseases.

53. **Ross Thyer**[1], Qiyao Wei[1], Andrew Gilmour[2], and Katie Leonard[3]
[1]Rice, Dept. of Bioengineering, [2]Rice, Systems, Synthetic, and Physical Biology Program, [3]Rice, Dept. of Chemical Engineering

An emulsion-based directed evolution platform to engineer selenocysteine biosynthesis.

Selenocysteine (Sec) is a rare, naturally occurring amino acid with unusual biophysical properties. Sec incorporation into proteins is an inefficient and convoluted process, which is further compounded by a lack of genetic reporters which can discriminate Sec from the canonical amino acid serine, its immediate biosynthetic precursor which forms a

competing incorporation pathway. Collectively, these issues make it challenging to interface the key biosynthetic enzymes with current high-throughput engineering methods, such as Compartmentalized Partnered Replication (CPR). CPR is emulsion-PCR method which utilizes water-in-oil emulsion droplets to partition individual cells encoding different library members, ensuring spatial separation. Genetic circuits can be constructed to establish a connection between the Gene-Of-Interest (GOI) and the expression or activity of a thermostable DNA polymerase, which can then selectively amplify the coding sequences for the most active GOI variants. We have recently engineered a thermostable DNA polymerase (SecPol) to be dependent on an arrangement of diselenide bonds, which enables us to leverage CPR to engineer and improved Sec biosynthesis and incorporation pathway. Using this system, we have identified several independent optimizations for the selenocysteine biosynthesis and incorporation pathway, along with broad improvements to the CPR methodology which reduce the presence of off-target amplicons and improve enrichment. CPR represents a promising platform for engineering challenging enzyme functions, especially within the context of intricate biosynthesis pathways or those not amenable to traditional workflows.

54. Andreas Torell, Alfred N. Larsson, Luke Odell, and **Daniel Fürth**
SciLifeLab/Uppsala University

Detection of protein-protein interactions by bio-orthogonal fluorogenic proximity probes

Detecting protein-protein interactions within cells is challenging. Transgenic approaches risk altering protein function via fluorescent tagging, while in situ methods lack in vivo compatibility. Here, we introduce fluorogenic probes with dual-tetrazine pegylated branched arms linked to xanthene dye. Activation requires both tetrazine arms to interact simultaneously with target proteins, enabling dual-substrate recognition. We applied our method to detect protein-protein interactions in both fixed and living cells, utilizing antibody conjugation for fixed cells and genetic code expansion for real-time detection in living cells. Our strategy ensures versatile applicability and seamless transition between fixed and living systems.

55. **Nick Fisk**, Jillyn M. Tittle, and Margaret A. Schmitt
University of Colorado Denver

Development of a Tunable Computational Model for the Translation of Expanded Genetic Codes

Despite continually improving experimental techniques and a growing store of experimental data, the relationship between system composition and the phenotypic behavior of natural and expanded translational processes have not been clearly established. Presently available computational models of translation do not account for changes in the composition of individual translational components as cellular growth rates change or

through the addition of orthogonal components into the system. We developed a Monte Carlo simulation of protein translation which follows the complement of *E. coli* and orthogonal tRNAs through 17 kinetic steps and 3 potential ribosomal exit points, ultimately leading to decoding a given codon. The model employs three sets of *in vitro* kinetic parameters corresponding to cognate, near-cognate, and non-cognate codon-tRNA interactions. The model determines cell composition-dependent, codon-specific translation time distributions and error rates at a level of detail beyond what has been experimentally evaluated. The combined calculated codon-level translation data produces estimates for overall translation rates that closely match the experimentally measured range of translation rates. The model makes many testable predictions about codon-dependent error rates, the contribution of tRNA concentrations to error rates, and ways in which error rates may change with cellular growth rate. Engineering translation requires understanding how system composition effects function. Integrated kinetics models of translation provide insight and guide experimental approaches to genetic code expansion.

