CRISPR 2023

27 June - 1 July 2023

Würzburg, Germany

DISCLAIMER: LIABILITY AND INSURANCE

The organizing committee will not assume any responsibility for damage or injuries to persons or property during the international meeting. It is recommended that participants and accompanying persons arrange themselves for personal travel and health insurance.

Dear CRISPR enthusiast,

Welcome to CRISPR2023, the 15th meeting of this long-running series on CRISPR-Cas systems! We are delighted to have you join us in Würzburg at the Institute of Molecular Infection Biology (IMIB) of the University of Würzburg and the Helmholtz Institute for RNA-based Infection Research (HIRI) from June 27th to July 1st, 2023. The meeting is a unique opportunity to share your science and interact with leading experts in this exciting and fast-paced field.

As has become the tradition, CRISPR2023 will feature a diverse and stimulating scientific program that covers various aspects of CRISPR biology and applications, ranging from genetics, biochemistry, structural biology, ecology, evolution, and technology development. You will also have the chance to enjoy the beautiful and historic city of Würzburg, with its stunning architecture, rich culture, and delicious cuisine. We have planned a boat trip for you and a wine tasting event in the Residenz, a UNESCO Heritage Site and major historical landmark in Würzburg, for your enjoyment.

We hope you are ready for a fun and productive week of cutting-edge science, inspiring discussions, and memorable experiences. Whether you are a seasoned CRISPR researcher or a newcomer to the field, we are sure you will find something to spark your curiosity and creativity.

We would like to thank our sponsors without whose generous support this meeting would not have been possible. Please visit their information booths and check out the promotional materials included in the delegate bag. A big thanks also to CRISPR2022 organizers April Pawluk, Erik Sontheimer and Feng Zhang for providing invaluable advice and assistance during the organization of this year's meeting as well as to our in-house organizing team working diligently in the background.

We wish you an exhilarating meeting with enough inspiration to keep you going until the next meeting in New Zealand.

Follow @CRISPR2023 on Twitter for more information as the meeting gets going.

The CRISPR2023 organizing committee

Chase Beisel
Helmholtz Institute for RNA-based Infection Research

Cynthia Sharma
University of Würzburg

Anita Marchfelder
University of Ulm

Stan Brouns
Delft University of Technology

Welcome to WÜRZBURG!

Würzburg is a picturesque city in the region of Lower Franconia (Unterfranken) in Bavaria that is situated on the Main river. Historically, it was a former Celtic territory that was settled by the Francs in the 6th and 7th century.

The city features many historic buildings with stunning architecture such as the Würzburg Residence, the Marienberg Fortress, and the St. Kilian Cathedral. The Würzburg Residence is one of Europe's most renowned baroque palaces and a registered UNESCO World Cultural Heritage Site. The palace was built by Balthasar Neumann and includes a much-acclaimed staircase with awe-inspiring frescos by the Venetian artist Tiepolo. The Marienberg Fortress is the most prominent feature of the city, towering above the Main river, amid the vineyards.

Würzburg and the surrounding regions are inextricably linked to wine and are home to a number of famous vineyards and wineries in Germany. For example, the historic wine cellar "Staatlicher Hofkeller" (at the Würzburg Residence) has been in existence for 875 years. Local wine can be enjoyed in many of the wine taverns in the city and also on the Old Main Bridge "Alte Mainbrücke", which offers views of the Marienberg Fortress and the pilgrimage church "Käppele".

Today, Würzburg is home to several internationally recognized institutions in higher education and research such as the Julius-Maximilians-Universität (JMU) Würzburg, the Technical University of Applied Sciences, and the University of Music, as well as several non-university research institutions.

Julius-Maximilians-Universität Würzburg (JMU)
Institute of Molecular Infection Biology (IMIB) / Research Center for Infectious Diseases (ZINF)

Founded in 1402, the Julius-Maximilians-Universität of Würzburg (JMU) is one of the largest universities in Germany and home to more than 28,000 students. Many eminent scholars and scientists have conducted their research in Würzburg. Among them are 14 Nobel laureates, including physicists Wilhelm Conrad Röntgen and Klaus von Klitzing, as well as chemist Hartmut Michel and physician Harald zur Hausen.

True to its motto *Science for Society*, JMU is committed to advancing research in fields that are relevant for the future. Infectious disease research has long been a major focus area of the biomedical sciences program of JMU. In 1993, the Institute of Molecular Infection Biology (IMIB) was founded together with the Research Center for Infectious Diseases (ZINF). The IMIB is an interdisciplinary research institution within the Medical Faculty of the University of Würzburg, with strong ties to the Faculty of Biology. Research groups at the IMIB study the molecular mechanisms of infections caused by bacterial and fungal pathogens as well as the host immune response. Research activities range from prokaryotic and eukaryotic cell biology and immunology to fundamental aspects of gene regulation and RNA biology, as well as the development of novel 3D infection models and diverse genomics technologies. Another focus of the Institute lies in the analysis of small RNAs and other non-coding RNA molecules in infection.

Helmholtz Institute for RNA-based Infection Research (HIRI)

The Helmholtz Institute for RNA-based Infection Research (HIRI) was established in May 2017 as a site of the Braunschweig Helmholtz Centre for Infection Research (HZI) in cooperation with the Julius-Maximilians-Universität Würzburg. Located on the Würzburg medical campus, the HIRI is the first research institution of its kind worldwide to fully focus on the role of RNA in infection processes.

Rising antimicrobial resistance, chronic infections, and (re-)emerging pathogens are among the major challenges facing humanity. As a federal institute, the HIRI pioneers an integrative approach to exploit the vast potential of RNA as a diagnostic, drug, and therapeutic target for new strategies to combat infectious diseases.

The HIRI focuses on three central areas: Basic research on bacterial pathogens, on viruses, and on the host response by the immune system. These three fields are complemented by applied research on diagnostics and RNA delivery for therapeutic purposes. The integration of emerging topics in RNA research, such as RNA modifications, will ensure a place for the HIRI at the forefront of infectious disease research. Core technologies, with a major focus on sequencing and CRISPR-based approaches, are harnessed for high-throughput exploration of RNA functions and will provide an opportunity to construct a systems-level view of the role of RNA during infection.

Agenda

Tuesday 27 June, 2023

17:00 - 18:30	Opening evening reception

Wednesday 28 June, 2023

09:00 - 09:10	Welcome and opening remarks by the organizers
09:10 - 10:00	KEYNOTE SPEAKER **Virginijus Šikšnys**, VILNIUS UNIVERSITY, LITHUANIA Exploring and harnessing the diversity of CRISPR-Cas systems

SESSION I-a: Class 1 CRISPR-Cas systems (Chair: Blake Wiedenheft)

10:00 - 10:25	**Malcolm White**, UNIVERSITY OF ST. ANDREWS Mechanistic Diversity of Type III CRISPR Systems
10:25 - 10:55	Coffee Break
10:55 - 11:15	**Gregor Hagelueken**, UNIVERSITY OF BONN Antiviral signaling by a cyclic nucleotide activated CRISPR protease
11:15 - 11:40	**Asma Hatoum-Aslan**, UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN Mechanisms of Type III-A CRISPR-Cas defense and counterattack by a phage-encoded protein
11:40 - 12:00	**Raymond Staals**, WAGENINGEN UNIVERSITY Type III-B CRISPR-Cas signaling-based cascade of proteolytic cleavages
12:00 - 13:30	LUNCH

SESSION I-b: Class 1 CRISPR-Cas systems (Chair: Chunyi Hu)

13:30 - 13:50	**Gintautas Tamulaitis**, VILNIUS UNIVERSITY Antiviral signaling by a cyclic nucleotide activates a RelE-like ribonuclease

13:50 - 14:15	**Lennart Randau**, UNIVERSITY OF MARBURG Functional and structural characterization of Type IV-A CRISPR-Cas-mediated CRISPR interference
14:15 - 14:35	**Ning Jia**, SOUTHERN UNIVERSITY OF SCIENCE AND TECHNOLOGY Type IV-A CRISPR-Csf complex: assembly, dsDNA targeting and CasDinG recruitment
14:35 – 14:55	**Ryan Jackson**, UTAH STATE UNIVERSITY Structure and Function of Type IV CRISPR systems and their anti-CRISPRs
14:55 - 15:25	Coffee Break
15:25 - 15:45	**Fabienne Benz**, PASTEUR INSTITUTE In trans adaptation and RNA-guided silencing by a Klebsiella plasmid-encoded type IV-A3 CRISPR-Cas

SESSION II-a: Class 2 CRISPR-Cas systems (Chair: Stephen Jones)

15:45 - 16:10	**Omar Abudayyeh**, MASSACHUSETTS INSTITUTE OF TECHNOLOGY New Molecular Technologies for Genome Editing and Cell Control
16:10 - 16:30	**Julene Madariaga-Marcos**, UNIVERSITY OF LEIPZIG Investigating the R-loop formation by CRISPR-dCas9 with single-molecule ultrafast twist measurements
16:30 - 17:00	Coffee Break
17:00 - 17:20	**Grace Hibshman & Jack Bravo**, UNIVERSITY OF TEXAS AT AUSTIN DNA interrogation by PAMless SpRY-Cas9
17:20 - 17:40	**Yan Zhang**, UNIVERSITY OF MICHIGAN CRISPR RNA and tracrRNA serve as a regulatory switch for apoCas9-mediated viral memorization in *Neisseria*
17:40 - 18:00	**Yibei Xiao**, CHINA PHARMACEUTICAL UNIVERSITY How Cas9-Csn2-Cas1-Cas2 supercomplex select prespacer during spacer acquisition
18:00 - 19:30	DINNER on-site
19:30 - 21:00	**POSTER SESSION #1 (ODD NUMBERS)**

Thursday 29 June, 2023

07:00 – 08:00	Fun Run with Vivlion
09:00 - 09:05	Opening remarks for Day 2

Session II-b: Class 2 CRISPR-Cas systems (Chair: Hong Li)

09:05 - 09:30	**Dipali Sashital**, IOWA STATE UNIVERSITY Metal-dependent specificity switching in CRISPR-Cas adaptation and interference
09:30 - 09:50	**Dina Grohmann**, UNIVERSITY OF REGENSBURG Allosteric activation of CRISPR-Cas12a requires the concerted movement of the bridge helix and helix 1 of the RuvC II domain
09:50 - 10:15	**Ekaterina Semenova**, RUTGERS UNIVERSITY tRNA anticodon cleavage by target-activated CRISPR-Cas13a effector
10:15 - 10:45	Coffee Break

Session III-a: CRISPR-derived technologies (Chair: Gil Westmeyer)

10:45 - 11:10	**Kira Makarova**, NATIONAL INSTITUTES OF HEALTH Comprehensive survey of novel CRISPR systems by mining the expansive genomic diversity of prokaryotic organisms
11:10 - 11:30	**Ailong Ke**, CORNELL UNIVERSITY Genome Editing Applications Based on the IscB-ωRNA structure
11:30 - 11:50	**Osamu Nureki**, UNIVERSITY OF TOKYO Minimal and most efficient genome editing Cas effector
11:50 - 12:10	**Chunlei Jiao**, HELMHOLTZ INSTITUTE FOR RNA-BASED INFECTION RESEARCH TracrRNA reprogramming enables direct, PAM-free detection of RNA with diverse DNA-targeting Cas12 nucleases
12:10 - 13:30	LUNCH
13:00 - 13:30	Lunch & Learn Ryan Leenay, ELI LILLY Therapeutic Gene Editing at a Large Pharma
13:30 - 15:00	**POSTER SESSION #2 (EVEN NUMBERS)**

Session III-b: CRISPR-derived technologies (Chair: Patrick Pausch)

15:00 - 15:20	**Adrian Molina Vargas**, UNIVERSITY OF ROCHESTER New design strategies for ultraspecific Cas13a-based RNA-diagnostic tools with single-nucleotide mismatch sensitivity
15:20 - 15:45	**Samuel Sternberg**, COLUMBIA UNIVERSITY Molecular innovation at the interface between transposons and CRISPR-Cas systems

15:45 - 16:05 **Morayma Temoche-Diaz**, METAGENOMI
Genome editing using novel reverse transcriptases discovered from metagenomics

16:05 - 16:25 **David Schwark**, PAIRWISE
A Novel Mechanistic Framework for Precise Sequence Replacement Using Reverse Transcriptase and Diverse CRISPR-Cas Systems

16:25 - 16:55 Coffee Break

Session IV-a: CRISPR ecology and evolution (Chair: Rolf Backofen)

16:55 – 17:15 **Raul Perez-Jimenez**, CENTER FOR COOPERATIVE RESEARCH IN BIOSCIENCES
Evolution of CRISPR-associated endonucleases as inferred from resurrected proteins

17:15 - 17:40 **Lotta-Riina Sundberg**, UNIVERSITY OF JYVÄSKYLÄ
Mucin induces CRISPR-Cas defense in an opportunistic pathogen

17:40 - 18:00 **Harry Edwards**, UNIVERSITY OF NOTTINGHAM
Linking the SOS response and CRISPR Cas adaptation

19:00 - 22:00 BOAT TRIP and DINNER

Friday 30 June, 2023

09:00 - 09:05 Opening remarks for Day 3

Session IV-b: CRISPR ecology and evolution (Chair: Wolfgang Hess)

09:05 - 09:25 **Owen Tuck**, UNIVERSITY OF CALIFORNIA BERKELEY
Genome expansion by a CRISPR trimmer-integrase

09:25 - 09:45 **Nathaniel Burman**, MONTANA STATE UNIVERSITY
Type III CRISPR-mediated Control of a Toxin-Antitoxin System

09:45 - 10:10 **Stineke van Houte**, UNIVERSITY OF EXETER
Understanding ecological determinants of CRISPR-phage interactions

10:10 - 10:30 **Hélène Chabas**, ETH ZURICH
CRISPR-Cas systems: a barrier for antibiotic-resistant conjugative plasmids?

10:30 - 11:00 Coffee Break

11:00 – 11:20 **Reetta Penttinen**, UNIVERSITY OF JYVÄSKYLÄ
CRISPR-harboring megaphages isolated from boreal freshwater environment

Session V-a: Beyond CRISPR (Chair: Alan Davidson)

11:20 - 11:40	**Joshua Modell**, JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE Prophages vaccinate CRISPR-Cas populations against lytic threats
11:40 - 12:05	**Joseph Bondy-Denomy**, UNIVERSITY OF CALIFORNIA SAN FRANCISCO Anti-CRISPR proteins as mediators of phage-host symbiosis
12:05 - 13:25	LUNCH
13:25 - 14:30	**POSTER SESSION #3 (OPEN)**
14:30 - 14:50	**Kevin Forsberg**, UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER The mysterious origins of anti-CRISPR proteins
14:50 - 15:15	**Peter Fineran**, UNIVERSITY OF OTAGO Regulation of anti-CRISPR production
15:15 - 15:35	**Alexander Meeske**, UNIVERSITY OF WASHINGTON Viral *cas* genes antagonize CRISPR immunity
15:35 - 16:05	Coffee Break

Session V-b: Beyond CRISPR (Chair: John van der Oost)

16:05 - 16:30	**Rotem Sorek**, WEIZMANN INSTITUTE Phages overcome bacterial immunity via diverse anti-defense proteins
16:30 – 16:50	**Giuseppina Mariano**, UNIVERSITY OF SURREY The novel anti-phage system Shield co-opts an RmuC domain to mediate phage defense across Pseudomonas species
16:50 - 17:15	**David Bikard**, PASTEUR INSTITUTE An anti-anti-CRISPR system: triggering cell death in response to phage counter-defenses
17:15 – 17:35	**Daan Swarts**, WAGENINGEN UNIVERSITY The diverse functions and mechanisms of prokaryotic Argonaute proteins
17:35 - 17:50	Closing remarks and announcement of CRISPR2024 in New Zealand
19:00 - 23:00	CONFERENCE DINNER with wine tasting in the Würzburg Residenz wine cellar

Saturday 1 July, 2023

FUN DAY in Würzburg

09:30 - 12:30	CITY TOUR
12:30 -	KILIANI VOLKSFEST

Thank you to our sponsors

Platinum

Lilly

arbor biotech

Gold

CAZYME

revvity

Silver

Arc Institute

vivlion
PRCISR™ CRISPR

The CRISPR Journal

GEN Biotechnology

EM Open Access Journal
ENGINEERING MICROBIOLOGY

HIRI HELMHOLTZ Institute for RNA-based Infection Research

Bronze

CARIBOU BIOSCIENCES®

Beam THERAPEUTICS

pairwise®

prime medicine

SNIPRBIOME
A CRISPR COMPANY

NEW ENGLAND BioLabs Inc.

Pioneering next-generation gene editing to put functional cures within reach.

arbor™
biotech

Learn More
About Arbor.

Caszyme is a biotech company based in Vilnius Lithuania. The company specializes in development and applications of CRISPR Cas technology and aims to develop new discoveries, innovative applications, and top-quality research in the field of **CRISPR based Molecular Tools**. **Caszyme** seeks to create outstanding value for researchers and companies practicing gene-editing worldwide.

WHAT WE OFFER?

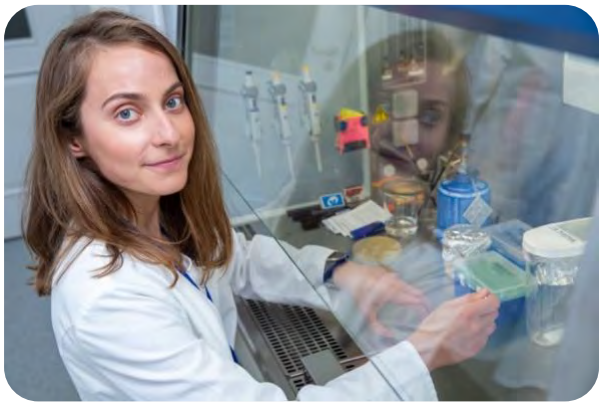
- CRIPSR research and development
- Cas nucleases
- Bioinformatic analysis
- Platform of characterized Molecular Tools
- Cas protein purification and analysis

ORTHOLOGS

Platform of Cas9, Type V and other orthologs for testing and licensing.

APPLICATIONS

- Human Therapeutics
- Diagnostics
- Industrial Biotechnology
- Agriculture
- Research Tools



Scan to find out more

revvity

Infinite reliability for functional genomics.

Dharmacon™ Reagents

Understanding functional genomics is critical to advance genetic research and therapeutic discovery. Dharmacon Reagents support researchers on this journey at the DNA, RNA, and protein level with the broadest range of functional genomic tools including industry leading gene modulation and gene editing solutions to help elucidate complex biological networks for functional genomic studies.

Known for almost 30 years as premier providers of synthetic oligonucleotides, Dharmacon Reagents were the first to offer algorithm designed synthetic siRNA for RNAi, and the first to offer guide RNA for CRISPR-Cas9 gene editing applications.

Dharmacon Reagents have you covered with a complete range of genomic solutions from single-gene experiments to whole genome screens facilitating advancements in genetic research and therapeutic discovery.

Scan me

For a complete listing of our global offices, visit www.revvity.com

1069850

ENGINEERING MICROBIOLOGY

Elsevier & Shandong University

Engineering Microbiology is a peer reviewed, open access journal. All Article Publishing Charge (APC) fees for accepted articles will be covered. In addition, any required language editing fees will be covered for all accepted articles.

EMAIL
engmicro@email.sdu.edu.cn

VISIT THE JOURNAL HOMEPAGE
https://www.journals.elsevier.com/engineering-microbiology

Engineering Microbiology covers the methodology, theory, mechanism, and application of engineering microbes with an ultimate goal of improving microbes for practical applications.

Engineering Microbiology covers fields including but not limited to:

- Microbial methodology and technology
- Microbial cell engineering
- Genetic engineering
- Metabolic engineering
- Enzyme engineering
- Microbial synthetic biology
- Microbial engineering for natural products and bulk/fine chemicals
- Medical and pharmaceutical microbiology and biotechnology
- Microbiota engineering
- Environmental microbial engineering
- Microbial genome editing technology
- Engineering of microbial regulatory networks
- Theories for microbial engineering
- Bioinformatics and computational analysis of microbial engineering

Scan to visit our homepage

Scan to check our WeChat official account

Editor-in-Chief

Dr. Youming Zhang

An academician of both the Academia Europaea and German Academy of Engineering

Why publish in *Engineering Microbiology*?

Gold open access

No open access publication fees for authors who submit and publish articles before December 31, 2024

Article types

Full length research papers | news
review articles | research highlights
short communications | commentaries

Easy to submit & Fast review

No formatting requirements for the 1st submission

1 week	Initial check
1 month	The first decision
3 months	Online

SPEAKER ABSTRACTS

Keynote Lecture

Virginijus Šikšnys

Vilnius University

From CRISPR-Cas to mobile genetic elements and beyond

In prokaryotes CRISPR-Cas system functions as an adaptive immune system that provides resistance against invading viruses. Deciphering the molecular mechanisms underlying the CRISPR-Cas immunity paved the way for development of novel tools for targeted genome engineering. Currently, RNA-programmable Cas9 and Cas12 effector nucleases of CRISPR-Cas systems are widely used for genome editing applications. Bioinformatic analysis identified TnpB proteins of IS200/IS605 transposon family as the likely predecessors of Cas12 nucleases. Biochemical experiments showed that TnpB indeed functions as the RNA-guided nuclease and can be adopted as a new class of genome editing tools. Recent cryo-EM structures revealed that TnpB represents the minimal structural and functional core of the Cas12 protein family. Taken together, these data show how parts of mobile genome elements were adapted by CRISPR-Cas systems to fight against phages.

Session I-a: Class 1 CRISPR-Cas systems

Malcolm White

University of St Andrews

Mechanistic Diversity of Type III CRISPR Systems

Type III CRISPR systems detect RNA from mobile genetic elements, resulting in the activation of the catalytic Cas10 subunit. Most Cas10s have a specialised PALM polymerase domain that synthesises a range of cyclic oligoadenylate (cOA) second messengers. A burst of cOA synthesis serves to activate ancillary effector proteins which tend to act non specifically, providing immunity by degrading crucial cellular components such as DNA or RNA. This can result in cell death / dormancy, but many type III CRISPR systems also encode “off switches” – enzymes that degrade the second messengers to reset the cell when invaders have been cleared. In this talk, I will present unpublished work on two divergent type III CRISPR systems, one encoded by a prophage in Vibrio cholerae and the second found in the CFB group of bacteria, often associated with the human microbiome. These studies have revealed some unexpected features which significantly expand the mechanistic diversity of type III CRISPR systems, both in terms of the functional repertoire of Cas10 and the associated effector proteins.

Gregor Hagelueken

University of Bonn

Antiviral signaling by a cyclic nucleotide activated CRISPR protease

Christophe Rouillon; Niels Schneberger; Haotian Chi; Katja Blumenstock; Stefano Da Vela; Katrin Ackermann; Jonas Moecking; Martin F. Peter; Bela E. Bode; Jonathan L. Schmid-Burgk; Dmitri Svergun; Matthias Geyer; Malcolm F. White; Gregor Hagelueken[1]

[1] University of Bonn

Type III systems can orchestrate a complex antiviral response that is initiated by the synthesis of cyclic oligoadenylates (cOAs) upon foreign RNA recognition. Among a large set of proteins that were linked to type III systems and predicted to bind cOAs, a CRISPR associated Lon protease (CalpL) caught our attention. The protein contains a sensor domain of the SAVED (SMODS-associated and fused to various effector domains) family, fused to a Lon protease effector domain. However, the mode of action of this effector was unknown. We determined the structure and function of CalpL and showed that the soluble protein forms a stable tripartite complex with two further proteins, CalpT and CalpS, that are encoded in the same operon. Upon activation by cA4, CalpL oligomerizes and specifically cleaves the MazF-homolog CalpT, releasing the extracytoplasmic function (ECF) sigma factor CalpS from the complex. This provides a direct link between CRISPR-based foreign nucleic acid detection and transcriptional regulation. Furthermore, the presence of a cA4-binding SAVED domain in a CRISPR effector reveals an unexpected link to the cyclic oligonucleotide-based antiphage signaling system (CBASS).

Asma Hatoum-Aslan

University of Illinois at Urbana-Champaign

Mechanisms of Type III-A CRISPR-Cas defense and counterattack by a phage-encoded protein

Type III CRISPR-Cas systems launch a multi-layered immune response against foreign nucleic acids using small CRISPR RNAs (crRNAs) in complex with multiple CRISPR-associated (Cas) proteins. Of the six subtypes currently identified (A-F), Types III-A and III-B are the best characterized. In these systems, crRNA binding to a complementary transcript triggers at least three catalytic activities by members of the effector complex: target RNA shredding by Cas7, DNA degradation by Cas10, and Cas10-catalyzed production of cyclic-oligoadenylates (cOAs), which subsequently bind and stimulate a variety of accessory nucleases. Additionally, our previous work showed that the model Type III-A CRISPR-Cas system in Staphylococcus epidermidis (CRISPR-Cas10) relies upon a variety of cellular 'housekeeping' nucleases to assist with degradation of foreign nucleic acids and ensure a successful defense. However, despite this robust immune response, we discovered a subset of S. epidermidis phages that have the capacity to overcome CRISPR-Cas10 immunity. This presentation will discuss our most recent insights into the mechanisms of CRISPR-Cas10 defense and corresponding phage-encoded countermeasures, with a focus on the cellular housekeeping pathways that mediate both processes.

Raymond Staals

Wageningen University & Research

Type III-B CRISPR-Cas signaling-based cascade of proteolytic cleavages

Jurre Steens[1]; Jack Bravo[2]; Stephan Köstlbacher[1]; Carl Raymund Salazar[1]; Caglar Yildiz[1]; Afonso Amieiro[1]; Thijs Ettema[1]; John van der Oost[1]; David Taylor[2]; Raymond Staals[1]

[1] Wageningen University & Research; [2] University of Texas at Austin

Type III CRISPR-Cas systems detect foreign RNA in a sequence-specific manner, and in response generate cyclic oligoadenylate (cOAs) signalling molecules. These cOAs activate ancillary proteins that carry the appropriate sensory domain: CARF (CRISPR-associated Rossmann fold) or SAVED (SMODS-associated and fused to various effector domains). CARF and SAVED proteins induce powerful immune responses that can lead to viral clearance, cell dormancy, or cell death. Here, we characterize a set of type III-associated genes that include two proteases, one of which is fused to a SAVED domain. We show that the SAVED-protease is activated by cOA and oligomerize to form long filaments that stabilize the protease's active sites. Activated SAVED-protease then specifically cleaves and activates a second protease. We further show this second protease can cleave other proteins in vitro. Finally, we demonstrate that this setup (type III system with the two proteases) provokes a strong abortive infection-like phenotype in vivo when challenged with a target RNA. Our findings suggest that type III CRISPR-Cas systems can employ a cascade of proteolytic events to mediate a strong immune response, with intriguing conceptual similarities to the eukaryotic signaling pathways leading to apoptosis.

Session I-b: Class 1 CRISPR-Cas systems

Gintautas Tamulaitis

Vilnius University

Antiviral signaling by a cyclic nucleotide activates a RelE-like ribonuclease

Prokaryotic type III CRISPR-Cas antiviral system employs a cyclic oligoadenylate pathway to activate a diverse range of auxiliary proteins. These CRISPR-associated proteins are very diverse and are comprised of the conserved signal sensing CARF-domain and variable effector domain (RNase, DNase, protease or others). Our performed bioinformatic analysis identified a large group of type III CRISPR-Cas associated proteins that contain a CARF-domain coupled to RelE toxin-like effector domain. However, the mode of action of these effectors is unknown. In order to understand the structure-function relationship of CARF-RelE protein, we performed structural studies of the effector and showed that cyclic nucleotide-activated CARF-RelE cleaves mRNA exposed in the ribosomal A-site both in vitro and in vivo. Here we propose a detailed mechanism for mRNA degradation by the effector.

This work was supported by RCL S-MIP-22-09.

Lennart Randau

Philipps Universität Marburg

Functional and structural characterization of Type IV-A CRISPR-Cas-mediated CRISPR interference

Our laboratory investigates Type IV-A1 CRISPR-Cas systems of Pseudomonas oleovorans strain DSM 1045 and Methylorubrum extorquens. Both organisms' Type IV CRISPR arrays are located on megaplasmids and reveal spacer sequences that match plasmid, transposon, and viral targets with a consensus 5'-AAG-3' PAM motif. Processing of CRISPR RNAs (crRNAs) was also observed for highly degenerated repeat sequences. P. oleovorans was found to produce crRNAs that target the host pilN gene. The deletion of this crRNA resulted in upregulated pilN expression, which impacts Type IV pilus generation. Synthetic crRNAs with engineered spacers were introduced into P. oleovorans as guide molecules for native effector complexes. Downregulation of their target gene expression was confirmed via tryptophan auxotrophy or pigment accumulation phenotypes. In addition, a reporter gene assay was established to analyze the (i) stringency and (ii) spatial coordination of CRISPRi effects on gfp expression. Recombinant Type IV-A CRISPR-Cas effectors were produced in Escherichia coli and showed activity against plasmid, virus, and host gene targets. Purified recombinant CRISPR ribonucleoproteins (crRNPs) consist of single copies of the Cas proteins Csf1 (Cas8-like), Csf3 (Cas5-family), the crRNA endonuclease Csf5 (Cas6-family), and five copies of Csf2 (Cas7-family) backbone proteins bound to the crRNA spacer. The helicase Csf4 (DinG) was found to be recruited to the effector crRNP upon target recognition, and a structure highlighting DinG and crRNP interactions was obtained. Our results highlight that Type IV-A CRISPR-Cas systems regulate a wide range of DNA targets in the absence of conventional DNase activities.

Ning Jia

Southern University of Science and Technology

Type IV-A CRISPR-Csf complex: assembly, dsDNA targeting and CasDinG recruitment

Type IV CRISPR-Cas systems, which are primarily found on plasmids and exhibit a strong plasmid-targeting preference, are the only one of six known CRISPR-Cas types for which the mechanistic details of their function remain unknown. Here, we provide high-resolution functional snapshots of type IV-A Csf complexes before and after target dsDNA binding, either in the absence or presence of CasDinG, revealing the mechanisms underlying Csf-crRNA complex assembly, 'DWN' PAM-dependent dsDNA-targeting, R-loop formation, and CasDinG recruitment. Furthermore, we establish that CasDinG, a signature DinG family helicase, harbors ssDNA-simulated ATPase activity and ATP-dependent 5'–3' DNA helicase activity. In addition, we show that CasDinG unwinds the nontarget strand (NTS) and target strand (TS) of target dsDNA from the Csf-crRNA complex. These molecular details advance our mechanistic understanding of type IV-A CRISPR-Csf function and should enable Csf complexes to be harnessed as genome-engineering tools for biotechnological applications.

Ryan Jackson

Utah State University

Structure and Function of Type IV CRISPR systems and their anti-CRISPRs

Type IV CRISPR systems are arguably the most diverse and poorly understood of all CRISPR types. Here we present structural, biochemical, and cell-based data of the Type IV-A and Type IV-B systems. These data provide novel insights into how the Type IV-A multi-subunit Csf complex combines with ancillary CasDinG protein to clear foreign plasmids. We also reveal three Type IV-A anti-CRISPRs identified with our cell-based assay. Biochemical and structural data with these anti-CRISPRs reveal putative anti-Type IV-A mechanisms that help us better understand Type IV-A function. In addition to our work on Type IV-A, we have begun to decipher the function of Type IV-B systems. A hallmark of Type IV-B systems is the protein called CsyH. Here we reveal biochemical functions of CsyH that suggest possible in vivo mechanism for the Type IV-B system.

Fabienne Benz

Institut Pasteur

In trans adaptation and RNA-guided silencing by a Klebsiella plasmid-encoded type IV-A3 CRISPR-Cas

Fabienne Benz[1]; Sarah Camara-Wilpert[2]; Rafael Pinilla Redondo[2]; Jakob Russel[2]; Katharina Wandera[3]; José Vicente Gomes Filho[4]; Frank Englert[3]; Johannes Kuehn[2]; Silvana Gloor[5]; Aline Cuenod[6]; Mònica Aguilà I Sans[2]; Lorrie Maccario[2]; Adrian Egli[6]; Lennart Randau[4]; Eduardo Rocha[1]; Chase L. Beisel[3]; Jonas Stenløkke Madsen[2]; David Bikard[1]; Alex R. Hall[5]; Søren Johannes Sørensen[2]; Rafael Pinilla-Redondo[2]

[1] Institut Pasteur; [2] Section of Microbiology, University of Copenhagen; [3] Helmholtz Institute for RNA-based Infection Research; [4] Philipps Universität Marburg; [5] ETH-Zurich; [6] University Hospital Basel, Switzerland

Type IV CRISPR-Cas systems are primarily encoded on plasmids and form RNA-guided multiprotein complexes with unknown biological functions. In contrast to other CRISPR-Cas types, they lack the archetypical spacer acquisition module and encode a DinG helicase instead of a nuclease component. Type IV-A3 systems are present in clinically relevant enterobacteria, carried by large conjugative plasmids that often harbor antibiotic-resistance genes. Although their CRISPR spacer contents suggest a role in inter-plasmid conflicts, this function and the underlying mechanisms have remained unexplored. Here, we demonstrate that an IncHI1B/FIB plasmid-encoded type IV-A3 CRISPR-Cas system co-opts the I-E adaptation machinery from its clinical Klebsiella pneumoniae host to acquire new spacers. Furthermore, we demonstrate that robust interference of conjugative plasmids and phages is elicited through crRNA-dependent transcriptional repression. By targeting plasmid core functions, type IV-A3 can prevent the uptake of incoming plasmids, limit their horizontal transfer, and destabilize co-residing plasmids, altogether supporting their involvement in plasmid competition. Collectively, our findings shed light on the ecological function and molecular mechanisms of type IV-A3 systems and have broad implications for understanding and countering the spread of antibiotic resistance plasmids in clinically relevant strains.

Session II-a: Class 2 CRISPR-Cas systems

Omar Abudayyeh

Massachusetts Institute of Technology

New Molecular Technologies for Genome Editing and Cell Control

The diversity of cell types and states in humans can be scalably measured and defined by expressed RNA transcripts and other cell-specific features, yet tools to control cells are lacking. We will describe a set of molecular technologies our lab is developing to address cell engineering at multiple levels, including large cargo DNA insertion and RNA sensors for programmable protein expression. In the first study, we present programmable addition via site-specific targeting elements (PASTE), a genome editing technology that enables the integration of large, diverse DNA cargo without relying on DNA repair pathways. PASTE utilizes a CRISPR-Cas9 nickase fused to both a reverse transcriptase and serine integrase for targeted genomic recruitment and integration of desired payloads. We demonstrated integration of sequences up to ~36 kilobases at multiple genomic loci in various human cell lines, primary T cells, and non-dividing primary human hepatocytes. We also discovered 25,614 serine integrases and cognate attachment sites, engineering orthologs with higher activity and shorter recognition sequences. In the second study, we investigated the type III-E CRISPR-Cas7-11 effector, which binds a CRISPR RNA (crRNA) and the putative protease Csx29, catalyzing crRNA-guided RNA cleavage. Cryo-electron microscopy structures of the Cas7-11-crRNA-Csx29 complex revealed tgRNA-induced conformational changes in Csx29. Biochemical experiments showed tgRNA-dependent cleavage of accessory protein Csx30 by Csx29, generating toxic protein fragments that cause bacterial growth arrest, regulated by Csx31. The Cas7-11-Csx29-Csx30 system was engineered for programmable RNA sensing in mammalian cells and is a flexible platform for RNA-triggered cell engineering. Our results establish new technologies, including novel CRISPR effectors and bacterial enzymes, as flexible platforms with wide applicability for studying cell states, therapeutics, and diagnostics.

Julene Madariaga-Marcos

Universität Leipzig

Investigating the R-loop formation by CRISPR-dCas9 with single-molecule ultrafast twist measurements

Julene Madariaga-Marcos[1]; Fabian Welzel[1]; Dominik J. Kauert[1]; Leonhard Jakob[2]; Andreas Schmidbauer[2]; Dina Grohmann[2]; Ralf Seidel[1]

[1] Universität Leipzig; [2] Universität Regensburg

The Type II CRISPR nuclease Cas9 is a revolutionary tool in genomic engineering due to its small size and its ability to cleave both DNA strands. dCas9, the cleavage-deficient variant of Cas9, can bind to dsDNA without cutting the strands after successful R-loop formation. This has the potential to increase the specificity of binding and offers a new range of applications in genome editing. However, the process of R-loop formation has a considerable tolerance for mismatches. To prevent off-targeting, quantitative modelling of the recognition process would be desirable, which requires knowledge about the energy landscapes of the R-loop formation. Our approach to determine such energy landscapes is to measure DNA unwinding during R-loop formation using ultrafast twist measurements. Using DNA origami nanostructures as rotor arms, directly reporting about twist changes on a millisecond time scale, we can resolve real-time R-loop dynamics of CRISPR-Cas effector complexes at the single base-pair level and construct corresponding energy landscapes. With this, we achieve unprecedented insight in the R-loop formation by dCas9 and the impact of mismatches on this process.

