196 - An NGS method for qualifying sequence impurities in therapeutic CRISPR sgRNAs

Kevin Holden[1]

[1]Synthego

Advances in the manufacture of chemically synthesized single-guide RNAs (sgRNAs) have made CRISPR a promising technology for use in gene therapy. As a clinical product, evaluating sgRNA reagent purity is of great importance for patient safety. Traditionally, analytical chemistry methods such as HPLC (high performance liquid chromatography) have been used to interrogate the purity of these chemically synthesized oligos; however, these methods do not provide any information about the sgRNA sequence. Due to process impurities, the presence of alternative sgRNA species in the sample could lead to deleterious patient effects. NGS (next-generation sequencing) technology has reshaped diagnostic testing and made in-roads in qualifying the purity of other clinical grade RNA products such as mRNA vaccines. Here, we describe an NGS-based method that provides sequence level information of sgRNA samples, including the detection of low level alternative sgRNA species. Extensive optimization has been performed to reduce background analytical artifacts that can confound signal. The method we describe can detect an alternative sgRNA species at a limit of 0.1% in a background of wild-type guide. This method can be used as an orthogonal purity metric to traditional HPLC while providing additional critical information regarding sgRNA sequence.

194 - Overview of the NIST Genome Editing Program

Samantha Maragh[1], NIST Genome Editing Program[1]

[1]NIST

The NIST Genome Editing Program (GEP) develops standards, methods, tools, technology, and community norms to advance the reliability of genome editing technology and foster confidence in measurements for the genome editing field. The NIST GEP actively supports this growing industry by: **1. Evaluating** measurement challenges related to implementing genome editing systems and understanding genome editing outcomes, **2. Qualifying** analytical methods being used to detect and assess genome editing outcomes, **3. Developing** new methods and standards to support confidence in detecting, interpreting, and reporting about genome editing outcomes. NIST GEP is organized into 3 focus areas: Physical Measurements, Data & Metadata and Documentary Norms & Standards. Active project areas include: qualification of assays for detecting genome wide off-target activity of genome editing molecules, single-cell manipulation and measurement, assays/control materials for assessing genome editing in animal biotechnology products, and the **NIST Genome Editing Consortium** (GEC) a public-private partnership with over 50 genome editing stakeholders to *define* measurement challenges for utilizing existing measurement capabilities to understand genome editing outcomes and *develop* shared solutions. The NIST GEC is now reporting results from a first interlab study evaluating DNA detection technologies used to report on- and off-target genome editing, developing metadata reporting norms, and has developed the first international standard for genome editing: *ISO 5058-1:2021 Biotechnology — Genome editing — Part 1: Vocabulary.*

193 - Polyplex-based CRISPR/Cas9 In Vivo Mutagenesis for Efficient Lung Cancer Modeling in Rodents

Irene Lara-Sáez[1], Ángeles Mencía[2], Enrique Recuero[2], Yinghao Li[1], Marta Garcia[3], Marta Oteo[4], Marta I Gallego[5], Ana Belén Enguita[6], Diana de Prado-Verdún[2], Sigen A[1], Wenxin Wang[1], Ramón Garcia-Escudero[2], Mirentxu Santos[2]

[1]University College Dublin, [2]Biomedical Innovation Unit, CIEMAT, [3]Biomedical Engineering Dpt, UC3M, [4]Biomedical Applications Unit, CIEMAT, [5]Unidad de Histologia,UCCTs, ISCIII, [6]Pathology Dpt, Hospital 12 Octubre

In recent years comprehensive genomic studies of human tumors have revealed thousands of genetic mutations linked with cancer. We have to find out a way to sift through this massive information to assess the role of these cancer-gene candidates. In this scenario, conventional transgenic technologies are extremely laborious with costly and exceedingly long model generation times. Here, we suggest a platform for lung mutagenesis using CRISPR/Cas9 RNPs that enables the introduction of a group of mutations in genes chosen for their potential to cause tumors into lung tissue. We use synthetic biomaterials for the delivery of the CRISPR/Cas9 reagents to adult lung epithelial cells.

First, we have evaluated a family of poly(β-aminoesters (PBAE) polymers for their capability to serve as carriers of CRISPR RNPs in the respiratory airways using a TdTomato reporter system and identified the cell types targeted as basal cells from trachea and bronchi; and Clara/club, neuroendocrine and alveolar type 1and 2 cells from lungs. Then, we have validated the effectiveness of this system by targeting a group of tumor suppressor genes, specifically *Rb1*, *Rbl1*, *Pten*, and *Trp53*, chosen for their potential to cause lung tumors, namely Small Cell Lung Carcinoma (SCLC). The disruption of these genes by means of in vivo gene editing leads to the development of SCLC. The SCLC obtained and their metastases show the same histologic, inmunohistochemical and genomic characteristics of the SCLC arisen in GEMMs models and human patients.

In conclusion, our CRISPR/Cas9 RNP delivery system utilizing cationic polymers offers an efficient approach for modeling lung tumorigenesis by simultaneously inactivating a set of tumor suppressor genes, in a manner independent of the mouse genetic background and using readily available reagents that do not require mouse germline manipulation or custom viral vector production.

This innovative strategy holds promise for faster and more cost-effective cancer modeling.

192 - Non-viral DNA Payloads for Gene and Cell Therapy-Total Solutions by GenScript

Fan Zhou[1], Lumeng Ye[2], Barbara Herr[3], Scott Pritchett[4]

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DNA payload plays a key role in gene editing and related gene and cell therapeutics development. Our team at GenScript has developed a comprehensive line of non-viral DNA template formats, each with unique advantages for different applications: GenExact™ single-stranded (ss)DNA, GenWand™ linear double-stranded (ds)DNA, and GenCircle™ circular double-stranded (ds)DNA. These tools have been designed to meet the diverse needs of modern genetic research. Through our poster, we share data from both internal tests and collaborators'

applications, which present superior knock-in efficiency and accuracy compared to conventional PCR products or plasmids. In addition to increased efficiency, safety is also improved by minimizing redundant sequences and backbones of DNA payloads. GenScript supports end-to-end GCT development from discovery to preclinical, clinical, and commercialization stages with high-quality RUO to cGMP services and products. Other useful tools such as GenCRISPR™ Cas9 enzymes, synthetic sgRNA, and our HDR knock-in design tool are also available to make your research easier and more efficient.

191 - High Efficiency CRISPR/Cas9 Cell Engineering with MaxCyte Electroporation

Andrew Mancini[1]

[1]MaxCyte

Genome engineering of primary human cells and iPSCs holds great promise for the treatment of diseases including cancer or genetic disorders. However, viral cell engineering has several limitations, including high cost, lengthy manufacturing processes and the risk associated with viral integration into the host genome. Therefore, a non-viral genome editing approach is gaining traction from basic to clinical research. MaxCyte® electroporation efficiently delivers genome editing tools in the form of DNA, mRNA and RNP into a wide variety of primary cells and stem cells. Here, we demonstrate high editing efficiency using MaxCyte electroporation to deliver CRISPR-Cas9 gene editing machinery into primary human keratinocytes, iPSCs, NK cells and activated T cells for knock-out and knock-in applications while maintaining high cell viability.

189 - Patient-derived Organoids to identify novel combination strategies using CRISPR-based Functional Genomics

Dario De Felice[1], Simon Vyse[1], Lambert Montava Garriga[1], Evangelia Kounatidou[1], Amna Shah[1], Ioritz Sorzabal Bellido[1], Chang Kim[1], Salome Adam[1], Grainne Gernon[1], Yinhai Wang[1], Laura Rosenberg[1], Luigi Aloia[1]

[1]AstraZeneca

Clinical translatability gap is one of the biggest hurdles in drug development. Despite giant improvements in pharmacokinetics and safety of new targets, potential drug candidates commonly fail in clinical validation due to lack of efficacy. Often the cause lies in wrong target selection as targets are identified, and further validated, using immortalized 2D cell lines, underestimating and overshadowing the relevance of the extracellular matrix and a 3D environment.Patient-derived organoids have emerged as a potential game-changer for drug discovery. As 3D organotypic cell structures, they retain organ architecture as well as genomic and transcriptional profiles of the tissue-of-origin, providing a more physiologically relevant platform to interrogate gene function and cellular responses to genetic and drug perturbations.Within AstraZeneca, the Functional Genomics department focuses on discovering and validating new putative disease targets for the development of safe and effective medicines leveraging our expertise in high-content CRISPR screenings.In the Advanced Cell Model team, we perform arrayed CRISPR screenings in 3D organoid models, coupled with the use of high-throughput workflows relying on automation (e.g. liquid handling platforms, high-content imaging and flow cytometer) as well as 3D culture-specific microwell plates and AI-based imaging analysis.The efforts to develop a cost- and time-efficient CRISPR screening approach using

patient-derived 3D organoids instead of the “gold-standard” 2D cell culture model, will enable us to pinpoint genes of interest with better likelihood for therapeutic potential, enhancing the accuracy of preclinical testing and, ultimately, reducing the translatability gap between the lab and the clinic.

188 - OligoSeq: NextGen Im-/Purity Profiling - Oligo Characterization by Next Generation Sequencing

Barbara Karolina Pfaff[1], Julius Buss[1], Ruven Jilly[1]

[1]BioSpring

The demand for high-quality synthetic oligonucleotides in therapeutics is rising, especially for applications like CRISPR/Cas. Verifying their quality requires precise analysis, particularly for longer sequences. Next Generation Sequencing (NGS) is a powerful tool for this, allowing not only identity confirmation but also characterization of impurities based on sequence. BioSpring and ecSeq Bioinformatics GmbH collaborated to develop an NGS method for oligonucleotides, including a tool called “Oligo Impurity Profiling Analysis”, which classifies composition and variants in a sample. Customized pipelines were developed to address data-specific challenges. Results are presented in an interactive HTML format, showing sequence variants and their relevance. Our poster showcases the library preparation workflow for 100mer NGS standard and the tool's analysis, providing a detailed view of oligo purity.

186 - GeneAbacusTM: A Novel PCR-free Assay for Gene Editing Validation

Iván Hernández[1]

[1]Countagen

Confirmation of successful gene targeting is a critical step for any CRISPR-based gene editing experiment. Methods traditionally used for this purpose are PCR-based which often requires lengthy and tedious optimization to achieve actionable results.

GeneAbacus is a digital quantification assay for genetic targeting validation using padlock probes and Rolling Circle Amplification (RCA) chemistry. Padlock probes recognize and differentiate edited and wild type sequences from a DNA sample with single base specificity. Single molecule amplification by RCA eliminates sequence bias and generates discrete signals that enable digital quantification using standard epifluorescence microscopy. The total workflow including analysis is completed within 5 hours and it is now packaged in a reagent kit commercialized by Countagen.

We performed a blind benchmarking of GeneAbacus against ddPCR and Amplicon Sequencing as a gold-standard with a set of 26 CRISPR/Cas9 edited one-cell stage mouse embryos. GeneAbacus resulted in a high correlation (R2=0.9485, n=26) against Amplicon Sequencing, in contrast to ddPCR which showed poor concordance (R2=0.5339, n=15). To further challenge the robustness of GeneAbacus to sequence context, we analyzed samples with gene editing targets of 66-84% GC content. GeneAbacus also scored high concordance with reference methods (R2=0.9955, n=10) without any workflow optimization required.

These results showcase the capability of GeneAbacus to perform robust and accurate genetic targeting validation in only 5 hours without optimization to enable streamlined gene editing discovery workflows.