56. Meg Schmitt[1], David G. Schwark[2], and John D. Fisk[1]
[1]University of Colorado Denver and [2] Pairwise

Evolution and deployment of an *M. barkeri* orthogonal pair for rapid evaluation of sense codon reassignment potential in *E. coli*

Genetic code expansion has largely focused on the reassignment of amber stop codons to insert single copies of non-canonical amino acids (ncAAs) into proteins. Increasing effort has been directed at employing the set of aminoacyl tRNA synthetase (aaRS) variants previously-evolved for amber suppression to incorporate multiple copies of ncAAs in response to *sense* codons in *E. coli*. Predicting which sense codons are most amenable to reassignment and which orthogonal translation machinery is best suited to each codon is challenging. Through evaluation of both targeted and error-prone PCR aaRS libraries, we evolved a highly efficient variant of the *Methanosarcina barkeri* pyrrolysyl orthogonal tRNA/aaRS pair that activates and incorporates tyrosine. The evolved *M. barkeri* tRNA/aaRS pair rivals the efficiency of the wild type tyrosine-incorporating *M. jannaschii* orthogonal pair. We deployed variants of the new orthogonal pair to rapidly probe the potential of sense codon reassignment using our previously developed fluorescence-based screen. Our screen utilizes the absolute requirement of tyrosine at position 66 of superfolder GFP for autocatalytic fluorophore formation. The introduced orthogonal tRNA competes with the endogenous translation machinery to incorporate tyrosine in response to a codon typically assigned another meaning in the genetic code. Significantly, every sense codon for which we were able to engineer an orthogonal tRNA with a Watson-Crick base pairing anticodon sequence is at least partially reassignable. Any detectable reassignment is a starting point for further improvement through additional directed evolution. We observe different patterns of sense codon reassignment efficiency for the *M. jannaschii* tyrosyl and *M. barkeri* pyrrolysyl systems, while holding other systemic conditions (e.g. *E. coli* strain, media composition, GFP and orthogonal translation machinery vector backbones)

constant, suggesting particular codons will be better suited to reassignment by different orthogonal pairs.

57. **Krittalak Chakrabandhu**[1], Laurent Ruel[2], Laurence Lavenant-Staccini[2], Valeriane Gaxotte[3], Christine Carapito[3], and Pascal Therond[2]
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Genetic Code Expansion Unveils Hedgehog Protein Interactions: Insights from Drosophila Models
Hedgehog (Hh) proteins play critical roles in embryonic tissue patterning, tissue maintenance, and regeneration. However, understanding the intricate interactions between Hh proteins and their partners, which are crucial for Hh distribution and responsiveness, has posed a significant challenge. Despite the abundance of components in the Hh pathway, only a limited number of direct interactions between Hh proteins and their partners have been confirmed. Complicating matters, certain interactions observed in vertebrates are absent in the commonly used Drosophila model, highlighting discrepancies between vertebrate and fly Hh pathways. To bridge this gap and facilitate a better understanding of Drosophila Hh signaling modulation, we embarked on a quest to uncover novel protein interactions vital to this process. Leveraging genetic code expansion and the incorporation of a photocrosslinking amino acid at specific sites on the Hh protein where partner binding had not been elucidated, we successfully captured transient Hh protein-protein interactions in live fly cells. Coupled with proteomics, our approach not only unveiled previously unknown proteins binding to Hh but also shed light on the role of a post-translational modification in the in vivo distribution of Drosophila Hh.

58. **Cory D. Dunn,** Meghan C. Reedy, Bharat V. Adkar, P. Benjamin Stranges
Chemistry Expansion, GRO Biosciences, Cambridge, Massachusetts 02139
Disruption-Activated Reporter Transcription: A method for rapid identification of aminoacyl-tRNA synthetases that incorporate non-standard amino acids
Incorporation of non-standard amino acids (NSAAs) into protein-based therapeutics can increase their stability, augment their efficacy, improve targeting to cells and organs, and facilitate evasion of the immune system. Expression systems permitting the incorporation of NSAAs require a translational machinery that can recognize and utilize these unusual amino acid substrates during protein synthesis. During directed evolution of NSAA-competent translational components, reporters that differentiate poorly between the desired NSAA and competing, endogenous standard amino acid (SAAs) are typically deployed, which often limits the power of initial rounds of selection. Here, we have developed an approach, entitled "Disruption-Activated Reporter Transcription" (DART) that

allows rapid enrichment for translational machinery variants that are mostly likely to allow NSAA incorporation. Within this improved method for the isolation of NSAA-directed translational machinery, expression of a reporter for which synthesis is agnostic to SAA or NSAA incorporation is controlled by a transcriptional repressor for which incorporation of a NSAA disrupts activity. By use of this AND-gate with two conditions on a translational machinery variant - general competence for reporter synthesis, as well as ability to add specific NSAAs to the polypeptide chain - we have isolated novel variants that allow for substantial incorporation of the NSAA 4-biphenylyl-L-alanine from a large pool of Methanocaldococcus jannaschii tyrosyl-tRNA synthetase mutants.