Grace Hibshman and Jack Bravo

University of Texas at Austin

DNA interrogation by PAMless SpRY-Cas9

Grace N. Hibshman, Jack P. K. Bravo, Hongshan Zhang, Tyler L. Dangerfield, Ilya Finkelstein, Kenneth A. Johnson, David W. Taylor

University of Texas at Austin

CRISPR-Cas9 is a powerful tool for genome editing, but its ability to target specific DNA sequences is limited by the requirement for a specific “NGG” protospacer-adjacent motif (PAM) sequence immediately adjacent to the DNA target. To overcome this, a "PAMless" engineered Cas9 variant called SpRY has been developed. However, the molecular mechanisms of how SpRY can recognize all potential PAM sequences and yet accurately identify DNA targets are not well understood. Here, we have combined enzyme kinetics, cryo-EM, and single molecule imaging to determine how SpRY interrogates DNA and recognizes cognate target sites for specific cleavage. We observe that divergent PAM sequences can be accommodated through conformational flexibility within the PAM-interacting region of SpRY, enabling SpRY to tightly associate with off-target DNA sequences. Once SpRY correctly identifies the DNA target, R-loop formation and subsequent DNA cleavage rates are ~1,000-fold slower than for SpCas9, enabling us to directly visualize multiple on-pathway intermediates states and rationally design mutants with improved DNA cleavage efficiency. Our findings shed light on the molecular mechanisms of PAMless genome editing with SpRY, providing a rational framework for designing future genome editing tools with improved versatility, precision and efficiency.

Yan Zhang

University of Michigan - Ann Arbor

CRISPR RNA and tracrRNA serve as a regulatory switch for apoCas9-mediated viral memorization in Neisseria

Xufei Zhou; Xin Li; Zhonggang Hou; Yan Zhang

University of Michigan - Ann Arbor

Prokaryotes adapt to viral challenges by acquiring CRISPR memories from invaders. The type II-A S. pyogenes Cas9 and its tracrRNA co-factor are essential for interference and immunization. Therefore, the dogma is Cas9 must complex with its RNA partners to be functional in adaptative immunity. Here we report a novel role of crRNA-tracrRNA pair in regulating Cas9-mediated adaptation.

We use type II-C system of N. meningitidis and meningococcal disease associated filamentous phage MDAΦ as model. During lysogenization, new spacers are preferentially generated from viral DNA and Cas9 is their PAM selector. Unexpectedly, in tracrRNA deletion and crRNA null strains, acquisition efficiency is greatly stimulated rather than diminished. Through extensive genetics and NGS analyses, we demonstrate that this super-adaptation phenotype is not due to interference defects or crispr-cas expression changes but driven by apoCas9 (i.e., RNA-free state). Our findings revealed a new function of crRNA-tracrRNA to rein in apoCas9's hyper-activity in acquisition. Such repression is important to mitigate auto-immunity risks caused by genomically derived self-targeting spacers.

Furthermore, we provided two examples where apoCas9 benefits its bacteria host: (i) biogenesis of neo-II-C CRISPR from a single repeat; and (ii) replenishing collapsed arrays. In meningococci, short CRISPR arrays produce minimum crRNAs, leading to apoCas9-augmented acquisition and expansion of the memory repertoire. Mechanistically, apoNmeCas9's nuclease lobe suffices for super-adaptation but requires the recognition lobe for RNA repression. Finally, using N. meningitidis as a surrogate we showed that the functions of apoCas9 and crRNA-tracrRNA in adaptation are conserved across multiple II-C orthologs.

Yibei Xiao

China Pharmaceutical University

How Cas9-Csn2-Cas1-Cas2 supercomplex select prespacer during spacer acquisition

Zhaoxing Li[1]; Yutao Li[1]; Meirong Chen[1]; Chunyi Hu[2]; Meiling Lu[1]; Min Luo[2]; Yibei Xiao[1]

[1] China Pharmaceutical University; [2] National University of Singapore

CRISPR-Cas systems confer adaptive immunity against exogenic elements in prokaryotes. Elucidating the RNA-guided DNA targeting and cleavage mechanism of Cas9 has revolutionized genome editing in diverse organisms. However, how Cas9 coordinates with other proteins in type II-A system to select PAM flanked prespacers in the adaptation process remains enigmatic. Here, we used cryo-electron microscopy (cryo-EM) to investigate the Enterococcus faecalis type II-A system and found that all four proteins form a 900 kDa Cas9/sgRNA2-Csn28-Cas18-Cas24 supercomplex. The two Cas14-Cas22 modules undergo drastic conformational changes and bridge two Csn2 tetramers, providing a framework for interacting with Cas9. Cas9 is sandwiched between two Cas1 dimers with its PAM-interacting domain sitting on top of Cas2 dimer, resulting in a novel open-state conformation that might facilitate prespacer sampling and selection. The supercomplex can specifically recognize a DNA duplex with a single-stranded PAM. We also captured a Csn28-Cas18-Cas24 structure showing that prespacer binding dissociates Cas9/sgRNA and rearranges Csn28-Cas18-Cas24 into a novel state ready for prespacer integration. These findings provide a foundation for understanding how Cas9 and Csn2-Cas1-Cas2 work together to acquire spacers in type II-A CRISPR-Cas system.

Session II-b: Class 2 CRISPR-Cas systems

Dipali Sashital

Metal-dependent specificity switching in CRISPR-Cas adaptation and interference

Giang Nguyen, Michael A. Schelling, Yukti Dhingra, and Dipali G. Sashital

Roy J. Carver Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA, USA

CRISPR-Cas immunity relies on the enzymatic activity of multiple metal-ion-dependent protein complexes. Although the metal-dependence of these enzymes is well-established, in vitro studies of their function and specificity have often been performed at metal-ion concentrations that far exceed reported physiological conditions. We show that physiological metal conditions strongly impact specificity of CRISPR-Cas complexes involved in both adaptation and interference. During adaptation, a Cas4-1 fusion protein switches nuclease activities depending on metal-ion identity. The Cas1 domain cleaves the non-PAM end of a prespacer through a Mg2+-dependent, ruler-based mechanism. The Cas4 domain cleaves the PAM end through a Mn2+-dependent, sequence-specific mechanism. This dual-cleavage activity enables prespacer processing and orientational integration that is completely contained within the adaptation machinery. During interference, Cas12a switches its specificity depending on metal ion concentration. Lowering Mg2+ concentration decreases cleavage defects caused by seed mismatches, while increasing the defects caused by PAM-distal mismatches. At physiological Mg2+ concentrations, PAM-distal mismatches can be as deleterious as seed mismatches, allowing escape from Cas12a-mediated immunity via emergence of PAM-distal mutations. While lowered Mg2+ concentrations improve the ability of Cas12a to bind targets with seed mismatches, metal ion concentration has no impact on binding to PAM-distal-mismatched targets. Instead, PAM-distal mismatches cause defects in cleavage following formation of the Cas12a-target complex. Overall, our results reveal the importance of physiological metal ion conditions on the function and specificity of CRISPR-Cas complexes.

Dina Grohmann

University of Regensburg

Allosteric activation of CRISPR-Cas12a requires the concerted movement of the bridge helix and helix 1 of the RuvC II domain

Elisabeth Wörle[1]; Anthony Newman[2]; Jovita D' Silva[2]; Leonhard Jakob[1]; Andreas Schmidbauer[1]; Gaetan Burgio[2]; Dina Grohmann[1]

[1] University of Regensburg; [2] The Australian National University

Recently, the single-effector nuclease Cas12a, which belongs to the type V CRISPR-Cas system, has been added to the Cas enzymes used for gene editing. Cas12a is a bilobal enzyme composed of the REC and Nuc lobe connected by the wedge, the REC1 domain, and the bridge helix (BH). In this study, we focused on Cas12a from Francisella novicida (FnCas12a) and investigated the functional roles of the BH and helix 1, a structural element that, together with the bridge helix (BH), connects the recognition and nuclease lobes of FnCas12a. Helix 1 is structurally linked to the lid domain, which opens upon DNA target loading, thereby activating the active site of FnCas12a. We generated BH and helix 1 variants and probed their structural states using single-molecule FRET measurements, revealing the previously unknown open and closed states of apoCas12a. BH variants preferentially adopt the open state in the apo form. The transition to the closed state of the Cas12a-crRNA complex is inefficient in BH variants, but the semi-closed state of the ternary complex can be adopted even when the BH is completely deleted. However, the FnCas12a variant that lacks helix 1 does not undergo the structural transitions required for efficient crRNA and DNA binding. We also assayed pre-crRNA processing and cis- and trans-DNA cleavage activity. We show that the BH affects the trimming activity and mismatch sensitivity of Cas12a, resulting in Cas12a variants with improved cleavage accuracy. Furthermore, we show that helix 1, rather than the bridge helix, is the predominant structural element that confers conformational stability to FnCas12a. Even small perturbations in helix 1 lead to a decrease in DNA cleavage activity, while structural integrity is unaffected. Our data, therefore, suggest that the concerted remodeling of helix 1 and the bridge helix upon DNA binding is structurally linked to lid opening and thus involved in the allosteric activation of the active site.

Ekaterina Semenova

Rutgers University

tRNA anticodon cleavage by target-activated CRISPR-Cas13a effector

Type VI CRISPR-Cas systems are the only CRISPR variety that cleaves exclusively RNA. The CRISPR RNA (crRNA)-guided, sequence-specific binding of target RNAs, such as phage transcripts, activates the type VI effector, Cas13, causes collateral RNA cleavage, which induces bacterial cell dormancy, thus protecting the host population from phage spread. In this work, we investigated the collateral activity of Cas13a from Leptotrichia shahii and determined that this protein (LshCas13a), when activated by the recognition of target RNAs, specifically cleaves tRNAs at anticodon loops in vivo and in vitro. tRNA species with anticodons containing uridines are the primary cleavage substrates for target-activated LshCas13a. We show that tRNA cleavage leads to protein synthesis inhibition and cell growth arrest. In phage-infected cells, RNA-targeting induces tRNA cleavage and limits infection. In addition, Cas13a collateral tRNA cleavage may indirectly activate the host stress response systems including cellular RNases encoded by toxin-antitoxin modules, which could provide a back-up defense mechanism. However, direct cleavage of tRNAs by target-activated LshCas13a is necessary and sufficient for cell growth suppression. Thus, tRNA cleavage is the primary mechanism through which the type VI-A CRISPR-Cas system from Leptotrichia shahii induces dormancy and limits the spread of phage infection through the population. The identified mode of action of Cas13a resembles that of bacterial anticodon nucleases involved in antiphage defense, which is compatible with the hypothesis that type VI effectors evolved from an abortive infection module encompassing an anticodon nuclease.

Session III-a: CRISPR-derived technologies

Kira Makarova

National Institutes of Health

Comprehensive survey of novel CRISPR systems by mining the expansive genomic diversity of prokaryotic organisms

Eugene V. Koonin[1], Han Altae-Tran[2], Soumya Kannan[2], Anthony J. Suberski[2], Kepler Mears[2], F. Esra Demircioglu[2], Lukas Moeller[2], Selin Kocalar[2], Rachel Oshiro[2], Kira S. Makarova[1], Rhiannon K. Macrae[2], Feng Zhang[2]

[1]National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA; [2]Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

Mining of genomic and metagenomic sequences has been instrumental for identification of diverse CRISPR variants. However, beyond the 6 major types, the novel CRISPR varieties remaining to be discovered appear to be rare even if likely interesting from the standpoints of both fundamental biology and application. To discover such rare CRISPR variants, the capacity to efficiently search sequence databases is indispensable. By establishing new large scale computational methods, we were able to carry out a comprehensive survey of CRISPR-associated systems. Multiple new variants of CRISPR systems and numerous new protein domains associated with CRISPR were discovered, indicating that there are substantially more biochemical functions coupled to adaptive immunity in prokaryotes than previously thought. We experimentally validate the activity of several of the newly discovered CRISPR systems.

Ailong Ke

Cornell University

Genome Editing Applications Based on the IscB-ωRNA structure

Ellie Tan[1], Hao Yan[2,3], Gabriel Schuler[1], Chengtao Xu[1], Weixin Tang[2,3], Ailong Ke[1]

[1] Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853, USA

[2] Department of Chemistry, The University of Chicago, Chicago, IL 60637, USA.

[3] Institute for Biophysical Dynamics, The University of Chicago, Chicago, IL 60637, USA

It has become clear that Class 2 CRISPR effectors Cas9 and Cas12 were evolved from RNA-guided nucleases in IS200/IS605 transposons. IscB is about 2/5 the size of Cas9 but shares similar domain organization. The associated ωRNA plays the combined role of crRNA and tracrRNA to guide dsDNA cleavage. We determined the 2.78 Å cryo-EM structure of IscB-ωRNA bound to dsDNA target, revealed the architectural and mechanistic similarities between IscB and Cas9 RNPs, and defined the target-adjacent motif recognition, R-loop formation, and DNA cleavage mechanisms at high resolution. We show that the transition from ancestral IscB to Cas9 involved dwarfing the ωRNA and introducing protein domain replacements. Only a fraction of the size of Cas9, IscB presents a fresh starting point to develop a new generation of genome editing tools, packageable into AAV. However, none of the reported IscB-ωRNAs exceeded 3% in editing efficiencies in human cells. We will describe the results from our systematic structure-guided rational design approaches to improve the editing efficiency of IscB-ωRNA in human cells. Over 50% editing efficiency have been achieved at multiple target sites. We will report additional data including the off-targeting profile and more.

Osamu Nureki

The University of Tokyo

Minimal and most efficient genome editing Cas effector

Satoshi N. Omura[1], Ryoya Nakagawa[1], Virginijus Siksnys[2], Tsukasa Ohmori[3], Atsushi Hoshino[4] and Osamu Nureki[1]

[1] Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo 113-0033, Japan.

[2] Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius, Lithuania.

[3] Department of Biochemistry, Jichi Medical University

[4] Kyoto Prefectural University of Medicine

SpCas9 and AsCas12a are widely utilized as genome editing tools in human cells. However, their relatively large size poses a limitation for delivery by cargo-size-limited adeno-associated virus vectors. The type V-F Cas12f from Acidibacillus sulfuroxidans is exceptionally compact (422 amino acids) and can function in human cells. However, the nuclease activity of wild-type AsCas12f is relatively low. We developed two AsCas12f activity-enhanced (enAsCas12f) variants, achieved by cryo-electron microscopy structural analysis and exhaustive mutant screening. Remarkably, the enAsCas12f variants exhibited comparable or higher genome editing activities in human cells than those of SpCas9 and AsCas12a. The structures revealed that the mutations stabilize the dimer formation and reinforce interactions with nucleic acids to enhance their DNA cleavage activities. Moreover, enAsCas12f packaged with partner genes in an all-in-one AAV vector exhibited efficient knock-in/knock-out activities and transcriptional activation in mice. Taken together, enAsCas12f could offer a minimal genome-editing platform for in vivo gene therapy.

Chunlei Jiao

Helmholtz Institute for RNA-based Infection Research

TracrRNA reprogramming enables direct, PAM-free detection of RNA with diverse DNA-targeting Cas12 nucleases

Chunlei Jiao[1] and Chase L. Beisel[2]

[1]Helmholtz Institute for RNA-based Infection Research, Helmholtz Centre for Infection Research

[2]Medical Faculty, University of Würzburg

CRISPR-Cas defense systems commonly utilize tracrRNAs as part of guide RNA biogenesis. We recently showed that tracrRNAs associated with Type II CRISPR-Cas systems could be reprogrammed to convert any RNA-of-interest into a guide RNA, linking the presence of that RNA to Cas9-based targeting of matching DNA. Here, we extend tracrRNA reprogramming to the diverse set of Type V CRISPR-Cas systems involving tracrRNAs, allowing the associated DNA-targeting nucleases to directly sense RNA without requiring a flanking PAM and unleash collateral single-stranded DNA cleavage. The reprogrammed tracrRNAs required distinct design rules depending on the system subtype. Following these rules, we could directly sense RNAs using Cas12b, Cas12e, and Cas12f nucleases using collateral cleavage of single-stranded DNA beacons. We further optimized the collateral assay by elevating the reaction temperature and altering the DNA target. The Cas12-Rptr platform allowed the detection and differentiation of 16S rRNA from 5 different pathogens using only one universal Rptr. These findings introduce Cas12 Rptrs that allow direct RNA sensing by DNA-targeting nucleases and greatly enhance the sensitivity of detection.

Session III-b: CRISPR-derived technologies

Adrian Molina Vargas

University of Rochester

New design strategies for ultraspecific Cas13a-based RNA-diagnostic tools with single-nucleotide mismatch sensitivity

Adrian M. Molina Vargas[1]; Raven Osborn[1]; Souvik Sinha[2]; Pablo Arantes[2]; Amun Patel[2]; Giulia Palermo[2]; Mitchell O'Connell[1]

[1] University of Rochester; [2] University of California Riverside

Molecular tools to dissect RNA function are valuable for studying the diverse roles RNA plays in cellular function, and as potential novel therapeutics, given thousands of dysregulated RNAs have been observed across a range of diseases. Recently described programmable nucleases, such as the prokaryotic CRISPR-associated nuclease Cas13, offer the potential to develop precise and flexible RNA-targeting technology. For Cas13 diagnostics, multiple efforts in recent years have focused on establishing powerful platforms that could couple nucleic acid amplification to Cas13-detection for higher sensitivity, streamline single-step protocols, perform multiplex testing or use various Cas13 orthologs. These robust platforms are promising to be deployed at point-of-care locations making testing accessible, scalable, faster, low-cost, and flexible, which is particularly relevant in the context of fast-developing outbreaks. However, exploration of the principles for crRNA design in the context of Cas13-based diagnostics has been limited, leaving it a case-by-case basis exploration of suitable crRNAs. This is particularly relevant when deploying Cas13 to distinguish a pathogen's genetic variation or whether a sample is pathological or not. Further progress in the diagnostic field requires a deeper understanding of the biophysical parameters that underlie Cas13 RNA-recognition and activation, which in turn will guide the rational design of more specific Cas13 RNA-diagnostics. By using various methods including mismatch tolerance profiling, crRNA length studies, crRNA architecture modifications and structure-guided engineering of Cas13a, we report new strategies and Cas13a variants that yield highly specific discrimination of RNA-targets down to single-nucleotide polymorphisms (SNP). We deployed this novel platform for the detection of single-nucleotide polymorphisms in SARS-CoV-2 variants of concern and showed its potential for disease diagnostics and epidemiological surveillance.

Samuel Sternberg

Columbia University

Molecular innovation at the interface between transposons and CRISPR-Cas systems

CRISPR-Cas systems protect bacteria and archaea from foreign invaders, including viruses and plasmids. Paradoxically, though, CRISPR-Cas evolution involved the repeated co-option of genes from another major class of mobile genetic elements: transposons. In turn, CRISPR-Cas systems have been co-opted by transposons to promote a new mode of mobilization involving RNA-guided DNA insertion. Thus, dynamic co-evolution at the host-transposon interface has had a profound influence on the diversification of prokaryotic adaptive immune systems and the functionalization of RNA-guided targeting enzymes. I will present our recent efforts to investigate the molecular functions of RNA-guided nucleases and nuclease-deficient CRISPR-Cas systems in transposition, focusing on TnpB-family enzymes and CRISPR-associated transposases (CASTs). By systematically screening and optimizing CAST systems, we further establish programmable transposases as a promising platform for kilobase-scale genome engineering in mammalian cells.

Morayma Temoche-Diaz PhD

Metagenomi

Genome editing using novel reverse transcriptases discovered from metagenomics

Morayma Temoche-Diaz; Leanna Monteleone; Sourab Kulkarni; Sarah Laperriere; Shailaja Chadha; Anu Thomas; Hannah Engel; Lisa Alexander; Kaitlyn Tsai; Ketaki Belsare; Cristina Butterfield; Cindy Castelle; Daniela Goltsman; Brian Thomas; Christopher Brown

Metagenomi

CRISPR technologies have the potential to revolutionize human therapeutic development. However, current methods that rely on creating double stranded breaks cannot address many mutations that lead to genetic diseases. Prime editing approaches overcome some of these challenges by encoding genomic corrections in guide RNAs that are incorporated into genomic sites using a reverse transcriptase (RT), such as Moloney murine leukemia virus reverse transcriptase (MMLV-RT). Despite the inherent advantages of this approach, the large size, genomic integration of guide RNA scaffolds, and RT priming requirements are major limitations that lead to suboptimal genome editing outcomes. Here, we explored the vast diversity of reverse transcriptases that exist in nature in order to discover novel enzymes that overcome these limitations. Millions of novel RTs were identified from metagenomic databases and selected RT candidates were screened for their ability to perform transversions, small insertions, and deletions. We identified numerous active systems that span across major classes of RTs including LTR retrotransposons, retroviral, group II introns, as well as several novel clades. Multiple novel RTs are comparable to or outperform MMLV-RT for genome editing. Notably, these high performing systems range in size from 250-750 aa and show diverse primer binding length requirements that enable more flexible guide RNA designs. Top candidates were engineered through rational design to enhance activity, binding properties, and to abolish RNaseH activity. These novel, small, versatile systems are promising technologies for therapeutic development.

David Schwark

Pairwise

A Novel Mechanistic Framework for Precise Sequence Replacement Using Reverse Transcriptase and Diverse CRISPR-Cas Systems

Y. Bill Kim[1]; Elizabeth Pierce[1]; Michael Brown[1]; Brenda Peterson[1]; Derek Sanford[1]; Justin Fear[1]; David Nicholl[1]; Ellyce San Pedro[1]; Grace Reynolds[2]; Joanne Hunt[1]; David Schwark[1]; Sathya Jali[1]; Nathaniel Graham[1]; Zoe Cesarz[1]; Tracey (Lincoln) Chapman[1]; Joseph Watts[1]; Aaron Hummel[1]

[1] Pairwise; [2] Current affiliation: Inceptor Bio

Prime editing utilizes reverse transcriptase (RT) fused to CRISPR-Cas domains to enable user-defined mutagenesis of the genome. Yet, only Cas9, a member of the type II CRISPR-Cas system has been shown to be compatible with reverse transcriptase so far. Here, we expand the utility of RT in precise genome editing by engineering a novel system that allows for the general utilization of reverse transcription-mediated sequence replacement with genome-targeting by diverse CRISPR/Cas systems, including type V CRISPR enzymes. This technology is termed REDRAW (RNA-encoded DNA replacement of alleles with CRISPR).

During REDRAW we apply an extended crRNA that allows for RNA-DNA hybridization and subsequent RT-mediated elongation of the target-strand (TS) DNA rather than the non-target strand (NTS) DNA as in Prime editing. First, we show that the REDRAW system tolerates fusion and non-fusion architectures with the RT. We also identified the optimal lengths of reverse transcriptase template and RNA/DNA complementarity.

We further improved the system by recruiting cellular factors that stabilize key intermediates required for RT-mediated polymerization, manipulating DNA mismatch repair to promote permanent integration of the edit into the genome, and stabilizing the extended crRNA structure through the attachment of a structured RNA motif at its 3’ end to mitigate degradation. Optimized versions of REDRAW can generate all forms of simple base conversions, including point mutations of multiple bases and precise insertions or deletions of varying lengths.

Taken together, REDRAW offers a mechanistically novel approach to harness the power of reverse transcriptase towards achieving sequence-specific DNA replacement, and demonstrates broad compatibility with several CRISPR-Cas systems, including Cas12a. Pairwise aims to leverage REDRAW to introduce consumer-oriented traits in fruits and vegetables that will make healthy food more convenient, affordable and sustainable.

Session IV-a: CRISPR ecology and evolution

Raul Perez-Jimenez

CIC bioGUNE

Evolution of CRISPR-associated endonucleases as inferred from resurrected proteins

Ylenia Jabalera; Sara Samperio; Raul Perez-Jimenez

CIC bioGUNE

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas9 protein is an effector that plays a major role in a prokaryotic adaptive immune system, by which invading DNA can be targeted and cut for inactivation. The Cas9 endonuclease is directed to target sites by a guide RNA (gRNA) where Cas9 can recognize specific sequences (PAMs) in foreign DNA, which then serve as an anchoring point for cleavage of the adjacent RNA-matching DNA region. Although the CRISPR-Cas9 system has been widely studied and repurposed for diverse applications (notably, genome editing), its origin and evolution remain to be elucidated. We have investigated the evolution of Cas9 from resurrected ancient nucleases (anCas) in extinct firmicutes species as old as 2600 myr to the current day. Surprisingly, we demonstrate that these ancient forms were much more flexible in their PAM and gRNA scaffold requirements compared to modern day Cas9 enzymes. In addition, anCas portrays a gradual paleoenzymatic adaptation from nickase to double-strand break activity, suggesting a mechanism by which ancient CRISPR systems could propagate when harboring Cas enzymes with minimal PAMs. The oldest anCas also exhibit high levels of activity with ssDNA and ssRNA targets, resembling Cas nucleases in related system types. Finally, we illustrate editing activity of the anCas enzymes in human cells. Prediction and characterization of anCas proteins uncovers an evolutionary trajectory leading to ancient enzymes with extraordinary properties.

Lotta-Riina Sundberg

University of Jyväskylä

Mucin induces CRISPR-Cas defense in an opportunistic pathogen

Gabriel Magno de Freitas Almeida, Ville Hoikkala, Janne Ravantti, Noora Rantanen, Lotta-Riina Sundberg

University of Jyväskylä

Parasitism by bacteriophages has led to the evolution of a variety of defense mechanisms in their host bacteria. However, it is unclear what factors lead to specific defenses being deployed upon phage infection. To explore this question, we co-evolved the bacterial fish pathogen Flavobacterium columnare and its virulent phage V156 in presence and absence of a eukaryotic host signal (mucin) for sixteen weeks. The presence of mucin led to a dramatic increase in CRISPR spacer acquisition, especially in low nutrient conditions where over 60% of colonies obtained at least one new spacer. Additionally, the presence of a competitor bacterium further increased CRISPR spacer acquisition in F. columnare. These results suggest that ecological factors are important in determining defense strategies against phages, and that the phage-bacterium interactions on mucosal surfaces may select for the diversification of bacterial immune systems.

Harry Edwards

University of Nottingham

Linking the SOS response and CRISPR Cas adaptation

Harry Edwards; Christopher Cannon; Ronald Chalmers

University of Nottingham

Prokaryotes have evolved multiple mechanisms to deal with the threat of bacteriophage infection. The CRISPR Cas system provides adaptive immunity through the acquisition of unique spacer sequences into the CRISPR array. The function of the Cas genes are well understood, but the role of other host factors are less well characterised. Here, I show that induction of Cas1-Cas2 gene expression results in cellular filamentation, as a consequence of induction of the SOS response and consequent sulA expression. Preventing induction of the SOS response, by either overexpression of recX or use of the lexA3 allele, reduces the rate of spacer acquisition. Furthermore, induction of the SOS response, via growth in sub-inhibitory antibiotic concentrations, increases the rate of adaptation. The overexpression of sulA causes spacer acquisition events to arise earlier and complements the rate of adaptation in the lexA3 background. Overall, these results reveal insight into the link between induction of the SOS response and spacer acquisition.

Session IV-b: CRISPR ecology and evolution

Owen Tuck

University of California, Berkeley

Genome expansion by a CRISPR trimmer-integrase

Joy Wang[1]; Owen Tuck[1]; Petr Skopintsev[1]; Katarzyna Soczek[1]; Gary Li[1]; Julia Zhou[1]; Basem Al-Shayeb[1]; Jennifer Doudna[2]

[1] University of California, Berkeley; [2] HHMI/University of California, Berkeley

CRISPR-Cas adaptive immune systems capture DNA fragments from invading mobile genetic elements and integrate them into the host genome to provide a template for RNA-guided immunity. CRISPR systems maintain genome integrity and avoid autoimmunity by distinguishing between self and non-self, a process for which the CRISPR-Cas1:Cas2 integrase is necessary but not sufficient. In some microbes, the Cas4 endonuclease assists CRISPR adaptation, but many CRISPR-Cas systems lack Cas4. We show here that an elegant alternative pathway employs an internal exonuclease to select and process DNA for integration using the protospacer adjacent motif (PAM). A natural Cas1:Cas2-exonuclease fusion (trimmer-integrase) catalyzes coordinated DNA capture, trimming and integration. Five cryo-EM structures of the CRISPR trimmer-integrase, visualized both before and during DNA integration, show how asymmetric processing generates size-defined, PAM-containing substrates. Before genome integration, the PAM sequence is released by Cas1 and cleaved by the exonuclease, marking inserted DNA as self and preventing aberrant CRISPR targeting of the host. Together, these data support a model in which CRISPR systems lacking Cas4 use fused or recruited exonucleases for faithful acquisition of new CRISPR immune sequences.

Nathaniel Burman

Montana State University

Type III CRISPR-mediated Control of a Toxin-Antitoxin System

Nathaniel Burman; Shishir Pandey; William Henriques; Trevor Zahl; Royce Wilkinson; Tanner Wiegand; Blake Wiedenheft

Montana State University

Recent studies have demonstrated that CRISPR systems form symbiotic relationships with Toxin-Antitoxin systems. We have identified a unique subclass of type III CRISPRs where the CRISPR-Cas loci encode an endoribonuclease toxin, while the corresponding antitoxin is fused to a subunit of the RNA-guided surveillance complex. This genetic fusion indicates that the Toxin-Antitoxin system has been co-opted by CRISPR-Cas to play a role in anti-phage defense. Here we use cryo-electron microscopy to determine the structure of a type III-B CRISPR-Antitoxin complex that suggests the activity of the associated toxin is under the master control of the CRISPR complex. This work provides structural insight towards a mechanism of type III CRISPR defense which exerts regulatory control over a Toxin-Antitoxin system.

Stineke van Houte

University of Exeter

Understanding ecological determinants of CRISPR-phage interactions

Many bacteria rely on CRISPR-Cas immunity to defend themselves against infections with bacteriophages, which in turn may use anti-CRISPR proteins to avoid immunity. In my talk will share our latest insights on how environmental factors impact the successful deployment of anti-CRISPRs. Furthermore, I will present our latest data from single-cell analyses that shed light on the consequences of CRISPR-Cas immunity for individual cells and the growth costs associated with mounting a successful immune response.

Hélène Chabas

ETH Zürich, Environmental System Sciences

CRISPR-Cas systems: a barrier for antibiotic-resistant conjugative plasmids?

Hélène Chabas; Berit Siedentop; Hannelore Mac Donald; Ricardo León-Sampedro; Sebastian Bonhoeffer

ETH Zürich, Environmental System Sciences

The discovery and the usage of antibiotics to cure bacterial infections is a massive success of modern medicine. Unfortunately, this success is threatened by the evolution of antibiotic resistant bacteria. A key-driver of the spread of these antibiotic resistances are conjugative plasmids i.e. widespread extrachromosomal DNA molecules that horizontally spread between bacteria and that frequently carry antibiotic resistance genes. Interestingly, it was shown that CRISPR-Cas systems that normally protect bacteria against phages can also target conjugative plasmids, as CRISPR-Cas systems can acquire spacers from them and degrade their DNA.

But it is unknown to which extent this molecular activity prevents the spread of an antibiotic resistant conjugative plasmid entering a bacterial population that carries CRISPR-Cas. To answer this question, we combine mathematical modelling with experimental evolution. We show that bacterial populations can employ CRISPR-Cas as a bet-hedging strategy: at equilibrium, CRISPR-Cas lets beneficial plasmid invade the population but exclude costly ones. We find that the speed with which the plasmid is driven to extinction is dependent on plasmid characteristics. Importantly, even if at equilibrium costly plasmids are excluded by CRISPR-Cas, reaching this equilibrium takes a considerable amount of time. During this time the plasmid is present at high frequency in the bacterial population, increasing the chance that the plasmid evolves or that the environmental conditions change, which would prevent plasmid extinction. In conclusion, our work shows that in many cases, CRISPR-Cas is not an efficient barrier for antibiotic-resistant plasmids.

Reetta Penttinen

University of Jyväskylä

CRISPR-harboring megaphages isolated from boreal freshwater environment

Reetta Penttinen[1]; Kati Mäkelä[1]; Elina Laanto[1]; Janne Ravantti[2]; Lotta-Riina Sundberg[1]

[1] University of Jyväskylä; [2] University of Helsinki

CRISPR defense is conventionally considered as bacterial defense mechanism against invading mobile genetic elements (MGEs), such as phages and plasmids. However, recent metagenomic and genomic studies have shown that also MGEs can harbor their own defense systems, putatively to manipulate the host metabolism or to prevent superinfection by other MGEs. Namely, several phage genomes resolved from genomic data are harnessed with CRISPR arrays some of which are accompanied with Cas gene cassettes. Yet, while these findings are based on genomic data and due to the lack of CRISPR-carrying phage isolates, it has remained undetermined what is the role of these systems in natural environments where phages have to compete for suitable host cells. We established a systematic 1-year sampling series from a boreal freshwater lake to isolate Flavobacterium -infecting phages. Genome analysis of these phages revealed five megaphage isolates (with a genome size of >600 kb) that harbored a CRISPR-Cas locus consisting of a partly conserved CRISPR array associated with putative Cas-encoding genes. Our further analysis suggests that based on the found Cas genes, the phage-encoded CRISPR systems resemble the CRISPR types I-Fv and II, however, each of them possessing a previously uncharacterized genetic organization of CRISPR-associated genes. With these findings we underline the importance of studying the phage ecology in natural environments via conventional culture-based techniques which may have sometimes remained overlooked. Further, the phage isolates allow studying the functionality and role of the phage-associated CRISPR defense in the inter-phage competition.

Session V-a: Beyond CRISPR

Joshua Modell

Johns Hopkins University School of Medicine

Prophages vaccinate CRISPR-Cas populations against lytic threats

Joshua Modell[1]; Nicholas Keith[2]

[1] Johns Hopkins University School of Medicine; [2] Johns Hopkins School of Medicine

CRISPR-Cas systems must discriminate self from non-self nucleic acids to provide bacteria with adaptive immunity against foreign agents, including bacteriophages. Prophages are simultaneously self and non-self elements; they reside within the bacterial chromosome and yet are mobile and can reactivate during the SOS DNA damage response, resulting in cell lysis. Prophage-targeting spacers have only been observed in cases where an anti-CRISPR (Acr) inactivates the CRISPR-Cas system to prevent auto-immune cleavage of the chromosome. It therefore remains unclear whether bacteria can generate functional spacers from their resident prophages in the absence of an Acr. Here, we show that prophages are indeed hotspots for CRISPR-Cas spacer acquisition. While most of these spacers result in auto-immune cell death, many cells survive by curing the targeted prophage. Furthermore, we demonstrate that these prophage-acquired cells can defend against naturally-arising lytic mutants that otherwise decimate the population. Our results suggest a new paradigm whereby spacers can be acquired on much longer timescales than the canonical model of a lytic sweep with a novel phage. This may explain why most known phage-targeting spacers target temperate phages. Finally, we propose a new role for CRISPR-Cas systems in bacterial evolution as mediators of prophage curing.

Joseph Bondy-Denomy

University of California, San Francisco

Anti-CRISPR proteins as mediators of phage-host symbiosis

Mobile genetic elements use anti-CRISPR proteins to neutralize CRISPR-Cas attack during invasion and to prevent self-targeting and spacer acquisition after establishment. This multi-faceted role of anti-CRISPR proteins demonstrates that these proteins are not simply used as invasion tools but as mediators of mutualism between MGE and self. Indeed, experiments from multiple systems and groups have revealed how detrimental self-targeting or acquisition from self can be. Perhaps most notably, CRISPR-Cas is the only known anti-phage system that will target an integrated prophage, and thus the continued expression of 1 or more Acr proteins is likely essential for stability. Here, I will discuss emergent anti-CRISPR mechanisms that support a key role in this symbiosis. Recently characterized anti-CRISPR mechanisms in our lab that impact Cas protein biogenesis of Cas9 (AcrIIA1) and Cas12 (AcrVA2) will be discussed. In each characterized case, the pairing of biogenesis inhibitors with targeting inhibitors likely enables effective invasion of MGEs and subsequent establishment and maintenance. I will also discuss our ongoing studies of a very common MGE-encoded anti-CRISPR enzyme AcrIF11, which ADP-ribosylates Cascade. Our work has revealed that this protein is a remarkably potent inhibitor in vivo (i.e. stronger than AcrIF1 sub-nanomolar stoichiometric binder) of Type I-F CRISPR-Cas during phage infection, but also during lysogeny, where all self-targeting is abolished, even upon crRNA or Cascade overexpression. Most Acr proteins, including those mentioned above, are encoded on temperate phages or plasmids. While anti-CRISPR proteins have been identified on lytic phages, they are comparatively rare. This is perhaps due to other potent CRISPR evasion mechanisms used by lytic phages such as base modifications, genomic segregation, and genomic repair. Therefore, while anti-CRISPR proteins are often imperfect solutions to CRISPR-Cas targeting, they present a simple and relatively effective mechanism to achieve MGE-host symbiosis.