185 - Pioneering Precision Medicines Using Microbial CRISPR Gene Therapy

Jonas Hink[1]

[1]SNIPR Biome

184 - CRISPR-Cas9 genome editing system for functional genetic screening in iPSCs

Nadya Isachenko[1], Dongfang Hu[1], Alex Chenchik[1], Paul Diehl[1], Donato Tedesco[1]

[1]Cellecta, Inc.

Induced pluripotent stem cells (iPSCs) are important in disease modeling, drug discovery, and the development of cell therapies, yet integrating an effective CRISPR/Cas9 system for functional genetic screening using them poses challenges. In this study, we utilized WTC11 human iPSCs to generate functionally validated, lentiviral-transduced Cas9-expressing lines. These lines exhibit high gene-editing activity and retain their capacity to differentiate. We employed flow cytometry to verify the presence of pluripotency markers Oct3/4, TRA1-60, and SSEA-4 in our iPSC-Cas9 cells, and further validated their pluripotency through their differentiation into ectoderm, mesoderm, and endoderm - the three germ layers. Additionally, we conducted a genome-wide transcriptome analysis to validate the expression of markers specific to each germ layer in the differentiated cells, confirming sustained Cas9 activity post-differentiation. This research demonstrates that iPSCs derived from patients can effectively be used in CRISPR/Cas9-based functional genetic screening in reconstituted patient-specific disease models or tissues.

183 - CRISPR/saCas9 and CRISPR/spCas9 systems for combiatorial genetic screens (CRISPR-KO, CRISPRa, CRISPRi)

Nadya Isachenko[1], Gayane Aleksanyan[1], Paul Diehl[1], Donato Tedesco[1]

[1]Cellecta, Inc.

This study explores the utilization of orthogonal CRISPR-based gene editing/modulation systems for combinatorial genetic screens using CRISPR knockout (CRISPR-KO), CRISPR activation (CRISPRa), and CRISPR interference (CRISPRi) functionalities. *S. aureus* (sa)Cas9 is an alternative nuclease to *S. pyogenes* (sp)Cas9 in scenarios where the latter cannot be used, or when multiple independent CRISPR systems need to be simultaneously expressed in the same cell. We set out to explore the feasibility of utilizing different combinations of saCas9 and spCas9 CRISPR systems to achieve the simultaneous inactivation (via CRISPR-KO or CRISPRi) and transactivation (via CRISPRa) of different target genes in the same host cell. For this purpose, a complete set of tools for CRISPR/saCas9 gene editing and gene modulation was developed, compatible with CRISPR/spCas9 coexpression. Specifically, we developed and validated optimized saCas9 and sg(sa)RNA lentiviral and AAV vectors, dual expression (sp)/(sa)sgRNA lentiviral library vectors, as well as (sa) CRISPR-KO, (sa)CRISPRi, (sa)CRISPRa fluorescence-based activity kits for the functional validation of saCas9 expressing cell lines. Results demonstrating the feasibility of the orthogonal screens will be presented, with different combinations of CRISPR-KO, CRISPRa, and CRISPRi

systems in multiple cell lines.

182 - A New Approach to Tackle Cancer Using Programmable Cytotoxic Nucleases

Michael Krohn[1]

[1]Akribion Genomics

Since the discovery of the CRISPR-Cas9 editing technology, numerous new Cas variants have been identified with the ambition to create alternatives or superior treatment approaches for various diseases including cancer. However, one major hurdle to overcome is to enable highly selective killing of cancer cells without affecting surrounding healthy tissue. Akribion Genomics set out to develop a proprietary CRISPR nuclease termed G-dase E, capable of RNA-triggered DNA and RNA destruction. G-dase E can elicit programmable and sequence-specific elimination of cancer cells expressing a target transcript without affecting the viability of the surrounding (non-target) cells. In order to demonstrate target specific depletion of human cells G-dase E RNP complexes are maturated with target-specific gRNAs and applied in mixed cell culture to specifically deplete the targeted cells e.g. cancer cells that express a 'cancerous' RNA Biomarker. In summary, we provide first evidence that G-dase E can be programmed to induce selective cell ablation 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 an innovative and novel therapeutic tool. Akribion's vision is to establish the G-dase E technology as an effective treatment platform in Oncology for amenable cancer patients with the vision to create a better quality of life.

181 - EMA's Experience & Support to the Development of Medicinal Products Using Genome Editing

Veronika Jekerle[1]

[1]European Medicines Agency (EMA)

Genome editing (GE) is a promising technology in the development of medicinal products. Clinical development mostly targets monogenetic diseases by introducing or removing associated mutations. The first CRISPR/Cas9 technology-based medicinal product, Casgevy (exagamglogene autotemcel), has recently been authorised in the EU and many more genome editing medicinal products (GEMPs) are in development. However, GE remains an emerging field with very limited scientific guidance available to developers to fit these complex products into the existing regulatory framework. This presentation is aimed at outlining the main regulatory and scientific challenges that developers encounter and what guidance and support pathways EMA offers to assist in bringing genome editing products to the benefit of patients.

180 - CRISPR Excellence Unleashed: Custom Nuclease Production Services to Propel Your GCT Research Forward!

Zhengzhi Li[1], Ada Guan[1], Grace Jiang[1], Sen Yang[1], Jeffery Shi[1], Kay Chuang[1]

[1]Biologics Department, GenScript USA Inc.

GenScript Biotech, a leading Contract Research Organization, has significantly advanced the field of gene editing by specializing in the production of CRISPR nucleases, including Cas variants, prime editing nucleases (PE), and base editing nucleases (BE) derived from E. coli. The adoption

of CRISPR-Cas9 technology has revolutionized gene editing, offering enhanced efficiency, accuracy, and reduced time consumption. GenScript's foray into CRISPR protein production leverages its customized enzyme production platform, yielding a wealth of expertise in CRISPR nucleases. The company has optimized the expression of CRISPR nucleases in E. coli through innovative vector design and employs mature purification processes that adhere to strict quality standards. GenScript's dedicated research platform allows for the swift assessment of CRISPR nuclease activity via in vitro cleavage assays, which is crucial for high-throughput expression screening of Cas variants. Additionally, GenScript provides comprehensive services, including the development of production processes and quality control measures that align with GMP standards. As a result, GenScript Biotech emerges as a pivotal collaborator in the progression of CRISPR technology for both research and therapeutic applications.

179 - Software Tools to Enable CRISPR Therapeutics Discovery & Design

Pawan Patel[1]

[1]Benchling

Software can play an important role in enabling CRISPR therapeutics research by enabling scientists with tools for in silico genome editing, experimental design and execution. In this presentation we will cover how software tools can specifically help with design and target identification. In this case software can be critical in supporting workflows via design of guide RNAs, cloning strategies and experimental planning in collaboration with multidisciplinary teams.

177 - CRISPR cell based functional assays for supporting pre-clinical drug target development and improved patient stratification

Arne Nedergaard Kousholt[1]

[1]VUS Diagnostics

For cancers, some gene variants found in tumours are clearly harmful and can be targeted with drugs, such as deletions in the tumour suppressors BRCA1 and BRCA2 that result in sensitivity to PARP inhibitors. However, for the majority of gene variants observed in patients, the clinical relevance and the functional impact of the gene variants are hard to predict. Patients with such gene Variants of Unknown clinical Significance (VUS) are often not eligible for associated biomarker-targeted drugs. Moreover, for introducing new drug targets in the clinical, drug resistance in heavily pre-treated patients is a huge challenge. In this setting, even well-established loss of function BRCA1 and BRCA2 gene variants can be VUS variants for the drug response. This makes it difficult to decide which patients to include for the clinical trials. As an example, a patient with an acquired deletion in BRCA1/2 might cause the expression of a truncated protein variant, and the question is whether the patient will benefit from PARPi treatment. To overcome the challenge of VUS variants and significantly help improve patient outcomes, we build cell models representing gene-specific patient subgroups and test these models in high throughput functional assays, thereby supporting pre-clinical drug target development and improving patient stratification. At our poster we will discuss the science behind our approach for supporting improved patient stratification and provide a few academic examples of assays developed by our founding team of scientists.

176 - Advances and Challenges of Next-Generation CRISPR Gene-Editing Technology for Drug Development

Alvin Luk[1,2] and Hui Yang[1,3]

[1]HuidaGene Therapeutics, Inc.

[1] HuidaGene Therapeutics Co., Ltd., Shanghai, China.
[2] Cholgene Therapeutics, Inc. (affiliate of HuidaGene), New Jersey, USA.
[3] Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China.

The CRISPR system can be used in disease models to precisely edit and manipulate genomes and to control the expression levels of genes, offering the potential to cure diseases as never before. Scientific aspects of employing CRISPR technology for gene-editing therapeutic applications will be discussed.

At HuidaGene, we developed CRISPR/Cas13 RNA-targeting therapy, HG202, to specifically suppress the expression of VEGF-A in the retina for age-related macular degeneration (AMD). Furthermore, we conducted the clinical study (NCT06031727) evaluating HG202 in AMD patients who are either responsive or have developed tachyphylaxis and treatment-resistant to anti-VEGF therapies. In addition, we also developed hfCas13Y-g*MECP2*, HG204, targeting *MECP2* duplication syndrome (MDS), a rare and fatal childhood neurodevelopment disorder characterized by duplication of the *MECP2* genes. Our humanized MDS transgenic mice data suggest efficient knockdown of the MECP2 expression and reversal to the normal level after the intracerebroventricular injection. Importantly, MDS mice injected with HG204 had a notable increase in median lifespan compared to littermates injected with PBS.

For the DNA editing, we engineered the natural Cas12i variant to develop the high-fidelity CRISPR-Cas12 (hfCas12Max) with high editing efficiency and specificity. We use a single adeno-associated virus (AAV) carrying hfCas12Max and gRNA, HG302, targeting the human dystrophin gene to treat Duchenne muscular dystrophy (DMD). Our *in-vivo* results show that HG302 efficiently restores dystrophin expression in the heart, diaphragm, and tibialis anterior and rescues motor functions in DMD mice.

The HG204 and HG302 programs have granted both orphan drug and rare pediatric disease designations by the US FDA.

175 - Epigenome Editing for the Effective Treatment of HBV

Brian Cosgrove[1]

[1]Tune Therapeutics

Chronic Hepatitis B (CHB) is a global health problem affecting over 300 million people, responsible for ~830,000 deaths per year worldwide, and the leading cause of hepatocellular carcinoma (Schweitzer et al., 2015). While existing treatments can reduce viral load in CHB patients, less than 5% achieve a functional cure of Hepatitis B Virus (HBV) through the use of antivirals alone, and the vast majority of infected patients suffer chronic health issues (EASL,

2017). These low functional cure rates - present in both current and pipeline HBV treatments - are in part due to the lack of direct, durable targeting viral cccDNA (covalently closed circularized DNA) - an extrachromosomal viral depot that drives HBV transcription and replication, and the effective source of viral latency. Here, we describe a new treatment approach for Chronic Hepatitis B that employs targeted epigenome editing approaches to disrupt the native epigenetic regulation of HBV, thereby shutting down transcription from HBV DNA sources. Through transient delivery of these epi-editors both *in vitro* and *in vivo*, we highlight how epi-editing enables the strong and durable epigenetic silencing of HBV, paving the way for a long-awaited functional cure.