59. Colin S. Burdette, Elizabeth W. Chandler, Yusra Naeem, Ryan E. Singer, and **Meghan E. Breen**
Furman University
Development of genetic code expansion tools for *Candida glabrata*
Candida glabrata is a pathogenic yeast that is a common cause of hospital acquired infections. High death rates for systemic infections coupled with increasing rates of resistance to frontline antifungal drugs has led the World Health Organization to classify *C. glabrata* as a priority pathogen. Resistance to azole class antifungal drugs is regulated by the transcription factor Pdr1. Previous work to characterize protein-protein interactions regulating Pdr1 activity have used co-immunoprecipitations, but these experiments can miss capturing weak or transient interactions and do not report on the specific sites of interaction. To address this, we are developing the first genetic code expansion tools to incorporate photocrosslinking noncanonical amino acids (ncAAs) into proteins in *C. glabrata*. This will enable the use of bioorthogonal reactions to covalently capture Pdr1 protein-protein interactions in their native environment. Proof of concept was demonstrated by incorporating *p*-benzoylphenylalanine (pBpa) into superfolder GFP at position Y151 using an orthogonal translation system consisting of an *E. coli* tyrosyl-tRNA synthetase ($_{Ec}$TyrRS) and tRNA$_{CUA}^{EcTyr}$ pair. Proper functioning of the bioorthogonal $_{Ec}$TyrRS/tRNA$_{CUA}^{EcTyr}$ pair was evaluated using western blots to quantify the expression of full-length protein when pBpa. We are now expanding this system to incorporate additional photoactivatable ncAAs and testing the incorporation of these ncAAs in the transcriptional activation domain of Pdr1. This genetic code expansion system will be applied by our lab to map protein-protein interactions regulating Pdr1 activity.

60. **Khwanthana Grataitong**[1] and Charoonroj Chotwiwatthanakun[2]
Navamintratiraj University[1], Mahidol university[2]
Development of genetically engineered chimeric virus-like particles via genetic code expansion and protein fusion for specific targeting cancer cells
Cancer is the most dangerous disease and leads to a high rate of death in humans worldwide. Several reports demonstrated that the upregulation of several receptors

disturbs cellular homeostasis and induces cell transformation through genetic alteration in several types of tumors. Virus-like particles (VLPs) are useful tools with a great impact on medical nanotechnology. Further modification of protein cages through genetic modifications of their exterior surface allows site-specific recognition and targeting delivery. This study aims to develop synthetic virus-like particles (VLPs) that have a significant impact on medical nanotechnology. Protein cages can be further altered genetically to target delivery and recognize specific sites on their external surfaces. Recombinant VLPs demonstrate the self-assembly for the desirable attribute of the nanocontainers. As previously reported note that the protruding domain functions as their binding site for recognizing host cells and is crucial for viral entry into cells. We aim to create chimeric VLPs functionalized with a specific peptide onto cancer cells through protein fusion and genetic code expansion (GCE) and conjugate the interior part to carry a specific therapeutic compound. Thus, this research offers a fundamental understanding of how viruses and cancer cells interact, which in turn stimulates the creation of drug delivery systems for delivering therapeutic medicines to specific cancer cells.

61. **Satpal Virdee[1],** Daniel Squair[1], Sunil Mathur[1], Adam Fletcher[2], Tim Clausen[3], and Juraj Ahel[3]
[1]University of Dundee, [2]University of Glasgow, [3]IMP Vienna

Engineered E2~Ub Conjugates for activity-based E3 ligase profiling

The ubiquitin system regulates proteome homeostasis where ~650 E3 ligase (E3) enzymes play a central role. Tasked with the covalent transfer of the small protein ubiquitin, E3s mark specific substrates for degradation. E3s regulate a host of cellular processes and their aberrant activity has been associated with diseases including cancer, neurodegeneration and disorders of the immune system. Crucial to delineating the functions of E3s, and assessing their suitability as therapeutic targets, are technologies for measuring their activity.