Kevin Forsberg

The mysterious origins of anti-CRISPR proteins

Samantha Sakells[1]; Danica Schmidtke[2]; Michael Knopp[3]; Dan Andersson[4]; Kevin Forsberg[1]

[1] UT Southwestern Medical Center; [2] Stanford University; [3] EMBL Heidelberg; [4] Uppsala University

CRISPR-Cas immune systems protect bacteria from infection by phages and other mobile genetic elements which, in turn, have developed inhibitors of these systems called anti-CRISPRs (Acrs). While the origins of CRISPR-Cas systems are well understood, less is known about the evolutionary history of Acrs. Many phages have independently evolved different Acrs, which often lack sequence or structural homology with one another or with other known proteins. Based on these observations, we hypothesized that some Acrs have evolved de novo from previously non-coding DNA.

De novo evolution is not specific to Acrs. Many small phage proteins are similarly diverse and enigmatic in their origin. However, Acrs can confer extremely high fitness advantages under tightly controlled settings, enabling us to use high-throughput functional selection to capture their de novo evolution in the laboratory. To approximate this de novo gene evolution, we screened plasmid libraries of >10^8 near-random polypeptides and identified two sequences capable of protecting a target plasmid from Streptococcus pyogenes Cas9.

One of these de novo genes protected both plasmids and phages from Cas9 interference. The encoded polypeptide was required for Acr activity and inhibited Cas9 by blocking its expression. Multiple Acrs in nature similarly inhibit their cognate Cas proteins by impacting expression. The de novo Acr blocked Cas9 expression after transcription but did not promote Cas9 degradation, suggesting it may act on other processes like translation or protein folding. Importantly, this Acr exhibits only mild toxicity, which suggests it does not globally block an essential cellular process. Indeed, the de novo Acr did not impact GFP expression, establishing its selectivity towards Cas9. Given the estimated 10^31 phages on Earth, an Acr discovery rate of ~1 in 10^8 suggests that the de novo emergence of Acrs in nature is a near statistical certainty.

Peter Fineran

University of Otago

Regulation of anti-CRISPR production

To counteract CRISPR–Cas defences, phages have evolved anti-CRISPR (Acr) proteins that can inhibit prokaryotic adaptive immunity. While Acrs are highly diverse, they are frequently encoded in an operon with a conserved anti-CRISPR-associated (aca) gene. Aca proteins contain a helix-turn-helix motif, which led to the proposal that Aca proteins might be regulators of their corresponding acr–aca loci. Indeed, we have demonstrated Aca2 represses the acrIF8–aca2 operon of temperate phage ZF40, which infects Pectobacterium carotovorum. We have also demonstrated that representatives of most classes of Aca proteins function as repressors. The purpose and molecular basis of this Acr regulation is poorly understood. We present here our discovery of robust Aca2-mediated anti-CRISPR regulation, including unexpected findings into how this regulation contributes to the control of anti-CRISPR activity.

Alexander Meeske

University of Washington

Viral cas genes antagonize CRISPR immunity

Prokaryotic CRISPR-Cas immunity is subverted via anti-CRISPRs (Acrs), small proteins that inhibit Cas protein activities when expressed during the phage lytic cycle or from resident prophages or plasmids. CRISPR-Cas defenses are classified into 6 types and 33 subtypes, which employ a diverse suite of Cas effectors and differ in their mechanisms of interference. As Acrs often work via binding to a cognate Cas protein, inhibition is almost always limited to a single CRISPR type. Furthermore, while acr genes are frequently organized together in phage-associated gene clusters, how such inhibitors initially evolve has remained unclear. Here we have investigated the Acr content and inhibition specificity of a collection of Listeria isolates, which naturally harbor four diverse CRISPR-Cas systems (types I-B, II-A, II-C, and VI-A). We observed widespread antagonism of CRISPR, which we traced to 9 novel and 4 known Acr gene families representing both narrow and broad-spectrum inhibitors. Among these were two Acrs that possess sequence homology to type I-B Cas proteins and inhibit Cascade-mediated target interference. Surprisingly, an additional type I-B Cas homolog did not affect type I immunity, but instead inhibited the RNA-targeting type VI CRISPR system through sequestration of crRNA. By probing the IMGVR database of viral genomes, we detected abundant orphan cas genes located within putative anti-defense gene clusters. Our observations provide direct evidence of Acr evolution via co-option of cas genes, and several new genes with potential for broad-spectrum control of genome editing.

Session V-b: Beyond CRISPR

Rotem Sorek

Weizmann Institute of Science

Phages overcome bacterial immunity via diverse anti-defense proteins

To overcome bacterial immunity, phages utilize proteins that inhibit CRISPR-Cas and restriction modification systems. It was recently shown that bacteria employ, apart from CRISPR-Cas and restriction systems, a considerable diversity of additional phage resistance systems, but it is largely unknown how phages cope with this multilayered bacterial immunity. The talk will present progress in understanding the diversity of mechanisms by which phages overcome bacterial defenses.

Giuseppina Mariano

University of Surrey

The novel anti-phage system Shield co-opts an RmuC domain to mediate phage defense across Pseudomonas species

Competitive bacteria-bacteriophage interactions have resulted in the evolution of a plethora of bacterial defense systems preventing phage propagation. In recent years, computational and bioinformatic approaches have underpinned the discovery of numerous novel bacterial defense systems. Anti-phage systems are frequently encoded together in genomic loci termed defense islands. Here we report the identification and characterisation of a novel anti-phage system, that we have termed Shield, which forms part of the Pseudomonas defensive arsenal. The Shield system comprises the core component ShdA, a membrane-bound protein harboring an RmuC domain. Heterologous production of ShdA alone is sufficient to mediate bacterial immunity against several phages. We demonstrate that Shield and ShdA confer population-level immunity and that they can also decrease transformation efficiency. We further show that ShdA homologues can degrade DNA in vitro and, when expressed in a heterologous host, can alter the organisation of the host chromosomal DNA. Use of comparative genomic approaches identified how Shield can be divided into four subtypes, three of which contain additional components that in some cases can negatively affect the activity of ShdA and/or provide additional lines of phage defense. Collectively, our results identify a new player within the Pseudomonas bacterial immunity arsenal that displays a novel mechanism of protection, and reveals a role for RmuC domains in phage defense.

David Bikard

Institut Pasteur

An anti-anti-CRISPR system: triggering cell death in response to phage counter-defenses

Anti-phage defense systems can be defeated by counter defenses carried by phages such as anti-restriction and anti-CRISPR proteins. We identified a defense system that seems to specialise in the recognition of anti-defense proteins and triggers cell death. This system called PARIS (phage anti-restriction-induced system) was found within a hotspot of genetic variation carried by P4-like satellites of P2 phages. It is composed of two genes, ariA and ariB. AriA is an ABC ATPase and ariB contains a TOPRIM domain of OLD family nuclease. Different PARIS systems defend against different phages suggesting that they employ different triggering mechanisms or that some phage escape PARIS immunity. We previously reported that PARIS-2 is triggered by T7-Ocr, an anti-restriction protein, that inhibits RM and BREX systems by mimicking the structure of DNA. To identify additional triggers of PARIS, we selected mutants of diverse phages that escape defense by these systems. Putative triggers were cloned and their ability to trigger cell death in the presence of PARIS was confirmed. Alphafold was used to predict the structures of these proteins, leading to the identification of structurally diverse PARIS triggers, including other DNA mimics and phage proteins of other functions. Strikingly, an anti-CRISPR protein (AcrIF9) was found to trigger PARIS systems. Future work will determine how PARIS systems can sense such a diverse set of phage protein triggers and how its activation leads to cell death.

Daan Swarts

Wageningen University

The diverse functions and mechanisms of prokaryotic Argonaute proteins

While eukaryotic Argonaute proteins (eAgos) are well known for their function in RNA silencing pathways, the function and mechanisms of the highly diversified prokaryotic Argonautes (pAgos) are less-well understood. In contrast to eAgos, certain pAgos utilize DNA guides and most pAgos characterized to date target DNA. As such, pAgos execute a variety of functions ranging from neutralizing invading DNA to stimulating homologous recombination. I will illustrate the diversity of pAgos and highlight recent discoveries about the function and mechanisms of so called ‘short pAgo’ systems.

POSTER ABSTRACTS

Class 1 CRISPR Cas systems

1| Type III CRISPR-Cas complexes act as protein-assisted ribozymes during target RNA cleavage

David Taylor

University of Texas at Austin

CRISPR-Cas systems are an adaptive immune system in bacteria and archaea that utilize CRISPR RNA-guided surveillance complexes to target complementary RNA or DNA for destruction. Target RNA cleavage at regular intervals is characteristic of type III effector complexes; however, the mechanism has remained enigmatic. Here, we determine the structures of the *Synechocystis* type III-Dv complex, an evolutionary intermediate in type III effectors, in pre- and post-cleavage states, which show metal ion coordination in the active sites. Using structural, biochemical, and quantum/classical molecular dynamics simulation, we reveal the structure and dynamics of the three catalytic sites, where the 2'-OH of the ribose on the target RNA acts as a nucleophile for in line self-cleavage of the upstream scissile phosphate. Strikingly, the arrangement of catalytic residues of most type III complexes resembles the active site of ribozymes, including the hammerhead, pistol, and Varkud satellite ribozymes. Thus, type III CRISPR-Cas complexes function as protein-assisted ribozymes, and their programmable nature has important implications for how these complexes could be repurposed for applications.

2| A new quantitative model for Type I-E CRISPR/Cas activation

Marko Djordjevic[1], Andjela Rodic[1], Magdalena Djordjevic[2], Marko Tumbas[1]*

[1]Quantitative Biology Group, Faculty of Biology, University of Belgrade, Belgrade, Serbia;
[2]Institute of Physics Belgrade, University of Belgrade, Belgrade, Serbia

A model system for CRISPR/Cas regulation (Type I-E in *E. coli*) is silent under standard conditions, and it is unclear if and how the system can be activated rapidly enough to protect the cell. Two qualitative models were proposed based on the fact that cas genes and CRISPR array are cooperatively repressed by H-NS (a pleiotropic transcription regulator). i) The first model assumes that foreign DNA generally has higher AT content and that H-NS prefers binding to AT reach sequences. When foreign DNA enters the cell, the concentration of free repressor is lowered due to binding to the foreign DNA sequence, relieving CRISPR/Cas repression. However, it is unclear if the perturbation of H-NS is sufficient to efficiently activate the system. ii) According to the second model, a transcription activator LeuO (H-NS antagonist) is activated upon virus entry, which relieves CRISPR/Cas repression. However, what exactly activates LeuO? We develop a new quantitative model based on the fact that leuO is strongly activated by BglJ, that both leuO and bglJ are repressed by H-NS, while LeuO also activates bglJ. Consequently, lowering H-NS concentration through the mechanism in model i) can lead to strong LeuO activation by a double-positive feedback loop so that CRISPR/Cas can be subsequently induced through the mechanism in model ii). To quantify the model, we apply a

combination of statistical thermodynamics and non-linear dynamics, which we previously showed can realistically explain measured experimental data. We obtain that combining HN-S binding cooperativity (leading to strong non-linearity) and positive feedback can rapidly produce crRNAs. We also perform a systematic bioinformatics analysis, which shows that the primary ingredient behind our model, i.e., the difference in AT content between the invasive DNA (phages and plasmids) and the genomes harboring CRISPR arrays, is widespread beyond *E. coli* and Type I-E systems.

3| Abstract withdrawn

4| Type III CRISPR-Cas provides protection against a phage with a hypermodified genome

Marina Mahler[1]; David Mayo-Muñoz[1]; Leah Smith[1]; Lucia Malone[1]; Juby Mathew[2]; Antony Fairbanks[2]; Stan Brouns[3]; Peter Fineran[1]

[1] University of Otago; [2] University of Canterbury; [3] Delft University of Technology

Bacteria have evolved diverse defense systems against their phage predators, such as the adaptive CRISPR-Cas systems. To evade this adaptive immunity, phages can encode anti-CRISPR proteins, protect their DNA within a nucleus-like structure or mask the DNA with covalent modifications. We isolated a *Serratia* phage related to *E. coli* phage T4 and discovered that it was completely resistant to the native host DNA targeting type I-F CRISPR-Cas system and partially resistant to the type I-E system. However, this phage remained

sensitive to the *Serratia* RNA targeting type III-A system. To identify the evasion mechanism of the phage we analysed the genome and found genes with the potential to generate DNA hypermodifications. These modifications were confirmed by chemical analysis to differ from glucose-hydroxmethlycytosines in T4 that were previously shown to provide differing levels of resistance to DNA targeting type I, II and V CRISPR-Cas systems. The DNA modification of the Serratia phage impedes interference by DNA targeting CRISPR-Cas systems but does not protect the phage from RNA targeting type III and VI systems.

5| Structure and mechanism of type I-G CRISPR system and its application

Qilin Shangguan[1]; Shirley Graham[1]; Ramasubramanian Sundaramoorthy[2]; Malcolm White[1]

[1] University of St Andrews, Scotland; [2] University of Dundee

To date, CRISPR systems have been widely discovered and applied in different microbiological fields. However, there is always an uncharted area of CRISPR systems in nature, awaiting exploration. The type I-G CRISPR system is one of the subtypes of type I CRISPR systems. Characterised by the enigmatic cas proteins Csb2 and Cas8g. the type I-G system possesses a unique mechanism in CRISPR recognition and interference. Here, we expressed and reconstructed a type I-G system from *Thioalkalivibrio sulfidiphilus*. We present key insights into the biochemistry and mechanism of the system, and a first view of the structure on the effector complex of type I-G is provided. Heterologous expression in E. coli provides immunity against mobile genetic elements. Repurposing type I-G for genome editing in E. coli with atypical Cas3 generates controllable editing. These observations provide an overview of the type I-G system, potentiating fundamental studies and further applications.

6| Dual control of a subtype III-Dv CRISPR-Cas system in the cyanobacterium *Synechocystis* sp. PCC 6803.

Raphael Bilger[1]; Dr. Angela Migur[2]; Dr. Matthias Riediger[3]; Dr. Alexander Wulf[4]; Prof. Dr. Henning Urlaub[4]; Prof. Dr. Wolfgang R. Hess[1]

[1] Albert Ludwigs University Freiburg; [2] Helmholtz Institute for RNA-based Infection Research (HIRI); [3] Otto-von-Guericke-University Magdeburg; [4] Max-Planck-Institute for Multidisciplinary Sciences

Prokaryotes have evolved CRISPR-Cas systems for defense against phages and mobile genetic elements. While different types of these systems share the same general steps: acquisition, expression and interference, their regulation is only beginning to be elucidated. In the cyanobacterium *Synechocystis* 6803, three separate CRISPR-Cas systems are located together on the megaplasmid pSYSA1. These systems were classified as a subtype I-D, a subtype III-Dv and a subtype III-Bv system. Here, we show that the expression of the subtype III-Dv system responds to environmental perturbations such as nitrogen starvation and changing light intensities. This regulation is mediated by the redox-responsive transcription factor RpaB, which binds to a HLR1 motif 5´-GTTAACAAatATTTACCT-3´, located 53 to 71 nt upstream of the transcription start site. While RpaB upregulates the transcription of the 5 gene operon encoding the Cas effector proteins at low light intensities, it does not directly control the

repeat-spacer array. However, RNA-protein UV-crosslinking and mass spectrometry revealed that the DEAD-Box RNA helicase CrhR interacts with the 125 nt leader transcript via six residues. Structure prediction based on the resolved structure of CshA, a helicase homolog from *B. subtilis* showed that four residues interacting with the leader transcript are located on the surface of the protein. Our results show that the expression of the III-Dv complex is regulated by the redox status at two different levels.

7| The type III CRISPR system of *Bacteroides fragilis* functions via a novel signal molecule

Haotian Chi [1]; Ville Hoikkala; Sabine Grüschow; Shirley Graham; Sally Shirran; Malcolm White

[1] University of St Andrews, Scotland

CRISPR-Cas systems are currently classified into at least five different types, among which Type III CRISPR system could detect invading RNA and respond by synthesising cyclic oligoadenylate (cOA) species as second messengers to activate accessory effectors to enhance immunity. Here we identified a novel type III-B CRISPR system from the human gut bacteria *Bacteroides fragilis* (BfaCmr) that associates with an uncharacterised CorA family membrane protein and a NrN family phosphodiesterase. BfaCmr can provide defence against mobile genetic elements when expressed in the heterologous host E. coli. Interestingly, we detected a novel signal molecule, not cOA or other known second messengers, when the BfaCmr system was activated. This signal molecule in turn presumably activates the membrane protein CorA, leading to membrane disruption and cell death. In addition, we found the phosphodiesterase NrN could degrade the signal molecule to switch off the signalling pathway. The discovery of the new type of signal molecule indicates the diversity of second messengers and expands our understanding of type III CRISPR-Cas-guided immunity.

8| CalpL is a cA4 activated SAVED domain containing protease that triggers a complex antiviral signaling cascade

Niels Schneberger[1]; Haotian Chi[2]; Katja Blumenstock[1]; Stella Arau Jakubzik[1]; Jonas Moecking[1]; Stefano Da Vela[3]; Jonathan Schmid-Burgk[1]; Dmitri Svergun[3]; Matthias Geyer[1]; Malcolm F. White[2]; Christophe Rouillon; Gregor Hagelueken[1]

[1] Universität Bonn; [2] University of St Andrews; [3] EMBL Hamburg

In type III CRISPR systems, detection of foreign RNA does not only trigger RNA and DNA cleavage, but also enables a highly complex and versatile immune response upon the synthesis of cyclic oligoadenylates (cOAs) in the Cas10 subunit. These cyclic second messengers are normally known to bind to the CARF domain of special effector proteins and thereby activate them. Numerous effector domains with different functions have been elucidated so far, ranging from RNases over transcription regulators to DNases. Bioinformatics studies reported on an unusual effector protein, a membrane-bound “CRISPR associated Lon protease” (CalpL). Here we present the structural and mechanistical analysis of this cA4 activated protease and

the associated phage defense system. Unlike predicted, we found CalpL to be a soluble monomeric protein that is activated by cA4 induced oligomerization. The activated protease cleaves the CalpT/S complex, which consists of two small proteins that are encoded in the same operon, directly upstream of CalpL. Multiple approaches to investigate the function of CalpT and CalpS finally led us to identify a bacterial sigma/anti-sigma factor system. We found that upon viral RNA detection cA4 triggers CalpL to cleave the anti-sigma factor CalpT, CalpS is released from the complex and can bind to RNA polymerase and by this regulate cellular transcription. This finding directly connects a type III CRISPR system to the transcription machinery of the cell. Our results reveal similarities to the CBASS system, recent reports on CRISPR activated caspases (Craspases) and even to mammalian systems such as the cGAS-STING pathway.

9| CryoEM studies of cyclic hexa-adenylate synthesis by a type III-A CRISPR-Cas Csm complex

Kenny Jungfer; Martin Jinek

University of Zurich, Zurich, Switzerland

Type III CRISPR-Cas systems equip prokaryotes with unique mechanisms to combat infections by invading genetic elements. A key feature of these systems is the sequence-specific recognition of target RNAs, which triggers the generation of cyclic oligoadenylate (cOA) second messengers by the Cas10 subunit of the type III Csm/Cmr effector complex. These serve as immune signaling molecules that bind and activate CRISPR-associated Rossman fold (CARF) domain-containing ancillary effector proteins to reinforce the host immune response. While different Csm/Cmr complexes are known to produce cOAs of various sizes (cAn), the molecular determinants of product size remain elusive. Here, we present cryoEM structures of the *Enterococcus italicus* Csm complex bound to different adenylate substrates, which capture intermediate states during the synthesis of cA6. Our observations reveal distinctive features of the EiCsm complex that dictate the specific size of its cOA products. Based on these findings, we refine and expand current mechanistic models of cAn formation, enhancing our understanding of type III CRISPR-Cas cOA signaling pathways.

10| Nuances of type III CRISPR-Cas of HB8 *Thermus thermophilus*

Aleksei Samolygo[1]; Matvei Kolesnik[1]; Konstantin Severinov [2]

[1] Skolkovo Institute of Science and Technology; [2] Waksman Institute, Rutgers, the State University of New Jersey

The type III CRISPR-Cas stands out with its unique set of biochemical activities. It has targeted ssRNase activity, ssDNase which targets the opposing strain, and non-specific RNase and nickase activities. The latter are activated in trans by cyclic oligoadenylate compounds that are produced by the cyclase domain of Cas10 upon detection of viral RNA. It is yet to be

understood which of this diverse set of activities is crucial for the overall response of type III CRISPR-Cas system.

Type III CRISPR-Cas system of thermophile bacteria *Thermus thermophilus* HB27c targets matrix RNA in vivo and leads to cell survival after phiKo phage infection, thus excepting the mechanism of altruistic suicide. However, it is not shown that all type III CRISPR-Cas systems work similarly, considering that slight changes in activities may lead, for example, to altruistic cell death. Harboring homologous type III CRISPR-Cas system, the HB8 strain of *Thermus thermophilus*, transformed with plasmid targeting P23-45 phage transcripts, has several orders of magnitude resistance to the phage. However, survival experiments show that most cells die after infection, although resistant cells survive statistically better than control cells. RNA sequencing of transcripts during phage infection does not show expected targeted RNA degradation, suggesting the prominent role of non-specific RNA degradation. While phage transcription is not completely stopped by the CRISPR-Cas system, it is likely that stochastic expression of phage lysis proteins leads to cell death. Moreover, new phage particles production is nearly completely impaired in resistant strains, suggesting that the key purpose of type III CRISPR-Cas system is to stop phage propagation rather than survival of a particular cell.

11| Exploiting Activation and Inactivation Mechanisms in Type I-C CRISPR-Cas3 for Genome Editing Applications

Mason Myers[1,2]; Xufei Zhou[2]; Zhonggang Hou[2]; Yan Zhang[2]; Ailong Ke[1]

[1] Cornell University; [2] University of Michigan

Type I CRISPR-Cas systems utilize the RNA-guided Cascade complex to identify matching DNA targets, and the nuclease-helicase fusion enzyme Cas3 to degrade them. Among six subtypes, Type I-C CRISPR-Cas is compact in size and highly active in creating large-sized genome deletions in human cells. Here we use four cryo-electron microscopy snapshots to define its RNA-guided DNA cleavage mechanism in high resolution. The non-target DNA strand (NTS) is accommodated by I-C Cascade in a continuous binding groove along the juxtaposed Cas11 subunits. Binding of Cas3 further traps a flexible bulge in NTS, enabling efficient NTS nicking. We identified two highly efficient anti-CRISPR proteins (AcrIC8 and AcrIC9) that strongly inhibited I-C Cascade-Cas3. Structural analysis showed that AcrIC8 inhibits PAM recognition through direct competition, whereas AcrIC9 achieves so through allosteric inhibition. Both Acrs potently inhibit I-C-mediated genome editing and transcriptional modulation in human cells. They serve as the first proof-of-concept for developing Acr off-switches for Type I -mediated eukaryotic genome editing.

12| Target Recognition by the *Thermotoga maritima* Type I-B CRISPR-Cas Effector Complex

Charles Lenihan[1]; Brian Learn [1]; John Mallon [2]; Scott Bailey [1]

[1] Johns Hopkins Bloomberg School of Public Health; [2] Brandeis University

In nature, bacterial and archaeal CRISPR-Cas systems provide adaptive immunity against invading foreign nucleic acids. On the basis of their sequence-specific, RNA-directed nucleolytic activity, various CRISPR-Cas effector complexes have been harnessed for laboratory and industrial applications. For use as a tool, a comprehensive and predictive understanding of target recognition is an absolute requirement. An important feature of DNA target recognition in CRISPR-Cas systems is avoiding cleavage of the host CRISPR array, which bears complementarity to the CRISPR RNA (crRNA) guiding the effector complex. To prevent cleavage of the host genome, CRISPR-Cas effector complexes have evolved a second requirement for cleavage: the presence of a short Protospacer Adjacent Motif (PAM) not present in the host CRISPR array. Here we present a 3.1 Å cryo-EM structure of the effector complex of the most common type of CRISPR-Cas system in nature, Type I-B, from *Thermotoga maritima* bound to target DNA. The structure bears remarkable similarity to the effector complexes of several other members of the Type I CRISPR-Cas family, namely those of I-A and especially I-C. Additionally, we identify a network of important interactions between bases of the PAM sequence and key residues in the effector complex, allowing us to rationalize in vitro binding and cleavage dependence on PAM sequence. Because the repurposing of endogenous CRISPR-Cas systems has been shown to often be substantially more efficient than the exogenous use of heterologous systems, we predict that this comprehensive structural characterization of the PAM recognition mechanism of the *T. maritima* CRISPR-Cas system will inform development of powerful gene editing tools for use in the wide array of industrially and medically relevant bacteria over which the common Type I-B systems are distributed.

13| DNA ligase A is essential for integration of prespacers into *Escherichia coli* type I-E CRISPR arrays

Sofiya Vinogradova[1]; Irina Balazs MD [2]; Olga Musharova [3]; Timofey Khvostikov[3]; Ivan Fedorov[3]; Konstantin Severinov [4]

[1] Moscow Institute of Physics and Technology; [2] Institute of Molecular Genetics, National Research Center "Kurchatov Institute"; [3] Skolkovo Institute of Science and Technology; [4] Waksman Institute, Rutgers, the State University of New Jersey

CRISPR-Cas systems provide prokaryotes with adaptive immunity against phages and plasmids. A CRISPR locus consists of multiple cas genes and an array of short sequences (spacers) derived from invader DNA, separated by identical repeats. The mechanism of CRISPR-Cas action can be divided into two parts: CRISPR adaptation (when a new spacer is acquired from the foreign DNA and integrated into the beginning of the array with generation of an additional copy of repeat) and CRISPR interference (when an effector complex composed

of Cas proteins and small RNA transcribed from CRISPR array identifies complementary foreign DNA and degrades it).

During the integration of a prespacer (precursor of a new spacer) into the CRISPR array, the prespacer's 3'-ends sequentially attack the upper and the lower strands of the first promoter-proximal repeat of the CRISPR array. In this way, so-called semi-integrated adaptation intermediates are formed. In these intermediates, prespacer 3'-ends are integrated into the CRISPR array while their 5'-ends remain free. DNA polymerases fill the gaps between the prespacer and nearby repeats, leaving nicks that have to be ligated. It has not been shown yet which of the two *E. coli* ligases, DNA ligase A or DNA ligase B, ligates the breaks in the CRISPR array after the filling-in reaction by a DNA polymerase is completed. Using high-throughput sequencing, we show that in the absence of ligA, there is a strong accumulation of semi-integrated adaptation intermediates, while there is no increase in CRISPR arrays with fully integrated spacers. Thus, DNA ligase A plays an essential role in CRISPR adaptation in *E. coli*.

14| Translation inhibiting effector in type III CRISPR-Cas defense

Irmantas Mogila; Giedre Tamulaitiene; Konstanty Keda; Albertas Timinskas; Audrone Ruksenaite; Giedrius Sasnauskas; Česlovas Venclovas; Virginijus Šikšnys; Gintautas Tamulaitis

Institute of Biotechnology, Life Sciences Center, Vilnius University

Type III CRISPR-Cas system provides prokaryotes with adaptive defense against viruses and invading plasmids by targeting foreign transcripts. It was recently discovered that these systems employ a specific signaling pathway to combat infections. When CRISPR-Cas effector complex binds to crRNA-complementary RNA it starts to synthesize cyclic oligoadenylates (cOA) from ATP. These molecules activate diverse CRISPR ancillary proteins which mostly consist of cOA-sensing CARF domain and effector domain: a nuclease, protease, deaminase, or other. Structures and functions have been elucidated for Csm6 RNases and Can1/Can2 nucleases degrading DNA or both RNA and DNA. These proteins slow down the gene expression of infected cell and may abort the infection by causing cell death. However, the mechanisms and functions of most other CARF proteins are still unknown.

Here we present structural and functional studies on ancillary proteins, named Cami1 (CRISPR-Cas associated mRNA interferase 1), consisting of CARF and RelE-like effector domain. We show that upon activation by cOA Cami1 proteins bind ribosomes to cleave mRNA. This causes ribosome stalling and growth arrest. We suggest a detailed mechanism of antiviral defense utilizing these effectors which expands the functional diversity of CARF ancillary protein network with type III CRISPR-Cas system at its core.

15| Type IV-A CRISPR-Cas activity of *Pseudomonas oleovorans*: Autoimmunity and natural CRISPRi regulation

Mariana Sanchez Londono[1]; Nathalie Klein[1]; Selina Rust[1]; Hannah Becker[1]; Xiaohan Guo[1]; Leah M. Immelmann[1]; Jose Vicente Gomes Filho [1]; Samuel Novak[2]; Lukáš Pekárek[2]; Neva Caliskan[2]; Lennart Randau[1]

[1] Philipps-Universität Marburg, Department of Biology, Marburg, Germany; [2] Helmholtz Institute for RNA-based Infection Research (HIRI), Helmholtz Centre for Infection Research (HZI), Würzburg, Germany

Bacterial Type IV CRISPR-Cas systems produce RNA-guided effector complexes with CRISPR RNAs (crRNAs) that mostly display plasmid-derived spacers. In *Pseudomonas oleovorans*, a type IV-A system was identified on a megaplasmid and its cas gene architecture did not indicate the presence of an apparent target nuclease. Recombinant type IV-A effector complexes were produced and found to exhibit PAM-dependent interference activity against plasmids and viruses. Additionally, the native CRISPR-Cas activity was shown to interfere with the expression of the host gene pilN which contains a protospacer that is fully complementary to the first spacer of the CRISPR array. Subsequently, we sought to obtain insights into the targeting mechanism of the effector complexes. First, we performed a mutational screen of protospacer-crRNA interactions which revealed relaxed target recognition and an increased off-targeting potential. Next, in vitro assays showed that type IV-A interference complexes specifically interact with the helicase DinG upon target recognition. Herein, we propose that the interference mechanism of the type IV-A systems resembles CRISPR interference (CRISPRi) activity. Thus, we compared the impact of the recombinant Type IV-A CRISPR Cas on gene regulation with the established dCas9-mediated CRISPRi methodology using Illumina sequencing of target transcripts. Our findings provide insights into the target interactions of type IV-A CRISPR-Cas systems and highlight their potential as gene regulation tools using engineered crRNAs.

16| Adapt or die: investigating coevolution between the I-F CRISPR-Cas system and anti-CRISPR proteins

Beatrice Fung[1]; Sabrina Stanley[1]; Alex Van Nynatten[1]; Joseph Bondy-Denomy[2]; Belinda Chang[1]; Alan Davidson[1]

[1] University of Toronto; [2] UCSF University of California San Francisco

The ceaseless evolutionary battle between bacteria and phages has resulted in a variety of defense and offense strategies. CRISPR-Cas systems are one such anti-phage defense mechanism. To counter this, phages encode anti-CRISPR proteins which are potent inhibitors of CRISPR-Cas. There exists immense diversity in CRISPR-Cas systems and their anti-CRISPR counterparts, which suggests that each have influenced the evolution and diversification of the other. Here we investigate I-F CRISPR-Cas systems and I-F anti-CRISPRs found in *Pseudomonas aeruginosa* to determine whether evidence of coevolution between CRISPR-Cas systems and anti-CRISPRs exists. We demonstrate that anti-CRISPR AcrIE3, a known inhibitor

of the I-E CRISPR-Cas system of *P. aeruginosa*, also inhibits the I-F CRISPR-Cas system of *P. aeruginosa* strain F11 but not strain PA14. Sequence comparison followed by mutation studies of the I-F CRISPR-Cas systems from *P. aeruginosa* strains F11 and PA14 showed that a single amino acid substitution in Cas8, R259Y, rendered the PA14 I-F CRISPR-Cas system susceptible to inhibition by AcrIE3. Interestingly, we also identified a diverse AcrIE3 homolog that inhibited the I-F CRISPR-Cas systems from both F11 and PA14. Together these results support the likelihood that anti-CRISPRs and CRISPR-Cas systems have coevolved, with each adapting to overcome the other. To provide additional support for this hypothesis, we determined whether Cas amino acid sites are under positive selection in *P. aeruginosa*, which is evidence of adaptive evolution. We found 16 Cas8 residues, including position 259, that showed significant evidence of positive selection, six of which interface with I-F anti-CRISPRs. Altogether, our findings demonstrate that AcrIE3 is a dual-activity anti-CRISPR, having the ability to inhibit both I-E and I-F CRISPR-Cas systems. In addition, analysis of Cas protein sequences from *P. aeruginosa* provides evidence of coevolution between anti-CRISPRs and CRISPR-Cas systems.

17| Cryo-EM structure and genomic editing profile of type I-B CRISPR-Cas3

Meiling Lu [1]; Chenlin Yu; Yuwen Zhang[1]; Zhenhuang Yang [2]; Yibei Xiao [1]

[1] China Pharmaceutical University; [2] Nanjing Drum Tower Hospital

CRIPSR-Cas system is an RNA-based adaptive immune system found exclusively in prokaryotes that targets and degrades foreign nucleic acids. Type I system is the most abundant. The stepwise activation mechanism to minimize off-target and the ability to cause long fragment deletions have led to its increasing concerns in CRISPR-Cas technologies. Type I-B CRISPR-Cas system is the most evolutionarily conserved subtype of type I but remains poorly studied. Hence, elucidating the molecular basis of type I-B system will greatly enhance our understanding of type I CRISPR-Cas systems and improve the feasibility of repurposing native type I systems into novel gene editing tools. Hereby, we performed functional analysis of type I-B system from *Synechocystis* sp. PCC 6714 (Syn) and initially determined its PAM preference. We also resolved the structures of Syn I-B in two functional states, which revealed the local conformational changes of the effector complex upon R-loop formation. This work also characterized the gene editing profiles of Syn I-B system in human CD3+ T cells. The mRNA-mediated delivery of Syn I-B led to unidirectional 4.5 kb deletion in TRAC locus and an editing efficiency of 41%. Our study lays foundation for harnessing and engineering type I-B system for long range gene editing in human cells.

18| Direct visualization of target search by RNA sensing and DNA sensing CRISPR Cas systems in live bacteria

Aswin Muralidharan[1]; Jochem Vink; Lucia Malone; Stan Brouns

[1] Delft University of Technology (TU Delft)

Fast and accurate recognition of foreign nucleic acid sequences by the CRISPR-Cas surveillance systems aids the host survival. To do so, microbes need to maintain sufficient number of surveillance systems at the right locations which can rapidly move during search phase while switch to a stably bound configuration upon recognition of invader target. Due to the inherent differences in the nature of the target, DNA and RNA sensing CRISPR-Cas systems employ different biophysical mechanisms for target search. We present single molecular tracking and super resolution imaging data of native DNA sensing Type I and RNA sensing Type III CRISPR-Cas systems in live bacteria. Our study reveals the differences in copy numbers, kinetics in the rates of target search and the localizations of the DNA and RNA sensing CRISPR-Cas surveillance complexes.

19| Structural basis of type IV-A mediated CRISPR interference

Rimvyde Cepaite[1]; Algirdas Mikšys[1]; Sarah Camara[2]; Nathalie Klein[3]; Fabienne Benz[4]; Lennart Randau[3]; Rafael Pinilla Redondo[2]; Lina Malinauskaitė[1]; Patrick Pausch[1]

[1] LSC-EMBL Partnership Institute for Genome Editing Technologies, Life Sciences Center, Vilnius University, Saulėtekio al. 7, 10257 Vilnius, Lithuania; [2] Department of Biology, Section of Microbiology, University of Copenhagen, Universitetsparken 15, 2100

CRISPR-Cas mediated DNA interference typically relies on sequence-specific binding and nucleolytic degradation of foreign genetic material. Divergent from this general mechanism, type IV-A CRISPR-Cas systems use a nuclease-independent CRISPR interference (CRISPRi) pathway to suppress gene expression for gene regulation and plasmid competition. To understand how the type IV-A system associated effector complex achieves CRISPRi, we determined cryoEM structures of two evolutionarily distinct type IV-A complexes bound to cognate double stranded DNA-targets in the absence and presence of the type IV-A signature DinG effector helicase. The structures reveal how the CRISPRi complexes sequence-specifically recognize the protospacer adjacent motif and target-strand DNA for formation of an R-loop structure. Additionally, we reveal how DinG engages the non-target DNA strand facilitated by interaction with Cas7 (Csf2). Remarkably, DinG recruitment appears to be independent of Cas8 (Csf1), which distinguishes type IV-A CRISPRi complexes from type I Cascades that recruit the nuclease-helicase Cas3 via Cas8. Our study provides a detailed view of type IV-A mediated DNA-interference and presents a structural foundation for the engineering of type IV-A-based genome editing tools.