174 - Our Karyotyping and FISH Assays as Tools to Evaluate the Genetic Stability of Cell Therapy Products - A Decade of GMP Experience

Vasileios Georgakakos[1]

[1]Clean Cells

In the last two decades, there has been a bloom in the development of cell-based medicinal products and especially in the immunotherapy field using modified immune cells against disease. During the long development and production cycle of each novel cell therapy product, the critical question is always the same: Is it safe? During this talk, we will share our decade of experience in evaluating the genetic stability of cell therapy products from their development and into Phase III trials and in preparation to their market authorization using our GMP-validated karyotype and FISH assays.

170 - Genome edited therapeutic T cells

Waseem Qasim[1]

[1]University College London (UCL) Great Ormond Street (GOS) Institute of Child Health

169 - Karyotyping and FISH for the characterization of cell & gene therapy products - A decade of GMP experience

Vasileios Georgakakos[1]

[1]Clean Cells

In the last two decades, there has been a bloom in the development of cell-based medicinal products and especially in the immunotherapy field using modified immune cells against disease. During the long development and production cycle of each novel cell therapy product, the critical question is always the same: Is it safe? During this talk, we will share our decade of experience in evaluating the genetic stability of cell therapy products from their development and into Phase III trials and in preparation to their market authorization using our GMP-validated karyotype and FISH assays.

168 - Genetic and transcriptional engineering of primary human blood cells

Rasmus O. Bak[1]

[1]Aarhus University

Our research primarily focuses on the CRISPR/Cas system for genetic engineering of blood cells. We aim to develop and apply novel CRISPR tools to facilitate their implementation into clinically relevant blood cells such as CD34+ hematopoietic stem cells. Here, I will present our efforts to develop CRISPR-based gene therapies to cure monogenic blood disorders. More recently, we have also developed CRISPR-based transcriptional modulation tools delivered as all-RNA reagents for transcriptional engineering of primary blood cells. This technology can impose transient transcriptional states into blood cells, which we hope can be implemented to improve different types of cell therapies.

167 - Flexible and Scalable Genetic Screens for Discovery and Characterization of Novel Therapeutic Targets

Paul Diehl[1]

[1]Cellecta

166 - Advancing Oncology Drug Discovery through Pooled CRISPR Screening

Nikhil Gupta[1], Sebastian Lukasiak[2], Alex Kalinka[1], Magdalena Strauss[3], Malwina Prater[1], Khalid Saeed[1], Angelos Papadopoulos[2], Andy Sayer[1], Carlos Company[4], Chara Mastrokalou[1], Curtis Hart[1], Marica Gaspari[1], Ultan McDermott[4], Gregory J. Hannon[5], Douglas Ross-Thriepland[2], David Walter[1]

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The Functional Genomics Centre (FGC), a collaborative endeavour between Cancer Research UK's (CRUK) Cancer Research Horizons and AstraZeneca, is committed to revolutionizing our understanding and treatment of cancer through the application of CRISPR-based gene editing. Additionally, it seeks to democratize access to these advanced research tools for CRUK-funded scientists and clinicians. With over 600 screens conducted across 90+ drug discovery projects, the FGC has made significant strides in identifying new targets, assessing on-target efficiency and off-target effects, and deepening our comprehension of cancer targets, all aimed at reducing attrition in clinical trials.

Within this framework, we highlight the FGC's pivotal role in advancing functional genomics technologies. At its core, the FGC focuses on optimizing CRISPR libraries. Through rigorous assessment of existing CRISPR tools, we have developed optimized, minimal genome-wide CRISPR libraries. These libraries enable a 50% reduction in the experimental scale of CRISPR screens, empowering our Target Discovery platform to conduct more efficient and cost-effective screens, thus accelerating innovation in cancer therapeutics.

Furthermore, a key initiative of the FGC is to provide researchers with a comprehensive understanding of cellular responses to genetic perturbations. Unlike traditional CRISPR screens, which often yield one-dimensional phenotypic data, Perturb-seq offers nuanced insights into

how genetic alterations influence cellular behaviour, thereby enriching our understanding of cancer biology. The FGC's successful validation of single-cell CRISPR knockout techniques lays the groundwork for exploring novel drug targets and therapeutic strategies.

Lastly, the FGC has been actively advancing the development of an in vivo CRISPR screening portfolio to enhance target identification and selection. Through diligent efforts in implementing in vivo CRISPR screens and ongoing work to establish guidelines for their execution and analysis, the FGC is poised to play a leading role in advancing cancer research methodologies, with the overarching goal of benefiting the broader scientific community.

165 - Pharmacological Interventions to Enhance Genome Editing Precision

Marcello Maresca[1]

[1]AstraZeneca

164 - Realizing the Promise of CRISPR Therapeutics

Laura Sepp-Lorenzino[1]

[1]Intellia Therapeutics

Intellia is leveraging CRISPR/Cas9 technologies for therapeutic genome editing. Our systemic lipid nanoparticle (LNPs) delivers a messenger RNA that encodes the Cas editing enzyme and a guide RNA that targets the desired genomic sequence to be edited. LNPs are given IV for in vivo genome editing, as well as used ex vivo, for engineering cell therapies.

NTLA-2001 is an investigational in vivo CRISPR-based therapy with the potential to be the first single-dose treatment for ATTR amyloidosis. NTLA-2001 is being evaluated in adults with hereditary transthyretin amyloidosis with polyneuropathy (ATTRv-PN) or transthyretin amyloidosis with cardiomyopathy (ATTR-CM). Preclinical and clinical data will be discussed.

NTLA-2002 is Intellia's in vivo investigational drug candidate for hereditary angioedema (HAE), designed to knock out the *KLKB1* gene in the liver with the potential to permanently reduce total plasma kallikrein protein and activity, a key mediator of the disease. This investigational approach aims to prevent attacks for people living with HAE by providing continuous reduction of plasma kallikrein activity, following a single dose, and to eliminate the significant treatment burden associated with currently available HAE therapies. NTLA-2002 is being in clinical studies in adults with Type I or Type II HAE. Clinical data including safety, kallikrein reduction and HAE attack rate data will be discussed. Preclinical and clinical data will be discussed.

We are also focused on engineering T cells against specific cancer antigens using chimeric antigen receptor and T cell receptors. We have developed a cell engineering platform using LNP, that enables introduction of multiple edits, with high efficiency, ensuring genomic integrity and desired quality attributes of expansion, memory phenotype, safety. We applied this technology to generate allogeneic platform that addresses all immunological requirements for an allogeneic approach, including resistance to elimination by natural killer cells. Preclinical data will be

discussed.

163 - Connecting Innovators: CA21113 2nd Year Journey in Fostering Collaborative Networks for Breakthroughs in Genome Editing to Treat Human Diseases (GenE-Humdi COST action)

Karim Benabdellah El Khlanji[1], Yonglun Luo[2], Rasmus O. Bak[2], Carsten W. Lederer[3], Ayal Hendel[4], Alessia Cavazza [5]

[1]GENYO, Centre for Genomics and Oncological Research, Pfizer/University of Granada/Andalusian Regional Government, Avenida de la Ilustración 114, 18016 Granada, Spain, [2]Department of Biomedicine, Aarhus University, 8000 Aarhus, Denmark, [3]Department of Molecular Genetics Thalassaemia, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus, [4]Institute of Nanotechnology and Advanced Materials, The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel, [5]Infection, Immunity and Inflammation Research and Teaching Department, Great Ormond Street Institute of Child Health, University College London, London, UK

Recent advances in genome editing (GE) technologies have unveiled unprecedented opportunities for treating diseases through precise modifications of patients' genomes. Despite promising results in animal models and ongoing clinical trials for genetic disorders, infectious diseases, and cancer, the insufficient integration of academic research into pharmaceutical companies' development strategies, limited interest in regulatory science, and the absence of established standards have hindered the widespread application of these technologies for treating human diseases. **Objective:** The GenE-HumDi COST action aims to overcome these barriers by fostering intensive collaboration among pharmaceutical companies, academic institutions, scientific and regulatory agencies, biotechnology firms, and patient advocacy groups. Our primary goal is to accelerate the translation of GE technologies for human disease treatment by addressing knowledge fragmentation and establishing standardized procedures and guidelines. Through the creation of a synergistic network of GE groups, GenE-HumDi coordinates the enhancement of current GE protocols, safety assessment, optimization of delivery methods, promotion of clinical translation, and the development of regulatory guidelines. This coordination is achieved through regular scientific meetings, training courses, and the sharing of data and materials, ensuring scientific quality and reproducibility. Establishing standard operating procedures for GE safety assessment as well as delivery and comparing approaches for off-target effect estimation are critical steps toward determining safe levels of off-target indels for GE approaches. The first annual GenE-HumDi meeting in Granada, Spain (2023), and the second in Limassol, Cyprus (2024), have facilitated the sharing of recent advancements and optimized communication among all relevant stakeholders. GenE-HumDi represents a pioneering effort to integrate efforts and knowledge in the field of genome editing, with the potential to revolutionize the treatment of various human diseases through an unprecedented collaboration between different sectors.

162 - Project Delta Force - Upregulating delta globin as a new avenue to treat hemoglobinopathies

Jan Nelis[1]

[1]Ariya Bio

The β-hemoglobinopathies, such as sickle cell disease and β-thalassemia, are among the most common genetic diseases worldwide and are caused by mutations affecting the structure or production of β-globin subunits in adult hemoglobin. Many gene editing efforts to treat β-hemoglobinopathies attempt to correct β-globin mutations or increase γ-globin for fetal hemoglobin production. δ-globin, the subunit of adult hemoglobin A2, has high homology to β-globin and is already pan-cellularly expressed at low levels in adult red blood cells. However, upregulation of δ-globin is a relatively unexplored avenue to increase the amount of functional hemoglobin. At Ariya Bio, we use homology-directed repair gene editing (HDR-editing) to insert various single or multiple transcriptional elements into the endogenous promoter region of δ-globin to increase overall expression of adult hemoglobin 2 (HbA2). We have developed multiple promoter constructs of varying strength to robustly upregulate expression of δ-globin from its endogenous locus in an erythroid cell line and human primary cells. Our β-hemoglobinopathy programs are currently in lead optimization, and we are currently in target identification for a follow-on asset. Our follow-on programs build on our capabilities to enable therapeutic protein expression in erythroid cell lineages.

160 - With technology to biology: The single-cell functional genomics revolution

Daniel Schraivogel[1]

[1]EMBL

Thousands of coding and non-coding genetic variants have been associated with complex traits. Despite this effort, a comprehensive understanding of how genetic variation governs phenotypic diversity and disease predisposition is still lacking. This is largely due to the technical challenge of mapping the mechanisms through which mutations shape cellular phenotypes, as well as the complex interplay of variants that forms the basis of most phenotypes of health and disease.

Recent functional genomics approaches fill this technological gap by combining genome-wide pooled CRISPR screens with scalable and sensitive single-cell readouts. I will present two of these technologies, targeted perturbation sequencing (TAP-seq, Schraivogel, Gschwind et al. Nat Meth 2020) and image-enabled cell sorting (ICS, Schraivogel et al. Science 2022), providing insights into the mode-of-action (via single-cell sequencing) and phenotypic consequences (via single-cell imaging) of genetic alterations.

These approaches transformed our approach to research and accelerated our understanding of the genetic basis of complex phenotypes, the mechanisms of transcription, and the molecular mechanisms underlying disease. I will present how we use these approaches to derive global rules of enhancer-mediated gene regulation, to dissect non-coding regulatory variants of immune diseases, and to identify novel drug targets for heart diseases. Single-cell functional genomics approaches contributed to an exciting new era of quantitative large-scale approaches to edit, write, and test genomes at unprecedented scale and resolution.