We have developed chemically augmented E2~Ub protein conjugates that function as sensors of E3 activity. Akin to activity-based probes, our protein sensors undergo covalent labelling of E3s when they are in their activated state. Through the incorporation of unnatural moieties into the E2~Ub conjugate, covalent labeling is achieved. We have incorporated photocrosslinking amino acids using genetic code expansion and developed semisynthetic strategies enabling concomitant E2~Ub conjugate assembly and electrophile installation. These probes have led to unexpected and striking biological discoveries and are being used to accelerate the discovery of drugs that modulate E3 activity.

62. Erkin Kuru[1,2], Helena de Puig[2,3], Allison Flores[1,2], Jonathan Rittichier[1,2], Christopher A. Ahern[4], Thomas G. Bernhardt[5,6], David Z. Rudner[5], James J. Collins[2,3,7], Marc Vendrell[8], George M. Church[1,2]
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Rapid discovery and evolution of nanosensors containing fluorogenic amino acids
Binding-activated optical sensors are powerful tools for imaging, diagnostics, and biomolecular sensing. However, new biosensor discovery is slow and requires tedious steps in rational design, screening, and characterization. Here we report on a platform that streamlines biosensor discovery and unlocks directed nanosensor evolution through genetically encodable fluorogenic amino acids (FgAAs). Building on the classical knowledge-based semisynthetic approach, we engineer ~15 kDa nanosensors that recognize specific proteins, peptides, and small molecules with up to 100-fold fluorescence increases and subsecond kinetics, allowing real-time and wash-free target sensing and live-cell bioimaging. An optimized genetic code expansion chemistry with FgAAs further enables rapid (~ 3 h) ribosomal nanosensor discovery via the cell-free translation of hundreds of candidates in parallel and directed nanosensor evolution with improved variant-specific sensitivities (up to ~250-fold) for SARS-CoV-2 antigens. Altogether, this platform will accelerate the discovery of fluorogenic nanosensors and pave the way to modify proteins with other non-standard functionalities for diverse applications.

63. Antje Kruger
Resilience
Cell-free protein synthesis for biologics development and manufacturing
Cell-free protein synthesis (CFPS) has become one of the most promising technologies for rapid biologics development and biomanufacturing. By leveraging cell lysates and their biochemical machinery, CFPS circumvents traditional bottlenecks in drug production and testing, such as cloning, cell transformation, and cell viability issues. This technology enables the rapid expression and testing of proteins, thereby accelerating development and optimization workflows. Utilizing lysates from genetically engineered microbial strains, CFPS supports the expression of a diverse array of proteins—ranging from microliters for high-throughput screening, milliliters for top drug candidate characterization, to over hundreds of liters for GMP scale-up. With its unparalleled flexibility, speed, and throughput, CFPS offers the opportunity to move from discovery to clinical scale and commercial

production at unprecedented speed. Here, we showcase National Resilience's advanced cell-free biologics development and biomanufacturing capabilities. We (i) present examples of diverse proteins and "unmanufacturable" biologics that we have successfully produced by customizing our cell- free protein expression system, (ii) demonstrate the capabilities of our Molecular Development platform in optimizing antibodies and synthetic peptide binders, and (iii) provide an overview of how our rapid cell-free biologics development and protein expression platform integrates into Resilience's comprehensive biologics development and biomanufacturing infrastructure. Additionally, we discuss how our capabilities can be harnessed to develop and manufacture ncAA-containing biologics.