20| Craspase is a CRISPR RNA-guided, RNA-activated protease

Sam van Beljouw [1]; Chunyi Hu; Alicia Rodríguez-Molina; Anna Haagsma; Ailong Ke; Stan Brouns

[1] Delft University of Technology

We discovered Craspase (for "CRISPR-guided caspase"), a protein complex composed of a CRISPR-Cas nuclease and a caspase-like protease. Craspase is capable of sequence-specific RNA binding, which is the trigger for activation of the protease. Activated Craspase site-specifically degrades a host protein, whereupon cleavage of the bound RNA by Craspase turns the protease off again. This self-regulatory capacity, combined with strict RNA activation requirements and precise proteolytic action, makes Craspase a unique player in bacterial immunity and an interesting candidate for protein-targeting technologies.

21| Type III-B CRISPR-Cas signaling-based cascade of proteolytic cleavages

Jurre Steens; Jack Bravo; Carl Raymund Salazar[1]; Stephan Köstlbacher; Constantinos Patinios; Caglar Yildiz; Afonso Amieiro; Andreas Bardis; Thijs Ettema; John van der Oost; David Taylor; Raymond Staals

[1] Wageningen University & Research

Type III CRISPR-Cas systems detect foreign RNA in a sequence-specific manner, and in response generate cyclic oligoadenylate (cOAs) signalling molecules. These cOAs activate ancillary proteins that carry the appropriate sensory domain: CARF (CRISPR-associated Rossmann fold) or SAVED (SMODS-associated and fused to various effector domains). CARF and SAVED proteins induce powerful immune responses that can lead to viral clearance, cell dormancy, or cell death. Here, we characterize a set of type III-associated genes that include two proteases, one of which is fused to a SAVED domain. We show that the SAVED-protease is activated by cOA and oligomerize to form long filaments that stabilize the protease's active sites. Activated SAVED-protease then specifically cleaves and activates a second protease. We further show this second protease can cleave other proteins in vitro. Finally, we demonstrate that an in vivo setup (type III system with the two proteases) provokes a strong abortive infection-like phenotype when challenged with a target RNA. Our findings suggest that type III CRISPR-Cas systems can employ a cascade of proteolytic events to mediate a strong immune response, with intriguing conceptual similarities to the eukaryotic signaling pathways leading to apoptosis.

22| Cyclic triadenylate-activated type III CRISPR-Cas-associated DNA nuclease

Dalia Smalakyte[1]; Algirdas Miksys[2]; Irmantas Mogila[2]; Lina Malinauskaite [2]; Gintautas Tamulaitis [2]

[1] Vilnius University, Life Sciences Center, Institute of Biotechnology; [2] Vilnius University, Life Sciences Center, EMBL Partnership Institute for Genome Editing Technologies

To defend against viruses that attack them, prokaryotes evolved various defense mechanisms. The mechanism that relies on cyclic oligonucleotides signaling pathway is a characteristic feature of CBASS (cyclic oligonucleotide-based antiphage signaling systems) and type III

CRISPR-Cas systems. Upon viral infection, a cyclase generates cyclic oligonucleotides, signaling molecules that activate downstream effector proteins which kill the infected cell or lead to cell growth arrest. Effector proteins are composed of a sensor domain fused with various effector domains. While proteins with a sensory CARF (CRISPR-associated Rossmann Fold) domain are commonly encoded adjacent to type III CRISPR-Cas systems, a highly diverged CARF homolog – SAVED (SMODS-associated and fused to various effector domains) domain is characteristic to CBASS effectors. However, SAVED domain-containing proteins are sometimes found neighboring type III CRISPR-Cas systems as well.

In this study, we aimed to understand the role and the mechanism of a type III CRISPR-Cas-associated effector that has a SAVED domain fused with an endonuclease-like domain. We demonstrate that this effector protein, together with the type III-A CRISPR-Cas system, protects against foreign nucleic acids in a heterologous *E. coli* host. Initial biochemical characterization reveals that cA3 binding triggers putative DNase activity.

This work was supported by the European Regional Development Fund (grant 01.2.2-CPVA-V-716-01-0001).

23| Characterization of anti-CRISPR proteins that inhibit a type I-E CRISPR-Cas system from Escherichia coli

Robert Wilson; Chantel Trost; Beatrice Fung; Alan Davidson

University of Toronto

Anti-CRISPR proteins are phage-encoded inhibitors of CRISPR-Cas systems. Anti-CRISPR proteins were initially discovered to block the type I-F CRISPR-Cas system of *Pseudomonas aeruginosa*. Through extensive biochemical and structural characterization, common themes of CRISPR-Cas inhibition by anti-CRISPRs have emerged. However, the study of anti-CRISPRs inhibiting the thoroughly characterized and highly prevalent I-E CRISPR-Cas system has lagged behind. To date, 9 anti-CRISPRs have been described to block the I-E CRISPR-Cas system of *Pseudomonas aeruginosa* with very little further biochemical investigation. This is because recombinant protein expression of the I-E CRISPR-Cas system from this species is not achievable. Further biochemical and structural investigation of anti-CRISPRs that block this highly divergent subtype of CRISPR-Cas system is important as it may reveal novel mechanisms of inhibition and highlight new weak points within CRISPR-Cas systems. This work outlines the discovery of novel I-E anti-CRISPRs capable of inhibiting an overlooked CRISPR-Cas system from *Escherichia coli* that is more widespread and highly divergent than that from E. coli K-12. An expression system was developed for this CRISPR-Cas system and is being harnessed to learn more about modes of CRISPR-Cas inhibition by I-E anti-CRISPR proteins. Biochemical experiments in vitro suggest that these anti-CRISPRs function by directly binding to the CRISPR-Cas effector complex, preventing it from accessing cognate target DNA. Further biochemical characterization of these anti-CRISPRs may reveal novel weak points within the I-E CRISPR-Cas effector complex.

24| CARFish: Identifying cOA-responsive effector proteins of type III CRISPR-Cas systems.

Jurre Steens[1]; Patrick Barendse[1]; Jasper Groen; Daan Swarts [1]; Afonso Amieiro[1]; Raymond Staals[1]

[1] Wageningen University & Research

Type III CRISPR-Cas is a particular class 1 CRISPR system that specifically targets RNA. When the effector complex finds a single-stranded RNA (ssRNA) complementary to its CRISPR RNA (crRNA), the Cas10 subunit starts producing cyclic oligoadenylates (cOA) signalling molecules that will be used to activate the ancillary modules of the type III complex. These ancillary modules are mainly composed of CRISPR-associated Rossmann Fold (CARF) domain proteins that, when triggered, will lead to cell death or dormancy through a diverse range of mechanisms. However, these downstream effectors are often not annotated and are difficult to predict bioinformatically . Therefore, a novel technique titled 'CAFRishing' was developed for discovering novel cOA -binding proteins. Briefly, this method is based on chromatography using a column with immobilized cOA molecules to bind proteins with affinity towards these molecules from cell lysates. From the strains tested, many conventional type III enzymes such as Csx1 and Csm6 were identified, demonstrating the validity of our approach. Furthermore, we identified many other, unexpected proteins, including a transcription regulator, an adenosine deaminase and an uncharacterized NYN domain protein. We purified the NYN domain protein, after which we investigate the potential role of this enzyme in the type III CRISPR immunity by performing in vitro reactions. Preliminary results indicated that this new NYN enzyme can interact with cOA molecules and carry out catatlytic functions. With CARFish, we hope to uncover the full repertoire of cOA-responsive effectors in type III systems.

25| Mechanisms of Type III-A CRISPR-Cas defense and counterattack by a phage-encoded protein

Olivia Howell; Lucy Chou-Zheng; Motaher Hossain; Tori Boyle; Asma Hatoum-Aslan; Barbaros Aslan

University of Illinois at Urbana-Champaign

Type III CRISPR-Cas systems launch a multi-layered immune response against foreign nucleic acids using small CRISPR RNAs (crRNAs) in complex with multiple CRISPR-associated (Cas) proteins. Of the six subtypes currently identified (A-F), Types III-A and III-B are the best characterized. In these systems, crRNA binding to a complementary transcript triggers at least three catalytic activities by members of the effector complex: target RNA shredding by Cas7, DNA degradation by Cas10, and Cas10-catalyzed production of cyclic-oligoadenylates (cOAs), which subsequently bind and stimulate a variety of accessory nucleases. Additionally, our previous work showed that the model Type III-A CRISPR-Cas system in *Staphylococcus epidermidis* (CRISPR-Cas10) relies upon a variety of cellular 'housekeeping' nucleases to assist with degradation of foreign nucleic acids and ensure a successful defense. However, despite this robust immune response, we discovered a subset of *S. epidermidis* phages that have the capacity to overcome CRISPR-Cas10 immunity. This poster will highlight our most recent insights into the mechanisms of CRISPR-Cas10 defense and corresponding phage-encoded

countermeasures, with a focus on the cellular housekeeping pathways that mediate both processes.

26| Protein-mediated genome folding allosterically enhances site-specific integration of foreign DNA into CRISPRs

Andrew Santiago-Frangos[1], William Henriques[1], Tanner Wiegand[1], Colin Gauvin[2,3], Murat Buyukyoruk[1], Ava Graham[1], Royce Wilkinson[1], Lenny Triem[1], Kasahun Neselu[4], Edward Eng[4], Gabriel Lander[5], Blake Wiedenheft[1,]

[1]Department of Microbiology and Cell Biology, Montana State University, Bozeman, MT, USA [2]Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT, USA [3]Thermal Biology Institute, Montana State University, Bozeman, MT, USA [4]Simons Electron Microscopy Center, National Resource for Automated Molecular Microscopy, New York Structural Biology Center, New York, NY, USA [5]Department of Integrative Structural and Computational Biology, The Scripps Research Institute, La Jolla, CA, USA

Bacteria and archaea acquire resistance to viruses and plasmids by integrating fragments of foreign DNA into the first repeat of a CRISPR array. However, the mechanisms by which the leader DNA upstream of the CRISPR regulates site-specific integration remain poorly understood. Here, we determine the structure of a 560 kDa integration complex that explains how upstream DNA recruits Cas (Cas1-2/3) and non-Cas proteins (IHF) to the CRISPR. These proteins fold 150 base-pairs of host DNA into a U-shaped bend and a loop that protrude from Cas1-2/3 at right angles. The U-shaped bend traps foreign DNA on one face of the Cas1-2/3 integrase, while the loop places the first CRISPR repeat in the Cas1 active site. Cas3 rotates 100-degrees to unveil Cas2, which recognizes leader motifs through shape-readout. The leader directs Cas1-2/3-mediated integration at downstream DNA, with few sequence requirements. These data suggest a structural model for primed acquisition, a scenario for the evolution of CRISPRs, and show DNA is used as a flexible scaffold to regulate DNA mobilization.

27| Nanopore detection of type III CRISPR-Cas produced oligo-adenylates and their stoichiometries

David Fuentenebro-Navas[1]; Jurre Steens[1]; Carlos de Lannoy[2]; Ben Noordijk[1]; Dick de Ridder[1]; Raymond Staals[1]; Sonja Schmid[1]

[1] Wageningen University; [2] Delft University of Technology

Cyclic oligo-adenylates (cOAs) are small signaling molecules that are produced by most type III CRISPR-Cas systems as well as CBASS immune systems. These cOAs are vital in the multifaceted immune response upon activation of the type III complex, allowing for allosteric activation of a variety of downstream effectors that lead to cell dormancy or death. Interestingly, cOAs with a variety of stoichiometries (3-6 adenosine moieties in the ring-like structure of these signaling molecules) have been observed in different type III systems. Elucidation of the nature of the produced cOA population is currently only possible with elaborate and sophisticated equipment. Here, we demonstrate a label-free, in vitro detection

platform for single cOA molecules using a protein nanopore assay. Using this setup, we achieved sensitive and reliable identification of the stoichiometry of cOA molecules from both synthetic and biological origin (i.e. those produced by type III complexes). To achieve this, a neural network was trained on mono-and polydisperse mixtures of synthetic cOAs and ultimately used to determine the stoichiometric composition of cOAs produced by the Csm and Cmr complex of *Thermus thermophilus*. This method could in the future be widely used to investigate the cOA production repertoire of type III and CBASS systems, both *in vitro* and *in vivo*.

28| Escape from Dormancy: A Type III saga

Amanda Shilton[1] *and Luciano Marraffini*[1,2]

[1]Laboratory of Bacteriology, The Rockefeller University, New York, NY, USA. [2]Howard Hughes Medical Institute, The Rockefeller University, New York, NY, USA.

Activation of the Cas10-Csm effector complex in Type III CRISPR immunity requires the recognition of a target sequence on newly transcribed RNA. Upon recognition, the complex is recruited to the site of transcription where co-transcriptional cleavage of invading DNA and RNA can occur. Most Type III systems also synthesize a second messenger molecule that activates CARF-domain effectors, such as the non-specific nuclease Csm6. Csm6 activation results in the degradation of both phage and host transcripts, halting phage replication and cell growth. The location of the target along the phage transcriptome matters for the outcome of Type III immunity. Spacers that match a target sequence on a gene that is transcribed early during infection provide robust immunity through the nuclease activity of Cas10, which results in the degradation of the viral genome and the survival of the infected cell. However, spacers that target a gene transcribed later in the phage replication cycle present a problem for rapid recognition and clearance. Unlike with early targets, the DNase activity of Cas10 is not sufficient to clear the phage DNA and Csm6 is necessary for immunity. It is presumed that Csm6 mediated immunity causes abortive infection due to collateral damage to the host cell through mass RNA degradation. We have found that cells harboring a late spacer enter a state of dormancy after phage infection, allowing uninfected cells in the population to proliferate. Surprisingly, using microscopy and single cell sorting, we demonstrate that some of the dormant cells can also escape their inactive state once the invading phage genome has been cleared through the DNase activity of Cas10.

Class 2 CRISPR Cas systems

29| Abstract removed

30| A DNA Unwinding Equilibrium Serves as a Checkpoint for CRISPR-Cas12a Target Discrimination

Jaideep Singh; Kevin Liu; Aleique Allen; Wei Jiang; Peter Qin

University of Southern California

CRISPR-Cas12a is a programable RNA-guided nuclease that has emerged as a powerful tool for genome manipulation and molecular diagnostics, and in-depth understanding on mechanisms of target discrimination is the foundation for continuing developments of Cas12a-based applications. In this study, we investigated Cas12a target recognition using a combination of site-directed spin labeling, fluorescent spectroscopy, and enzyme kinetics. With a fully matched RNA guide, the data revealed an inherent equilibrium between a DNA unwound state and a DNA-paired duplex-like state. Experiments with off-target RNA guides and pre-nicked DNA substrates identified this equilibrium as a mismatch sensing checkpoint prior to the first

step of DNA cleavage. The data sheds light on the distinct targeting mechanism of Cas12a and the finding may better inform CRISPR based biotechnology developments.

31| Identification, characterization and development of a novel CRISPR system utilizing an alternative mechanism for Genome Editing

Rebekka Medert; Christian Zurek; Rebecca Heger; Julia Noske; Michael Krohn; Paul Scholz

Akribion Genomics AG

Despite a vast amount of genetic information available in public databases, only a small number of CRISPR associated nucleases usable for genome editing have been identified so far. In addition, a more wide-spread distribution of CRISPR technologies pervading all areas of commercial applications in life sciences are hindered by unresolved patent disputes and intertwined license options specifically for the CRISPR-Cas9 system.

In order to expand the toolbox of CRISPR nucleases and to provide alternatives for application areas where Cas9 usage may be obstructed, we created a proprietary treasure box of more than 2000 novel CRISPR nuclease sequences. These sequences were identified in a metagenomics screening from rationally selected and species specific enriched samples with a total sequencing depth of more than 3 Tbps (Tera-basepairs). Besides from novel sequences related to families of CRISPR nucleases already known to the public, several sequences with very low or no sequence homology to classical CRISPR nucleases have been identified and two main families of nucleases are currently advancing as novel genome editing tools.

The lead candidate of these nucleases is termed G-dase E, which is a novel type of Class 2 CRISPR system with no sequence homology to the classical CRISPR nucleases and a novel molecular mechanism to target both DNA and RNA. In contrast to classical CRISPR systems, the G-dase E nuclease induces collateral DNA and RNA degradation after an initial, guide-RNA specific activation. Despite this non-classical mode of action, highly efficient genome editing using a homology-directed repair (HDR) enrichment strategy has been evaluated in various prokaryotic, yeast and fungal cells with additional organisms currently in development. Furthermore, the novel mode of action opens up application fields where classical CRISPR nucleases cannot be employed, for example in diagnostics or specific cell depletion & enrichment in oncology.

32| Pipeline for improving the target efficiency of sgRNAs of CRISPR-Cpf1 system

Pragya Kesarwani [1]; Dhvani Sandip Vora [2]; Dr. Deepak T. Nair[1]; Dr. Durai Sundar[2]

[1] Regional Centre for Biotechnology; [2] Indian Institute of Technology Delhi, India

Targeted nucleases are widely used for altering the specific location of the genome with precision. The endonucleases facilitate efficient genome editing via designing a guide RNA (gRNA) consisting of a 23-nucleotide target sequence. gRNA preferably binds to the target

location, but the on-target and off-target activity of gRNAs varies widely. Off-target activity due to mismatch tolerance in the CRISPR-Cas system is a major factor inhibiting its clinical applications. Ensuring on-target efficiency and minimizing off-targets for a target sequence is the major objective of this study. A pipeline has been designed to predict potential off-target sites in the human genome for a target sequence and a multilayer perceptron (MLP) has been used to predict the cleavage efficiency of the potential off-target sites. An MLP classifier was trained with sequence and base-dependent binding energy-associated features for AsCpf1 and LbCpf1 to predict the target efficiencies. Positional preferences of nucleotides, distribution of mismatches, and classification-dependent feature importance between high-activity and low-activity off-targets were also studied. Positional preference of nucleotides revealed that Thymine is highly disfavored at positions adjacent to Protospacer Adjacent Motif (PAM) whereas Guanine is favored in high-activity off-targets. Mismatch distribution analysis revealed that mismatches were more prominent in the trunk and promiscuous region and transition type mismatches were more preferred at 16, 17, and 18 nucleotides position. Thermodynamics-associated features such as low to the moderate melting temperature of the non-seed region and base-dependent PAM binding energy were predicted as best predictors by the MLP classifier for high activity off-targets. GC content, dinucleotide frequencies, number of bulges, and mismatches in the seed and trunk region were other characteristic features between high-activity and low-activity off-targets for both AsCpf1 and LbCpf1.

33| Discovery and engineering of a miniature CRISPR-Cas type V-L system

Lauren Alfonse; Alexa Ornstein; Erin Doherty; Jenna Rachman; Tomoaki Sasaki; Blake McMahon; Quinton Wessells; Anthony Garrity; Gabrielle Munoz; Zachary Maben; Mishti Bose; Paul Yan; Amy Biasella; David Cheng; Tia DiTommaso; John Murphy; Gregory Hoffman

Arbor Biotechnologies

Employing a metagenomic search, we identified a family of miniature CRISPR-Cas type V-L systems capable of RNA-guided dsDNA target cleavage without a tracrRNA. A bacterial depletion screen revealed several active systems which were subsequently shown to have low-level editing in mammalian cells. Among the systems with activity in mammalian cells, we selected a 745aa nuclease effector as a candidate for engineering. An unbiased mutational scanning approach was applied to identify single substitutions that increase indel activity in mammalian cells. Indel-enhancing single substitutions were then screened in combination, with a combinatorially engineered variant demonstrating indel editing activity comparable to SpCas9 in HEK293T cells. Structure determination via cryo-EM revealed the domain organization and nuclease mechanism of the ternary complex. Due to its small size and robust editing levels, this novel miniature CRISPR-Cas type V-L system is an attractive therapeutic candidate for single- or dual-guide excision via all-in-one AAV delivery.

34| Prophages vaccinate CRISPR-Cas populations against lytic threats

Nicholas Keith[1]*; Joshua Modell*

[1] Johns Hopkins University School of Medicine

CRISPR-Cas systems must discriminate self from non-self nucleic acids to provide bacteria with adaptive immunity against foreign agents, including bacteriophages. Prophages are simultaneously self and non-self elements; they reside within the bacterial chromosome and yet are mobile and can reactivate during the SOS DNA damage response, resulting in cell lysis. Prophage-targeting spacers have only been observed in cases where an anti-CRISPR (Acr) inactivates the CRISPR-Cas system to prevent auto-immune cleavage of the chromosome. It therefore remains unclear whether bacteria can generate functional spacers from their resident prophages in the absence of an Acr. Here, we show that prophages are indeed hotspots for CRISPR-Cas spacer acquisition. While most of these spacers result in auto-immune cell death, many cells survive by curing the targeted prophage. Furthermore, we demonstrate that these prophage-acquired cells can defend against naturally-arising lytic mutants that otherwise decimate the population. Our results suggest a new paradigm whereby spacers can be acquired on much longer timescales than the canonical model of a lytic sweep with a novel phage. This may explain why most known phage-targeting spacers target temperate phages. Finally, we propose a new role for CRISPR-Cas systems in bacterial evolution as mediators of prophage curing.

35| Endogenous RNA targeting by Cas9 in the foodborne-pathogen Campylobacter jejuni

Sahil Sharma [1]*; Cynthia Sharma*[2]

[1] Department of Molecular Infection Biology II, Institute of Molecular Infection Biology (IMIB), University of Würzburg, Germany; [2] Institute for Molecular Infection Biology (IMIB), University of Würzburg

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) systems are naturally occurring adaptive immune systems found in bacteria and archaea. There has been emerging evidence that Cas9 might have cellular roles beyond genome defence and can impact gene regulation or virulence. Recently, we have uncovered that Cas9 from the human pathogen *Campylobacter jejuni* (CjCas9) can bind and cleave endogenous mRNAs in a crRNA-dependent manner. Using RNA-immunoprecipitation and sequencing (RIP-seq) in natural isolates of *C. jejuni* with different spacer complements, we confirmed endogenous RNA-binding as a common feature of CjCas9 and revealed additional unexpected modes of RNA-binding. These include duplex formation between tracrRNA and cellular RNAs from outside the CRISPR-Cas9 locus, generating so-called non-canonical CRISPR RNAs (ncrRNAs). We leveraged our discovery of ncrRNAs to develop a novel diagnostic tool for multiplexable RNA detection termed LEOPARD (2). Despite this novel application, it remained unclear so far whether formation of different cellular CjCas9 ribonucleoprotein (RNP) complexes play any role in endogenous gene regulation. Knowing that Cas9-mediated RNA

cleavage requires crRNA-mRNA interactions *in-vivo*, we performed RIP-seq on engineered strains harbouring deletions of either individual crRNA, trans-activating crRNA (tracrRNA) or the entire CRISPR locus which revealed previously unknown mode of RNA-binding by CjCas9, independent of both crRNA and tracrRNA. Further, using RIP-seq of CjCas9 in complementation strains for either active (WT) or catalytically-dead (dCas9) Cas9, we identify ‘‘hidden’’ RNA targets, enriched only with dCas9. These different modes of RNA-binding hint towards an unexpected role of Cas9 nuclease as a potential RNA effector protein in *C. jejuni*. Overall, our findings provide new insights into varied modes of RNA-binding by CjCas9, uncovering its indelible impact on endogenous gene regulation.

36| Structures of Cas12a R-loop propagation on pathway to DNA cleavage

Isabel Strohkendl; David W. Taylor

The University of Texas at Austin

CRISPR-Cas12a recognizes target DNA through initial PAM binding and a 20-base pair (bp) R-loop between the complementary crRNA and target strand, providing a mechanism for precise DNA targeting that has benefitted genome editing applications. Previous kinetic characterization of Cas12a DNA targeting showed directional R-loop formation is the key specificity-determining step for DNA binding and cleavage. Despite the importance of R-loop formation in recognizing on-targets, rejecting off-targets, and triggering RuvC-mediated DNA cleavage, little is known as to how the R-loop forms within Cas12a. Here, we use cryo-electron microscopy to capture a series of kinetically trapped *Acidaminococcus* sp. Cas12a R-loop structure intermediates to elucidate how Cas12a forms its 20bp R-loop and delivers the target DNA into the RuvC active site. Cas12a first interrogates 5bp of target DNA using a kinetic seed, followed by dramatic Rec domain mobility to accommodate R-loop extension. Propagation through the middle R-loop is driven by base pair energetics as Rec domain flexibility limits protein contacts to the nascent R-loop. Only during formation of the final R-loop base pairs does Rec2 dock, enabling contacts throughout the R-loop. R-loop completion also brings the nontarget strand across the RuvC domain, leading to lid engagement and coordination with the active site. Following nontarget strand cleavage, subsequent melting of the R-loop heteroduplex and sharp bending of the DNA bring the target strand to the RuvC active site. Together, our kinetically captured Cas12a structural intermediates reveal conformational checkpoints that exist during directional R-loop formation to ensure high targeting specificity and demonstrate how both DNA strands reach the RuvC active site for cleavage.

37| Harnessing the diversity of CRISPR-Cas proteins for genome editing

Giedrius Gasiunas[1], Tomas Urbaitis[1], Laima Budenaite[1, 4], Aida Vitkeviciene[1], Migle Stitilyte[1,4], Liz Van Ginkel[2], Lindsey Lange[2], Vesna Djukanovic[2], Stephanie Lex[2], Stephen Gasior[2], Zhenglin Hou[2], Pierluigi Barone[2], Jeffrey Sander[2], Jennifer Curcur[3], Ryan T. Fuchs[3], Megumu Mabuchi[3], G. Brett Robb[3], Joshua K. Young[2]

[1]CasZyme, Vilnius, LT-10257, Lithuania. [2]Corteva Agriscience™, Johnston, IA 50131, USA. [3]New England Biolabs, Ipswich, MA, 01938, USA. [4]Life Sciences Center, Vilnius University, Vilnius, Lithuania.

The Cas9 protein, derived from bacterial CRISPR-Cas systems, has become a widely studied and used genome editing tool due to its versatility and efficacy. However, several constraints have been identified for its use. These include its relatively large size, protospacer adjacent motif (PAM) requirements, and insufficient specificity for some applications. To address these limitations, we explore the natural diversity of CRISPR-associated (Cas) enzymes to develop novel RNA-guided tools with potentially beneficial properties. Here, we report our efforts to functionally characterize and engineer a diverse collection of Type II and V Cas effectors. Our findings reveal that Cas nucleases display a wide range of sizes, structural features, biochemical properties, PAM recognition, and DNA cleavage capabilities in vitro and in cellular environments. Furthermore, utilizing a variety of engineering approaches, we show that divergent RNA-guided nucleases can be developed as editing tools for use in human cells. Overall, our results suggest that the natural diversity of Cas proteins provides a promising wellspring for the development of novel gene editing tools.

38| Mechanistic and evolutionary insights into a type V-M CRISPR-Cas effector enzyme

Satoshi Omura[1]; Ryoya Nakagawa; Wen Wu; Christian Sudfeld; John van der Oost; Osamu Nureki

[1] The University of Tokyo

RNA-guided type V CRISPR-Cas12 effectors provide adaptive immunity against mobile genetic elements in bacteria and archaea. Among diverse Cas12 enzymes, the recently identified Cas12m2 (CRISPR-Cas type V-M) is highly compact and has a unique RuvC site. Although the non-canonical RuvC triad does not permit dsDNA cleavage, Cas12m2 still protects against invading mobile genetic elements through transcriptional silencing by strong DNA binding. However, the molecular mechanisms of RNA-guided genome inactivation by Cas12m2 remain unknown. Here, we report the cryo-electron microscopy structures of two states of Cas12m2–crRNA–target DNA ternary complexes and Cas12m2–crRNA binary complex, revealing structural dynamics during crRNA–target DNA heteroduplex formation. The structures revealed that the non-target DNA strand is tightly bound to a unique arginine-rich cluster in the REC domains and the non-canonical active site in the RuvC domain, ensuring the strong DNA binding affinity of Cas12m2. Furthermore, structural comparison of Cas12m2 with TnpB, a putative ancestor of Cas12 enzymes, suggests that interaction of the characteristic coiled-coil REC2 insertion with the PAM distal region of the heteroduplex is crucial for Cas12m2 to engage in adaptive immunity. Collectively, our findings improve the mechanistic

understanding of diverse type V CRISPR-Cas effectors and provide insights into the evolution of TnpB to Cas12 enzymes.

39| The Influence of Activity Inhibition on the Conformation of CRISPR-Cas13a analysed by EPR distance measurements

Catrin Allar; Maria Francesca Vicino; Olav Schiemann

University Bonn

The class 2, type VI ribonuclease CRISPR-Cas13a is known for its diagnostic application. Although it is already in use in nucleic acid detection, the conformational mechanistics are not fully resolved yet. Some crystal structures of its mechanistic route from the apo, via the pre-crRNA- and crRNA-, to the target—crRNA-bound state are known, however, these structures stem from multiple different bacteria and contain different levels of truncation. Our aim is to resolve the structure of Cas13a from *Leptotrichia buccalis* without any cut-offs, using EPR distance measurements. In this regard, I am focussing on the influence of activity inhibition on both the distances during apo and crRNA-containing measurements of Cas13a.

To investigate this, we are combining Site-Directed Spin Labelling (SDSL) and Pulsed Electron-Electron Double Resonance Spectroscopy (PELDOR or DEER). PELDOR is a pulsed EPR method that provides distance distributions between spin labels and thus information on the conformational distribution of, e.g., a protein. We attached two nitroxide spin labels site specifically to Cas13a by exchanging naturally occurring cysteins and introducing new ones at the site of interest by means of site directed mutagenesis. The cysteines are then reacted with MTSSL, covalently linking the label to Cas13a via disulphide bridges. Placing the labels at different sites and measuring the distance distributions between the labels with PELDOR yields a picture of the conformational distribution of Cas13a. Based on the previous works of East-Seletsky et al., we inhibited the active centres of the pre-crRNA maturation and the target RNA degradation. Here, we will report on the construct design and the observed conformational changes resulting from activity modification.

40| Following conformational changes in LbuCas13a from apo to the cr- and target RNA bound state with EPR spectroscopic distance measurements

Maria Francesca Vicino; Catrin Allar; Olav Schiemann

University of Bonn, Clausius Institute for physical and theoretical chemistry

The type VI CRISPR effector protein Cas13a is an RNA nuclease, catalyzing two RNA cleavage events: First, the maturation of pre-crRNA and second, the cleavage of target RNA, activating a non-specific collateral cleavage. Although all four structures of Cas13a on route from apo, via the pre-crRNA bound complex (Cas13a-precr), the crRNA bound complex (Cas13a-cr), and the crRNA-target RNA bound complex (Cas13a-cr-target) are known, they origin from proteins of different organisms and contain different extends of truncation. To identify and follow

conformational changes on a molecular level, structures of Cas13a in all states from one organism are needed.

We aim to resolve static structures and follow conformational changes by site directed spin labelling in combination with Pulsed Electron-Electron Double Resonance (PELDOR) spectroscopy, which enables to measure distance distributions between spin labels in e.g., biomolecules in the range of 1.6-16 nm.

To this end, we labeled *Leptotrichia buccalis* Cas13a with pairs of nitroxide spin labels at different positions and tested their influence on the functionality. All active constructs were subjected to PELDOR experiments. We compared the resulting distance distributions with and without RNA to in silico generated distributions. These were calculated with the software mtsslWizard and are based on the experimental RNA bound structures as well as the AlphaFold2 structure as input. We show that the unknown apo Cas13a conformation has a very flexible, and wide-open REC-lobe conformation and a preorganized NUC-lobe. Upon pre-crRNA addition a rigidification and conformational selection occurs. The unknown Cas13a-precr conformation is very similar to the Cas13a-cr conformation. Both, the conformation and conformational changes between Cas13a-cr and Cas13a-cr-target fit to the calculated ones and to the known structures.

41| AcrVIB1 inhibits the Cas13b nuclease prior to RNP complex formation

Katharina Wandera; Angela Migur; Anuja Kibe; Neva Caliskan; Chase Beisel

Helmholtz Institute for RNA-based Infection Research (HIRI)

Anti-CRISPR proteins (Acrs) are small phage-derived proteins that inhibit the adaptive immune system CRISPR-Cas. Over the years, several Acrs have been discovered for various CRISPR-Cas subtypes, with little to no sequence or structural similarity between these small proteins. This diversity is reflected not only in their amino acid composition, but also in the exhibited mode-of-action. One common feature among known Acr mechanisms is inhibition of the formed RNP complex, with mechanisms ranging from binding to the DNA-binding pocket of the RNP complex (AcrIIA4) to acetylation of an amino acid residue that is required for recognition of the protospacer-adjacent motif (AcrVA5). Here, we show that AcrVIB1 inhibits the Cas13b nuclease prior to RNP complex formation. In vitro, AcrVIB1 strongly interacts with PbuCas13b but not the crRNA, while the interaction is reduced when binding to the Cas13b-gRNA RNP. Surprisingly, binding AcrVIB1 enhances binding of Cas13b to its guide RNA, although the crRNA does not undergo processing, and the Cas13b does not cleave collateral substrates in the presence of an RNA target. In vivo, the crRNA undergoes degradation in the presence of AcrVIB1. Thus, we propose a novel mechanism in which AcrVIB1 binds to PbuCas13b, promoting partial or altered crRNA binding. This process prevents crRNA processing hence target recognition and exposes the bound RNA to host ribonucleases. This mechanism expands Acr-based inhibitory mechanisms to upstream of RNP formation.

42| Statistical analysis of CRISPR-Cas13b array leaders shows widespread RNA structures

Maximilian Feussner[1]; Zasha Weinberg[1]; Angela Migur[2]; Omer S. Alkhnbashi[3]; Rolf Backofen[4]; Chase Beisel[2]

[1] University Leipzig; [2] Helmholtz Institute for RNA-based Infection Research (HIRI), Helmholtz Centre for Infection Research (HZI), Würzburg, Germany; [3] King Fahd University of Petroleum and Minerals; [4]University Freiburg

CRISPR-Cas systems provide adaptive immunity to plasmids and phages. They store small fragments of the invader's genetic material as "spacers" in a CRISPR array. Each spacer is sandwiched between fixed repeats. Each spacer-repeat pair gives rise to a CRISPR RNA (crRNA) that directs a nuclease to cleave nucleic acid sequences corresponding to the spacer, thus defending against the corresponding invader. With repeats on either side of each spacer, there is one more repeat than spacer, and the extra repeat is not paired with a spacer. While this extra repeat is often used to acquire new spacers, it would result in a crRNA with a spacer from outside the array. This "extraneous" crRNA (ecrRNA) would not target an invader and could interfere with crRNA biogenesis. We investigated type VI-B systems, whose extra repeats are located proximal to the array leader. Using several lines of experimental evidence, we showed that the leader base pairs with the extra repeat and forms an interfering RNA secondary structure to inhibit its processing. The RNA secondary structure between the leader and the extra repeat interferes with the functional RNA structure of the repeat and prevents processing of Cas13b. This conference submission focuses on work to bioinformatically determine whether this mechanism is widespread in type VI-B systems. We designed a statistical test to computationally analyze the pairing potential between leaders and the extra repeat in several type VI-B systems, and calculated an aggregate p-value on an independent test set of 9×10-3 for type VI-B. This significant p-value suggests that many type VI-B systems use this strategy to silence their ecrRNAs, thereby amplifying the experimental results.

43| The leader RNA in CRISPR-Cas13b systems enhances CRISPR RNA production by inhibiting processing of the first repeat

Angela Migur[1]; Maximilian Feußner[2]; Chunyu Liao[1]; Zasha Weinberg[2]; Chase Beisel[1]

[1] Helmholtz Centre for Infection Research - Helmholtz Institute for RNA-based Infection Research (HIRI-HZI), Würzburg, Germany; [2] University of Leipzig, Leipzig, Germany

CRISPR-Cas systems store CRISPR (cr)RNAs used for sequence-specific immune defense in CRISPR arrays comprising conserved repeats with intervening invader-derived spacers. Recently, the leader RNA upstream of the transcribed CRISPR array was shown to optimize crRNA production in type II-A systems by forming a hairpin with the first repeat [1] . Here, we report an analogous mechanism in the wholly unrelated type VI-B systems. Unlike II-A systems, VI-B systems rely on the RNA-targeting nuclease Cas13b and do not utilize tracrRNAs for crRNA processing [2]. However, like II-A systems, the first repeat in the VI-B CRISPR array is expected to yield to an invader-independent “extraneous” (e)crRNA. Building on this

commonality, we predicted leader-repeat hairpins across VI-B systems. Using the VI-B system from *Porphyromonas gingivalis*, we showed that hairpin formation prevented proper folding of the first repeat for binding and processing by Cas13b. Mutating the hairpin restored ecrRNA formation and impinged on production of the crRNA targeting the most recently encountered invader. These findings suggest that CRISPR-Cas systems broadly possess mechanisms to optimize crRNA production and prevent ecrRNA formation.