159 - Deciphering the code of cancer: A deep dive into variants with saturation prime editing

Zhenya Ivakine[1]

[1]Hospital for Sick Children Toronto

High-throughput functional characterization of genetic variants at their endogenous loci has traditionally been constrained by the limited efficiency of homology-directed repair methods. In our study, we present an advanced application of CRISPR prime editing, coupled with a novel haploidization strategy, to enhance the classification of genetic variants. This approach, termed saturation prime editing (SPE), was exemplified through its application to the NPC1 gene, implicated in Niemann-Pick disease type C, and demonstrated a notable sensitivity of this gene to genetic perturbations. Furthermore, extending SPE to the BRCA2 gene showcased the method's versatility across different genes, given an appropriate cellular assay. Our findings underscore SPE's capacity for the efficient and precise functional characterization of genetic variants, offering a significant leap forward in the elucidation and potential treatment of genetic disorders.

158 - Base editing based on Cas12a variants

Stefano Stella[1]

[1]Ensoma

Base editing is a minimalist gene editing system that can induce specific base conversions within the DNA target. It includes a nickase (such as nCas9) that binds and cuts one strand of the DNA target and an enzyme (such as deaminases) that catalyzes the conversion of DNA bases. CRISPR-Cas12a is a type V CRISPR system containing a single DNA cleavage site where Cas12a undergoes protein rearrangement to sequentially bring each strand of the DNA target into the catalytic site to be cleaved. Thus, generating a highly efficient nickase enzyme with Cas12a scaffold can be challenging. However, because of its multiplex ability and high specificity, Cas12a is a desirable scaffold for developing a safe and easy-to-deliver gene editing system. At Ensoma, using a structurally guided approach, we have generated and screened thousands of Cas12a variants to develop an Engenious base editing platform that induces high level of base editing, maintaining the multiplex ability and the high specificity of the Cas12a scaffold. Furthermore, our editor does not induce INDELS on the edited site making our Engenious base editing safer for *in vivo* gene editing on progenitor cells such as hemopoietic stem cells.

157 - Improved synthetic RNA-guided nucleases for human therapeutic use

Andre Cohnen[1]

[1]Bayer

Novel gene editing enzymes can either be created through engineering approaches, or by leveraging naturally occurring genetic diversities. These novel enzymes promise more flexibility and novel features in comparison to existing benchmarks. The widely used *Streptococcus pyogenes* (Spy)Cas9 is specific for a short non-degenerate PAM sequence. However, the large size of SpyCas9, together with its sgRNA and expression elements presents a challenge to the 4.5 kb DNA AAV packaging limit, as well as to the synthesis of long mRNA templates and their stable formulation into LNPs. The best-characterized smaller Cas9s frequently recognize degenerate and longer PAMs, reducing the number of addressable genomic targets. We demonstrate that enzyme diversity can be identified through microbial database mining. We further evaluated four related, previously uncharacterized small Cas9. Surprisingly, most were specific for a common NNGG-PAM that enables addressing the same targets as SpyCas9.Using protein-engineering

approaches, we altered these genes to generate synthetic RNA-guided nucleases (sRGNs) and demonstrated superior editing in human cells. Analyses using all possible, single-nucleotide mismatched off-targets of a DNA substrate indicated high overall specificity in cell free experiments. Interestingly, different clones displayed a higher specificity for different mismatches at different sites along the target. Comparing the activity of sRGNs with SpyCas9 across 12 targets, we observed overall editing efficiencies on par with the larger SpyCas9. LNP packaging and *in-vivo* performance vs. SpyCas9 were comparable. Thus, we have generated a set of novel, small sRGNs, from which improved nucleases can be selected for particular targets of interest. These engineered RNA-guided nucleases are expected to be valuable additions to the canon of known genome-editing nucleases that can be employed for human gene therapy applications.

156 - Charting New Horizons in guide RNA Manufacturing

Raoul Hennig[1]

[1]BioSpring

Pioneering advancements in guide RNA manufacturing since 2016, BioSpring is a global supplier of commercial, clinical, and preclinical guide RNA. In this talk, our leading manufacturing expert will guide you through what it takes to scale GMP guide RNA manufacturing for clinical and commercial use, the extensive development involved, and the engineering and innovation that goes into evolving reliable high resolution analytical methods and achieving high purity, even in long and complex guide RNA constructs.

155 - Enabling GMP Production of sgRNA for CRISPR-based Cell and Gene Therapies

Kevin Holden[1]

[1]Synthego

Synthego supports the development of CRISPR-based cell and gene therapies, from discovery to first in human clinical trials. We have developed platforms for synthesizing CRISPR single-guide RNAs (sgRNA) to support preclinical research. Here, we present our work to develop a new, state-of-the-art GMP facility that launched in mid-2023 and is specifically designed to support scientists and clinicians with clinical-grade CRISPR sgRNA for Phase I clinical trials. In addition, we will highlight a CRISPR gene therapy program at an academic medical center that we have supported from discovery research to an upcoming clinical trial slated to begin in early 2024. Looking forward, we will share our efforts to combine analytical chemistry methods and sequencing to interrogate the purity of chemically synthesized sgRNAs.

154 - Reinventing Cardiovascular Disease Treatment with Single-Course Gene Editing Medicines

Andrew Bellinger[1]

[1]Verve Therapeutics

High cumulative exposure to blood cholesterol is a primary causal driver of atherosclerotic cardiovascular disease (ASCVD), the leading global cause of death. Several classes of pills and injections exist to reduce low-density lipoprotein cholesterol (LDL-C) levels, but gaps in the

chronic care model arising from sub-optimal adherence, access, and cost combine to generate poor real-world outcomes for LDL-C control. VERVE-101 is an investigational *in vivo* base editing medicine composed of an mRNA encoding an adenine base editor and a guide RNA targeting the *PCSK9* gene packaged in a lipid nanoparticle. It is designed to be a single-course treatment that permanently inactivates PCSK9 production by the liver. The *PCSK9* target is pharmacologically validated and human genetic data show that complete absence of *PCSK9* leads to low LDL-C levels and reduced ASCVD risk with no apparent deleterious effects. VERVE-101 is being investigated in a first-in-human clinical trial in high-risk patients with ASCVD and a genetic form of high cholesterol, heterozygous familial hypercholesterolemia. In an interim analysis of the first 10 participants in the trial, VERVE-101 led to dose-dependent LDL-C reductions that were durable for six months with follow-up ongoing. These results provide the first proof-of-concept for *in vivo* base editing in humans. Two additional programs expected to advance into the clinic in 2024 will be reviewed, a second program targeting PCSK9 (VERVE-102), and a program targeting a second lipid gene ANGPTL3 (VERVE-201).

153 - Implementation of gene editing to correct hematopoietic stem cells from Fanconi anemia patients

Paula Rio[1]

[1]CIEMAT

Division of Hematopoietic Innovative Therapies. Centro de Investigaciones Energéticas Medioambientales y Tecnológicas and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIEMAT/CIBERER), Madrid 28040, Spain.

Gene editing has emerged as a promising strategy for the acute correction of a wide range of disease-associated mutations. Although homologous directed repair (HDR) is the conventional mechanism used to correct specific mutations, diseases associated with severe defects in this DNA repair mechanism, such as Fanconi anemia (FA), require alternative gene therapy approaches. In this context, we have previously shown that NHEJ-CRISPR/Cas9-mediated gene editing can remove/compensate for specific mutations in different FA genes.

The description of novel and safer genome editing tools opens the possibility to potentially correct most of the mutations described in FA patients, with the ultimate goal of generating precise drugs for FA patients.

To this end, we have optimized the use of base editing (BE) and prime editing (PE) to correct different mutations in FA hematopoietic stem and progenitor cells (HSPCs). As a proof-of-concept, we focused on a common mutation in Spanish FA patients that results in a stop codon in exon 4 of FANCA (c.295 C>T). Using BE technology, we generated a therapeutic base conversion in >50% of BM HSPCs from FA patients harboring this mutation. The efficacy of the genetic conversion was confirmed by the correction of the characteristic phenotype of FA cells. Using an optimized Prime editing approach, combining the use of a PEmax architecture in synergy with the PE3 system, various mutations in FA lymphoblastic cell lines were precisely corrected. Moving to our target cells, HSCs, we also confirmed the efficiency of PE to target long-term repopulating HSCs from healthy donors. Finally, we demonstrated for the first time the ability to correct HSPCs from FA patients by reverting the mutation to the wild-type

sequence.

Taken together, our results indicate that the optimization of base and prime editing in HSPCs will pave the way for the development of personalized medicines for FA patients.

152 - Transforming Gene Therapy From the Few to the Many

Karina Thorn[1]

[1]Novo Nordisk

RNA and DNA therapies have gained significant attention and hold great promise in the field of medicine. These emerging RNA and DNA drug modalities involve delivery of therapeutic molecules directly into the cells to target specific disease-causing mechanisms and enable temporarily or permanently genetic modifications, such as modulation of gene expression or point mutations corrections.

Clinical validated RNA modalities, such as small interfering RNA (siRNA) for loss-of-function (LoF) modalities and messenger RNA (mRNA) for temporary gain-of-function (GoF) modalities, have already shown potential in treating a range of diseases including rare genetic disorders, cardiometabolic diseases, viral infections, and certain types of cancers.

Achieving precision genetic treatment requires optimal cargo/delivery balance and continuing innovation to unlock organ, cell and gene specificity or redosability of all RNA and DNA drug modalities will accelerate future treatment and curative opportunities in both rare indications and more prevalent diseases.

151 - From Allogeneic CAR-T Cells to SMART-CART to Fight Solid Tumors

Julien Valton[1]

[1]Cellectis

Adoptive cell therapy based on chimeric antigen receptor-engineered T (CAR T) cells has been transformational for selective heme malignancies. However, its therapeutic efficacy in solid tumors is severely hampered by several factors. Prominent among these is a complex tumor microenvironment (TME), the components of which subvert immune clearance by inhibiting intra-tumor T cell infiltration and establishing an immunosuppressive milieu. Furthermore, tumor antigen heterogeneity as well as low level expression of CAR-directed tumor-associated antigens (TAA) in normal tissues can result in antigen-escape and on-target off-tumor cytotoxicity, respectively, raising significant concerns about therapeutic safety and relapse.

Thus, endowing chimeric antigen receptor (CAR) T cells with additional potent functionalities is of utmost importance for improving their therapeutic efficacy and specificity in the context of solid tumor. However, because potency could be deleterious without control, these additional features need to be tightly regulated. Immune pathways offer a wide array of tightly regulated genes that can be repurposed to express potent functionalities in a highly controlled manner. We first explored this concept by repurposing TCR and/or PD1, two major players of the T cell

activation pathway using TALEN-mediated gene editing. We inserted the CAR into the TCRα gene, and IL-12P70 into PDCD1 genes to create an IF/THEN logic gate circuit in primary T-cells. This strategy results in transient, antigen concentration-dependent IL-12P70 secretion, increases CAR T cell cytotoxicity and extends survival of tumor-bearing mice.

Following that proof of concept, this IF/THEN logic gate circuit was further optimized and adapted to develop two SMART CAR T-cells scaffolds specific for solid tumor treatment.

The first one consists in developing a SMART CAR T-cell scaffold to address triple negative breast cancer (TNBC) and overcome its hostile tumor microenvironment. This scaffold was designed to express a constitutive CAR targeting the MUC1 TAA, to secret IL-12P70 after MUC1-CAR engagement and was endowed with TGFBRII KO to overcome the inhibitory effect of TGFB1 present in the TME. Using a comprehensive *in vitro* and *in vivo* characterization, we demonstrate that this scaffold enhanced both the proliferation of CAR T-cells and their infiltration in TNBC tumors expressing TGFB1, significantly reducing tumor burden and extending survival in orthotopic TNBC animal models.