64. **Rachel Franklin**[1], Cat Hoang Vesely[2], Phillip Zhu[3], Joseph Meeuwsen[1], and Richard B. Cooley[2]
[1]Agilent Technologies, Corvallis, Oregon, United States, [2]Oregon State University- GCE4ALL Center, Corvallis, Oregon, United States, [3]Oregon Health and Science University, Portland, Oregon, United States
Using Top-Down Mass Spectrometry to Characterize Proteoforms Created via Genetic Code Expansion
Protein post-translational modifications (PTMs) play crucial roles in cellular processes, yet studying PTMs presents significant challenges. Mass spectrometry (MS) is considered a gold standard analytical technique for defining protein molecular weights, sequences, and modification composition. Top-down mass spectrometry (TDMS) provides rich information about proteins and protein complexes. Alternative fragmentation techniques such as electron capture dissociation (ECD) are crucial for top-down analysis because they enable sequence analysis of large protein ions while preserving important modifications such as phosphorylation. Genetic code expansion (GCE) creates site-specifically modified proteins, offering a method to probe the effects of PTMs using authentic proteoform standards. By combining GCE and TDMS, we can generate and analyze site-specifically modified proteins, providing unique insights into protein structure and function. In this presentation, we highlight two recent applications using top-down ECD MS to characterize genetically modified protein systems. First, we investigate the sequential phosphorylation of the SARS-CoV-2 nucleocapsid serine/arginine (SR)-rich domain by glycogen synthase kinase (GSK-3β), a key regulatory mechanism controlling viral replication. Our ECD fragmentation results identified nine phosphorylation sites within the SR-rich region of the nucleocapsid protein. We also compare kinase activation with genetically encoded phosphoserine and a non-hydrolyzable phosphoserine mimic which has important implications for studying phosphorylation within cells. We further applied top-down analysis to a larger protein, Bcl-XL, and its binding to the peptide BAD. TDMS revealed impurities in the protein samples which were used to inform downstream binding analysis with isothermal titration calorimetry (ITC). Impurities were identified using de novo sequence analysis. The location of ECD fragments generated from the Bcl-XL/BAD complex suggests that native TDMS can be used to investigate the structural features of the protein complex. This information compliments binding characterization studies using other

biophysical techniques. This work demonstrates the potential of using TDMS to characterize genetically engineered proteins. The field of top-down mass spectrometry is rapidly developing as technological advances have enabled more effective analysis of large proteins. In the future, genetically engineered proteins will likely serve as valuable standards for top-down analysis.

65. Patrick J. Almhjell, Micah B. Olivas, Polly M. Fordyce, and Dan Herschlag
Stanford University

Dissecting enzyme function with synergistic advances in high-throughput microfluidics and genetic code expansion

Genetic code expansion (GCE) has emerged as a promising technique to systematically modify enzymes and other proteins with extraordinary precision. With GCE, it is possible to install subtly perturbed residues (e.g., in pK_a or charge distribution), sensitive biophysical probes, bio-orthogonal handles, and post-translational modifications (PTMs) at positions of interest via the site-specific incorporation of noncanonical amino acids (ncAAs). However, the extraordinary potential of GCE has been limited by costly materials, labor-intensive synthesis, and imperfect translational fidelity, often making it difficult to obtain a sufficient quantity of homogeneous ncAA-containing protein for biochemical analysis. I overcome these limitations by combining GCE with the recently developed high-throughput microfluidic enzyme kinetics (HT-MEK) platform, which can determine fundamental thermodynamic and kinetic constants of enzymes across >1,000 reaction chambers at once. HT-MEK requires only attomoles of material per reaction chamber and can express, purify, and repeatedly assay >1,000 enzyme variants in parallel, thereby making it feasible and routine to collect thousands of biochemical measurements using less material and effort than typically required to collect a single such measurement. I describe progress towards a robust, high-fidelity *in vitro* transcription/translation system for generating homogeneous populations of ncAA-containing proteins on the HT-MEK device, even for ncAAs that are only subtly different from native amino acids. I further demonstrate parallel expression and purification of enzymes generated through amber suppression across 662 individual chambers. I apply this system to the study of enzymatic general base catalysis at carbon, a critical task of natural and engineered enzymes, using the pyridoxal 5’-phosphate (PLP)-dependent enzyme alanine racemase (AlaR). This implementation of GCE provides biophysical “knobs” that can reveal how enzyme scaffolds tune critical reaction parameters—here, pK_a, Brønsted coefficients, and reactive intermediate stability—to achieve their prodigious rates and selectivities. The synergistic advance provided by combining HT-MEK and GCE will unlock new discoveries across the biological and chemical sciences, revealing previously invisible aspects of enzyme function.