[1] Liao, et al. (2022) Spacer prioritization in CRISPR-Cas9 immunity is enabled by the leader RNA. Nat. Microbiol., 7, 530–541

[2] Smargon, et al. (2017) Cas13b Is a Type VI-B CRISPR-Associated RNA-Guided RNase Differentially Regulated by Accessory Proteins Csx27 and Csx28. Mol. Cell, 65(4):618-630.e7

44| Characterization of compact CRISPR-Cas12 effector complexes

Greta Bigelyte; Brigita Duchovska; Rimante Zedaveinyte; Arunas Silanskas; Tautvydas Karvelis

Institute of Biotechnology, Life Sciences Center, Vilnius University, Lithuania

In recent years, CRISPR-Cas9 and Cas12a nucleases have revolutionized the genome editing field. Being guided by an RNA to cleave double-stranded (ds) DNA targets, Cas9 and Cas12a offer unprecedented flexibility for DNA targeting. However, the delivery of these nucleases to cells in vivo is limited by the capacity of commonly used adeno-associated viruses (AAVs). Packaging large Cas12a (900-1500 aa) and Cas9 (1000-1600 aa) nucleases encoding genes into these vectors remains complicated. As a result, recent efforts have focused on expanding the number of characterized CRISPR-Cas systems to identify novel and smaller nucleases, which could be better candidates for in vivo delivery using AAV vectors. However, the functional diversity of these compact nucleases within each subtype remains poorly explored.

In this study, we describe a set of novel Cas12 proteins, ranging 500-700 aa in size. Using biochemical methods, we identified RNA molecules required for effector complex formation and key elements for DNA target recognition in vitro. Further investigation of selected Cas12 proteins revealed their ability to provide DNA interference in cells. Altogether, our results indicate that the natural diversity of Cas proteins provides a source of potential novel gene editing tools with unique characteristics.

45| CRISPR-Cas9 limits the spread of a meningococcal disease-associated filamentous phage

XIN LI; Xufei Zhou; Rucheng Diao; Peter Freddolino; Zhonggang Hou; Yan Zhang

University of Michigan - Ann Arbor

N. meningitidis is the main causative agent of bacterial meningitis and septicemia. In this pathogen, DNA natural transformation is the main source of genetic variation that underlies antibiotic resistance, immune evasion, and virulence; very little is known about its bacteriophages. Recently, comparative genomics identified putative filamentous prophages

in meningococcal strains. Our informatic analysis also found that ~30% of natural spacers of *Neisseria* CRISPR-Cas9 loci can match to these prophage sequences, hinting at functional interplay. One of these phages is known as the meningococcal disease associated phage MDAΦ, due to its linkage to hypervirulence and increased colonization of human epithelial cells. Here using MDAΦ as model, we demonstrated the role of CRISPR-Cas9 in limiting the spread of *Neisseria* filamentous phages.

MDAΦ lysogenizes the CRISPR+ meningococcal strain 8013 in a type IV pili-dependent manner. By characterizing the resulting transductants, we found that MDAΦ establishes infection primarily as a non-integrative episome but can also integrate into dRS3 sites of host genome as a prophage. Importantly, *Neisseria* CRISPR that encodes a pre-existing MDAΦ-targeting spacer can prevent MDAΦ infection. To examine if CRISPR can cure MDAΦ from a lysogen population, we built an inducible Cas9 system that allowed us to activate Cas9 after MDAΦ lysogenization. Our induction assays suggest that Cas9 can effectively clear the MDAΦ episomes without killing the infected hosts. Collectively, our findings shed new light on the role of CRISPR-Cas9 in modulating the filamentous phage content and pathogenicity in bacteria.

46|Temperate phage tolerance by the type VI-A CRISPR-Cas system in *Listeria seeligeri*

Marshall Godsil[1]; Nova Wei[2]; Alex Meeske[2]

[1] University of Washington - Seattle; [2] University of Washington

Temperate phages are a widespread class of bacteriophages that employ a biphasic replication strategy. During the lytic phase, progeny virions are produced which lyse the cell, and escape to new hosts. In the lysogenic phase, the phage integrates into the host genome where it is maintained passively. Viral integration into the host chromosome as a strategy for immune evasion has been studied in eukaryotic viruses, but analogous interactions in prokaryotic systems remain understudied. Since lysogeny plays an important role in gene transfer, immune tolerance of lysogeny may be an adaptive trait. Rather than lose immune function to accommodate lysogeny, bacteria can employ immune systems that restrict lytic replication and tolerate lysogeny by targeting hallmarks of lytic replication. Lysogen tolerant immunity was previously observed in the RNA-sensing type III CRISPR-Cas system (Goldberg et al. 2014). By sensing RNA, the type III CRISPR system can be exclusively activated during lytic transcription, allowing lysogenic replication. However, this lysogen tolerance is unstable due to the DNA-cleaving activity of this system (Goldberg et al 2018). In contrast, the type VI CRISPR system, senses and cleaves only RNA. By lacking DNA cleavage activity, type VI CRISPR immunity may be more tolerant of lysogeny and enforce a stable relationship with temperate phages. Here we show that the native type VI-A host *Listeria seeligeri* restricts the phage lytic program when equipped with spacers targeting lytic genes, and that these same spacers permit lysogen establishment. Further, we found that these self-targeting lysogens are stable and can restrict prophage reactivation. In the future, we aim to characterize whether and how cells surviving reactivation retain their prophages.

47| Diversity of CRISPR-Cas type II-A systems in *Streptococcus anginosus*

Richard Bauer; Dorina Haider[1]; Aline Grempels; Rebecca Roscher; Stefanie Mauerer; Barbara Spellerberg

[1] Ulm University Medical Center

Streptococcus anginosus is a commensal streptococcal species that is often associated with invasive bacterial infections. However, little is known about its molecular genetic background. Many streptococcal species, including *S. anginosus*, harbor CRISPR-Cas systems and a CRISPR-Cas type II-A system as well as a type II-C system have been reported for this species. To characterize the CRISPR-Cas type II systems of *S. anginosus* in more detail, we conducted a phylogenetic analysis of Cas9 sequences from CRISPR-Cas type II systems with a special focus on *Streptococci* and *S. anginosus*. In addition, a phylogenetic analysis of *S. anginosus* strains based on housekeeping genes included in MLST analysis, was performed. All analyzed Cas9 sequences of *S. anginosus* clustered with the Cas9 sequences of CRISPR type II-A systems, including the Cas9 sequences of *S. anginosus* strains reported to harbor a type II-C system. The Cas9 genes of the CRISPR-Cas type II-C systems of other bacterial species separated into a different cluster. Moreover, analyzing the CRISPR loci found in *S. anginosus*, two distinct csn2 genes could be detected, a short form showing high similarity to the canonical form of the csn2 gene present in *S. pyogenes*. The second CRISPR type II locus of *S. anginosus* contained a longer variant of csn2 with close similarities to a csn2 gene that has previously been described in *Streptococcus thermophilus*. Since CRISPR-Cas type II-C systems do not contain a csn2 gene, the *S. anginosus* strains reported to have a CRISPR-Cas type II-C system appear to carry a variation of CRISPR-Cas type II-A harboring a long variant of csn2.

48| Cas12a2 nucleases form three functionally-distinct clades

Oleg Dmytrenko[1]; Xiye Chen[1]; Chase Beisel[1]; Ryan Jackson[2]

[1] Helmholtz Centre for Infection Research - Helmholtz Institute for RNA-based Infection Research (HIRI-HZI), Würzburg, Germany; [2] Utah State University

Cas12a2 is a novel group of CRISPR-Cas nucleases. We have previously characterized a Cas12a2 ortholog from *Sulfuricurvum* sp., an RNA-targeting nuclease with collateral activity towards dsDNA, ssDNA, and RNA. We demonstrated that SuCas12a2 can enact population-level immunity by degrading non-targeted nucleic acids, including chromosomal DNA, and, therefore, eliminating the infected bacteria upon recognizing an infectious target. Since then, we identified over seventy Cas12a2 orthologs that form three distinct phylogenetic clades: Su, Sm, and Ap. Orthologs within each clade share unique amino acid motifs and predicted structural domains, while orthologs between the clades can share as little as 8% amino acid identity. For instance, the Sm and Ap clade orthologs lack many of the residues found in SuCas12a2, such as the aromatic amino acids required for collateral DNA cleavage. Based on these differences, we hypothesize that Cas12a2 orthologs from the three clades are functionally distinct, especially with regard to their collateral activity. To investigate this

diversity, we first identified a set of active Cas12a2 nucleases from each clade through screens in cell-free extract (TXTL) and *E. coli*. We next purified orthologs from the Sm and Su clades and showed that they maintain activity in TXTL. However, only the Su clade orthologs showed activity in vitro under the conditions previously established for SuCas12a2, indicating that the Sm orthologs have distinct substrate requirements and functional properties. Further characterization of representative orthologs from the three clades is ongoing, with the focus on determining their target range, specificity, and preference for collateral substrates. This work highlights the previously unknown functional diversity of Cas12a2 nucleases and informs their potential downstream applications.

49| Abstract removed

50| A *Streptococcus thermophilus* phage gene cluster encodes proteins that in combination are required for Cas9 inhibition

Bianca Garcia-Garcia [1]; Yurima Hidalgo-Reyes; Chantel Trost; Genevieve Rousseau; Sylvain Moineau; Alan Davidson

[1] University of Toronto, Canada

Bacteria, and the viruses that infect and destroy them (bacteriophages), are engaged in an endless arms race. Consequently, bacteria have developed a variety of mechanisms to evade phage attack, including CRISPR-Cas systems. To counter this defense, bacteriophages (phages) have evolved small proteins called anti-CRISPRs (Acrs). Although more than 90 distinct families of Acrs have been described, most share little sequence similarity and differ mechanistically. Since the genes that encode Acrs are frequently positioned next to genes that encode transcriptional repressors called Acr-associated (aca) genes, acas are often used as genetic

landmarks for identifying new Acrs. However, there are a few examples of acrs that are not associated with acas, such as the previously identified *Streptococcus thermophilus* anti-CRISPR, AcrIIA5. In this study, we report a novel aca gene that is unusually positioned four open reading frames (ORF) downstream of the gene encoding AcrIIA5. Using this aca gene as marker, we identified several genes in *Streptococcus* genomes that encode functional Acrs. Additionally, the four genes separating this novel aca and AcrIIA5, denoted D4276-4G, co-occur in many different genomic contexts and are associated with phage proteins. Surprisingly, we found that D4276-4G, which contain two big ORFs with predicted enzymatic activity, is a potent inhibitor of several type II-A and II-C CRISPR-Cas9 systems. Moreover, one *Streptococcus thermophilus* Cas9 protein, Sth3Cas9, is only fully inhibited if the four genes are expressed together, revealing a previously undescribed approach for the inhibition of the CRISPR-Cas systems. Using D4276-4G as a genetic landmark, we further identified other acrs and putative acas near this gene cluster in other species. Our findings reveal the first example of CRISPR system inhibition by more than one protein and expand the repertoire of arsenals that phages use to counteract bacterial immunity.

51| Translation-dependent downregulation of Cas12a mRNA by an anti-CRISPR protein

Nicole Marino[1], Alexander Talaie[1], Heloise Carion[1]*, Yang Zhang[1], Sukrit Silas[1], Yuping Li[1], and Joseph Bondy-Denomy[1,2,3]*

*These authors contributed equally to this work. [1]Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA 94158, USA; [2]Quantitative Biosciences Institute, University of California, San Francisco, San Francisco, CA 94158, USA 3Innovative Genomics Institute, Berkeley, CA 94720, USA

Bacteria have evolved multiple defense systems, including CRISPR-Cas, to cleave phage and other mobile genetic elements (MGEs). In turn, phage have evolved mechanistically diverse anti-CRISPR (Acr) proteins that use novel and co-opted mechanisms to block cleavage. Here, we report that an anti-CRISPR (AcrVA2) unexpectedly inhibits Cas12a biogenesis by binding its nascent polypeptide and triggering destruction of its mRNA. By recognizing conserved amino acids near the Cas12a N-terminus, AcrVA2 specifically downregulates the mRNA of diverse Cas12a orthologs. Mutating four amino acids in Cas12a abrogates binding and downregulation by AcrVA2; however, these mutations decrease Cas12a anti-phage activity, suggesting that this recognition mechanism constrains Cas12a escape. AcrVA2 is found on diverse MGEs across numerous bacterial classes, typically where Cas12a systems are not found, suggesting similar molecular outcomes may be achieved against other targets. These findings expand our understanding of the microbial arms race and may enable polypeptide-specific gene regulation in prokaryotes and beyond.

52| Cleave it or leave it? – Predicting CRISPR-Cas9 activity with a kinetic model

Hidde Offerhaus; Martin Depken

Delft University of Technology, Bionanoscience Department

Many applications of CRISPR technology come with stringent requirements on nuclease efficiency and fidelity. One must choose an appropriate enzyme and guide RNA to meet these requirements, and many on- and off-target activity prediction tools exist to assist in making this choice. We present a new tool that is the first to make quantitative and physically interpretable predictions of CRISPR-Cas9 cleavage and binding activity. Our predictions have been shown to outperform state-off-the-art off-target scoring schemes. The tool relies on a mechanistic model of target recognition where the R-loop hybridization kinetics follow from an energy landscape, which we obtain through machine learning. The details of this landscape give insights into the target recognition process, which might guide future efforts to engineer high-precision Cas9 variants. We currently work on applying the model to more CRISPR enzymes, and we are investigating how to integrate sequence dependency into the model framework. By extending our kinetic model, we hope to develop a tool that supports effective CRISPR applications and sheds new light on these fascinating proteins.

53| Basecamp Research: Leveraging a knowledge graph of global biodiversity for sequence-independent discovery and annotation of new gene-editing systems

Marcus Leung; Philipp Lorenz

Basecamp Research

With more than 99.9% of biodiversity remaining unknown, nature provides untapped potentials for the discovery of gene-editing systems with novel functions or improved performance. To address this knowledge gap, Nagoya-compliant sampling of environmental metagenomes and comprehensive metadata across five continents covering 50% of global biomes was conducted. This enabled the construction of a knowledge graph with 3.5 billion relationships, contextualising protein and genome sequences with hundreds of biological, chemical, and environmental metadata types. Searching this resource based on graph algorithms and the context captured around proteins and genomes has enabled the identification and annotation of gene editing systems in a sequence-independent manner.

CRISPR derived technologies

54| Predicting prime editing efficiency and product purity by deep learning

Nicolas Mathis; Ahmed Allam; Lucas Kissling; Kim Marquart; Lukas Schmidheini; Cristina Solari; Zsolt Balázs; Michael Krauthammer; Gerald Schwank

University of Zurich

Prime editing is a versatile genome editing tool that requires experimental optimization of the prime editing guide RNA (pegRNA) to achieve high editing efficiency. In this study, we conducted a high-throughput screen to analyze prime editing outcomes of 92,423 pegRNAs on a highly diverse set of 13,349 human pathogenic mutations, including base substitutions, insertions, and deletions. Based on this dataset, we identified sequence context features that influence prime editing and trained PRIDICT (PRIme editing guide preDICTion), an attention-based bidirectional recurrent neural network.

PRIDICT reliably predicts editing rates for all small-sized genetic changes with a Spearman's R of 0.85 and 0.78 for intended and unintended edits, respectively. We validated PRIDICT on endogenous editing sites as well as an external dataset and showed that pegRNAs with high (> 70) versus low (< 70) PRIDICT scores exhibited substantially increased prime editing efficiencies in different cell types in vitro (12-fold) and in hepatocytes in vivo (10-fold). This highlights the value of PRIDICT for both basic and translational research applications.

55| Toxin-based selection of CRISPR knock-in to differentiate induced pluripotent stem cells into skeletal myocytes

Antje Rottner; Marcello Maresca; Grzegorz Sienski

AstraZeneca

Scalable and robust protocols to differentiate human pluripotent stem cells (hiPSC) are still lacking for many cell types. Novel genome engineering tools can support the development of improved differentiation strategies and accelerate cellular model generation. Our recently developed strategy to select for bi-allelic CRISPR mediated knock-ins (“Xential”) at the HBEGF locus based on diphtheria toxin selection provides an efficient platform to enable transcription factor-driven direct conversion of hiPSC into cell types of interest without the need for clonal isolation. We applied this strategy to develop a skeletal myocyte differentiation pipeline, a cell type whose differentiation has so far been difficult or expensive and not genotype specific in the case of purchasable induced skeletal myocytes. We engineered hiPSC to inducibly express the muscle-master regulator MYOD1 along with shRNA-based downregulation of the pluripotency factor OCT4. Upon small molecule mediated transcription activation, hiPSC differentiate and mature into skeletal myocytes within 10 days. The differentiated myocytes present an elongated and spindle-like morphology with characteristic multinucleation. They demonstrate a protein and RNA profile confirming their skeletal myocyte identity with key characteristic myogenic markers such as Desmin, Myogenin, Troponin and MHC. The obtained skeletal myocytes further developed a characteristic functional signature by increasing their

glucose uptake two-fold upon stimulation with insulin. This novel pipeline can generate skeletal myocytes at scale at less than 1% of the cost of commercial inducible skeletal myocyte alternatives. The principle based on inducible expression of transcription factors demonstrated here could be further expanded to derive many other cell types cost-efficiently.

56| SPOT-ON – a high-performing Cas9 nuclease with exceptional intrinsic fidelity

Aleksandar Zdravkovic; Dmitrii Degtev; Burcu Bestas; Sandra Wimberger; Alexandra Madsen; Aikaterini Emmanouilidi; Michelle Porritt; Pinar Akcakaya; Grzegorz Sienski; Marcello Maresca

AstraZeneca AB

Cas9 proteins have demonstrated great potential in therapeutic gene editing. However, only a limited number of Cas9s display substantial levels of activity in vivo, and only a subset of these have progressed to clinical trials. Furthermore, there are always safety concerns with the therapeutic use of Cas9 systems due to potential off-target editing events.

Recently, we discovered a new member of the type II-B family of Cas9 from the human gut microbiome metagenomic database, which we named SpOT-ON. We biochemically characterized SpOT-ON and demonstrated its gene editing activity in vitro and in vivo. Importantly, SpOT-ON was also shown to cause less off-target events compared to the widely used SpCas9, as demonstrated by our recently developed technique based on DUPLEX-sequencing.

Taken together, our findings suggest that SpOT-ON could serve as a safe and effective candidate for clinical use. The discovery of SpOT-ON will be presented as well as our latest engineering efforts that made this protein as efficient as SpCas9 yet with better intrinsic fidelity.

57| Xential selection for precise genome insertion

Oi Kuan Choong; Songyuan Li; Nina Akrap; Grzegorz Sienski; Marcello Maresca

AstraZeneca AB

Advancements in DNA engineering and genome editing have significantly impacted the fields of medicine and drug development, offering new avenues for generating disease models and therapeutic applications. Despite their immense potential, the low efficiency of genome editing presents a major challenge in selecting and scaling up cells with desired edits from a pool of unedited cells. To overcome this challenge, we have developed Diphtheria Toxin (DT)-based selection method to enrich for genetically engineered cells named “Xential” for “recombination (X) at a locus conditionally essential for cell survival” hereafter. Through screening, we have identified mutations in the receptor for Diphtheria Toxin (DT) that confer protection to human cells from DT. Selective enrichment of cells with an edited DT receptor variant allows co-selection for simultaneously introduced, precisely targeted second-site gene

modifications at the desired locus, resulting in the generation of a pool of accurately edited cells. This method enables the rapid generation of a homogeneous cell population with biallelic integration of a DNA cassette at the selection locus, without the need for clonal isolation.

Nowadays Xential engineering plays a pivotal role in many projects in the AstraZeneca portfolio and has revolutionized modern drug development overall. With Xential, we have engineered thousands of transgenes to the genome of human cells covering applications as diverse as cellular reporters monitoring protein folding or tricks to aid lineage differentiation programs. During the meeting, I will present some of the highlights of our unpublished work.

58| Expanding the Scope of CRISPR-Cas Systems for Gene Editing through Ancestral Sequence Reconstruction

Sara Samperio; Ylenia Jabalera; Raul Perez-Jimenez

CIC bioGUNE Center of Excellence Severo Ochoa

CRISPR-Cas systems have revolutionized molecular biology by providing a powerful toolkit for genetic editing, faster and more accessible than traditional methods. However, the need for a specific PAM sequence and the design of gRNAs, remain major challenges that limit their use in more advanced applications beyond the laboratory. Moreover, immune responses to CRISPR enzymes in humans can restrict their therapeutic potential.

Recently, our laboratory has utilized the ancestral sequence reconstruction (ASR) technique to resurrect ancestral forms of the Cas9 enzyme from *Streptococcus pyogenes* (1). These ancestral forms exhibit greater flexibility in their PAM requirements and higher promiscuity in the use of different gRNA scaffolds. Additionally, these ancestral enzymes have demonstrated tolerance to a wider range of temperatures, pH values, low immunogenicity and activity in different substrates. These results corroborate that ASR is a promising technique for generating novel orthologues that may overcome the current limitations of Cas enzymes.

In this study, we extended our scope to other subtypes and variants of the CRISPR systems, such as SaCas9 and FnCas12, by reconstructing the sequences of ancestral nodes through molecular evolution models. The ancestral nodes obtained differ significantly from the modern Cas enzymes, accumulating many mutations that generate distinct new enzymes. We characterized these enzymes bioinformatically and selected those with greater biotechnological interest for laboratory reconstruction. The reconstructed enzymes are being characterized at the molecular level to identify potentially unique features. Our main goal is to expand the arsenal of gene editing tools by providing new orthologues, thereby facilitating the application of genome editing in the therapeutic field.

1. Alonso-Lerma, B., Jabalera, Y., Samperio, S. et al. Evolution of CRISPR-associated endonucleases as inferred from resurrected proteins. Nat Microbiol 8, 77–90 (2023)

59| Abstract Removed

60| Targeted cell depletion using the collateral activity of a novel CRISPR/Cas nuclease in human cell culture

Torsten Fauth[1]; Robin Back; Dirk Ritzmann; Rebekka Medert; Dirk Sombroek; Michael Krohn; Paul Scholz

[1] Akribion Genomics AG

The identification and further engineering of novel Cas nucleases allows the development of diverse applications including more targeted and advanced cancer therapies. However, one major hurdle to overcome is to ensure highly selective killing of cancer cells without affecting surrounding healthy tissue.

Here we report that our proprietary Cas nuclease termed G-dase E, which possesses an RNA-guided, 'collateral' nuclease activity, can be employed in tissue culture cells to induce selective killing of cell populations that are defined by a specific marker RNA. First, we assessed the selective targeting of a GFP transgene in standard HEK293 cells. Furthermore, we addressed the potential of G-dase E to specifically target clinically relevant oncogenes in cancer cells by

using the cervix carcinoma cell line HeLa, which expresses the human papilloma virus HPV18-E6 and E7 oncogenes.

Several guide RNAs were designed against different regions of the GFP and HPV18-E6/E7 mRNAs and assembled with G-dase E into functional RNPs, which were delivered into tissue culture cells by electroporation. To determine the target-specific cell depletion we employed fluorescence microscopy, FACS analysis and crystal violet staining as well as sulforhodamine cytotoxicity assays. We could show that RNPs specifically directed against the GFP mRNA lead to killing of GFP expressing HEK293 cells. Moreover, it was possible to selectively deplete GFP-positive cells from a mixed pool consisting of GFP and RFP expressing HEK293 cells. Strikingly, we also succeeded in killing HeLa cells by transfecting RNPs that target the HPV18-E6/E7 mRNA, whereas HEK293 cells that do not carry the oncogenes remained viable.

In summary, we provide first evidence that G-dase E can be programmed to induce selective cell killing by targeting a user-defined or disease-specific marker RNA. This offers the potential of developing targeted cancer therapies based on G-dase E as a novel therapeutic tool.

61| CRISPRon-ABE: Deep learning CRISPR adenine base editing design from data generation

Ying Sun[1]; Kunli Qu[2]; Christian Anthon; Xiaoguang Pan[3]; Yonglun Luo[2]; Jan Gorodkin [1]

[1] University of Copenhagen; [2] Lars Bolund Institute of Regenerative Medicine / Aarhus University; [3] Lars Bolund Institute of Regenerative Medicine / Copenhagen University

The development of the CRISPR base editing technology holds the promise to meet the challenges posed by the DNA repair of the double strand-break resulting from “classical” CRISPR-based editing. For example, in the Adenine Base Editor (ABE) Adenines inside an editing window of ~8 nt of the DNA target can with different likelihoods be changed to Gs. The design task is to predict in part with which frequency these A to G outcomes appear and to predict the guide RNA (gRNA) efficiency. Current methods are evaluated separately on the A to G outcome proportions and on the total gRNA efficiency which can lead to suboptimal choices as the overall low efficiency may hamper the intended edits. To better support construction of enhanced prediction models, we generated new data using our SURRO-seq technology [2] which doubled the higher efficiency gRNAs from the ~5,000 in the public domain [3] ~10,000 gRNAs and used these to construct a deep learning-based ABE predictor CRISPRon-ABE using the same strategy as our state-of-the-art Cas9/gRNA editing efficiency prediction tool, CRISPRon [1]. To simultaneously evaluate gRNA efficiency and the outcome frequencies, we use the RK (K=2) correlation coefficient [4]. For CRISPRon-ABE we obtain R2=0.80 clearly exceeding R2=0.72 by the current best method DeepABE [3] evaluated on the same independent test set with gRNAs not used in the training either model. We generated similar data for Cytosine Base Editing and are currently developing a corresponding computational model.

References:

[1] Xiang, X. et al. Nat Commun 12, 3238, (2021). https://www.nature.com/articles/s41467-021-23576-0
[2] Pan et al. Nat Commun 13, 4049 (2022). https://www.nature.com/articles/s41467-022-31543-6
[3] Song, M. et al. Nat Biotechnol 38, 1037-1043, (2020). https://www.nature.com/articles/s41587-020-0573-5
[4] Gorodkin, J. Comput Biol Chem 28, 367-374, (2004). https://linkinghub.elsevier.com/retrieve/pii/S1476927104000799

62| Increased in planta gene targeting efficiency by fusion of 5´ exonucleases to CRISPR endonucleases

Tom Schreiber [1]; Anja Prange[1]; Petra Schäfer[1]; Thomas Iwen; Sylvestre Marillonnet [1]; Alain Tissier[1]

[1] Leibniz Institute of Plant Biochemistry (IPB)

Application of homology-directed repair (HDR) for the efficient modification of plant genomes is still challenging. Using a transient assay in *Nicotiana benthamiana* for knock-ins by HDR, we tested a number of exonuclease fusions to CRISPR endonucleases. Among these, several 5'-exonucleases from different viruses gave a massive increase in gene targeting frequencies when fused to Cas9 and Cas12a. These were then tested in *Arabidopsis thaliana* for the stable targeted integration at a specific locus. A 10-fold increase was observed with the 5'-exonuclease-Cas9 fusion over the control with Cas9. These fusions provide a useful tool for the efficient targeted insertion and replacement of sequences in plant genomes and can be used for various applications in plant breeding.

63| Design and characterization of novel CRISPR-associated endonucleases using ancestral sequence resurrection

Ylenia Jabalera; Sara Samperio; Raul Perez-Jimenez

CIC bioGUNE

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonucleases have revolutionized biotechnology field due to their potential to be applied as programmable genome manipulators. Our group has utilized ancestral sequence reconstruction (ASR) to study the evolutionary history of CRISPR-Cas9 from *Streptococcus pyogenes* (SpCas9) and discovered a promising ancestor, the Firmicutes Common Ancestor (FCA), which lived 2.6 billion years ago (1). FCA displays exceptional characteristics that position it as a potential valuable tool in biotechnology, including lower PAM restriction, reduced immunogenicity, high substrate promiscuity, and nickase activity. However, the molecular mechanisms behind these traits remain unknown. Therefore, in this work, we have characterized and investigated the molecular basis of these ancestral features to create new synthetic endonucleases. The ultimate goal is to develop a versatile, all-in-one endonuclease with a wide range of potential biotechnological applications, such as genome editing and nucleic acid detection platforms.

(1) Alonso-Lerma, B., Jabalera, Y., Samperio, S. et al. Evolution of CRISPR-associated endonucleases as inferred from resurrected proteins. Nat Microbiol 8, 77–90 (2023). https://doi.org/10.1038/s41564-022-01265-y

64| Novel compact RNA-guided nucleases from nonpathogenic soil microbes provide new tools for efficient and robust editing in human cells

Hui-Chia Yu-Kemp[1]; Steven Goodson[1]; David Wiley[1]; Michael Coyle[1]; Drew Kelso[1]; Shaorong (Ron) Chong[1]; Gunjan Arya[1]; Philip Borden[1]; Alexandra Crawley; Adrian Pickar-Oliver[1]; Lisle Mose[1]; Salem Faham[1]; Michael Vanden Over[1]; Ariel Vitenzon[1]; Lei Ying[1];Tedd Elich[1]

[1] Life Edit Therapeutics, an ElevateBio company

Bacterial adaptive immunity utilizes RNA-guided systems comprising effectors and guide RNAs (gRNA) to target foreign nucleic acids for degradation. These systems serve as versatile tools to modulate the genome in a target-site specific manner and potentially provide new therapeutic options. Mining through our unique soil nonpathogenic bacterial genome database, we continue to discover many new RNA-guided nucleases which are evolutionary distant from other widely used CRISPR systems. We have several lead nucleases that cover diverse PAM sequences allowing broad genomic access. In addition, the nucleases are small, which allows us to package and deliver via Adeno-Associated Virus (AAV) systems for in vivo therapeutic studies. Using our nucleases, we are building a library of proprietary base editors. To test the editing function of our systems, we targeted an assortment of disease-relevant genes in a variety of cell types (human hematopoietic lineage cells, human primary T-cell, human iPCS, etc.) and demonstrated the editing robustness and efficiency. In human primary T cells, we performed multiplexed editing that resulted in efficient protein knock-down at multiple targets with both our nuclease and base editors. To optimize editing efficiency, we applied different engineering approaches focusing on our nuclease and the gRNA backbone, resulting in up to 2-fold improvement in editing. Our engineered gRNA strategy generally improved guide stability and rescued otherwise ineffective gRNAs. Importantly, we evaluated the targeting specificity of our nucleases and did not detect off-target editing. Our discovery and engineering pipelines have expanded the CRISPR toolbox for future sophisticated therapeutic use.

65| Base editing of key residues in the BCL11A-XL-specific zinc finger domains de-represses fetal globin expression

Vignesh Rajendiran[1]; Nivedhitha Devaraju[1]; Nithin Sam Ravi[1]; Lokesh Panigrahi[1]; Joshua Paul[1]; Chandrasekar Gopalakrishnan[2]; Stacia Wyman[3]; Keerthiga Ariudainambi[4]; Gokulnath Mahalingam[1]; Yogapriya Moorthy[1]; Kirti Prasad[1]; Anila George[1]; Dhiyaneshwaran Sukumaran[2]; Sandhiya Gopinathan[1]; Aswin Anand Pai[1]; Ryo Kurita[5]; Yukio Nakamura[6]; Poonkuzhali Balasubramanian[1]; Rajasekaran Ramalingam[2]; Saravanabhavan Thangavel[1]; Shaji R Velayuthan[1]; Jacob E Corn[7]; Merlin Crossley[8]; Srujan Marepally[1]; Alok Srivastava[1]; Kumarasamypet M Mohankumar[1]

[1] CHRISTIAN MEDICAL COLLEGE AND HOSPITAL; [2] Vellore Institute of Technology (VIT), Vellore; [3] University of California at Berkeley; [4] Thiruvalluvar University; [5] Japanese Red Cross; [6] RIKEN; [7] ETH Zurich; [8] University of New South Wales

Reactivation of fetal globin (HbF) levels ameliorates the severity of -hemoglobinopathies. BCL11A is a major fetal globin gene (HBG1/2) repressor and is being targeted in clinical trials. Its longest isoform, BCL11A-XL, has three distinct Zinc Finger (ZnF) domains and directly binds the fetal globin (HBG1/2) gene promoters. Several patients with mutations that disrupt these ZnF domains have neurodevelopmental disorders, and elevated fetal globin (HbF) with otherwise unremarkable hematology. We disrupted the BCL11A-XL-specific ZnF domains using CRISPR/Cas9 and observed high HbF induction, but defects in Hematopoietic Stem and Progenitor Cells (HSPC) engraftment and erythroid maturation. Then, instead of ablating the domains, we introduced more subtle base substitutions in the ZnF-domains using base editing, and effectively upregulated HbF with greatly reduced hematological side effects. In particular, a ZnF4 mutation resulted in significant HbF levels, minimal changes to erythroid gene expression, no impact on erythroid maturation in vitro, and only a modest reduction in HSPC engraftment in vivo. A modification of ZnF6 also elevated of HbF levels, which was unexpected as this region does not directly interact with DNA at the HBG1/2 promoters. Our strategy of specifically altering ZnF domains of BCL11A-XL has identified residues in ZnF4 that are critical for fetal globin repression but are less important for the other hematological functions of BCL11A, and thus provides insights into its molecular mechanism and suggests new targets for treating of -hemoglobinopathies.

66| Digital single cancer biomarker detection using CRISPR/Cas sensing: towards dynamic profiling for CpG methylation quantification

Jeanne Elisabeth van Dongen ; Johanna Berendsen ; Jan Eijkel ; Loes Segerink

University of Twente

Cancer, as a disease, starts with a genetic change in the genome that results in downregulating genes involved in “normal” cell behavior. This genetic change can be induced by a difference in the DNA code (mutation) or epigenetic alterations. The hypermethylation of cytosine is a critical hallmark in many cancer cells: the loss of expression of genes occurs about ten times more often by hypermethylation of promoter CpG islands than by other mutations.

For early cancer diagnostics, accurate and rapid detection of these epigenetic CpG methylation mutations involved in tumor development is crucial. Previously, we have presented an amplification-free in vitro diagnostic tool to discriminate single CpG site methylation in DNA by using methylation-sensitive restriction enzymes (MSREs) followed by Cas12a-assisted sensing. While this method showed much potential in terms of specificity, selectivity, and ease of use, the detection limit presented (100 pM) is still far away from the concentrations of DNA found in liquid biopsies such as urine. Since all commonly available pre-amplification techniques used for CRISPR sensing result in a loss of epigenetic modifications, other methods need to be exploited to lower the detection limit of this Cas12a assay while keeping methylation selectivity.

We suggest using a "digital" CRISPR method, where absolute quantification can be achieved without external standards. Unlike most digital methods, we extract information about the target sequence by dynamically following single-molecule CRISPR reactions. In this way, we hope to enable absolute quantification of the methylation % of a single CpG methylation site. We are currently working on distinguishing between droplets containing methylated DNA and those containing non-methylated DNA fragments by following the increase in fluorescence of a single droplet over time. After we confirm this, we will continue with liquid biopsy samples of patients in collaboration with AmsterdamUMC.

67| Genome Editing of Veterinary Relevant Mycoplasmas Using a CRISPR-Cas Base Editor System

Thomas Ipoutcha; Fabien Rideau; Géraldine Gourgues; Yonathan Arfi; Carole Lartigue; Alain Blanchard; Pascal Sirand-Pugnet

INRAE - UMR BFP

Mycoplasmas are minimal bacteria that infect humans, wildlife, and economically relevant livestock. Mycoplasma infections cause chronic inflammatory diseases, which can lead to death in some animals. However, the lack of efficient recombination and genome engineering tools limits the production of mutant strains for the identification of virulence factors and the development of improved vaccine strains. This has hampered functional studies of many species and left several questions about the molecular basis of their pathogenicity unanswered. To address this issue, we adapted an efficient Cas9-Base Editor system to introduce targeted mutations into four major pathogenic species that span the phylogenetic diversity of these bacteria: the avian pathogen *Mycoplasma gallisepticum*, the small ruminant's pathogen *Mycoplasma agalactiae* and the two most important bovine mycoplasmas, *Mycoplasma bovis* and *Mycoplasma mycoides* subsp. *mycoides*. Using an inducible SpdCas9-pmcDA1 cytosine deaminase system, we disrupted several major virulence factors in these pathogens. We evaluated various induction times and inducer concentrations to optimize editing efficiency. The optimized system was powerful enough to disrupt 54 of 55 insertion sequence transposases in a single experiment. Lastly, we successfully disrupted two different genes in *Mycoplasma bovis* in a single assay, using an all-in-one multiple gRNA system. Whole-genome sequencing of the edited strains showed that off-target mutations

were limited, suggesting that most variations detected in the edited genomes are Cas9-independent. Our findings present, for the first time, an effective, rapid, and easy-to-use genetic tool for the study of these important animal pathogens and likely the entire class Mollicutes.