The second one consists in developing an Inducible Dual SMART CAR T-cells scaffold to overcome the challenges of the 'cold' TME and off-tumor toxicity. This scaffold was designed to express a constitutive CAR, targeting FAP expressed by cancer associated fibroblasts (CAFs) present in solid tumors and a second CAR integrated at the PDCD1 locus, targeting the TAA named mesothelin. FAP-CAR engagement specifically in the CAF-positive solid TME induces expression of the mesothelin-CAR, establishing an IF/THEN-gated circuit sensitive to dual antigen sensing. Using comprehensive *in vitro* and *in vivo* strategies, we demonstrate that TME-restricted co-expression of FAP and mesothelin CAR enhances anti-tumor cytotoxicity, while limiting "on-target off-tumor" toxicity at bystander sites expressing mesothelin alone.

150 - Preclinical development of gene editing therapies

Alessia Cavazza[1]

[1]University College London's Greater Ormond Street Institute of Child Health

The CRISPR/Cas toolbox has recently emerged as a powerful means for tackling blood disorders when applied to hematopoietic stem cells. With conventional treatments for primary immunodeficiency diseases (PIDs), such as allogeneic stem cell transplantation or autologous gene therapy, still facing important challenges, the rapid development of genome editing technologies to more accurately correct the mutations underlying the onset of genetic disorders has provided a new alternative, yet promising platform for the treatment of such diseases. Dr Cavazza will discuss her team's recent efforts in trying to bring gene editing-based therapeutics for PIDs from bench to bedside, highlighting the plethora of aspects that need to be investigated when assessing the feasibility of a gene editing platform at the preclinical level.

149 - "PRCISR CRISPR: How prior knowledge can drive hit confidence in perturbation genomics."

Manuel Kaulich[1]

[1]Goethe University Frankfurt / Vivlion

CRISPR-Cas9 screens enable the systematic study of gene function across genomes, with compact and uniform gRNA libraries being crucial for screens in sparse cell models or combinatorial formats. In my presentation, I will present our experimental evidence on how prior knowledge of gRNA performance allows the selection of phenotypically agreeing gRNAs and what consequences this has on confident candidate calling. I will then present evidence that in silico prediction tools and their gRNA sequence scores, plus predicted off-target effects are independent of gRNA agreement, and conclude that the phenotypic agreement among gene-specific gRNAs is a hitherto unappreciated gRNA quality metric for the unbiased investigation of gene effects.

147 - Advancing CRISPR medicine by probing the detection, design, and delivery

Yonglun Luo[1]

[1]Aarhus University

The CRISPR-based genome editing technologies continue to evolve. Promising preclinical and clinical trials in CRISPR therapy have been achieved. Recently, the first CRISPR-based drug has been approved. Despite all these promising progresses, technical challenges limit the widespread adoption of CRISPR-based therapeutics. Only a small proportion of patients can benefit from the existing CRISPR therapeutic products, primarily due to their personalized nature. In most cases, CRISPR-based therapy is personalised, requiring the design of a specific CRISPR guide RNA (gRNA), with or without a donor repair template, to target and correct disease-causing mutations in individual patients. This approach necessitates the generation of millions of unique CRISPRs to address the diverse clinical variants. Additionally, genome editing by CRISPR is not always of full fidelity, and unintended cleavage may occur in other genomic loci with sequences like the target locus. To overcome these challenges, we have developed methods for large-scale detection of CRISPR editing outcomes (both on-target and off-targets) in cells. These large amounts of editing outcome data have been further used to train deep-learning-based models for the design of CRISPR gRNAs with high activity and specificity. Another significant challenge in CRISPR therapy is the delivery of therapeutic CRISPR components to specific cells and organs in vivo. We are exploring viral and non-viral-based methods, particularly mRNA delivery with lipid nanoparticles (LNP), for effective and safe in vivo CRISPR delivery, aiming to advancing the development of CRISPR medicine.

146 - New Insights Into On- And Off-Target Effects of Genome Editing Tools

Toni Cathomen[1]

[1]University Freiburg

In recent years, several gene- and base-editing approaches for treating congenital and acquired diseases have been developed and successfully translated into the clinic, with the first CRISPR-based drugs approved. Before gene-edited products can be applied to patients, the genome of the edited cells must be carefully evaluated, as genome editing is associated with extensive on-target insertions and deletions, as well as potential off-target activities leading to chromosomal translocations. I will briefly discuss the methods used to assess off-target effects, and present CAST-Seq, an assay we have developed to detect on- and off-target activities, including chromosomal rearrangements, in clinically relevant cell types ex vivo or in vivo. I will

illustrate how a detailed assessment of the collateral damage in cells edited with CRISPR-Cas nucleases, nickases or base editors has provided us with a better understanding of the underlying mechanisms. Finally, I will draw some conclusions on how to reduce the risk of genotoxicity and improve the safety of these new therapeutic approaches.

145 - Precisely Edited Primary Human Muscle Stem Cells as an ATMP in Muscular Dystrophies

Simone Spuler[1]

[1]Charité Universitätsmedizin Berlin

Skeletal muscle is equipped with a dedicated population of adult muscle stem cells fully capable to regenerate muscle until very old age. In muscular dystrophies, devastating diseases characterized by progressive muscle degeneration and atrophy, muscle stem cells lose their capacity to regenerate muscle. Muscular dystrophies are monogenic disorderscaused by mutations in one of 40 different genes. We utilized CRISPR/Cas9 - derived gene editing tools to precisely correct disease-causing frequent mutations leading to limb-girdle muscular dystrophies. Gene editing was performed ex vivo in primary human muscle stem cells. Corrected mutations include SGCA c.157 G>A, CAPN3 c.550del A, DYSF c.4872_4876delinsCCCC, and DMD c.9 G>A. Robust regenerative effects as well as repopulation of the stem cell niche with corrected muscle stem cells are demonstrated in animal models. We are setting up a first-in-human clinical trial transplanting genetically corrected primary human muscle stem cells in an autologous manner (Basket-trial;clinicaltrial.gov: NCT05588401). Preclinical safety studies are currently being performed as well as transfer of our patented methods to propagate muscle stem cells in a pharmaceutical environment (ATMP, advanced therapeutic medicinal product). Geneticallycorrected muscle stem cells will have a long-lasting regenerative effect on dystrophic skeletal muscle.

144 - INDUCE-seq: Ensuring the safe development of cell and gene therapies by gene editing

Simon Reed[1]

[1]Broken String Biosciences

Advances in genome editing are making it increasingly possible to develop new cell and gene therapies. Synthetic genes and modifications to existing ones can be made in a site-specific manner, while immune-compatible cells created for use in allogeneic patients enable production of ‘off-the-shelf’ cellular therapies. However, gene editing is a new way of treating disease, requiring new tools to ensure their safe and efficacious design and use in patients. Genome editing comes with risk, including malignant transformation of target cells caused by culturing cells outside the body. Gene editing can directly cause genomic instability resulting in selection for outgrowth of clones with oncogenic mutations. Furthermore, the gene editing tools themselves can cause genomic instability in the form of DNA breaks at sites other than the intended target. Such ‘off-target’ editing can activate proto-oncogenes, or disrupt tumor suppressors thus driving carcinogenesis. ‘Off-target’ mutagenesis may also generate neoantigens, triggering autoimmunity, or other types of cellular dysfunction. Furthermore, rare penetrant mutations are now known to confer severe risk of common disease, underscoring the

importance of identifying 'off-target' gene editing to assess risk. More precise methods are needed for testing 'off-target' editing during all phases of therapeutic development, including post-treatment follow-up. At present standardised assays to accurately assess the precision of gene editing in cell and gene therapies are lacking. Here, we describe the development and characterization of INDUCE-seq to address this. CRISPR-Cas9-based gene editing of five well-studied genetic targets, including two that have recently been evaluated by regulators, was conducted by two independent industry partners using two different cell-types. On and off-target gene editing was assessed by measuring DNA breaks in the genome using INDUCE-seq and the genetic mutations caused by editing at these locations were subsequently measured using Duplex-sequencing, which sensitively detects rare mutations. The results of this HESI-sponsored validation study will be presented to demonstrate the reproducibility of the assays for measuring intra and inter laboratory 'on-' and 'off-target' editing, the importance of conducting this analysis in the intended cell/tissue targets and the discovery of confirmed off-target gene editing in cells at genomic locations not predictable by *in silico* analysis alone.

143 - Xdrop®: changing the approach to gene editing validation and single-cell functional assays

Sidsel Alsing[1]

[1]Samplix

Xdrop is a benchtop instrument for preparing DNA and cells for highly accurate assays.

Xdrop enables precise assessment of CRISPR-edits and CAR cassette integration sites.

Xdrop facilitates high-throughput functional screening of single cells and cell-cell interaction studies, yielding detailed biological insights.

Xdrop can yield results that support the development of gene and cell therapies.

142 - CRISPR-Based Functional Genomic Characterization of Mechanisms of Action of Degraders for Targeted Protein Degradation

Seung Wook Yang[1]

[1]AMGEN

Targeted protein degradation (TPD) utilizing Proteolysis Targeting Chimeras(PROTACs) and molecular glue degraders has emerged as a potent therapeutic paradigm to eliminate disease-associated proteins that have traditionally posed significant challenges for targeting using conventional small molecules. These approaches involve heterobifunctional or monovalent small molecules that induce the proximity of target proteins with ubiquitin E3 ligases, leading to the subsequent ubiquitination and degradation of the target proteins via the proteasome. Although TPD presents an appealing approach for broadening the druggable potential, the utilization of small molecules for TPD applications has focused on a limited subset of E3 ligases, considering that the human genome encodes over 600 E3 ligases. RNA-guided CRISPR (clustered regularly interspaced short palindromic repeat)- Cas protein systems are powerful tools for targeted genome editing, particularly valuable in conducting comprehensive screening into

genomic function at a large scale using single guide RNA (sgRNA) libraries. Given the advantages of TPD, to overcome the current limited utilization of E3 ligases and fully understand the therapeutic potential as the drug discovery modality, here we'll discuss how leveraging CRISPR-Cas9 knock-out screening can lead to significant findings inidentifying unexploited ubiquitin E3 ligases and characterizing MOA of degradersfor therapeutic target degradation aimed at overcoming treatment resistance.

141 - Updated outcomes of variant detection and quantitation from the first NIST Genome Editing Consortium Interlab Study

Samantha Maragh[1]

[1]National Institute of Standards & Technology (NIST)

The U.S. National Institute of Standards and Technology (NIST) Genome Editing Consortium (GEC) convenes experts across academia, industry, and government to collaboratively address precompetitive genome editing measurements and standards needed to increase confidence in evaluating genome editing and utilizing these technologies in research and commercial products.

We present here the newly expanded results of the first NIST GEC Interlab Study, composed of organizations in the genome editing field partnering with NIST to understand the performance of DNA detection technologies being used for confirming on- and off-target genome editing. For this interlab study, NIST designed mixture schemes, using highly genomically characterized genomes from the Genome in a Bottle consortium. Mixture samples consisted of DNA variant benchmarks from single nucleotide to indels tens of kilobases long, and were present at frequencies ranging from >30% to 0.1 - 0.25%; with two optional samples of ~0.01% and ~0.001%. 14 Participants blinded to variant sequence and frequency returned their results after being provided samples and a list of genomic positions of interest to be analyzed by any type of DNA detection process currently utilized by the participant.