68| Innovative single gRNA enhanced-deletion genome editing-based correction of aberrant splicing due to pathogenic deep-intronic variants

Pietro De Angeli[1]; Stefanida Shliaga; Arturo Flores-Tufiño; Shibu Antony; Katarina Stingl; Laura Kühlewein; Valentina D'Agostino; Bernd Wissinger; Susanne Kohl

[1] Universität Klinikum Tübingen

Although successful in correcting aberrant splicing caused by deep intronic variants (DIV), the standard CRISPR/Cas9 approaches, involving the generation of a deletion containing pathogenic DIV and intronic sequences, has several drawbacks. These include increased risk of off-target effects, chromosomal rearrangements, activation of P53-related pathways when using multiple gRNAs, and the need for more components for delivery. To overcome these issues, we developed Enhanced-Deletion Cas (EDCas) molecules, where a RNA-guided endonuclease is fused to the human three prime repair exonuclease (TREX2). EDCas molecules, generated from SpCas9 or a smaller, AAV-packable ED-synthetic RNA-guided endonuclease (EDsRGN), are effective in generating larger deletions at the targeted sites and successfully prevent recognition of faulty splicing sequences by the splicing machinery. This results in consistent splicing correction using only a single gRNA.

In our current project, we demonstrate that EDCas molecules are effective in correcting aberrant splicing caused by the USH2A:c.7595-2144A>G DIV in both minigene assays and patient-derived fibroblasts. Using six single different gRNAs coupled to EDSpCas9, we achieved higher splicing rescue rates (53.5±11.8% - 88.0±1.9%) compared to SpCas9 (34.0±25.3% - 71.8±30.0%) in HEK293T cell minigene assays. Four lead gRNAs were further validated in patient-derived USH2A:c.7595-2144A>G homozygous fibroblasts, showing high splicing rescue rates (85.7±3.7% - 92.4±4.8%), consistent with the minigene assay results. Deletion profiles obtained clearly demonstrate that EDSpCas9 is capable of inducing enhanced and directional deletions compared to SpCas9, with a marked 3'-5' deletion directionality on the non-target strand. Our preclinical results are promising and transferable to advanced disease models. Ongoing experiments include assessing potential off-target effects and the implementation of EDsRGN to allow AAV delivery of the editing system.

69| The Joint AstraZeneca - Cancer Research Horizons Functional Genomics Centre: Using pooled CRISPR screening to drive oncology drug discovery

Nikhil Gupta [1]; Sebastian Lukasiak[2]; Alex Kalinka[1]; Daniel Barrel[3]; Lu Li[3]; Malwina Prater[1]; Carlos Company[3]; Chara Mastrokalou[1]; Marica Gaspari[1]; Andy Sayer[1]; Ultan McDermott[3]; Gregory Hannon[4]; Douglas Ross-Thriepland[2]; David Walter[1]

[1] Cancer Research Horizons & Joint AstraZeneca-Cancer Research Horizons Functional Genomics Centre; [2] BioPharmaceuticals R&D, AstraZeneca & Joint AstraZeneca-Cancer Research Horizons Functional Genomics Centre; [3] Oncology R&D, AstraZeneca & Joint AstraZen

The emergence of CRISPR technologies has enabled rapid and precise gene editing opportunities. The Functional Genomics Centre (FGC) – a joint venture between Cancer Research UK's (CRUK) drug discovery engine, Cancer Research Horizons, and AstraZeneca has been launched to better utilise and improve the CRISPR technology within cancer research. Located in Cambridge Biomedical Campus, the FGC is comprised of 25 cancer and computational scientists. With the goal of accelerating the discovery of new cancer medicines, the FGC aims to be world's leading centre of excellence in genetic screening, cancer modelling and big data processing.

The FGC leverages the strengths of both CRUK's and AstraZeneca's world-class oncology research and drug discovery experience, with the potential to drive scientific innovation in how new drug targets are identified and validated, and uniquely democratise access to the latest cutting-edge research tools and expertise for many thousands of scientists and clinicians funded by CRUK.

Here we will highlight how the FGC is leading the development and improvement of genetic screening tools: designed/optimised minimal genome-wide CRISPR libraries through independent evaluation of publicly available CRISPR tools and development/deployment of new CRISPR technologies. These advancements will allow us to delve deeper into mechanistic understanding of cancer biology and enable our Target Discovery platform to impact more projects, faster, at a lower cost, sustainably and importantly with robust output.

70| First step towards whole genome cloning of *Bacillus subtilis* in yeast by CReasPy-Fusion

Julien Barret[1]; Gabrielle Guesdon[1]; Géraldine Gourgues[1]; Anne-Gaëlle Planson [2]; Pascal Sirand-Pugnet[1]; Vincent Sauveplane[2]; Etienne Dervyn[2]; Matthieu Jules[2]; Carole Lartigue[1]

[1] INRAE - UMR BFP; [2] INRAE, Micalis Institute, AgroParisTech, Université Paris-Saclay

In 2010, promising synthetic biology technologies have emerged that use yeast as a platform for the assembly and engineering of synthetic bacterial genomes prior their transplantation into a recipient cell. These technologies opened up new avenues towards the construction of cells with fully controlled biological properties.

The transfer of these tools to microorganisms of industrial interest, such as the Gram+ bacterium *Bacillus subtilis* (Bsu), will be a central advance in the field of biotechnology. The

INRAE consortium has set out to clone the whole Bsu genome in yeast using CReasPy-Fusion, a newly developed method based on direct fusion between bacterial protoplasts and yeast spheroplasts preloaded with a CRISPR Cas9 tool. Efforts to date have demonstrated: (1) cell-to-cell fusion between Bsu and yeast, a phenomenon never described before; (2) the efficiency of a CRISPR Cas9 system to capture and modify a shuttle plasmid during Bsu/yeast fusion; and (3) the efficiency of the CRISPR Cas9 system to capture a 130-kb fragment of the Bsu genome. Since then, larger Bsu genome fragments have been cloned in yeast; their capture being facilitated by the addition of ARS elements along the Bsu chromosome. We now aim to clone the ~3 Mb genome of a genome-reduced Bsu strain in yeast.

71| Glow-in-the-Dark Infectious Disease Diagnostics Using CRISPR-Cas9-Based Split Luciferase Complementation

Harm van der Veer[1]; Eva van Aalen[1]; Claire Michielsen[1]; Eva Hanckmann[1]; Jeroen Deckers[1]; Marcel van Borren[2]; Jacky Flipse[2]; Anne Loonen[3]; Joost Schoeber[3]; Maarten Merkx[1]

[1] Eindhoven University of Technology; [2] Rijnstate Hospital; [3] Fontys University of Applied Sciences

Nucleic acid detection methods based on CRISPR and isothermal amplification techniques show great potential for point-of-care diagnostic applications in infectious disease. However, most current methods rely on fluorescent or lateral flow assay readout, requiring external excitation or post-amplification reaction transfer. Instead, we developed a bioluminescent nucleic acid sensor (LUNAS) platform in which target dsDNA is sequence-specifically detected by a pair of dCas9-based probes mediating split NanoLuc luciferase complementation. LUNAS is easily integrated with recombinase polymerase amplification (RPA), providing attomolar sensitivity in a one-pot assay. A calibrator luciferase is included for a robust ratiometric readout, enabling signal detection by a digital camera. We designed a reverse transcription (RT)-RPA-LUNAS assay for detecting SARS-CoV-2 RNA without the need for cumbersome RNA isolation and demonstrated the diagnostic performance for COVID-19 patient nasopharyngeal swab samples. Detection of SARS-CoV-2 from samples with viral RNA loads of ~200 cp/µL was achieved within ~20 minutes, showing that (RT)-RPA-LUNAS is attractive for point-of-care infectious disease testing. Using corresponding primers and guide RNAs, this method can be readily adapted to other pathogens, enabling rapid development of diagnostics for emerging epidemic threats. Moreover, recently developed LUNAS colour variants allow for multiplex RPA-LUNAS assays detecting multiple targets in a single reaction.

72| Generation of multi-component CRISPR complexes for systematic PE-mediated insertions in mammalian cells

Gil Westmeyer [1]; Dong-Jiunn Jeffery Truong[2]; Julian Geilenkeuser[1]

[1] Technical University of Munich; [2] Technische Universität München / Helmholtz Munich

Given the intricate spatial arrangement of the nCas9-RT fusion proteins, the complex orchestration of the various molecular functions, the delicate ratio of affinities between the

self-complementary prime editing gRNA (pegRNA) and the primer binding site (PBS), and the downstream cellular processes leading to the incorporation of the intended edit, it is remarkable that prime editing has already proven to be such an efficient gene editing technique. To better understand the critical PE processes, we developed a multicolor fluorescent hISPC reporter line capable of distinguishing all resulting gene edits by FACS, enabling the systematic optimization of prime editors. We then developed prime editor variants with optimized protein fusions that conferred additional functionalities to the complex. In particular, adding specific nucleases to the editor complex significantly increased editing efficacy without compromising editing precision by introducing secondary nicks that risk double-strand breaks. The up to a 4-fold increased editing performance compared to current best editors across all loci and cell types was particularly notable for longer insertions up to 45 bps, including recombination sites and sequences encoding epitopes or protein components for fluorescence complementation. In addition, we quantified the editing speeds of prime editors compared to other CRISPR-based editing techniques using arrayed single-cell microscopy experiments to systematically characterize the required editing durations and match optimal gene delivery methods for respective mammalian cell types. Our systematic analyses have identified a prime editor variant with a several-fold increase in editing efficacy without compromising precision, significantly advancing the only available CRISPR-based editing technique that enables long insertions also in post-mitotic cells such as hIPSC-derived neurons or cardiomyocytes, making it directly useful for biotechnological applications.

73| CRISPRgate improves loss-of-function genetic approaches

Jannis Stadager[1]; Zeynab Najafova [2]; Steven Johnsen [2]; Julian Jude [3]; Philipp Rathert [1]

[1] University of Stuttgart; [2] Robert Bosch Centre for Tumour Diseases; [3] Twist Biosciences

Loss-of-function (LOF) genetic studies investigate the direct regulatory functions of genes and are used for large-scale whole-genome screening. With increased gene depletion efficiency and specificity, CRISPR replaced the most commonly used RNAi technology, reaching a new milestone in LOF studies.

Despite the significant advances of the CRISPR technology in gene knockout (CRISPRko) and interference (CRISPRi), there are still limitations that hinder its full potential for applications involving patient-derived samples, spatial transcriptomics or in-vivo experiments. As a result of genomic diversity i.e. alternative splicing, in-frame repair, multiple TSSs, alternative start-codons and the persistence of strong epigenetic marks, the full effectiveness of CRISPR in these applications is still limited.

To overcome these limitations, we developed a new method called CRISPRgate and first monitored the depletion of non-essential genes (CD13 and CD33) compared to standard CRISPRko and CRISPRi methods. The effectiveness of CRISPRgate was further validated by targeting BUB1, a gene whose function and essentiality is not fully understood. Finally, we conducted a CRISPR screen demonstrating an improved depletion efficiency and a higher gene depletion rate when using CRISPRgate compared to other published pooled screening approaches which demonstrate, that CRISPRgate exceptionally improves the biological effect

of the loss of function. The variance in sgRNA performance observed for single CRISPRi or CRISPRko sgRNAs is drastically reduced, resulting in more significant gene hits and much higher consistency in the biological effect of sgRNAs targeting the same gene. Our results indicate that CRISPRgate will be the best available CRISPR LOF tool expanding the usage of CRISPR screens to future applications.

74| Detection of CRISPR on-target effects using 'SAFE' DNA donors carrying diagnostic substitutions

Martin Lackner[1]; Nelly Helmbrecht[1]; Svante Pääbo[1,2], Stephan Riesenberg[1]

[1]Max Planck Institute for Evolutionary Anthropology, Leipzig, Sachsen, 04103, Germany; [2]Okinawa Institute of Science and Technology, Onna-son, Okinawa, 904-0495, Japan

Cellular repair of the CRISPR/Cas9-induced double-strand breaks can result in unintended effects at the target locus, such as larger deletions or loss of heterozygosity due to gene conversion. Detection of such unintended genome alterations is critical as they may have biological effects that could be erroneously attributed to the intended mutation, but cannot be reliably achieved by sequencing of the target site alone. Here, we present a strategy for detecting unintended effects and generating control cells that carry wild-type alleles but have demonstratively undergone editing at the target site. Our approach, called sequence-ascertained favorable editing (SAFE), uses DNA donor mixtures containing desired nucleotide substitutions or wild-type alleles, along with additional 'diagnostic' substitutions that are unlikely to have functional consequences. When the target site is sequenced, two distinct sequences indicate successful editing of both chromosomes with 'SAFE' donors containing different sets of 'diagnostic' substitutions, whereas a single sequence indicates loss of the second allele due to a deletion or gene conversion. Using 'SAFE' donors, we could detect all copy number changes and almost all instances of gene conversion among more than 850 edited human stem cell clones. These results demonstrate the potential of 'SAFE' donors to provide easy and reliable detection of on-target effects.

75| Predominant precise genome editing by inhibition of DNA repair pathways avoids the need for single-cell cloning

Stephan Riesenberg; Philipp Kanis; Dominik Macak; Tomislav Maricic

Max Planck Institute for Evolutionary Anthropology

Major hurdles of the CRISPR-Cas9 editing technology are I.) low efficiency for precise introduction of nucleotide substitutions, II.) unintended byproducts, and III.) time-consuming single-cell cloning. Also, clone-to-clone variation often complicates comparative analyses of unedited and edited cellular clones. We find that the combined inhibition of two DNA repair pathways either by mutations or by inhibitory substances results in that up to 93% of chromosomes in populations of cells will carry desired point mutations introduced by

template based homology-directed repair (HDR). Insertions, deletions and rearrangements at the target site as well as unintended changes at other off-target genomic sites are largely abolished. We validate this approach for 69 different target sites and show that it outcompetes standard prime editing by metrics of efficiency and absence of editing byproducts. When testing repeated cell bulk editing to enrich for precisely edited cells, absolute HDR ranged from 80% to 96%. Thus, cell populations carrying high proportions of precisely edited cells can be produced using HDRobust without the isolation of cellular clones.

76| Genome editing using a split prime editor

Jure Bohinc; Vida Forstnerič; Duško Lainšček; Roman Jerala

Kemijski Inštitut

The CRISPR-based DNA editing technology called prime editing (PE) composed out of Cas9 nickase (Cas9 H840A) fused to reverse transcriptase (RT) enables us to create a wide range of precise and accurate edits on the genome without causing double-stranded breaks. Introduced changes are encoded within the prime editing gRNA (pegRNA), another essential part of PE. PE can induce transition and transversion mutations and small insertions and deletions or their combinations. The technology was further improved using different strategies, such as using two pegRNAs, engineering the prime editor (PE2) protein or the pegRNA, resulting in even higher editing efficacies and wider scope of possible editing outcomes. Since the PE2 protein is a large fusion protein, one of the challenges of prime editing in vivo to show therapeutic potential is the delivery of the PE2 protein by using common vectors such as AAVs. We developed a variant of a compact prime editor. Using custom reporter systems, we tested our prime editor on plasmids and at the genomic level, where we showed that the editing efficacy of our prime editor is comparable to the original PE2. We are further developing our approach to prime editing and testing it using different strategies of delivering either the plasmids or ribonucleoproteins to cells. Our system can also be used with other prime editing strategies like PE3. The results indicate that the novel compact prime editor is a viable contender among the prime editing systems already reported in the literature, which may be used for editing clinically relevant mutations.

77| Automated identification of sequence-tailored Cas9 proteins using massive metagenomic data

Matteo Ciciani[1]; Michele Demozzi[1]; Eleonora Pedrazzoli[1]; Elisabetta Visentin[1]; Laura Pezzè[2]; Lorenzo Federico Signorini[3]; Aitor Blanco-Miguez[1]; Moreno Zolfo[4]; Francesco Asnicar[1]; Antonio Casini[2]; Anna Cereseto[1]; Nicola Segata[1]

[1] University of Trento; [2] Alia Therapeutics; [3] Tel Aviv University; [4] Okinawa Institute of Science and Technology

The identification of the protospacer adjacent motif (PAM) of Cas9 nucleases is crucial for their use as genome editing tools and represents a major limitation toward efficiently navigating

through the natural Cas9 diversity present in microbial genomes. Here we develop a computational pipeline that was used to interrogate a massively expanded dataset of metagenome and virome assemblies for accurate and comprehensive PAM predictions.

From 1,083,368 bacterial and archaeal genomes and 378,361 phagic genomes, we identified 92,140 CRISPR-Cas9 loci and generated PAM predictions for 61,095 Cas9s (66.3%), grouped in 2546 clusters. We validated our approach by confirming PAM preferences of 26 already reported Cas9 proteins and of 4 novel ones from our database, obtaining 85% correct predictions. From our prediction pipeline it emerges that PAMs can be grouped in 32 clusters which can be associated with specific clades of Cas9 proteins.

A key application of our PAM prediction pipeline is the identification of uncharacterized Cas9 nucleases recognizing PAM sequences generated by pathogenic mutations, which could offer specific targeting options for the mutated allele without targeting the wild-type one. By interrogating the ClinVar database we estimated that 98.6% of small pathogenic mutations are included in at least one of the PAMs predicted by our analysis, while only 76.1% could be potentially targeted by Cas nucleases already used in the genome editing field. Thus the PAM diversity identified by our analysis provides a nearly complete coverage of small pathogenic mutations that was previously lacking. As a proof of concept, starting from the disease-causing mutation P23H in the rhodopsin gene, we isolated and experimentally validated a Cas9 which uses a PAM sequence corresponding only to the mutated allele.

Our PAM prediction pipeline will be instrumental to generate a Cas9 nuclease repertoire responding to any PAM requirement.

78| Characterization and deployment in vivo of FiCAT genome writer

Jon Martínez de Lagos Marcos; Natalia Artigas; Jessica Jaraba Wallace; Avencia Sanchez Mejias; Marc Güell[1]

[1] Universitat Pompeu Fabra, Barcelona

Find Cut-and-Transfer (FiCAT) is a robust gene writing platform, combining the precision of CRISPR-Cas9 systems, with an engineered piggyBac transposase with donor DNA processing and high transfer capacity. Here, with the aim to demonstrate FiCAT activity in vivo, we performed precise gene delivery to mouse models liver cells using viral-free delivery vectors via systemic administration. We were able to efficiently deliver FiCAT to mice liver targeting genomic safe harbors, together with a reporter transposon in DNA form. Stable transduction was confirmed by qPCR analysis of the inserted transgene and at 6 weeks after transduction. Lipid nanoparticles (LNPs) have been extensively validated both pre-clinically and clinically for the delivery of different nucleic acid cargos. Once optimized FiCAT mRNA-based LNP formulation, specific cut capacity in mice liver was analyzed by targeted NGS. In this work, we were able to successfully deliver mRNA cargos via LNPs in vivo, and optimize DNA cargos delivery for codelivery with FiCAT machinery; for enabling gene writing in vivo. Our work validates the feasibility of doing viral free stable gene transfer in vivo; which is one of the challenges in our desire to deliver safely and efficiently therapeutic genes into the target cells

and achieve precise and efficient in vivo targeted insertion of DNA fragments in mammalian genomes.

79| LAMP-CRISPR/Cas12a assays for detecting *Leishmania* spp. in human clinical samples

Eva Dueñas[1], Ingrid Tirado[1], Percy Huaihua[2], Jose A. Nakamoto[1], Luis Cabrera-Sosa[1], Alexandra Poma[1], María Cruz[3], Jorge Arévalo[2], Pohl Milón[1], Vanessa Adaui[1]

[1]Laboratory of Biomolecules, Faculty of Health Sciences, Universidad Peruana de Ciencias Aplicadas, Lima, Peru; [2]Laboratory of Patho-antigens, Laboratories for Research and Development, Faculty of Science and Philosophy, Universidad Peruana Cayetano Heredia, Lima, Peru; [3]Hospital Nacional Adolfo Guevara Velasco, Cusco, Peru

Leishmaniasis is a neglected parasitic disease affecting mainly low-resource countries. Molecular diagnostics offer high sensitivity and specificity over microscopy or culture, but since they require infrastructure and specialized equipment, they are only available at research centers. CRISPR-Cas systems have been repurposed for next-generation molecular diagnostics. Usually CRISPR-based assays need a previous amplification step of the target nucleic acid to achieve highly sensitive detection. That is accomplished through conventional PCR or isothermal amplification techniques. This step provides the first round of specificity. The crRNA sequence that recognizes the target DNA brings a second layer of specificity. Here, we have developed LAMP-CRISPR/Cas12a assays targeting *Leishmania* kinetoplast DNA (kDNA) or 18S ribosomal DNA (rDNA). These assays detect at least 2x10^0 *Leishmania* parasite genome equivalents. The analytical specificity is being tested using DNA of different species of Leishmania, *Trypanosoma*, and other microbial pathogens to evaluate cross-reactions. For the LAMP step, we will compare the performance of an in-house Bst-LF DNA polymerase with respect to the commercial enzyme (WarmStart LAMP Kit, NEB). For calculating the diagnostic sensitivity and specificity, we will process 100 human clinical samples, which include 50 negative samples for *Leishmania* infection, and 50 positive samples based on a quantitative PCR (qPCR) assay targeting kDNA. Our previously developed PCR-CRISPR assays achieved 100% concordance with the qPCR assay. We expect that a highly sensitive pre-amplification step by LAMP coupled to CRISPR-based detection will allow to achieve high target detection sensitivity, with the advantage that these LAMP-CRISPR assays are more close to be adapted to a point-of-care test. Cas12a-mediated detection will be analyzed via fluorescence and lateral flow readouts.

80| Pin-pointTM technology: a versatile base editing solution for the generation of cell therapies

Bronwyn Joubert[1], Robert Blassberg[1], Jennifer Harbottle[1], Olga Mielczarek[1], Paul Russell[1], Jesse Stombaugh[1], Robin Loesch[1], John Lambourne[1], Kevin Hemphill[1], Matthew Perkett[1], Michael Rushton[1], Žaklina Strezoska[1], Anja van Brabant Smith[1], Immacolata Porreca[1]

[1] Horizon Discovery

CRISPR base editing is the new generation of CRISPR technology. CRISPR-Cas systems introduce double strand DNA breaks at the target site and rely on non-homologous end joining to disrupt gene function. Base editing introduces a point mutation, which can turn a gene off by creating a stop codon but can also edit a causative mutation to repair a defective gene. Base editing has been used to edit point mutations that are causative of rare diseases and create CAR-T treatments for cancer with an improved safety profile compared to nuclease-based technologies. The first cohort of base editing therapeutics entered clinical trials only a few years after the development of the technology.

Horizon's modular Pin-point base editing system efficiently and precisely converts target nucleotides in the genome by recruiting DNA modifying deaminases via an aptamer encoded in the guide RNA (gRNA) of the sequence-targeting Cas component. We optimized design and delivery conditions of the Pin-point editing machinery to apply base editing to therapeutically relevant cell types. We targeted a set of therapeutically relevant genes for the development of allogeneic CAR-T achieving high knockout efficiency and editing purity at all sites simultaneously with a safer editing profile and enhanced cell viability compared to traditional nuclease systems. Our technology also enables robust simultaneous targeted knock-in and multiplex knockout without the requirement of additional sequence-targeting components. The ability to perform complex genome editing in multiple cell types safely, efficiently, and precisely opens the door to the application of the Pin-point system in a range of advanced cell therapies.

81| CRISPR-based high-throughput identification of causal genes in yeast hybrids

James Boocock[1,2,3,4], Leslie Alamo Tapia[1,2,3,4], Shivani Patel[1,2,3,4], Joshua Bloom[1,2,3,4], Leonid Kruglyak[1,2,3,4]

[1]Department of Human Genetics, University of California, Los Angeles, Los Angeles, United States
[2]Department of Biological Chemistry, University of California, Los Angeles, Los Angeles, United States
[3]Howard Hughes Medical Institute, University of California, Los Angeles, Los Angeles, United States
[4]Institute for Quantitative and Computational Biology, University of California, Los Angeles, Los Angeles, United States

Genetic mapping approaches have been used to discover thousands of loci that influence traits, but identifying the causal genes and variants that underlie these loci remains challenging. Genetic associations may arise from multiple linked single-nucleotide and/or structural variants in a region that are jointly responsible for the observed signal. There is a need for high-throughput approaches to directly test the effects of gene-scale haplotypes at associated loci on traits. To address this need, we developed a CRISPR-based method that generates gene-sized regions of homozygosity in heterozygous diploids of the yeast *Saccharomyces cerevisiae*. Our approach uses CRISPR to target one allele of a variant site in a diploid yeast strain; this introduces a double-stranded break that is repaired via homologous recombination from the uncut chromosome. To test this approach, we designed 15 allele-specific guide RNAs to target variant sites in a diploid hybrid between lab (BY) and industrial (CEN.PK) strains of yeast. We transformed these guide RNAs into a BYxCEN.PK diploid and

induced Cas9. We performed whole-genome sequencing of 159 strains and found that 143 (90%) of the strains were homozygous for the allele that was not targeted with CRISPR. Of the correctly edited strains, 29 (20%) also picked up a large homozygosity event up to 0.8 million base-pairs (bp) in size starting near the cut site and continuing to the end of the chromosome. The remaining 114 (80%) strains contained a local homozygosity event around and including the site that was targeted by CRISPR. These events ranged in size from 1 bp to 55,022 bp, with a median length of 2,326 bp. The average yeast gene is approximately 1,700 bp long, suggesting that our method could be used to test the impact of gene-sized haplotypes on traits at scale.

82| Picking up the Pieces: The Fragmented Process of Cas13 Mediated RNA Cleavage in Human Cells

Brandon Davis Burris[1,3], Brandon Park[2,3], Ning Tong[1,3], and Mitchell O'Connell[1,2,3]

[1]University of Rochester School of Medicine and Dentistry; [2]Department of Biochemistry and Biophysics; [3]Department of Biomedical Genetics; [4]Center for RNA Biology

Prokaryotes use Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and CRISPR Associated (Cas) proteins to target and cleave foreign genetic elements in an RNA-guided manner. Type VI CRISPR-Cas systems contain a single effector ribonuclease, Cas13, that processes and binds to a guide-RNA, forming an RNA-guided RNA-targeting effector complex. Previous studies have shown that Cas13 can be engineered to target and modulate RNA processes in human cells, illustrating the versatility and specificity of Cas13 as an RNA Knockdown (KD), splicing, or imaging tool. However, we have made a surprising observation that upon Cas13 mRNA cleavage the resultant 5' and 3' fragments appear to experience differential RNA decay. This affects the interpretation of Cas13-mediated knockdown experiments by RT-qPCR and may partly explain the difficulty in implementing Cas13 as an RNA-knockdown tool. We have since optimized conditions for RNA targeting experiments using RfxCas13d, providing a standard protocol for researchers to follow.

Additionally, there is evidence in the literature that the generation of these fragments can stimulate endogenous activity linked to cellular stress and immunity. This artificial stimulation potentially reduces the reliability of long-term stable knockdown experiments with Cas13, illustrating the need for rapid clearance of these fragments post cleavage. To this end, we are using single molecule RNA microscopy to visualize fragment generation and we plan to use cell lines stably expressing RfxCas13d and targeting gRNAs to measure the kinetics of fragment generation. Lastly, we are developing a novel Cas13 fusion to facilitate rapid clearance of Cas13 cleaved RNAs to mitigate downstream effects, expanding the CRISPR toolbox.

83| PRCISR™ CRISPR: Vivlion's CRISPR-enabled discovery platform

Martin Wegner[1], Micheal Murphy[1], Manuel Kaulich[1,2,3,4]

[1]Vivlion GmbH, 60598, Frankfurt am Main. [2]Institute of Biochemistry II, Faculty of Medicine, Goethe University Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany[3]Frankfurt Cancer Institute, 60596 Frankfurt am Main, Germany. [4]Cardio-Pulmonary Institute, 60590 Frankfurt am Main, Germany.Exploring the enormous space of the human genome with CRISPR-based target identification strategies is challenging, particularly for large scale combinatorial approaches that aim to edit multiple genes in a single cell. PRCISR™ CRISPR is Vivlion's proprietary monogenetic and combinatorial CRISPR-based discovery platform. It combines expert knowledge and proprietary library generation technology to facilitate the application of uniformly distributed single and combinatorial CRISPR libraries at scale. PRCISR™ CRISPR is unique in maximizing gene editing efficiency and hit-retention rates, enabling powerful screen parallelization for the identification of combinatorial phenotypes by enhancing throughput and innovative target identification. PRCISR™ CRISPR begins with expert consultation on study design, followed by the generation of highly uniform CRISPR libraries, and their screening in different contexts. PRCISR™ CRISPR then delivers sample sequencing and state-of-the-art computational analyses. Our PRCISR™ CRISPR pipeline is optimized and validated to find true positive hits, enabling the discovery of robust candidates for accelerated validation and mechanistic follow-up.

84| Optimized metrics for combinatorial and orthogonal CRISPR screens

Ronay Cetin[1]; Martin Wegner[1]; Leah Luwisch[1]; Sarada Saud[1]; Tatjana Achmedov[2]; Sebastian Süsser[1]; Antonella Vera-Guapi[1]; Konstantin Müller[1]; Yves Matthess[1]; Eva Quandt[3]; Simone Schaubeck[1]; Chase Beisel[2]; Manuel Kaulich[1]

[1] Institute of Biochemistry II, Faculty of Medicine, Goethe University Frankfurt, Theodor-Stern-Kai 7, Frankfurt am Main, Germany; [2] Helmholtz Institute for RNA-based Infection Research (HIRI), Helmholtz-Centre for Infection Research (HZI), Würzburg, Germany; [3] Faculty of Medicine and Health Sciences, Universitat Internacional de Catalunya, Barcelona, Spain

Efficient and robust CRISPR-associated (Cas) nucleases are essential for unbiased investigations of genotype-to-phenotype associations in single and combinatorial gene dependencies at scale. While SpCas9 and AsCas12a are widely used for single, combinatorial and orthogonal screenings, systematic side-by-side comparisons are scarce. In this study, we compared the combinatorial SpCas9, AsCas12a, and CHyMErA in hTERT-immortalized retinal pigment epithelial (RPE1) cells, and identified performance-critical parameters. Our analyses revealed that SpCas9 was superior to AsCas12a, and CHyMErA was largely inactive under the tested conditions. To improve AsCas12a and CHyMErA applications, we used arrayed dual-gRNAs, which negatively affected the effect size range of combinatorial AsCas12a applications but enhanced CHyMErA performance. However, the performance improvement was limited to AsCas12a dual-gRNAs, as SpCas9 gRNAs remained largely inactive. To avoid using hybrid gRNAs for orthogonal applications, we developed a multiplex SpCas9-enAsCas12a approach (multiSPAS) that avoids RNA processing for efficient orthogonal gene editing.

85| RepFluo, a fast fluorescent in-vitro assay of Cas9 activity exploiting melting curve analysis.

Filippo Fronza[1]; Roberto Verardo [2]; Claudio Schneider[1]

[1] Università degli Studi di Udine; [2] Laboratorio Nazionale CIB

Although ex vivo applications of CRISPR have been widely investigated, particularly for genome editing purposes, the interest in in vivo applications of the endonuclease family has being emerging more recently, particularly in the field of molecular diagnostic.

This surge in interest necessitates the development of new tools to facilitate the creation of novel technologies and enable rapid design iteration. However, we encountered a challenge in evaluating Cas9 activity in vitro due to the limited number of available techniques, which are typically time-consuming, labor-intensive, and often require specialised materials.

To address these limitations, we have developed a novel methodology that employs the melting curve of a fluorescent reporter DNA (RepFluo) as a readout for Cas9 activity in vitro. Our technique can be performed in a single step, within the precise time and temperature control of a PCR thermocycler, and with minimal sample manipulation after the reaction has been initiated.

Using our approach, we were able to evaluate Cas9 activity under different conditions and obtain valuable insights into the time required to perform a SpCas9 cleavage in vitro, as well as the effect of high temperatures on the protein's activity.

86| Directional dependencies in chemo-resistant non-small cell lung cancer

Loren Antonella Vera-Guapi; Ronay Cetin; Sebastian Süsser; Yves Matthess; Manuel Kaulich

Institute of Biochemistry II, Goethe University Frankfurt - Medical Faculty, University Hospital, Frankfurt, Germany

Cancer is a complex and multifaceted disease with its appearance and progression not depending on single genes but instead from complex genetic interactions between them. In fact, during the process of cellular adaptation to drug exposure (resistance development), cancer cells gain combinations of mutations which we hypothesize can represent genetic vulnerabilities. This may also imply the existence of directional dependencies in which the occurring order of mutations dictates the phenotypic outcome. While the mechanistic understanding of such phenotypes would greatly improve our knowledge about genetic interactions and sequential dependencies, major technical limitations remain towards their identification, including scalable technologies to mimic the directional occurrence of mutations in functional genomic approaches.

Here, we propose to mimic the directional occurrence of mutations and investigate genetic vulnerabilities associated with them. To do so, we will focus on clinically relevant cancer druggable genes and develop an orthogonal CRISPR system to timely-separate combinatorial edits in cells. In order to distinguish genetic interactions from directional dependencies, we

will perform parallel (simultaneous edits) and directional (timely-separated edits) screens. Our proof-of-concept study already identified three directional dependencies among tumor-suppressor and core-essential genes (TP53→C3orf17, ARNT→XRN2, NF2→DIMT1), suggesting an uncontrolled proliferation to generate a dependency on selected essential genes

87| Overcome barriers to phage reboot for the construction of synthetic *Pseudomonas aeruginosa* tailed phages using yeast platform.

Thomas Ipoutcha; Ratanachat Racharaks; Stefanie Huttelmaier; Erica M Hartmann

Northwestern University

Pseudomonas aeruginosa (PA) is a Gram-negative bacterium responsible for 51,000 infections with 2,700 deaths in the US every year and approximately 559,000 deaths globally in 2019. Antimicrobial resistance has increased concern surrounding bacterial infections, particularly PA, because of its diverse ways of resisting antibiotics. Phage therapy is a promising solution to antimicrobial resistance, but the use of natural phages for therapy has some limitations. In our study, we developed a synthetic biology approach to construct synthetic phages for PA treatments. We focused on two lytic tailed phages, JG024 and DMS3, both capable of infecting the clinically relevant PA14 strain. Using transformation-associated recombination (TAR) cloning, we demonstrated the feasibility of constructing JG024 and DMS3 genomes in yeast, where many tools are available for genome engineering. Next, we successfully rebooted the synthetic phage DNA using PA to produce viable phage particles. In optimizing this step, we identified bacterial factors that were inhibiting phage reboot. This method opens up many possibilities for phage therapy in PA. Currently, we are attempting to engineer the phage to deliver a CRISPR-based editor system that will target and inactivate genes involved in biofilm production. Future work will expand the use of this system to other phage-host pairs and explore additional possibilities for phage-delivered therapeutics.

88| Improved specificity of AsCas12a programmed with modified crRNA.

Nikita Shebanov[1]; Anne-Marie Heckel[1]; Natalia Nikitchina[1]; Inna Pyshnaya[2]; Mariya Meschaninova[2]; Ivan Tarassov[1]; Nina Entelis[1]

[1] Strasbourg University; [2] Institute of Chemical Biology and Fundamental Medicine SB RAS

Pathogenic mutations in human mitochondrial DNA (mtDNA) cause severe incurable diseases. In most cases, these mutations are heteroplasmic, i.e. the simultaneous presence of mutant and wild type mtDNA molecules in the cell. Mitochondrial targeting of CRISPR/Cas systems aiming to reduce mutant mtDNA load is challenging, mostly due to inefficient import of crRNAs into mitochondria. Previously we developed a mitochondrial CRISPR/Cas9 system, but a shift of the heteroplasmy level has not been achieved (1). We now consider Type V AsCas12a endonuclease as one of the most promising candidates for mitochondrial genome editing since it requires relatively short crRNA (40-bp), which can be a better substrate for mitochondrial import. We have recently shown that AsCas12a endonuclease can be

programmed with split version of crRNA, consisting of two 20-bp moieties (2), which can improve its targeting into the mitochondria due to reduced size.

The therapeutic potential of mitochondrial CRISPR/Cas systems depends on their ability to discriminate between wild type and mutant mtDNA molecules. Thus, in this study we designed various versions of crRNA targeting pathogenic point mutations in ND5 gene, G13513A and A13514G, and tested if AsCas12a programmed with these crRNAs can cleave in a specific manner mutant mtDNA molecules. By in vitro cleavage assay we demonstrated the increased specificity of AsCas12a programmed with split crRNAs. To further improve the on-target cleavage, we introduced deoxyribonucleotides into various regions of crRNA, and compared the ability of the system to discriminate between wild type and mutant mtDNA. Here we show that modified split crRNA variants can cleave specifically mutant mtDNA molecules, which opens the way for further in vivo applications.

(1)Loutre et al., 2018
(2)Shebanova et al., 2022
This study is supported by the Région Grand Est, IdEx Unistra [ANR-10-IDEX-0002], SFRI-STRAT'US project [ANR 20-SFRI-0012], EUR IMCBio [ANR17-EURE-0023] (France)

89| The AHR:ARNT complex prevents cell transformation by blocking MAGE:TRIM28-mediated p53 degradation.