The presentation will detail these results including: accuracy of variant detection and frequency reported, differences as well as trends across analysis workflows, and measurement challenges identified. Datasets, metadata, and results will be released in a manuscript and made publicly available. Further in-progress NIST GEC work includes development of clonal engineered cell lines to be used both as DNA/cell based controls and for a second interlab study.

140 - Deciphering Cas9 immunogenicity

Roberto Nitsch[1]

[1]AstraZeneca

Interest in using CRISPR-Cas9 for treating monogenic human disorders is growing, and several medicines are advancing to clinical trials. However, the widely used Streptococcus pyogenes Cas9 (SpCas9) has the potential to trigger host immune response. To overcome this, we engineered a modified SpCas9, called Fast Degrading Cas9 (fdCas9), with a single amino acid substitution to enhance lysosomal degradation and reduce the cytosolic persistence. The highly reduced protein amount makes fdCas9 very challenging to be presented on the MHC-I and thus, nearly invisible to the immune system. Although fdCas9 showed low intracellular levels, it

retained high editing efficiency and this makes it an attractive enzyme for in vivo CRISPR therapies, broadening its clinical applications.

139 - CRISPR and human induced pluripotent stem cells - the magic duo for medical research

Pia Johansson[1]

[1]Lund University

CRISPR and human induced pluripotent stem cells (iPSCs) constitute a transformative duo which has nothing less than revolutionized medical research. This combination enables scientists to precisely edit genes, allowing for disease modelling, screening as well as loss- and gain-of-function studies in iPSC-derived model systems.

However, maintaining high standards of research necessitates standardized methodologies and quality control, which underscores the pivotal role of core facilities. These facilities serve as bastions of expertise, providing standardized protocols, and rigorous quality assurance. This is particularly vital when it comes to iPSCs as they are highly sensitive cells, prone to both pluripotency drift and genomic instability, especially after the clonal expansion that often follows custom gene-edits. This talk focuses on the possibilities of the CRISPR/iPSC combination but also the quality controls needed for consistency, reliability, and reproducibility in the field.

138 - Base and prime editing strategies to re-write CFTR mutations causing cystic fibrosis - validation in patient derived cell models

Marianne Carlon[1]

[1]KU Leuven Faculty of Medicine

Considering the more than 2000 variants reported in the *CFTR* gene, of which 719 confirmed causing CF (CFTR2.org), the most straightforward way seemingly would be to develop a single gene therapy agent for all CF-causing mutations. Several such strategies are currently in clinical testing with promising first results. The advent of highly versatile gene editing tools, such as CRISPR-Cas9 and its derivatives base and prime editing, has opened up a new era for treating genetic diseases by allowing targeted genome modifications. Importantly, these methodologies allow to preserve endogenous gene expression and regulation, in contrast to gene addition, and thus avoid ectopic CFTR expression as only in cells naturally expressing CFTR, the edit will become visible on the protein level.

Generally speaking, the base editor up till now has conferred higher editing outcomes of targeted *CFTR* corrections than the prime editor, although only a few studies so far have investigated the prime editor's potential in a CF context. Prime editing is perhaps the most versatile gene editor of all due to its ability to rewrite at least in theory, up to 93% of CF-causing mutations. We applied base and prime editing to several drug-refractory CFTR variants. Our workflow consists of a pipeline with preclinical testing in models with increasing complexity, including patient-derived cell models. Off-target (OT) edits were analyzed by Guide-Seq and targeted deep sequencing, revealing no OT edits. For improved translational delivery, we delivered the gene editors through virus-like-particles (VLPs), which conferred rapid (3h) and high

(~90%) editing in various primary cell models.

137 - Precision Genetic Engineering of Hematopoiesis by Gene Editing Tools

Luigi Naldini[1]

[1]San Raffaele Telethon Institute for Gene Therapy (TIGET)

Genetic engineering of hematopoietic stem cells (HSC) with lentiviral vectors has been providing substantial benefit to growing numbers of patients affected by primary immunodeficiencies, hemoglobinopathies and storage disorders. Long-term follow up shows stable hematopoietic reconstitution by high numbers of corrected HSC without signs of clonal expansion or exhaustion, providing a reassuring molecular picture underlying the long-lasting clinical benefit. Precise engineering by gene editing may further improve the reach and safety of HSC gene therapy by achieving in situ gene correction or targeted transgene integration. We reported the first targeted gene editing of human HSC followed by studies highlighting barriers constraining its efficacy and strategies overcoming them. Homology-driven editing, however, remains limiting in long-term HSC and the genetic outcome at target sites heterogenous and, for some by-products, potentially genotoxic. Template delivery by Integrase-defective lentiviral vectors rather than AAV6 and the use of lipid nanoparticles instead of electroporation may increase safety and efficiency of the procedure. Moreover, the emergence of base and prime editors that bypass the requirement for DNA double-strand breaks (DSB) allows editing single/few mutant nucleotides with limited activation of DNA damage response. We have shown, however, that DSBs are significantly lowered but not abrogated. Moreover, the expression of constitutive deaminase domains within the editors may impact the mutagenic load of treated cells. While these potentially genotoxic outcomes can be mitigated by optimizing expression and culture conditions, they should be better investigated and monitored in emerging clinical applications.

Another long-sought goal of HSC GT is to make space for the infused cells without relying on genotoxic conditioning, which entails acute and chronic serious adverse effects. We have shown that HSC mobilization opens a window of opportunity for engraftment of donor cells, which can effectively outcompete those in the circulation for engraftment in the depleted niches. Competitive advantage results from the rescue in culture of a detrimental impact of mobilizing agents on HSC and can be further enhanced by transient over-expression of engraftment effectors. These findings were obtained in human hematochimeric mice and are currently being investigated in non-human primates.

Overall, our work should advance HSC gene therapy by a combination of transformative approaches leveraging on precision genetic engineering while alleviating the morbidity of the procedure, broadening application to several diseases and patients worldwide.

136 - Functional genomics tools to dissect genetic networks of rejuvenation

Jin Chen[1]

[1]Altos Labs

Recent advancements in functional genomics have provided powerful tools to untangle the complex genetic networks underlying diverse biological processes. In particular, cellular

"rejuvenation" is emerging as a complex interplay of multifaceted pathways, including epigenetic reprogramming and cytoplasmic stress response pathways, that remain poorly understood (and poorly defined). I will present several vignettes to introduce CRISPR-based tools and functional genomic concepts that can be applied to enable the systematic dissection of the genetic components orchestrating rejuvenation, highlighting the development of CRISPR-based epigenetic modulators, applications of high-throughput single-cell screens, and the role of non-coding transcriptomic regions. By unraveling these intricate genetic networks, we hope to gain insights that not only deepen our fundamental understanding of the biology of aging but also hold promise for the development of therapeutic strategies against age-related diseases**.**

135 - Computational CRISPR/Cas9 gRNA design

Jan Gorodkin[1]

[1]University of Copenhagen

Utilizing CRISPR/Cas9 for gene editing involves selecting a 20-nucleotide spacer sequence that complements the DNA at the intended cut site. As a result, to edit a specific region there are typically multiple guide RNAs (gRNAs) to choose from. However, these gRNAs may exhibit varying on-target cleavage efficiencies and distinct off-target effects, resulting in unintended cuts elsewhere in the genome. Therefore, designing CRISPR/Cas9 gRNAs is essentially a process of evaluating multiple candidates within a given genomic region, considering both on-target efficiencies and off-target effects. As an alternative to cutting the DNA, base editors have been introduced, enabling direct editing of the DNA. However, these editors present a challenge, as bystander nucleotides may also undergo unintended edits. Consequently, there is a general interest in predicting the entire outcome of such editing processes. I will present CRISPRon, a deep learning-based method for designing Cas9 gRNAs, and similar deep learning tools for predicting the outcomes of adenine and cytosine base editors.

134 - Less is More: Efficient Novel Non-Viral Immune Cell Engineering With Precise Genomic Integration

Howard Wu[1]

[1]Full Circles Therapeutics

The toolbox for genome edi1ng in basic research and therapeu1c applica1ons is rapidly expanding. While efficient targeted gene abla1on using nuclease editors has been demonstrated from bench to bedside, precise transgene integra1on remains a technical challenge. AAV6 has been a prevalent donor carrier for homology-directed repair (HDR) mediated genome engineering but has reported safety issues, manufacturing constraints, and restricted applica1ons due to its 4.5 Kb packaginglimit. Non-viral targeted gene1c knock-ins rely primarily on double-stranded DNA (dsDNA) and linear single-stranded DNA (lssDNA) donors. Both dsDNA and lssDNA have been previously demonstrated to have low efficiency and cytotoxicity. Here, we developed a non-viral genome wri1ng catalyst (GATALYSTTM) system which allows produc1on of ultrapure, minicircle single-stranded DNAs (cssDNAs) up to ~20 Kb as donor templates for highly efficient precision transgene integra1on. cssDNA donors enableknock-in efficiency of up to 70% in induced pluripotent stem cells (iPSCs), superior efficiency in mul1ple clinically relevant primary cell types, and at mul1ple genomic loci implicated for clinical applica1ons with various nuclease

editor systems. When applied to immune cell engineering, cssDNA engineered CAR-T and CAR-NK cells exhibit potent and durable an1-tumor efficacy. The excep1onal precision and efficiency,improved safety, payload flexibility, and scalable manufacturability of cssDNA unlocks the full potential of genome engineering with broad applica1ons in therapeu1c development, disease modeling and otherresearch areas.

133 - Identification of novel oncology targets using a combination of functional genomics approaches & machine learning tools

Fiona Behan[1]

[1]GSK

Identification of novel Oncology targets is an ongoing challenge for the field. Efforts such as the Cancer Dependency Map have demonstrated the value of large-scale genetic perturbation approaches aiming to identify new therapeutic opportunities. Our work aims to bring this type of approach to the next level with the introduction of multi-gene perturbation methodology in order to address more complicated biological questions. One area where this strategy could add real value is in the search for synthetic lethal interactions in cancer. The concept of synthetic lethality is where a cancer cell has lost functionality of a gene/pathway which creates a dependency on a second gene/pathway. Targeting this dependency should selectively kill cancer cells as normal cells retain full functionality of the original gene/pathway. CRISPR perturbation is an ideal tool to identify such interactions. This project has established a complex perturbation framework consisting of experimental and complementary machine learning approaches. As the space of possible multi-gene interactions is incredibly vast, machine learning algorithms are used to predict potential interesting gene interactions, which are then experimentally tested. The experimental strategy developed uses dual CRISPR knockout in relevant cancer cell models with a selection of endpoints (e.g. viability, cell health via imaging, post-perturbation transcriptomics). Resulting data is then used to refine & optimise performance of machine learning models to increase probability of successfully identifying important gene interactions. This presentation will cover key considerations for combining functional genomics at scale with machine learning approaches.