Konstantin Mueller; Dorothea Maneta; Manuel Kaulich

University Hospital Frankfurt, Goethe University Frankfurt

The tumor microenvironment (TME) is comprised of more than cellular interactions, thus, being able to represent it properly within experimental conditions is of utmost importance. Elements such as hypoxia, nutrients as well as the ability to grow in three-dimensional conditions play an important role in the invasiveness of a tumor. To be able to better mimic the physiological conditions, we developed a 3D soft-agar system that allows us to investigate mono- and multi- genetic traits to map tumor suppressor phenotypes and identify synergistic gene interactions. By performing such experiments, we identified a hitherto unknown genetic interaction between TP53 and the AHR/ARNT complex that prevents cellular transformation upon TP53 loss-of-function mutations. The AHR/ARNT transcription factor complex is best described to function in the signaling of xenobiotics, yielding transcriptional responses that include the upregulation of cytochrome CYP1B1 to assist in xenobiotic inactivation. Exploring the underlying mechanism of the TP53 genetic interaction, we generated stable gene knockouts and quantified global transcriptional changes by RNA-seq. This revealed strong enrichment of Melanoma Antigen Gene (MAGE) family genes in TP53 and AHR mutant cells. MAGE genes are known to be highly expressed in a variety of cancers. Of particular relevance is MAGEC2 which is known to regulate p53 stability by forming a complex with the E3 ubiquitin ligase TRIM28. Thus, we present a model in which loss-of-function mutations in TP53 and AHR/ARNT induce the upregulation of MAGE genes, ultimately leading to the degradation of residual p53 levels and thereby causing cellular transformation and clonal expansion.

90| Novel CRISPR/Cas Editing Strategies for Unbiased Rescuing of Common Splicing Defects in ABCA4 gene.

Pietro De Angeli; Arturo Flores-Tufiño; Susanne Kohl; Bernd Wissinger

Institute for Ophthalmic Research, Tübingen University Hospital

Inherited retinal disorders like Stargardt disease (STGD1) are caused by mutations in genes like ABCA4 that result in faulty mRNA splicing, leading to abnormal protein translation. There is currently no cure for STGD1, but gene therapy using CRISPR/Cas technology is a promising approach to permanently correct genetic defects. Our aim is to test novel CRISPR/Cas-based strategies to correct splicing defects due to common pathogenic ABCA4 variants, restoring correct mRNA processing and protein translation.

To achieve this, minigene models harboring the common ABCA4 c.5461-10T>C, c.5461-10T>G, c.5196+1013A>G, c.5196+1056A>G, and c.768G>T variants were cloned, and validated in HEK293T by mRNA splicing analysis. To generate stable cell lines expressing the variants, lentiviral "landing pad" vectors were created to allow the cloning of validated ABCA4 fragments downstream of an antibiotic resistance marker and upstream of a fluorescent protein. Correct splicing is required for the expression of the fluorescent protein, streamlining the validation of CRISPR/Cas approaches by flow cytometry analysis.

A gRNA library composed of all possible single gRNAs encompassing sequences involved in faulty splicing will then be generated. The established cell lines expressing the splicing defects will be transduced with the gRNA library. Novel Cas variants will be unbiasedly screened to assess their effectiveness in correcting splicing defects in combination with all designed gRNAs.

The mutant ABCA4 minigenes were found to have mRNA missplicing, consistent with prior research. The developed lentiviral “landing pads” vectors have the capability to multi-clone the validated ABCA4 minigenes, which can be efficiently transduced into HEK293T cells to ease the screening of editing strategies. This study provides a robust platform for consistently and objectively evaluating the efficacy of genome editing strategies in correcting splicing defects.

91| Use of the type V CRISPR-Cas system to induce a deletion in human mitochondrial DNA

Natalia Nikitchina [1]; Anne-Marie Heckel[1]; Ilya Mazunin[2]; Ivan Tarassov[1]; Nina Entelis[1]

[1] UMR7156 – Molecular Genetics, Genomics, Microbiology, University of Strasbourg, Strasbourg, 67081, France; [2] Center of Life Sciences, Skolkovo Institute of Science and Technology, Moscow, 143026, Russia

Many human neuromuscular diseases can be caused by mutations in the mitochondrial genome (mtDNA), which encodes a few proteins essential for the production of energy in the form of ATP through oxidative phosphorylation (OXPHOS). The severity of mitochondrial diseases is depended on heteroplasmy level – a state when mutant and wild-type (WT) mtDNA molecules coexist in the same cell. One approach to decrease the mutant mtDNA level may

consist of inducing the degradation of such molecules by targeted cleavage. CRISPR-Cas systems seem to be perspective for this purpose due to their reliance on DNA–RNA interactions for specific mtDNA site recognition (Jinek, M., et al., 2012). For this, nuclease and crRNA components should be modified for the effective import into mitochondria (Loutre, R., et al., 2018).

Here we adapted CRISPR-Cas12a system, which recognizes AT-rich PAM sequences, for its effective import and functioning in human mitochondria. We created a stable human cell line with an inducible expression of the mito-hAsCas12a nuclease and demonstrated its mitochondrial localization. To evaluate the activity of the system in mitochondria, we transfected this cell line with two crRNAs targeting mtDNA which should induce a deletion. To inhibit degradation of linearized mtDNA, the mitochondrial exonuclease (MGME1) was inactivated with siRNA as described in (Peeva V. et al., 2018). We then analyzed total DNA isolated from cells using Southern hybridization and Sanger sequencing of the mtDNA fragment. For the first time, we demonstrated the presence of a deletion in human mtDNA induced by a CRISPR-system.

These findings open a possibility to establish a system that can be used for mitochondrial genome editing and specific elimination of mutant mtDNA in vivo.

Keywords: mitochondria, Cas12a, mitoCRISPR.

This study was supported by IdEx Unistra [ANR-10-IDEX-0002], SFRI-STRAT'US project [ANR 20-SFRI-0012], EUR IMCBio [ANR17-EURE-0023] and the Région Grand Est (France).

92| Structure-based engineering of FnCas9 to increase its efficiency

Isabel Weisheit; Grzegorz Sienski; Dmitrii Degtev

Discovery Biology, Discovery Sciences, R&D, AstraZeneca, Gothenburg, Sweden

Therapeutic genome editing requires highly efficient and - at the same time also - safe editing approaches. While the focus of the most widely used Cas9 protein from *Streptococcus pyogenes* (SpCas9) has been on improving the safety of the enzyme, the picture is different for the Cas9 enzyme from *Francisella novicida* (FnCas9): This Cas protein of the Class II B family has previously been reported to have high intrinsic fidelity with low tolerance to single mismatches within the target sequence. The widespread use of FnCas9, however, has so far been prevented by it's low editing activity in human cells. We therefore applied structure-based rational protein design to generate new, highly-active protein variants. Furthermore, we developed an in vitro reporter system to analyze the editing activities of multiple variants in a high-throughput manner. With this, we developed engineered (e) FnCas9, which shows highly increased editing activity at endogenous targets. This new eFnCas9 is a promising candidate to develop efficient and safe gene therapies.

93| Pergamon is a highly active and compact human CRISPR knockout library

Sebastian Süsser; Ronay Cetin; Antonella Vera-Guapi; Manuel Kaulich

Goethe University Frankfurt, Medical Faculty

Genome-wide CRISPR screenings are a powerful tool to assign function to genes and identify synthetic gene circuits. However, their implementation remains experimentally expensive with large demands on cell culture. Here, we present the newly-designed and generated highly-uniform, genome-wide CRISPR knockout library "Pergamon" and use it to screen for genetic vulnerabilities associated with increased cell replication stress. Pergamon contains 2 highly-active gRNAs that are selected based on robust performance across >1000 genome-wide CRISPR screens, and is highly-uniform to support genome level screening in hard-to-handle cell models (e.g. organoids, iPSs, primary or immune cells). Pergamon will be available from Addgene.com shortly.

The retinoblastoma tumor suppressor gene (RB1) plays a crucial role in cell cycle regulation, and its loss is associated with a variety of human malignancies. To identify RB1-dependent genetic interactions and vulnerabilities, we applied "Pergamon" and performed a series of genome-wide screens in unperturbed and RB1-depleted hTERT-immortalized RPE1 cells. As part of our analysis, we demonstrate the robust and superior performance of Pergamon in identifying core essential genes. Comparing unperturbed with RB1-depleted RPE1 cells informs about common RPE1-specific vulnerabilities and identifies previously known within-pathway genes. Most interestingly, our analysis reveals hitherto unknown in-between pathway genes that, collectively, suggest the RB1 cell cycle entry pathway to be somehow be connected to the functionality of these genes/pathways. Taken together, we present the novel and compact CRISPR knockout library "Pergamon", demonstrate its excellent performance in calling core essential genes, and identify RB1-dependent synthetic sick and buffering gene relationships.

94| Generation of CRISPR knockout mouse megakaryocytes

Gabriel Araujo; Zoltan Nagy; Bernhard Nieswandt

University of Würzburg

Background

Platelets are small, anucleated cells essential for blood clotting. Platelets are produced by megakaryocytes, which develop from haematopoietic stem cells (HSCs) in the bone marrow. We have recently identified the inhibitory receptor G6b-B as a critical regulator of megakaryocyte maturation (Becker, Nagy et al. Blood Advances 2022). The roles of gene products involved in platelet biogenesis are traditionally investigated using knockout mouse strains, however, this approach is time consuming and costly. CRISPR-editing of human HSCs and their differentiation into mature megakaryocytes has recently been described (Montenont et al. Blood Advances 2021) and could provide a rapid alternative to identify new genes regulating platelet production.

Aims

We have set out to adapt the protocol to mouse HSCs to enable the generation of CRISPR KO megakaryocytes. We have selected G6b-B, as the first target gene to ablate using the adapted protocol.

Methods

To enrich native bone marrow HSCs from C57BL/6 mice, we utilized negative depletion and cell separation with the Dynabeads magnetic system. Non-viral gene editing of HSCs using CRISPR/Cas9 ribonucleoprotein nucleofection with CRISPR guide RNA, trans-activating CRISPR RNA, and Cas9 nuclease was performed on day 0 on a 4D-Nucleofector system. We used non-targeting guide RNA transfected HSCs as controls. Cells were cultivated in the presence of thrombopoietin and the efficiency of G6b-B protein ablation on in vitro-differentiated CRISPR-edited megakaryocytes was assessed by flow cytometry on day 7.

Results

We found that one of the tested guide RNAs resulted in a close to complete G6b-B ablation. These results are similar to those reported for human CD34+ cell-derived megakaryocytes.

Conclusions

Our results show that the adapted CRISPR protocol is able to effectively ablate proteins from mouse megakaryocytes, indicating its high potential for rapid screening of genes, which are implicated in platelet biogenesis.

95| Engineered bacteriophage for in situ delivery of CRISPR RNA-guided DNA transposases

Avery Roberts[1]; Benjamin Adler[2]; Brady Cress[2]; Jillian Banfield[2]; Jennifer Doudna[2]; Rodolphe Barrangou[3]

[1] North Carolina State University; [2] University of California, Berkeley and Innovative Genomics Institute, University of California, Berkeley; [3] North Carolina State University and Innovative Genomics Institute, University of California, Berkeley

Bacteriophage can be used as a vector to deliver engineered DNA to bacteria in diverse microbial communities. Previously, we deployed engineered temperate phage lambda for base editing of plasmid and chromosomal loci in *Escherichia coli* in a synthetic microbial community context. Here, we aim to engineer bacteriophage lambda to harbor and deliver CRISPR RNA-guided transposons (CASTs) for phage-delivered, RNA-guided DNA transposition in E. coli. Using CRISPR-Cas13a phage engineering methods, we are replacing non-essential regions of the lambda genome with the sizable CAST payload and a donor transposon that will be mobilized into the host *E. coli* genome. We have selected lambda strains with lifestyle- and lysis-altering mutations for controlled phage activity. Currently, we are studying cell lysis, lysogeny, and targeted genome editing outcomes using mutant phage lambda and wild-type or Amber suppressor mutant *E. coli* host strains. Our efforts aim to determine optimal combinations with minimal or no lysogeny and undisturbed host viability for targeted,

efficient host genome editing. By engineering phage with precise host-range specificity, we strive to surmount barriers for DNA-editing all-in-one RNA-guided CRISPR-Cas transposase (DART) system delivery and achieve targeted editing in microbial communities. This research contributes to the development of improved phage-mediated methods for in situ, site- and species-specific genome editing in mixed communities. Future efforts will be applied to investigate and manipulate the composition and function of rhizosphere microbial communities.

96| Developing and Optimizing Editing Tools for Non-Model Microbes

*Leo Song[1], *Sophia E. Swartz[1], Amanda Alker[1], Jennifer A. Doudna[1], Benjamin E. Rubin[1]

University of California, Berkeley

CRISPR-associated transposons (CASTs) hold unique potential in applying targeted genetic edits to complex microbial communities. However, little is known about the molecular mechanism and regulation of CAST integration and their widespread utilization is limited by low efficiency across diverse, non-model bacteria. To overcome these limitations and expand the range and applicability of type I-F CASTs as editing tools, we employed a whole genome mutant screening approach to identify putative regulators of CAST transposition in established model systems in which CAST is known to integrate. Candidate regulator hits identified through the screens were individually validated, with a particular focus on well-characterized genes and proteins involved in known mechanisms and pathways. Finally, we leveraged our findings by constructing vectors that incorporate these key regulators to increase editing efficiency. These results will shed light on the molecular mechanisms underlying CAST integration and enable more efficient editing in diverse non-model microorganisms.

97| Tipping the balance between DNA rehybridization and RNA:DNA duplex maintenance enables Cas9 to catalyze multiple-turnover reactions

Juan Pan; Megumu Mabuchi; G. Brett Robb

New England Biolabs

The RNA-guided Cas9 endonuclease from *Staphylococcus aureus* (SauCas9) can catalyze multipleturnover reactions whereas Cas9 from *Streptococcus pyogenes* (SpyCas9) is a single-turnover enzyme. Here we dissect the mechanism of multipleturnover catalysis by SauCas9 and elucidate its molecular basis. We show that the multiple-turnover catalysis does not require more than stoichiometric RNA guides to Cas9 nuclease. Rather, the RNAguide loaded ribonucleoprotein (RNP) is the reactive unity that is slowly released from product and recycled in the subsequent reaction. The mechanism that RNP is recycled formultiple-turnover reaction entails the unwinding of the RNA:DNA duplex in the R-loop. We argue that DNA rehybridization is required for RNP release by supplementing the energy cost in the process. Indeed, turnover is arrested when DNA rehybridization is suppressed. Further, under higher salt conditions, both SauCas9 and SpyCas9 showed increased turnover, and engineered

SpyCas9 nucleases that form fewer direct or hydrogen bonding interactions with target DNA became multiple-turnover enzymes. Thus, these results indicate that for both SpyCas9 and SauCas9, turnover is determined by the energetic balance of the post-chemistry RNP-DNA interaction. Due to the conserved protein core folds, the mechanism underpinning turnover we establish here is likely operant in all Cas9 nucleases.

98| Analytical approaches for the detection of gene doping with CRISPR/Cas for doping control purposes

Alina Paßreiter[1]; Nana Naumann; Andreas Thomas; Nicolas Grogna; Philippe Delahaut; Mario Thevis

[1] Manfred-Donike Institute for Doping Analysis (MDI e.V.)/German Sport University Cologne

The CRISPR/Cas tool kit constitutes one of today's most frequently used gene editing techniques since editing of virtually any DNA sequence can be realized, due to the quickly progressing research into different Cas effectors and their ever-expanding range of targets. However, the simplicity and cost-effectiveness of those CRISPR tools can also facilitate the illicit utilization of CRISPR/Cas in order to achieve performance enhancements amongst athletes. This abuse is classified as gene doping, which is banned in sports according to the Prohibited List of the World Anti-Doping Agency (WADA). Consequently, there is an urgent need for the direct detection of misused CRISPR/Cas techniques in doping control samples. Hence, two promising analytical approaches for the detection of Cas9 and its respective sgRNA associated with the bacterium Streptococcus pyogenes are presented herein. The identification of the exogenous protein Cas9 was accomplished by means of a bottom-up analytical approach via immunoaffinity purification, tryptic digestion, and subsequent detection by HPLC−HRMS/MS. Complementary to that, Specific High Sensitive Enzymatic Reporter UnLOCKing (SHERLOCK) was utilized for targeted nucleic acid detection of sgRNA in combination with reverse transcriptase-recombinase polymerase amplification (RT-RPA) and an additional, gel-based screening procedure. A proof-of-concept administration study simulating a hypothetical gene doping scenario with lipid mediated CRISPR ribonucleoprotein (RNP) complexes employing an in vivo mouse model revealed a detection window of up to 8 h post administration for both analytes in plasma, supporting the principal applicability of those proposed testing strategies to authentic doping control samples in the future and encouraging ongoing research and method optimization.

99| An Adaptable Platform for Continuous Directed Evolution in Human Cells

Amanuella Mengiste[1]; Samuel Hendel [1]; Andrew Sabol[1]; Zachary Severence [2]; Sreekanth Vedagopuram [2]; Santosh Chaudhary [2]; Amit Choudhary [2]; Matthew Shoulders [1]

[1] MIT; [2] Broad Institute of MIT & Harvard

At present, most directed evolution experiments are undertaken in vitro, in bacteria or in yeast, even when the resultant biomolecules are bound for use in mammalian cells. Unfortunately, as a direct consequence of the discrepancy between the environments where

biomolecules are optimized and the mammalian cellular milieu, where they are intended to function, many evolved bioproducts fail. In other words, differences between cellular environments—in cellular compartmentalization, trafficking, post-translational modifications, intermolecular interactions, and folding—hamper the preservation and transferability of evolved function.

A compelling solution to this challenge is to use the mammalian cell itself as the design, engineering and quality-control setting for the directed evolution of biomolecules destined for use in mammalian cells. However, the complexity of human cells presents formidable challenges at every step of a traditional directed evolution workflow. For instance, there are far fewer tools for genetic manipulation in human cells compared to bacteria/yeast, and human cells have longer doubling times, which simultaneously slows down amplification and dramatically reduces the total number of variants that can reasonably be screened.

Specifically, we developed an adenovirus-based system for the directed evolution of biomolecules in mammalian cells. This approach allows us to exploit the ease of inducing mutations on viral DNA and the rapid progression of viral infections to produce thousands of genetically diverse virions per cell, without compromising the "evaluation" of biomolecules in the human cellular environment. Using this approach, we are engineering CRISPR-Cas genome editors, which hold immense potential for treating or curing previously intractable genetic diseases.

100| SIBR-2.0: Strict and inducible expression of any gene of interest in mesophilic prokaryotes and eukaryotes

Simona Della Valle[1]; Enrico Orsi [2]; Belen Adiego-Perez [3]; Sjoerd Creutzburg [3]; Evangelia Pentari[2]; Harrison Steel [1]; John van der Oost [3]; Pablo Ivan Nikel [2]; Raymond Staals [3]; Nico Claassens [3]; Wei Huang[1]; Constantinos Patinios[3]

[1] University of Oxford; [2] Technical University of Denmark; [3] Wageningen University and Research

After the successful implementation of SIBR to control the activity and cytotoxicity of Cas12a in *Escherichia coli* MG1655, *Pseudomonas putida* KT2440 and *Flavobacterium* IR1, we sought to expand the application portfolio of SIBR towards other organisms, proteins and functions. To achieve this, we began by developing SIBR-Cas9 and SIBR-Cas12a genome engineering tools in the difficult-to-engineer chemolithoautotrophic bacterium *Cupriavidus necator*. Surprisingly, whilst SIBR-Cas9 was strictly and inducibly expressed in *C. necator*, SIBR-Cas12a appeared to be leaky. Through a series of experimental approaches, we discovered that alternative translation initiation sites exist within the SIBR sequence. These sites allow or inhibit translation depending on the succeeding genomic context; something that may limit the portability of SIBR to certain genes. To overcome this bottleneck, we devised the transfer of SIBR from its original position (directly after the start codon) towards the open reading frame of the gene of interest (GOI). This approach successfully resulted in a strict and inducible system (termed as SIBR-2.0) that can be easily transferred to any GOI in mesophilic prokaryotes. Using the obtained knowledge, we used SIBR-1.0-Cas9 and SIBR-2.0-Cas12a to successfully knockout two genes in *C. necator*. Following, we used SIBR-2.0 to control the

expression of a T7 polymerase-GFP cascade system in *E. coli*, showing the functionality of SIBR across different genes and for different purposes. Lastly, we expanded the applicability of SIBR-2.0 to the yeast *Saccharomyces cerevisiae*. Due to our novel SIBR-2.0 design, we overcame the non-sense mediated decay mechanism that is inherent by all eukaryotes, therefore making SIBR-2.0 a valuable tool for strict and inducible gene expression also in eukaryotes. We foresee that SIBR-2.0 will be used to control the expression of highly toxic genes in both prokaryotes and eukaryotes, simplifying cloning and characterization of such genes.

CRISPR Ecology and Evolution

101| Uncovering the Dynamics of CRISPR Array Evolution with a Spacer Order Respecting Maximum Likelihood Approach

Axel Fehrenbach[1]; Alexander Mitrofanov; Omer Alkhnbashi; Rolf Backofen; Franz Baumdicker[1]

[1] University of Tübingen

CRISPR arrays rapidly evolve due to spacer insertions and deletions. Insertions in the CRISPR array are notable for occurring predominantly at one end of the array, therefore they provide a chronology of foreign invasions.

Comprehending the evolution of CRISPR arrays holds significance in a wide area of research. For example, close monitoring of phylogenetic relationships and prompt tracking of epidemic outbreaks is possible, due to the shorter timescale of CRISPR array evolution. Moreover, it allows to infer the evolutionary history of individual spacers of interest and thereby aid in the exploration of the ecology of bacteria, archaea and their phages.

Commonly used tools for general ancestral reconstruction do not consider the spacer insertion order and thus, relying on them will result in misleading conclusions.

We introduce SpacerPlacer, a tool that utilizes probabilistic models of CRISPR array evolution and a maximum-likelihood approach to reconstruct ancestral states of a group of CRISPR arrays while respecting the insertion order.

Using SpacerPlacer, we examine CRISPRCasdb to estimate the evolutionary behavior of the contained CRISPR arrays and compare between CRISPR types and different species. Furthermore, we find that neighboring spacers are likely to be deleted in blocks rather than exclusively individually by using a likelihood ratio test. Moreover, contrary to a common notion, analysis of spacer deletion positions in our reconstructions indicates that spacers are deleted evenly along the array.

102| Evaluating the efficiency of conjugative CRISPR-Cas9-based antimicrobial for eradicating ESBL genes from a synthetic community

Ilmur Jonsdottir; Cindy Given; Krista Norvasuo; Petra Paananen; Pilvi Ruotsalainen; Matti Jalasvuori; Reetta Penttinen

University of Jyväskylä

Antimicrobial resistance (AMR) has emerged as a significant and growing global health threat. Conjugative plasmids are key players in the dissemination of AMR genes among bacterial populations, making them attractive targets for intervention. Further, harnessing the promiscuity of these mobile genetic elements (MGEs) to deliver CRISPR editing components to resistant bacterial communities may allow for efficient AMR targeting and eradication. Our CRISPR-Cas9-based antimicrobial relies on the conjugative delivery of ESBL-targeting CRISPR system into multi-resistant bacteria wherein it effectively cleaves the target resistance gene.

In this research, we assessed the efficiency of the CRISPR-antimicrobial to remove ESBL genes from clinical *Escherichia coli* isolates. In order to evaluate the ecological and evolutionary effects that may determine the success of CRISPR-antimicrobial treatment, we studied its effectiveness within a multispecies community in a 10-days serial culture microcosm experiment. Conjugative CRISPR-antimicrobial was introduced into 4 different synthetic gut microbial communities inhabited by an ESBL-*E. coli* strain carrying either chromosomal or plasmid-based blaCTX-M-15 gene. We followed the presence and amount of blaCTX-M-15 gene in the communities with qPCR analysis. Our results show that the outcome of the CRISPR-antimicrobial treatment is highly determined by the genetic background of the targeted ESBL gene and may even allow for the escape of the ESBL plasmid into a host with better fitness. Further, the results show that while CRISPR tools show great potential as a weapon against the spread of antibiotic resistance, a broader understanding of the ecological and evolutionary factors that may restrain their efficient usage is still warranted.

103| CRISPR-Cas on plasmids: Quantifying the relevance of location for phage protection efficiency

Berit Siedentop; Dario Rüegg; Sebastian Bonhoeffer; Hélène Chabas

ETH Zürich, Environmental System Sciences

CRISPR-Cas systems are adaptive immune systems that protect bacteria against phages. It has been shown that those systems are not only found on the bacterial chromosome but are also frequently carried by plasmids. However, the importance of the CRISPR-Cas location on the protection efficiency against virulent phage is unclear. In order to investigate the impact of CRISPR-Cas location on the protection efficiency against virulent phages we use mathematical modeling considering plasmid, CRISPR-Cas and phage characteristics. We find that for CRISPR-Cas systems carried by plasmid the protection efficiency against virulent phages is decreased in certain conditions compared to CRISPR-Cas systems located on the chromosome. The magnitude of the decrease in efficiency is not solely determined by plasmid traits but by an interplay of plasmid, CRISPR-Cas and phage characteristics. We identify that the driving mechanism for the decreased protection efficiency against virulent phages is evolutionary emergence, i.e. phage outbreaks caused by phage mutants that are able to escape a spacer.

104| Cryo-EM structure of the transposon-associated TnpB enzyme

Ryoya Nakagwa[1]; Hisato Hirano[1]; Satoshi Omura[1]; Suchita Nety[2]; Soumya Kannan[2]; Han Altae-Tran[2]; Wen Wu[3]; John van der Oost[3]; Feng Zhang[2]; Osamu Nureki[1]

[1] The University of Tokyo; [2] Broad Institute of MIT & Harvard; [3] Wageningen University & Research

The class 2 type V CRISPR effector Cas12 is thought to have evolved from the IS200/IS605 superfamily of transposon-associated TnpB proteins. Recent studies have identified TnpB proteins as miniature RNA-guided DNA endonucleases. TnpB associates with a single, long RNA (omegaRNA) and cleaves double-stranded DNA targets complementary to the omegaRNA

guide. However, the RNA-guided DNA cleavage mechanism of TnpB and its evolutionary relationship with Cas12 enzymes remain unknown. Here we report the cryo-electron microscopy (cryo-EM) structure of *Deinococcus radiodurans* ISDra2 TnpB in complex with its cognate omegaRNA and target DNA. In the structure, the omegaRNA adopts an unexpected architecture and forms a pseudoknot, which is conserved among all guide RNAs of Cas12 enzymes. Furthermore, the structure, along with our functional analysis, reveals how the compact TnpB recognizes the omegaRNA and cleaves target DNA complementary to the guide. A structural comparison of TnpB with Cas12 enzymes suggests that CRISPR–Cas12 effectors acquired an ability to recognize the protospacer-adjacent motif-distal end of the guide RNA–target DNA heteroduplex, by either asymmetric dimer formation or diverse REC2 insertions, enabling engagement in CRISPR–Cas adaptive immunity. Collectively, our findings provide mechanistic insights into TnpB function and advance our understanding of the evolution from transposon-encoded TnpB proteins to CRISPR–Cas12 effectors.

105| CRISPR-Cas targeting promotes gene exchange in *Haloferax volcanii* by triggering homologous recombination

Deepak Kumar Choudhary; Israela Turgeman-Grott; Uri Gophna

Tel Aviv University

Archaea possess multiple defense systems to protect themselves from viral encounters. Such defenses can affect gene exchange and evolutionary processes. For example, the CRISPR-Cas, and restriction-modification systems restrict gene exchange in several bacterial and archaeal species. Unlike bacteria, in archaea, the molecular mechanisms of action of these defense systems and how they influence gene exchange and evolution remain understudied. Here, we examine the role of the archaeal CRISPR-Cas immune system in gene exchange. Surprisingly, in *Haloferax volcanii*, within-species CRISPR-Cas targeting significantly increases mating efficiency in comparison to the non-targeted control. Furthermore, we observed that CRISPR-Cas-targeted genome cleavage increased homologous recombination (HR) frequency, and there is a connection between CRISPR-Cas systems and the DNA repair machinery following archaeal mating. Specifically, we focused on several key components of the HR pathway: RadB, Mre11, and Rad50. In *H. volcanii*, RadB is essential for efficient DNA repair by HR, and MRE11-RAD50 reduces the frequency of homologous recombination. Interestingly, like CRISPR-Cas targeting, disruption of together MRE11 and RAD50 increased mating efficiency in H. volcanii. Thus, within-species CRISPR-Cas targeting increases homologous recombination (HR), which may promote future gene transfer, indicating the role of DNA repair machinery and CRISPR–Cas systems in microbial evolution. Additionally, when we performed mating experiments between *H. volcanii* strain with cas genes and a strain without cas genes (CRISPR-Cas-KO,) and then selected recombinant colonies, we observed that all the mating products were CRISPR-Cas positive. This suggests a dominance of CRISPR-Cas-encoding plasmids in mating scenarios.

106| Horizontal transfer of adaptation and effector modules as a potential mechanism for overcoming CRISPR-Cas system inhibitions

Riccardo Rosselli[1]*; Noemi Guzmán; Javier Espinosa; Francisco Mojica*

[1] University of Alicante

Diverse CRISPR-Cas types act as adaptive immune systems in prokaryotes by intercepting, recording, and degrading mobile genetic elements (MGEs) that enter the cytoplasm. As a result, these systems undergo evolutionary dynamics that are typical of host-parasite interactions; their genes rapidly adapt by following the antagonistic relationship between players, and lateral gene transfer can provide a substantial contribution in balancing the dominance of competitors.

Our study focuses on the evolution of the subtype I-E CRISPR-Cas system, which is widely distributed among Bacteria and Archaea. Specifically, we mapped subtype I-E in 4,310 microbial genomes and analyzed the evolution of adaptation and effector modules separately. The result uncovered phylogenetic discrepancies between individual modules and their corresponding coding genomes. To explore the adaptive advantages arising from modular exchanges, we conducted spacer acquisition experiments by expressing *S. enterica* Cas1-Cas2 and *E. coli* Cas1-Cas2 coding plasmids in both *E. coli* and *S. enterica* strains. The data showed that the *S. enterica* adaptation module was not functional when expressed in its native microbial strain. However, *S. enterica* Cas1-Cas2 elicited efficient acquisition in *E. coli*. This outcome highlights the strong regulation of Cas1-Cas2 in *S. enterica* mediated by inhibitory factors potentially including anti-CRISPR molecules (Acr) and suggests that the acquisition of cas modules might be sufficient to neutralise the inhibition.

We are currently investigating whether the acquisition of functional modules from different bacterial clades represents an evolutionary mechanism for microbial cells to overcome CRISPR-Cas evading strategies, such as Acr proteins.

107| Screening Metagenomic DNA for Proteins Affecting the Rate of Spacer Acquisition

Christopher Cannon

University of Nottingham

CRISPR-Cas systems are widely distributed in prokaryotes where they defend against mobile genetic elements, particularly phages. The interference arm has provided several valuable molecular tools, which rely on the exquisite target-specificity of effector proteins, most notably Cas9.

Interference relies on prior adaptation by acquisition of a spacer sequence from an invading element. This process is complex and has not yet been full reconstituted in vitro. It is also likely to be influenced by the physiological state of the cell.

The influences have been difficult to address owing to the limitations of the PCR-based Spacer Integration (SPIN) assay. My host laboratory has developed a genetic assay capable of screening large numbers of clones simultaneously. I have used the assay to screen 10 Gbp of metagenomic DNA for BAC clones that either increase or decrease the rate of spacer acquisition in the E. coli Type 1E CRISPR system. Following isolation, BACs will be subcloned and phenotypes validated.

108| From Ancestry to Orientation: CRISPR Array Transcriptional Direction Prediction from an Evolutionary Perspective.

Axel Fehrenbach[1]; Alexander Mitrofanov[2]; Omer Alkhnbashi; Rolf Backofen[2]; Franz Baumdicker[1]

[1] University of Tübingen; [2] University of Freiburg

Predicting the orientation of CRISPR arrays, i.e. its transcriptional direction, is crucial for various applications, such as the investigation of the mechanisms of adaptation, CRISPR RNA processing, and interference. Numerous subsequent analyses depend on the correct identification of the crRNA-encoding strand, including leader region detection and identification of protospacers and the corresponding protospacer-adjacent motifs (PAMs). Existing tools predict the CRISPR orientation individually for each array based on the corresponding repeat sequence and its neighboring region. In contrast to the single-array-based approach, a comparison of closely related CRISPR arrays can leverage the population genetic information generated by the polarized insertion of new spacers.

We present a new tool that utilizes this evolutionary perspective to predict the orientation of CRISPR arrays. For both possible orientations of the arrays, the tool reconstructs the most likely ancestral spacer insertions and deletions along a phylogenetic tree. Based on the alternative reconstructions, we implemented a likelihood ratio test to identify the correct orientation.

Our approach fundamentally expands the existing toolkit for orientation prediction, as the evolutionary approach uses completely independent information compared to the single-array-based approaches. The novel evolutionary approach can thus improve the confidence of existing orientation prediction, and can also indicate where single-array-based predictions might need a revision.

Applying our method to arrays from CRISPRCasdb we were able to predict the orientation of 75% of the arrays that other tools can not determine. In addition, we find with high confidence that roughly 15-30% of existing orientation predictions in CRISPRCasdb need to be reversed. Interestingly, our approach also indicates that the performance of existing orientation prediction tools strongly depends on the type of the corresponding CRISPR system.

109| TnpB structure reveals minimal functional core of Cas12 nuclease family

Tautvydas Karvelis [1]; Giedrius Sasnauskas[1]; Giedrė Tamulaitienė[1]; Gytis Druteika[1]; Arturo Carabias[2]; Arūnas Šilanskas[1]; Darius Kazlauskas[1]; Česlovas Venclovas[1]; Guillermo Montoya[2]; Virginijus Šikšnys[1]

[1] Vilnius University, Life Sciences Center, Institute of Biotechnology; [2] Structural Molecular Biology Group, Novo Nordisk Foundation Centre for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen

The widespread TnpB proteins of IS200/IS605 transposon family have recently emerged as the smallest RNA-guided nucleases capable of targeted genome editing in eukaryotic cells. Bioinformatic analysis identified TnpB proteins as the likely predecessors of Cas12 nucleases, which along with Cas9 are widely used for targeted genome manipulation. Whereas Cas12 family nucleases are well characterized both biochemically and structurally, the molecular mechanism of TnpB remains unknown. Here we present the cryogenic-electron microscopy structures of the *Deinococcus radiodurans* TnpB–reRNA (right-end transposon element-derived RNA) complex in DNA-bound and -free forms. The structures reveal the basic architecture of TnpB nuclease and the molecular mechanism for DNA target recognition and cleavage that is supported by biochemical experiments. Collectively, these results demonstrate that TnpB represents the minimal structural and functional core of the Cas12 protein family and provide a framework for developing TnpB-based genome editing tools.

Beyond CRISPR

110| Enhanced Detection and Comprehensive Assessment of Terminal Repeat Mutations in CRISPR Arrays

Alexander Mitrofanov; Omer Alkhnbashi; Rolf Backofen

Universität Freiburg

In recent years, the CRISPR-Cas adaptive immune system has garnered significant attention due to its potential applications in various fields. A range of tools has been developed to identify and classify distinct components of the CRISPR-Cas systems. However, accurate detection of CRISPR arrays remains a challenging task, primarily due to several complicating factors. One particular challenge is the identification of mutations at the terminal end of the array.

In this study, we present an extension to the CRISPRidentify algorithm that enhances its capacity to detect mutated repeats, which often remain unidentified by other existing tools. Leveraging a comprehensive dataset, we conducted an extensive analysis and gathered statistical data on the behavior of such mutated repeats across different Cas subtypes.

Furthermore, we investigated the self-targeting potential of spacers found in the corresponding CRISPR arrays. This analysis enabled us to formulate a hypothesis that some of the observed candidates may possess functions beyond traditional immune defense. This insight may contribute to a more nuanced understanding of the CRISPR-Cas system, expanding its potential applications and facilitating further research in this rapidly evolving field.