132 - Novel DNA Payloads and One-Stop CRISPR Toolbox Accelerate Non-Viral Gene Editing Therapeutics Development

Fan Zhou[1]

[1]GenScript

As CRISPR gene editing technology becomes increasingly mature and perfect, more and more cell therapy developers are turning to more cost-effective and safer non-viral CAR-T cell therapy preparation solutions. They combine gene editing technology with non-viral DNA vectors to carry out allogeneic cell therapy and develop treatment options for malignant tumors other than blood tumors. Since non-viral DNA drug-encoding vectors can be prepared quickly and in small doses, with simple processes, free of animal source, and sterility, this rapid and economical solution also greatly promotes the discovery and verification of new targets. GenScript has been committed to the development of non-viral DNA payloads and providing synthesis services of various DNA formats. The GenExact™ ssDNA has lower cytotoxicity. The single-stranded form

can effectively reduce NHEJ repair and reduce the off-target probability. Combined with CTS fragments, the efficiency of fragment typing can be greatly improved. The GenWand™ dsDNA has covalently close-ended structure, which is more stable and less toxic than traditional PCR products. The GenCircle™ circular DNA has extremely short backbone sequence and equivalent efficient productivity and supercoiled form to conventional plasmids. Compared with traditional plasmid templates, it has lower cytotoxicity and higher knock-in efficiency. GenScript has established a one-stop factory from gene synthesis to large-scale production, equipped with experienced project management and regulatory affairs teams to meet the needs of non-viral payloads at different stages from drug discovery to preclinical research to clinical trials and commercialization, which will accelerate the development of non-viral gene editing therapeutics**.**

131 - CRISPR functional genomics as a tool in drug discovery

Bernhard Schmierer[1]

[1]Karolinska Institutet

Perturbing a system to understand its function underpins modern biology. We owe much of our understanding of genes and genomes to mutant phenotypes, both natural and induced. These efforts continue today, with CRISPR technology allowing genome-scale functional genomics studies in human cells at unprecedented scale and resolution. Classical screens target entire genes or regulatory elements, while sequence diversification screens using base-editors create and interrogate a large number of genetic variants. These genetic perturbation methods can help address some of the most challenging problems in drug discovery, such as mechanism-of-action elucidation and drug-target interaction.

130 - Engineered CRISPR Technologies to Improve Genome Editing

Ben Kleinstiver[1]

[1]Massachusetts General Hospital and Harvard Medical School

User-specifiable genome modifications are now possible by using gene editing approaches. Despite the vast potential of CRISPR technologies for editing genomes, many properties of Cas enzymes remain suboptimal for laboratory or therapeutic use. We are therefore deploying protein engineering strategies to improve the intrinsic properties of CRISPR enzymes, including their ability to access the genome efficiently and safely (with high precision and accuracy). Our recently developed Cas enzyme, named SpRY, offers unparalleled access to the genome for various applications[1,2]. We are exploring the utility of engineered Cas enzymes as base editors in preclinical experiments to treat several monogenic disorders (including spinal muscular atrophy[3] and others). Despite our observations of high levels of *in vivo* editing and robust phenotypic benefits, for most diseases, the necessity of developing bespoke editing strategies for each patient-specific mutation imposes challenges that impact the scalability of these approaches[4]. Therefore, we are also developing CRISPR technologies capable of larger kilobase-scale edits. We recently improved the natural properties of CRISPR-associated transposases (CASTs), which are capable of programmable RNA-guided genomic integration of DNA cargos[5]. Finally, we are developing other genome editing approaches that leverage the advantageous properties of DNA-dependent DNA polymerases (DDPs) to install nearly any short, medium, or large sized DNA edit[6]. These new technologies, termed ‘click editors’, utilize simple DNA oligonucleotides tethered

to a nCas9-bound target site as a template for genome writing (permitting the installation of edits encoded on the template DNA molecule). Together, these engineered enzymes offer new capabilities for generating small and large genetics edits, simplifying editing strategies towards the development of new tools and genomic medicines.

129 - Find-and-replace CRISPR Genome Editing HDR2.0: a Promising Therapeutic Strategy

Ayal Hendel[1]

[1]Bar-Ilan University

RAG2-SCID is a primary immunodeficiency caused by mutations in *Recombination-activating gene 2 (RAG2)*, a gene intimately involved in the process of lymphocyte maturation and function. Ex-vivo manipulation of a patient's own hematopoietic stem and progenitor cells (HSPCs) using CRISPR-Cas9/rAAV6 gene editing could provide a therapeutic alternative to the only current treatment, allogeneic hematopoietic stem cell transplantation (HSCT). Here we show an innovative *RAG2* correction strategy that replaces the entire endogenous coding sequence (CDS) for the purpose of preserving the critical endogenous spatiotemporal gene regulation and locus architecture. Expression of the corrective transgene leads to successful development into $CD3^{+}TCR\alpha\beta^{+}$ and $CD3^{+}TCR\gamma\delta^{+}$ T cells and promotes the establishment of highly diverse TRB and TRG repertoires in an in-vitro T-cell differentiation platform. Thus, our proof-of-concept study holds promise for safer gene therapy techniques of tightly regulated genes.

128 - FiCAT gene writing platform for advanced therapies

Avencia Sanchez-Mejías[1]

[1]Integra Therapeutics

In our previous work we developed a robust gene delivery tool called FiCAT (Pallarès et al; 2021), combining the precision of Cas9 DNA scanning and targeting DNA with an engineered piggyBac transposase with donor DNA processing and transfer capacity. A double-strand break in a specific site of the genome that could be recognized and repaired by the transposase followed by the insertion of the desired therapeutic gene. Here, with the aim to demonstrate FiCAT activity *in vivo*, we performed precise gene delivery to the liver using non-viral carriers that trigger less immunological responses compared to viral-based approaches. At this point, lipid nanoparticles (LNPs) have been extensively validated for the delivery of different nucleic acid cargos. To validate our gene writing platform in vivo, we first optimized both, DNA and RNA LNP formulations. With DNA-based nanoparticles, a biodistribution assessment was performed to ensure that transgene expression was restricted in the liver. For that, different lipid mixtures and formulation parameters, as well as different ratios between nucleic acids were assessed. We precisely inserted the DNA transposon codifying our gene of interest by co-delivering FiCAT machinery to mice liver targeting Rosa26 genomic safe harbor. Next, and with the aim of improving our delivery capacity, we optimized different all-in-one formulations to keep all the nucleic acid cargos in the same nanoparticle with Acuitas Therapeutics, screening different lipid mixtures and flow rate. Targeted insertion in vivo was also validated together with high editing levels. Finally, Hemophilia-A has emerged as a good candidate to validate our gene writing technology in vivo. This genetic disorder is caused by dysfunction of a large gene (hFVIII, around 9kb) that triggers clotting disorders and hemorrhagic issues. To assess that, we first tested 3

different payload encoding for hFVIII. The best performing one was tested in WT animals using our insertion machinery. This study included detailed characterization of on-target and off-target insertional events, potential immune response, plasma hFVIII levels (ELISA) and determination of in vivo targeted cell types. Furthermore, we extended this analysis to HA mouse models in vivo including the assessment of therapeutic benefit and, toxicity assessment (IL6, AST, ALT). Here, we aimed to safely deliver DNA and mRNA cargos via LNPs *in vivo* thanks to our gene writing machinery (FiCAT). This novel technology opens a window for the treatment of several genetic disorders, including Hemophilia A, making an important breakthrough in the gene therapy field**.**

127 - End-to-End Tools for Interrogation of CRISPR-Cas Associated Genotoxicity

Ashley Jacobi[1]

[1]Integrated DNA Technologies

Identifying and verifying low frequencies of genomic alterations resulting from off-target editing, gRNA synthesis errors, cross-contamination, or other unintended gRNA activity is critical to preventing unexpected genotoxic effects for the gene editing therapeutic community. However, assembling the necessary components and expertise for genotoxicity characterization studies is expensive and labor intensive. To better enable the gene editing community, we demonstrate a series of tools, workflows, and services that can be leveraged to perform genotoxicity characterization of CRISPR reagents. First, we demonstrate an empirical *in vivo* tag-based off-target nomination service rooted in our RNaseH2-dependent amplification technology as a method to generate an off-target list for a target gRNA. Then, we demonstrate how outputs of this service can be fed into rhAmpSeq to identify and classify indels and chromosomal re-arrangements at on/off-target loci with increased effectivity compared to existing methods. Finally, we demonstrate a method to sensitively identify gRNA cross-contamination down to sub-0.1% gRNA contamination levels by leveraging unique NHEJ DNA repair fingerprint information of select gRNAs. Using this method, we demonstrate that editing attributable to a characterized gRNA activity can be identified at frequencies far below detection limits of canonically utilized tools, and we further use this method to characterize the ability of IDT's synthesis platform to prevent cross-contamination reproducibly. From this work, we demonstrate the importance of manufacturing processes in mitigating unintended genomic alterations and present new tooling/services to improve genotoxicity characterization in gene therapy workflows.

126 - A novel portfolio approach to CRISPR-based gene therapies with tailored advantages for human therapeutics

Antonio Casini[1]

[1]Alia Therapeutics

CRISPR-based therapeutics represent a paradigm shift in gene therapy, as demonstrated by the recentapproval of the first gene edited therapeutic product and the increasing number of clinical trials exploitingCRISPR medicines. However, limitations mainly connected to the safety, deliverability and genomic targetingcoverage of the current toolbox are hampering the full exploitation of this technology for human therapeutics.We tackled these limitations by sourcing novel CRISPR systems from human microbiome reservoirs througha proprietary discovery

engine which includes a highly efficient in silico PAM prediction pipeline and in housecapabilities for rapid hit to lead screening coupled with directed evolution approaches, with immediateadvantages for clinical translation.We exploited our novel editors to advance a therapeutics pipeline exploring both in vivo and ex vivoapproaches. We addressed the genetic heterogeneity (more than 200 mutations) of RHO-dependentautosomal dominant retinitis pigmentosa through a first-in-class mutation-independent and allele-specifictargeting strategy for the in vivo treatment, which could be potentially extended to many other conditions.We further characterized our portfolio of editors for efficacy and safety in multiplex ex vivo editing of primaryhuman T cells.Overall, our portfolio approach yielded CRISPR tools exquisitely customized to each unique targeting need todeliver superior editing accuracy and specificity through a range of different and novel PAM motives, includinga range of smaller CRISPR editors selected for efficient and optimal in vivo delivery using AAV vectors.

125 - Identification and Evolution of Novel CRISPR-Cas9 Systems From the Human Microbiome

Anna Cereseto[1]

[1]Department of Cellular, Computational and Integrative Biology, University of Trento, Trento, Italy

CRISPR technologies are transforming the bio-medicine field by providing new therapeutic concepts for the treatment of diseases through genetic repairs, modification of the immune cells in cancer and deployment of disease protecting factors. Nonetheless, the current variety of CRISPR nucleases and derived technologies are limited to address the complexity of genome modification in living cells. Challenges are imposed by specific properties of CRISPR tools which includes high molecular weight which limits the compatibility with most commonly vectors for delivery including lipid nanoparticles, target sequence constraints, immunogenicity and unpredictable efficiency and precision throughout the genome. We recently focused on the development of new technologies by retrieving CRISPR systems from a large databank of the human microbiome and through directed evolution approach to enhance the activity of the prokaryotic enzymes to eukaryotic environment. This work led us to the discovery of new CRISPR systems and the enhancement of Cas nucleases with compelling features for genome editing applications.