111| Regulators of type V-K CRISPR-associated transposon (CAST) and their atypical tracrRNAs

Marcus Ziemann[1]; Viktoria Reimann[1]; Tao Zhu[2]; Yue Shi[2]; Yajing Liang[2]; Xuefeng Lu[2]; Wolfgang Hess[1]

[1] Universität Freiburg; [2] Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences

Type V-K CAST (CRISPR-Cas associated transposon) systems represent a unique CRISPR-Cas variant. Their genetic set-up includes a minimal CRISPR system encoded on the left, a transposase operon on the right, as well as a variable number of cargo genes in between. The CRISPR component contains a single complex-building gene (cas12k), followed by the tracrRNA locus and a short CRISPR-array. Fundamental to this structure is furthermore a single gene for a regulatory protein always located upstream of cas12k in reverse orientation. These regulators do not belong to a single coherent family, but rather to three different groups based on their sequence and potential protein structure. They were named Arc_1 and Arc_2, small Arc_repressors able to bind DNA via a ribbon-helix-helix-domain and CvkR, a MerR-like factor that targets DNA via its helix-turn-helix-motif [1]. CvkR was analyzed in more detail and found to directly repress the expression of cas12k and of the transposase gene tnsB, thus being able to suppress its own genetic mobility. It also regulates the abundances of tracrRNA and crRNA

indirectly via the amount of Cas12k [1]. We furthermore found unique aspects of V-K associated tracrRNAs. In Anabaena 7120, the tracrRNA is transcribed from a single promoter in a joint primary transcript with the CRISPR array. Both elements are quite conserved and show a remarkably consistent distance of ~200 nt from each other [1,2]. The tracrRNA-CRISPR promoter contains a DNA motif we found conserved within the leaders of other CRISPR arrays. The function of this motif is currently unknown but it indicates a connection between the CAST and the more classical CRISPR systems in different organisms.

[1] Ziemann M., et al. (2023) CvkR is a MerR-type transcriptional repressor of class 2 type V-K CRISPR-associated transposase systems. Nat. Comm. 14, 924.
[2] Mitrofanov A., et al. (2022) CRISPRtracrRNA: Robust approach for CRISPR tracrRNA detection. Bioinformatics 38, ii42–ii48.

112| Repair of double strand breaks induced by CRISPR targeting

Anna-Lena Sailer[1]; Julia Wörtz[1]; Aris-Edda Stachler[1]; Laura Mitchell[2]; Victoria Smith[2]; Thorsten Allers[2]; Anita Marchfelder[1]

[1] Ulm Universität; [2] Nottingham University

Double-strand breaks (DSBs) are one of the most serious DNA damages. It is generally accepted that there are two ways to repair DSBs in our model organism Haloferax volcanii: cost intensive homologous recombination (HR) or error-prone microhomology end joining (MMEJ). MMEJ is based on the exposure of microhomology regions that anneal, followed by flap removal, DNA resynthesis, and ligation [1]. However, knowledge about the individual proteins involved in MMEJ is limited.

In the present project, we will further investigate MMEJ and the proteins involved using a technique known as self-targeting [2]. This uses the organism's own CRISPR-Cas I-B system and a plasmid encoding a crRNA targeting an endogenous gene to induce a DSB in the non-essential crtI gene required for the synthesis of reddish pigments. The DSB will be then repaired by MMEJ leaving behind deletions flanked by microhomology regions. The resulting deletions cause colonies to appear white instead of red and their number as well as sizing of the homology regions can be used to monitor repair capability and efficiency.

We established and characterised several deletion strains lacking proteins thought to be involved in MMEJ and performed self-targeting with these strains. Absence of some proteins causes longer microhomology regions indicating impaired MMEJ repair. To further unravel the interplay of proteins during MMEJ we also generated multi-deletion strains. This way, we found several proteins to functionally overlap, amongst others Cas1 and Fen1 previously demonstrated to display equivalent functions during DNA repair [3]. An influence of Cas1 on the outcome of MMEJ is another fascinating link between the cellular repair machinery and the CRISPR-Cas system.

1. Pérez-Arnaiz et al., 2020. Open Biol. 10, 200293.
2. Stachler, A.-E. et al., 2017. Nucleic Acids Research 45, 5208–5216.
3. Wörtz, J. et al., 2022. Front. Microbiol. 13, 822304.

113| Compelling cellular reactions and gene expression patterns of *Saccharolobus solfataricus* upon deletion of a type III CRISPR-Cas system

Erika Wimmer[1]; Isabelle Anna Zink [2]; Melina Kerou [1]; Logan Hodgskiss [1]; Christa Schleper[1]

[1] University of Vienna (Austria); [2] Wageningen University and Research (Netherlands)

Sharing their environment with many conjugative plasmids and viruses, Sulfolobales harbor extensive CRISPR-Cas systems and are important model organisms for studying CRISPR-Cas functions. Besides three Type I-A systems, *Saccharolobus solfataricus* harbors two Type III systems of subtypes B and D. Theoretically, type III effector complexes hold the potential to regulate cellular processes post-transcriptionally as they specifically target mRNA molecules. There are indeed indications for additional roles of type III complexes apart from virus defense: they are often not genomically linked to CRISPR arrays or adaptation modules, thus relying on co-occurring CRISPR-Cas systems for a canonical immune response, and exhibit high numbers of accessory genes potentially constituting a functional connection to other cellular processes. In this study, the endogenous type I CRISPR-Cas system was harnessed to knock out the type III-B module in *S. solfataricus* in order to discern its putative links to other processes and alternative roles besides immunity. Effects of the genomic deletion of the type III interference module were analyzed using RNAseq. Among the upregulated genes in the absence of the type III-B system, there is an enrichment of genes associated with energy production and conversion, as well as with carbohydrate or amino acid transport and metabolism, such as ABC transporters. Generally, a significant accumulation of genes encoding transmembrane proteins in the upregulated proportion of the transcriptome suggests interconnections between the type III-B CRISPR-Cas system and various membrane-associated processes. Interestingly, the knockout mutant displayed a reduced generation time compared to the wild-type strain when grown on mixed carbon sources. However, when switching to defined carbon sources, the knockout strain would consistently display lengthier adaptation times compared to the wild-type, indicating that the regulatory flexibility is impaired.

114| Transcriptional regulation of type V-K CRISPR-associated transposase systems

Tao Zhu [1]; Yue Shi[1]; Marcus Ziemann[2]; Viktoria Reimann[2]; Yajing Liang[1]; Xuefeng Lu[1]; Wolfgang Hess[2]

[1] Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences; [2] University of Freiburg, Faculty of Biology, Institute of Biology III, Genetics and Experimental Bioinformatics

CRISPR-associated transposases (CASTs) are promising new genomic editing tools which merge the activities of Tn7 transposition with RNA-programmed DNA insertion. These systems exist in different groups of bacteria, including certain cyanobacteria, which contain type V-K CAST systems. Most of these systems harbor tnsB, tnsC, and tniQ transposase genes at the left

end with cas12k, a minimal CRISPR array, and an atypical repeat-spacer at the right end. Cargo genes are sandwiched between these elements, ranging from several to multi-kilobase pairs.

How the activity of these systems is controlled in situ has remained largely unknown. Here we investigated the native expression pattern of the key components of CAST V-K in the cyanobacterium *Anabaena* sp. PCC 7120 (AnCAST)[1]. A novel MerR-type transcriptional regulator encoded by alr3614 was identified. We found both alr3614 and the cas12k coding gene all3613 are translated from leaderless mRNAs. Transcriptional analyses revealed that Alr3614 represses the expression of CRISPR components as well as the T7 transposase genes of AnCAST. We found a number of Alr3614 homologs across cyanobacteria and suggested naming these regulators CvkR for Cas V-K repressors. Further biochemical analyses identified a widely conserved CvkR binding motif 5'-AnnACATnATGTnnT-3'. Crystal structure of CvkR at 1.6 Å resolution reveals that it comprises distinct dimerization and potential effector-binding domains and that it assembles into a homodimer, representing a discrete structural subfamily of MerR regulators.

Ligand recognition under certain cellular trigger(s) is essential for the transcriptional regulation of MerR regulators. Thus, the ligand molecule and the functionality of this hypothetical signaling input sensed by CvkR are matters of further research.

Reference:

1. Ziemann M., et al. CvkR is a MerR-type transcriptional repressor of class 2 type V-K CRISPR-associated transposase systems. Nature Communications 2023, 14:924.

115| Proviruses in *Haloferax volcanii*

Nadia Di Cianni [1]; Jutta Brendel[1]; Lisa-Katharina Maier[1]; Friedhelm Pfeiffer[2]; Mike Dyall-Smith[3]; Thorsten Allers[4]; Björn Voß[5]; Anita Marchfelder[1]

[1] Ulm Universität; [2] Max-Planck-Institute of Biochemistry; [3] University of Melbourne; [4] Nottingham University; [5] University of Stuttgart

According to PhiSpy tool, six proviruses are predicted in *H. volcanii* named as Halfvol1, Halfvol2, Halfvol3, Halfvol4, Halfvol5 and Halfvol6 (Friedhelm, 2018, unpublished). The first four proviruses are located on the main chromosome while the other two on the mini-chromosome pHV4. Halfvol1 and Halfvol3 are annotated as pleolipoviruses. Halfvol1-3 are flanked by a tRNA gene. We were able to generate a deletion strain where Halfvol1-4 were delated as well as single deletion strains of the proviruses.

We demonstrated that Halfvol1, Halfvol2, Halfvol3 can excise from the genome at low temperature (30°C) and that deletion provirus strains shows a different phenotype in comparison to the wild-type.

We examined the properties of the provirus strains using microscopy, swarming assay and biofilm formation. Halfvol3 seems to influence swarming motility and cell morphology of *H. volcanii*, since the ΔHalfvol3 strain shows an elongated cell shape and a defect in swarming

motility in contrasted to the wild type. Biofilm formation at 30°C is decreased for the strain ΔHalfvol1-4, further investigation will aim to attest which provirus generates this phenotype.

116| Prokaryotic Argonautes stimulate homologous recombination

Patrick Barendse; Daan Swarts

Wageningen University

Prokaryotic Argonautes (pAgos) are diverse nucleic acid guided nucleases involved in defense against mobile genetic elements. Recently, a pAgo from *Natronobacterium gregoryi* (NgAgo) has been shown to stimulate homologous recombination between genomic and invading plasmid DNA. On this poster we describe our investigation of pAgo-stimulated homologous recombination.

117| Mechanistic insights into transposon cleavage and integration by TnsB of ShCAST system

T. Zeng [1], J. Yin [1], Z. Liu [1], Z. Li [1], Y. Zhang [1], Y. Lv [1], M. Lu [1], M. Luo [2], M. Chen [1], Y. Xiao [1]

[1] China Pharmaceutical University [2] National University of Singapore (NUS)

The Type V-K CRISPR-associated transposons (CASTs) hold great potential as a programmable tool for site-specific gene insertion, using RNA guidance to achieve DNA integration. While the core components of CAST have been structurally characterized, how the transposase TnsB recognizes donor DNA and the impact of TnsB's association with the AAA+ ATPase TnsC on donor DNA cleavage-integration have yet to be elucidated. Besides, from the application respect whether Cas12k is replaceable in CAST is worth exploring. In this study, we demonstrate that a TniQ-dCas9 fusion can direct the site-specific transposition by TnsB/TnsC in ShCAST. TnsB exhibits a 3'-5' exonuclease function that specifically cleaves the donor DNA at the terminal repeats' end, integrating the left end before the right end with marked nucleotide preference. Additionally, we observed an increase in the association between TnsB and TnsC when they were in a half-integration state. In summary, our findings offer critical insights into the mechanisms of CRISPR-mediated site-specific transposition via TnsB/TnsC and expand the potential range of its applications.

Novel Defense systems

118| The structural basis of hyperpromiscuity in a core combinatorial network of Type II toxin-antitoxin and related phage defence systems

Karin Ernits[1]; Chayan Kumar Saha[1]; Tetiana Brodiazhenko[2]; Aditi Shenoy[3]; Julián Duque-Pedraza[1]; Veda Bojar[1]; Jose Nakamoto[1]; Tatsuaki Kurata[1]; Artyom Egorov[1]; Olena Shyrokova[1]; Marcus Johansson[1]; Toomas Mets[2]; Aytan Rustamova[2]; Jelisaveta Džigurski[2]; Tanel Tenson[2]; Abel Garcia-Pino[4]; Arne Elofsson[3]; Vasili Hauryliuk [1]; Gemma Atkinson[1]

[1] Lund University; [2] University of Tartu; [3] Stockholm Univeristy; [4] Université libre de Bruxelles

Toxin-antitoxin (TA) systems are a large group of small genetic modules found in prokaryotes and their mobile genetic elements. Type II TAs are encoded as bicistronic (two-gene) operons that encode two proteins: a toxin and a neutralising antitoxin. Using our tool NetFlax (standing for Network-FlaGs for toxins and antitoxins) we have performed a large-scale bioinformatic analysis of proteinaceous TAs, revealing interconnected clusters constituting a core network of TA-like gene pairs (1).

To understand the structural basis of toxin neutralisation by antitoxins, we have predicted the structures of 3,419 complexes with AlphaFold2. Together with mutagenesis and functional assays, our structural predictions provide insights into the neutralising mechanism of the hyperpromiscuous Panacea antitoxin domain (2). In antitoxins composed of standalone Panacea, the domain mediates direct toxin neutralisation, while in multidomain antitoxins the neutralisation is mediated by other domains, such as PAD1, Phd-C and ZFD. We hypothesise that Panacea acts as a sensor that regulates TA activation.

We have experimentally validated 16 novel NetFlax TA systems and demonstrated that three of them mediate phage defence. Using functional domain annotations and experimental assays, we uncovered their potential mechanisms of toxicity (e.g. disruption of membrane integrity, inhibition of cell division and abrogation of protein synthesis) as well as biological functions (e.g. antiphage defence). The interactive version of the NetFlax TA network that includes structural predictions can be accessed at http://netflax.webflags.se/.

References:

1. Ernits et al. (2023) The structural basis of hyperpromiscuity in a core combinatorial network of Type II toxin-antitoxin and related phage defence systems. BioRxiv, doi.org/10.1101/2023.03.22.533649

2. Kurata et al. (2022) Panacea: a hyperpromiscuous antitoxin protein domain for the neutralisation of diverse toxin domains. PNAS 119 (6) e210221211

119| Inactive prokaryotic Argonautes confer immunity by activating diverse effectors

Wenyuan Han

Wuhan City, Hubei Province

Argonaute proteins (Agos) bind short nucleic acids as guides and are directed by them to recognize and/or cleave target complementary nucleic acids. More than ~75% of prokaryotic Agos (pAgos) are inactive due to the mutation of the catalytic site, of which the functions have been mysterious for a long time. According to their architecture features and phylogenetic status, these inactive pAgos include long-B pAgos with the canonical four-domain architecture, and short and (pseudo)short pAgos that only have the C-terminal two domains. We previously demonstrated that an archaeal (pseudo)short Ago activates a trans-membrane (TM) effector to mediate membrane depolarization upon sensing viral invasion, which kills the infected cells and suppresses viral propagation. Most recently, we characterized three long-B pAgos that are equipped with different effectors, i.e. nuclease, Sir2-domain-containing protein and TM protein, respectively. In particular, the long-B pAgo-nuclease system senses invading plasmid by RNA-directed target DNA recognition and activates the nuclease for indiscriminate DNA cleavage. This results in genomic DNA degradation and extensive cell death, thus removal of the invader from the cell population. In addition, the long-B pAgo-Sir2 system and long-B pAgo-TM system also trigger cell death via the Sir2 NADase and the TM effector, respectively. Together, out data, as well as the recent findings about the short pAgo-NADase systems, show that inactive pAgos share a conserved mechanism to mediate immune response against mobile genetic elements via abortive infection.

120| Deepdefense: Annotation of immune systems in prokaryotes using DeepLearning

Sven Hauns; Omer Alkhnbashi; Rolf Backofen

University of Freiburg

Due to a constant evolutionary arms race, archaea and bacteria have evolved an abundance and diversity of immune responses to protect themselves against phages.

Since the discovery and application of CRISPR-Cas adaptive immune systems, numerous novel candidates for immune systems have been identified. Previous approaches to identifying these new immune systems rely on HMM-based homolog searches or use labor-intensive and costly wet-lab experiments. To aid in finding and classifying immune systems, we use machine learning to classify already known immune system proteins and discover potential candidates in the genome. Neural networks have shown promising results in classifying and predicting protein functionality in recent years.However, these approaches frequently rely on the assumption of a closed world, which is often different in practice. In this work, we explore neural networks for immune protein classification and deal with different methods for rejecting unrelated proteins in a genome-wide search, and establish a benchmark. Then, we

optimize our approach for accuracy. Based on this, we develop an algorithm called Deepdefense to predict immune cassette classes based on a genome. Deepdefense can automatically detect genes and define the cassette annotation and classification using two model classifications. Finally, we test our approach for detecting immune systems in the genome against an HMM-based method.

121| Phage accessory genes reveal noncanonical functions of restriction-modification systems

Sukrit Silas[1]; Héloïse Carion[1]; Kira Makarova[2]; Eric Laderman[1]; David Sanchez Godinez[1]; Matthew Johnson[1]; Andrea Fossati[3]; Danielle Swaney[3]; Michael Bocek[4]; Eugene Koonin[2]; Joseph Bondy-Denomy[1]

[1] University of California San Francisco; [2] National Institutes of Health; [3] J. David Gladstone Institutes; [4] Twist Biosciences

Facing constant phage and mobile genetic element attack, bacteria have evolved a vast repertoire of defense systems. In turn, phages employ diverse counter-defense mechanisms to counteract bacterial immunity. Many defense strategies are shared broadly across bacteria (such as restriction-modification and CRISPR-Cas), but the overall phage-resistance landscape of any specific bacterial niche likely also features a large contribution from more specialized antiviral mechanisms. Here, we built a platform to characterize bacterial defense systems and phage counter-defenses (both specialist and generalist strategies) in a high-throughput manner. We posit that studying phage-encoded antagonists of bacterial defenses can reveal physiologically relevant immunity pathways in natural bacterial isolates. We selected 200 uncharacterized accessory genes (AGs) through an analysis of thousands of *Enterobacteriophage* genomes and expressed them in 20 wild *E. coli* strains challenged with 8 model phages. A prominent class of AG functions we observed was antagonism of restriction-modification (R-M) systems. The T4 internal head protein II (IpII) was first suspected to target host-immunity 50 years ago. Our screen revealed that IpII inhibits a fused type IV R-M enzyme GmrSD. We also identify a type III R-M system that performs both targeted antiphage defense as well as abortive defense in response to R-M inhibitors. Finally, we find the first example of decoy immunity in bacteria, wherein a type I R-M-derived system has replaced its restriction endonuclease with a toxin that triggers abortive immunity in the presence of R-M inhibitors. Together, these results reveal noncanonical functions of R-M systems and support the utility of phage accessory genes as a tool to probe host biology.

122| Distribution of Prokaryotic Immune Systems

Lisa Olijslager[1]; Daan Swarts

[1] Wageningen University & Research

Despite the rapidly increasing number of prokaryotic immune systems identified and characterised, little is known about what determines their environmental distribution. PADLOC and DefenseFinder are two recently developed bioinformatical tools that facilitate

mining genomic datasets for prokaryotic immune system. In this study, we compare the performance of PADLOC and DefenseFinder in phylogenetically curated databases and applied these tools to investigate if there are traits linked to the environmental distribution of immune systems.

123| Engineering of prokaryotic Argonautes to improve their double stranded DNA cleavage efficiency

Isabelle Anna Zink[1], Youri Peeters[1], Jan Fiala[2,3], Albert J.R. Heck[2,3] and John van der Oost[1]

[1]Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, Wageningen, the Netherlands; [2]Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands; [3]Netherlands Proteomics Center, Padualaan 8, 3584 CH Utrecht, The Netherlands.

Active prokaryotic Argonautes (pAgos) are nucleases that recognize target DNA through specific base pairing by a short DNA guide. Unlike CRISPR-Cas, pAgos have the advantage of being able to target any desired sequence without requiring a recognition motif on the target sequence adjacent to the guide-complementary region. However, pAgos are inefficient at cleaving double stranded DNA (dsDNA) targets with high GC content (> 40% GC) in vitro because they lack helicase activity. In vivo, the unwinding of pAgo target sites may be enabled by host proteins. In this study, we characterize native interaction partners of archaeal pAgos and use them to engineer archaeal and bacterial pAgos towards more efficient cleavage of high GC-dsDNA targets. Pull-downs of the Argonaute from the hyperthermophilic archaeon *Pyrococcus furiosus* (PfAgo) identified DNA binding proteins, such as an OB-fold (oligonucleotide/oligosaccharide) containing protein, as native interaction partners. Supply of the purified Pf-OB-fold partner protein - and even homologs from other thermophilic archaeal species – to PfAgo was found to improve the cleavage efficiency of high GC-dsDNA targets at 77°C. The same was observed when mixing archaeal OB-fold proteins with the bacterial CbAgo (Clostridium butyricum) operating at 37°C. Based on these findings, we generated a series of synthetic fusion enzymes consisting of an archaeal OB-fold protein and either PfAgo or CbAgo. All the fusion proteins tested were more efficient in cleaving plasmid DNA or linear dsDNA targets than the respective pAgos alone and were able to target high GC targets (> 52% GC tested). We here present standalone pAgo enzymes engineered to more efficiently cleave high GC content dsDNA. We believe that these proteins hold great promise as tools for various in vitro and in vivo applications, including genome engineering in mesophilic and thermophilic organisms.

124| Tandem mobilization of anti-phage defenses alongside SCCmec cassettes

Motaher Hossain; Barbaros Aslan; Asma Hatoum-Aslan

University of Illinois at Urbana-Champaign

Bacterial viruses (phages) and the immune systems targeted against them significantly impact bacterial survival, evolution, and the emergence of pathogenic strains. While recent research has made spectacular strides towards discovering and validating new defenses in a few model organisms, the inventory of immune systems in clinically-relevant bacteria remains underexplored, and little is known about the mechanisms by which these systems horizontally spread. Such pathways not only impact the evolutionary trajectory of bacterial pathogens, but also threaten to undermine the effectiveness of phage-based therapeutics. Here, we investigate the battery of defenses in *staphylococci*, opportunistic pathogens that constitute leading causes of antibiotic-resistant infections. We show that these organisms harbor a variety of anti-phage defenses encoded within/near the infamous SCC (staphylococcal cassette chromosome) mec cassettes, mobile genomic islands that confer methicillin resistance. Moreover, we demonstrate that SCCmec-encoded recombinases mobilize not only SCCmec, but also tandem cassettes enriched with diverse defenses. Further, we show that phage infection potentiates cassette mobilization. Our collective findings reveal that beyond spreading antibiotic resistance, SCCmec cassettes play a central role in disseminating anti-phage defenses. This work underscores the urgent need for developing adjunctive treatments that target this pathway to preserve the effective lifetime of the burgeoning phage therapeutics.

125| HRAMP – The mysterious CRISPR variant of Haloarchaea

Jan Kohnle[1]; Pascal Märkle[1]; Julia Wörtz[1]; Sabine König[2]; Christopher-Nils Mais[3]; Gert Bange[3]; Henning Urlaub[2]; Anita Marchfelder[1]

[1] Ulm University; [2] Max Planck Institute for Biophysical Chemistry, Göttingen, Germany; [3] LOEWE Center for Synthetic Microbiology (SYNMIKRO), Philipps-Universität Marburg

The number and forms of CRISPR-Cas variants are rapidly increasing - some just a reverberation of the original. One of these new forms is a haloarchaea-specific CRISPR-Cas variant, called halobacterial repeat-associated mysterious protein (HRAMP) system, which was identified by Makarova et al.1 This system is derived from class 1 CRISPR Cas systems and is present in many but not all haloarchaea. The HRAMP system occurs in different forms but always consists of the three core genes cas7-like, casX and cas5-like and can furthermore be present in combination with different nucleases (DEDDy & HNH). Neither CRISPR arrays nor adaptation proteins can be found in this system. HRAMP can occur as a stand-alone (*Natrialba magadii*) or in combination with other type I systems like type I-B/I-D (*Halorubrum lacusprofundi*). Given the similarity of the core genes to type I Cas proteins and the presence of nucleases, a defense-related role of the system is highly likely. However, the exact function of HRAMP remains to be determined.

We are studying the HRAMP system of *H. lacusprofundi* to explore its role in viral defence or beyond. We want to clarify the role of the core proteins Cas7-like and Cas5-like. Both have been shown to co-purify with RNA partners and RNAseq analysis reveals binding of CRISPR-Cas I-D crRNAs to Cas5-like. This points to a shared crRNA pool and thus interplay of defence systems in *H. lacusprofundi*.

Complex formation by the HRAMP core proteins is under investigation using Cas7-like epitope fusions. Overproduction of the core proteins and pull-down of tagged Cas7-like results in a high molecular weight fraction containing Cas7-like proteins alongside other binding partners. Nature and function of these interaction partners are currently under investigation.

1. Makarova et al., 2019, FEMS Microbiology Letters 366, fnz079

126| Elucidating the basis for phage specificity in the Nhi defense system

Tori Boyle[1]; Asma Hatoum-Aslan [2]

[1] University of Illinois at Urbana Champaign; [2] University of Illinois at Urbana-Champaign

Staphylococci are common skin-dwelling commensals that are also leading causes of hospital-acquired infections. Bacterial viruses (phages) have profound impacts on the survival and pathogenic potential of staphylococci, which highlights the critical need to explore the immune mechanisms that staphylococci utilize to overcome viral infections. Recently, we identified an enzyme that prevents phage DNA accumulation through nuclease and helicase activities, which we refer to as Nhi (Nuclease-Helicase Immunity). Nhi restricts members from all three morphological families of staphylococcal phages, but the basis for its specificity to phage-derived DNA remains unknown. Here, we investigate the molecular requirement(s) for Nhi recognition and targeting of phages. Based on previous work, we hypothesize that phage single-stranded DNA binding proteins comprise the recognition element for Nhi defense. We test this hypothesis using in vivo genetics and in vitro biochemical approaches. Since Nhi homologs are distributed across three bacterial phyla, these efforts will inform studies on diverse phage-host relationships.

127| Record-seq - Noninvasive assessment of gut function using transcriptional recording sentinel cells

Florian Schmidt[1], Jakob Zimmermann[2], Tanmay Tanna [1,3], Rick Farouni [1], Tyrrell Conway [4], Andrew J. Macpherson [2,5] & Randall J. Platt [1,5,6]

[1]Department of Biosystems Science and Engineering, ETH Zurich, Mattenstrasse 26, 4058 Basel, Switzerland. [2]Department of Visceral Surgery and Medicine, Inselspital, Bern University Hospital, University of Bern, Switzerland. [3]Department of Computer Science, ETH Zurich, Universitätstrasse 6, 8092 Zurich, Switzerland. [4]Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK 74078, USA. [5]Botnar Research Center for Child Health, Mattenstrasse 24a, 4058 Basel, Switzerland. [6]Department of Chemistry, University of Basel, Petersplatz 1, 4003 Basel, Switzerland.

Bacteria within the gut continuously adapt their gene expression to environmental conditions that are associated with diet, health, and disease. Noninvasive measurements of bacterial gene expression patterns throughout the intestine are important to understand in vivo microbiota physiology and pathophysiology. Current methods do not offer sufficient information about transient or proximal events within the intestine without using indirect or invasive approaches that disturb normal physiology and are inapplicable to clinical practice.

Transcriptional recording by CRISPR spacer acquisition from RNA (Record-seq) enables engineered bacteria to continuously record the history of gene expression in a population of bacteria. Over time, snippets of intracellular RNA are converted into DNA and integrated as a historical record of spacer sequences within CRISPR arrays through the action of an integration complex that contains a reverse transcriptase Cas1 fusion protein (RT-Cas1) and Cas2. Here, using a refined Record-seq methodology, we used transcriptional recording Escherichia coli sentinel cells to reveal intestinal and microbiota physiology under different dietary and disease contexts along the length of the unmanipulated mouse intestine. Microbial sentinels that traverse the gastrointestinal tract capture a wide range of genes and pathways that describe interactions with the host, including quantitative shifts in the molecular environment that result from alterations in the host diet, induced inflammation, and microbiome complexity. We demonstrate multiplexed recording using barcoded CRISPR arrays, enabling the reconstruction of transcriptional histories of isogenic bacterial strains in vivo. Record-seq therefore provides a scalable, noninvasive platform for interrogating intestinal and microbial physiology throughout the length of the intestine without manipulations to host physiology and can determine how single microbial genetic differences alter the way in which the microbe adapts to the host intestinal environment.

128| Functional mechanism of the Craspase substrate Csx30

Babatunde Ekundayo, Dongchun Ni, Henning Stahlberg

Laboratory of Biological Electron Microscopy, IPHYS, SB, EPFL, and Dept. Fundamental Microbiology, Faculty of Biology and Medicine, UNIL, Cubotron, Rt. de la Sorge, 1015 Lausanne, Switzerland.

The recently discovered member of the CRISPR toolbox, Craspase, is a ribonucleoprotein complex composed of Cas7-11, crRNA and TPR-CHAT. These components assemble into a multifunctional complex capable of target RNA sensing and cleavage. Target RNA sensing activates the protease activity of TPR-CHAT, which cleaves its target substrate protein Csx30 to cause cell toxicity. However, the mechanism by which Csx30 mediates cell toxicity remains unknown. Here we combine biochemical and biophysical approaches to dissect the functional mechanism of Csx30 as the Craspase substrate and thus revealing its role in cell toxicity and abortive immunity.

129| Adeno associated virus (AAV) and CRISPR-Cas9 targeted inhibition of iBALT target genes to prevent COPD progression

Shruthi Kalgudde Gopal[1], Chahat Suri, Aicha Jeridi[1], Thomas M Conlon[1], Ali Önder Yildirim[1]

Institute of Lung Health and Immunity (LHI), Comprehensive Pneumology Center (CPC), Helmholtz Munich, [1]Member of the German Center for Lung Research (DZL).

One of the hallmarks of COPD progression is the formation of iBALT (induced bronchus associated lymphoid tissue). iBALT is an organized tertiary lymphoid structure that develops in response to infection or under chronic inflammatory conditions (Conlon M T, Nature 2020). A recent emerging concept is the role of oxysterols (downstream cholesterol metabolites), in particular 25-hydroxycholesterol (25-HC) and 7α, 25-dihydroxycholesterol (7α, 25-HC), as important regulators of immune function. In our translational study in EMBO Molecular Medicine (Jia et al. 2018) COPD patients and CS-exposed mice demonstrated two important oxysterol enzymes to be significantly upregulated and localized in airway epithelial cells, regulating CS-induced iBALT formation. To further support a pathogenic role in COPD, we clearly demonstrated mice deficient in target genes or EBI2 the receptor for 7α, 25-OHC, exhibited protection from CS-induced COPD. We therefore propose that developing a, therapeutic to target the Oxysterol enzymes will be crucial to block iBALT and mitigate COPD progression.

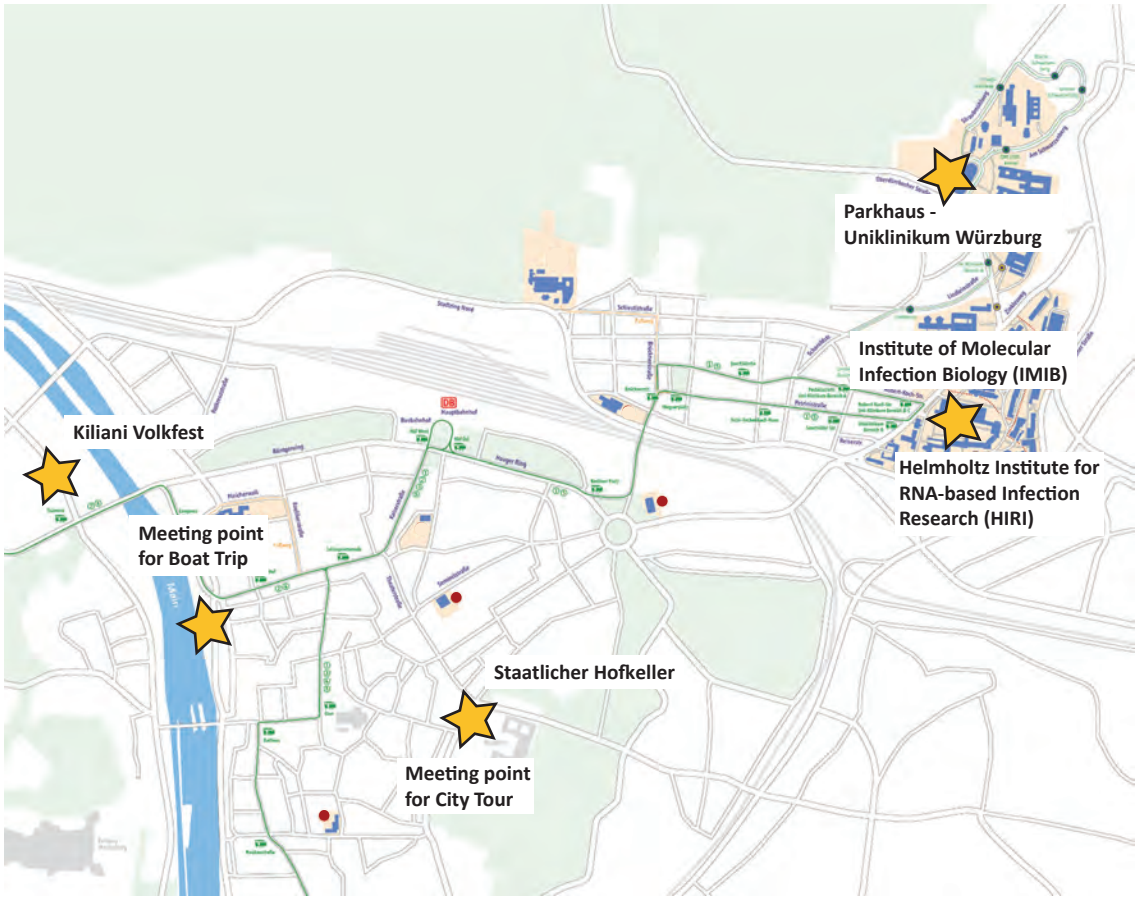
In order to overcome the risks, challenges and outcomes involved in a global gene knock out system and the systemic use of immune modulating agents, we are developing an approach to locally inhibit target expression in lung tissue. AAV based therapeutics have recently emerged as the safest and most effective gene delivery tools to drive long-term transgene expression in gene therapy. Here, we are combining the Adeno associated virus and CRISPR-Cas9 based therapeutic gene-editing approach in our CS-induced murine model of COPD to inhibit the expression of Oxysterol enzyme, and thereby reduce iBALT formation and prevent COPD development.

130| An unusual constellation of Cas4 nucleases in a new archaeal isolate.

Sefkan Polat, Richard Stöckl, Robert Reichelt, Dina Grohmann

Department of Microbiology and Archaea Centre, University of Regensburg, Germany.

"Clustered regularly interspaced short palindromic repeats (CRISPR) refers to DNA sequences found in the genomes of prokaryotic organisms. These sequences (spacers) are derived from DNA fragments of mobile genetic elements (MGEs), which the organism encountered. Multiple CRISPR associated (Cas) proteins are involved in the acquisition of spacers and the subsequent immune response against MGEs. Spacer acquisition is enabled by Cas1 and Cas2 proteins. However, some organisms harbour an additional protein called Cas4. Though the function of Cas4 is not elucidated yet entirely, Cas4 proteins are suggested to be involved in pre-spacer processing events for spacer acquisition. In many cases, Cas4 genes are encoded adjacent to Cas1 or even fused to the Cas1 protein. Cas4 proteins contain two conserved motifs: a RecB-like nuclease domain and four conserved Cysteine residues, coordinating an Fe-S cluster. It was shown that Cas4 proteins have endonuclease and/or exonuclease activity cleaving ssDNA in 5’ to 3’ and 3’ to 5’ direction. Sometimes, Cas4 is encoded outside the CRISPR locus. Notably, in some bacteria and archaea no CRISPR-Cas system but a single Cas4 protein can be found in the genome. In this case, they are referred to as solo-Cas4. Further, Cas4 is encoded in viruses as well and are referred to as viral-Cas4. Solo- and viral-Cas4 proteins often have the nuclease domain and Fe-S cluster found in CRISPR-associated Cas4 proteins. Here, we performed an initial study of a solo- and viral-Cas4 variant from the archaeal strain Mex13E-Lk6-19. This hyperthermophilic archaeon has recently been isolated and characterized at the Archaea Centre Regensburg. The functional role of solo-Cas4 and viral-Cas4 has not been elucidated yet."



Institute of Molecular Infection Biology (IMIB)
Helmholtz Institute for RNA-based Infection Research (HIRI)
Address: Josef-Schneider-Straße 2/D15, 97080 Würzburg
IMIB secretary office: +49 931 31 82441
HIRI secretary office: +49 931 31 83321

Parkhaus - Uniklinikum Würzburg
Address: Oberdürrbacher Straße, 97080 Würzburg

Meeting point for Boat Trip
Ship Tourism Georg Schiebe and Steffen Kurth
Address: Kranenkai 2, 97070 Würzburg

Conference Dinner with wine tasting
Staatlicher Hofkeller
Address: Residenzplatz 3, 97070 Würzburg

Meeting point for City Tour
Franconia Fountain (Frankoniabrunnen)
Address: Residenzplatz 2A, 97070 Würzburg

Kiliani Volkfest
Address: Talaveraplatz, 97082 Würzburg

CRISPR 2023

27 June - 1 July 2023

Würzburg, Germany