124 - Exploiting Targeted Epigenome Editing for Therapeutic Applications

Angelo Lombardo[1]

[1]San Raffaele Telethon Institute for Gene Therapy and Vita-Salute San Raffaele University, Milan, Italy

Epigenome editing is emerging as a powerful new strategy to silence gene expression without altering its primary DNA sequence. In this regard, we and others have previously shown that transient delivery of Engineered Transcriptional Repressors (ETRs) can lead to efficient, long-term stable and specific epigenetic silencing of endogenous genes in both human and mouse cell lines. The ETRs are chimeric proteins composed of a programmable DNA binding domain, such as CRISPR-Cas9 or ZFPs, fused to either one or more of the following epigenetic repressive domains: KRAB, the catalytic domain of DNMT3A, and DNMT3L. Whether the ETR technology

could program efficient and long-lasting gene silencing in clinically relevant cell types and *in vivo* remains unknown. During my talk, I will present our efforts to improve and characterize the technology towards its *in vivo* application and further describe application of the platform for cancer immunotherapy.

123 - In Vivo Correction of a Genetically Humanized Fanconi Anemia Mouse Model Using Digital Editing Technologies

Colette B. Rogers[1, 2, 3, 4], Joseph J. Peterson[1, 2, 3, 4], Paige E. Carlson[1, 2, 3, 4], Cassandra J. Butterbaugh[1, 2, 3, 4], John E. Wagner[1, 5], Beau R. Webber[1, 2, 3, 4], Branden S. Moriarity[1, 2, 3, 4]

[1]Department of Pediatrics, University of Minnesota, Minneapolis, MN 55455, USA, [2]Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA, [3]Center for Genome Engineering, University of Minnesota, Minneapolis, MN 55455, USA, [4]Stem Cell Institute, University of Minnesota, Minneapolis, MN 55455, USA, [5]Division of Blood and Marrow Transplantation, University of Minnesota, Minneapolis, MN 55455, USA

Fanconi anemia (FA) is a rare genetic disease caused by mutations in certain DNA repair genes (i.e. FA genes), whose gene products make up the FA pathway for interstrand crosslink (ICL) repair. FA patients are predisposed to bone marrow failure (BMF) and cancer development. The current standard-of-care for the >90% of FA patients that develop BMF or hematopoietic malignancy is allogeneic hematopoietic stem cell transplant (HSCT). Significantly, many FA patients do not have a suitable donor, increasing the rates of graft-versus-host-disease and cancer occurrence. As such, allogeneic HSCT can result in a non-uniform outcome with side effects causing morbidity and mortality. Therefore, there is significant interest in the development of an effective and safe gene therapy approach for FA. While the development of CRISPR/Cas9-mediated gene editing has made it possible to induce site-specific double-strand breaks (DSBs) and correct gene mutations through homology-directed repair (HDR), this approach is ineffective in FA cells because of their underlying defect in HDR. Furthermore, there is a paucity of hematopoietic stem and progenitor cell (HSPC) targets impeding *ex vivo* gene editing. To overcome these limitations, we are utilizing cutting-edge, 'digital' base editors (BEs) and prime editors (PEs), neither of which require DSB repair, to correct the most common FA mutation (*FANCA* Spanish founder mutation; c.295 C>T) *in vivo*. Our lab pioneered the successful deployment of BEs to correct the *FANCA* Spanish founder mutation in patient-derived cells *in vitro* (PMID: 35955545) and we now hypothesize that *in vivo* correction of the *FANCA* c.295 C>T mutation using BEs or PEs will rescue the phenotypic manifestations of FA. To test this, we are developing a novel FA mouse model in the 129 S/v background that (i) is genetically humanized at *Fanca* exon 4 and harbors the *FANCA* Spanish founder mutation, and (ii) is made prone to genotoxic ICLs by loss of *alcohol dehydrogenase 5* (*Adh5*). Using these mice, we will measure correction of the *FANCA* Spanish founder mutation *in vivo* by adenosine base editor (ABE) and PEmax delivered by recombinant adeno-associated viruses (rAAVs) or lipid nanoparticles. Furthermore, we will assess FA phenotypes after *FANCA* correction (i.e. bone marrow HSPC population size, function, sensitivity to ICLs, and repopulation capacity). Completion of our studies will generate preclinical data demonstrating proof-of-concept for the use and superiority of *in vivo* digital gene editing of bone marrow HSPCs for treatment of FA.

122 - New Approach for Designing Split Genome Editors Without Compromising Editing Efficiency

Jure Bohinc[1, 2], Vida Forstnerič[1], Duško Lainšček[1, 3], Roman Jerala[1, 3]

[1]Department of Synthetic Biology and Immunology, National Institute of Chemistry, Ljubljana, Slovenia, [2]Graduate School of Biomedicine, University of Ljubljana, Ljubljana, Slovenia, [3]EN-FIST Centre of Excellence, Ljubljana, Slovenia

Recent advancements in CRISPR-based gene editing technologies predominantly involve RNA-guided nucleases coupled with DNA modifying enzymes. Notable among these are Base Editors, comprising either a deactivated Cas9 or a Cas9 nickase linked to a deaminase enzyme; Prime Editors, integrating a Cas9 nickase with reverse transcriptase; and Click Editors, which are combinations of a Cas9 nickase and a DNA polymerase, among others. However, the substantial size of these fusion proteins presents challenges for delivery via vectors such as AAVs, necessitating the development of more compact versions. While these fusion proteins can be split into distinct domains, this often results in reduced editing efficiency. Our research introduces a method for constructing split editor proteins that maintain robust DNA editing capabilities. This is achieved through the use of synthetic, small dimerizing domains that reassemble the split proteins non-covalently within the cell. We demonstrate this technique with a split prime editing system, where the nickase and reverse transcriptase components are separated and linked to dimerizing domains. Tests on various reporters and genomic sites show that our dimerizing split prime editor not only preserves but, in some cases, enhances editing efficiency. These findings suggest that our novel split prime editor is a promising addition to the existing arsenal of prime editing tools, with potential applications in correcting clinically relevant mutations. Further, we are extending this innovative approach to other DNA editing methods, such as base editing.

121 - Necessity Is the Mother of Innovation, the Ace Pico Protocol Redefining Cell Therapy Delivery

Dr. Alaa Abdelkarim Mohammed[1]

[1]EW ACE Cells Lab UK

Ace Pico protocol is a groundbreaking protocol that has the potential to revolutionize the way cell therapy is administered. As cell therapy continues to gain traction as a promising treatment option for a variety of medical conditions, the need for efficient and effective delivery systems has become increasingly important.

As we learned in cell therapy, it is dose-dependent, and to achieve complete healing, multiple doses are required [*Francois, M., Copland, I. B., Yuan, S., Romieu-Mourez, R., Waller, E. K., & Galipeau, J. (2012)*]. However, administering multiple doses in short periods is not feasible, representing one of the obstacles in cell therapy.

In addition, the pharmacokinetics of stem cells are the subject of many studies [*Lee, R. H., Pulin, A. A., Seo, M. J., Kota, D. J., Ylostalo, J., Larson, B. L., ... & Prockop, D. J. (2009)*]. Complications that might arise from this phenomenon cast doubt on the use of stem cells via intravenous (IV) injection, which is the most commonly used route of administration

The ACE Pico protocol offers a solution to the limitations of current cell therapy administration methods, providing a means to disseminate cells into tiny particles smaller than the body

capillaries to avoid cardiovascular events when injected intravenously. Furthermore, this protocol also opens the door to more efficient delivery systems, such as the sublingual or topical routes, ultimately leading to improved patient compliance and fewer side effects.

The current limitations of cell therapy administration, such as the inability to give multiple doses daily or weekly, can significantly impact patient compliance and overall treatment outcomes. The ace pico protocol addresses these limitations, offering a means to deliver cells in a way that minimizes the risk of cardiovascular events and opens the door to alternative delivery routes. This protocol has the potential to increase patient compliance, improve treatment outcomes, and reduce the likelihood of side effects associated with current cell therapy administration methods.

Many studies were done to validate the ACE Pico protocol, histological studies, biomolecular studies, chromatographic analysis, sterility tests, tissue culture for safety and toxicity, tissue cultures for efficacy, animal tests for safety, toxicity and efficacy in addition to some clinical studies.

We would like to share these results to the conference scientists to exchange the ideas and get the needed feedback.

In conclusion, the ace pico protocol represents a significant advancement in the field of cell therapy administration. By addressing the limitations of current administration methods and offering a more efficient and flexible means of delivering cell therapy, this protocol has the potential to improve patient compliance, treatment outcomes, and overall safety. As cell therapy continues to evolve as a promising treatment option for a wide range of medical conditions, the importance of an effective delivery system cannot be overstated. The ace pico protocol stands to play a crucial role in the future of cell therapy, offering a safer, more efficient, and more patient-friendly means of administration.

119 - Necessity is the mother of innovation, The ACE Pico Protocol Redefining Cell Therapy Delivery

Dr. Alaa Abdelkarim Mohammed[1]

[1]EW ACE Cells lab Limited

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118 - Programmable Multi-Kilobase RNA Editing Using CRISPR-Mediated Trans-Splicing

Jacob Borrajo[1], Kamyab Javanmardi[1], James Griffin[1], Susan St. Martin[1], David Yao[1], Kaisle Hill[1], Paul Blainey[2], Basem Al-Shayeb[1]

[1]Amber Bio, [2]MIT

Current gene editing approaches in eukaryotic cells are limited to single base edits or small DNA insertions and deletions, and remain encumbered by unintended permanent effects and significant challenges in the delivery of large DNA cargo. Here we describe Splice Editing, a generalizable platform to correct gene transcripts *in situ* by programmable insertion or replacement of large RNA segments. By combining CRISPR-mediated RNA targeting with endogenous cellular RNA-splicing machinery, Splice Editing enables efficient, precise, and programmable large-scale editing of gene targets without DNA cleavage or mutagenesis. RNA

sequencing and measurement of spliced protein products confirm that Splice Editing achieves efficient and specific targeted RNA and protein correction. We show that Splice Editors based on novel miniature RNA-targeting CRISPR-Cas systems discovered and validated in this work can be packaged for effective delivery to human cells and affect different types of edits across multiple targets and cell lines. By editing thousands of bases simultaneously in a single reversible step, Splice Editing could expand the treatable disease population for monogenic diseases with large allelic diversity without the permanent unintended effects of DNA editing.

116 - Efficient sortase-mediated assembly of CRISPR-Cas9

Seyed Hossein Helalat[1], Rodrigo Coronel Tellez[1], Helga Thora Kristinsdóttir[1], Astrid Dolinger Petersen[1], Yi Sun[1]

[1]Technical University of Denmark

A major limitation of the application of CRISPR-Cas9 for therapeutic purposes is the large size of the *cas9* gene (>4 kb) and the potential of offtarget editings. Lentiviruses and recombinant adeno-associated viruses are commonly employed as CRISPR-Cas9 delivery systems but their limited packaging capacity and risk of undesired genomic mutagenesis represent significant drawbacks. To facilitate and improve the delivery of the CRISPR-Cas9 complex into target cells we developed a split-Cas9 system, using two split forms of the Cas9 nuclease (C- and N- terminal) together with a sortase enzyme enabling specific ligation of the two fragments *in situ*. Different combinations of plasmids were employed for the expression of Sortase A (SrtA), and either Cas9 C- or N-terminal harboring the SrtA recognition and ligation sites LPXTG/poly-G.
Sortase-mediated *in situ* assembly of the two Cas9 fragments and consequent activation were evaluated *via* knockout of the *luc* and *itgal* genes in HEK293-luc and Jurkat cells, respectively. Knockouts of both *itgal* and *luc* genes were confirmed by FACS and luciferase assays, demonstrating the successful assembly and function of Cas9 nuclease without affecting cell viability. Moreover, the addition of a nuclear localization signal (NLS) on SrtA increased the efficiency of Cas9 activity. Overall, the proposed sortase-mediated splitCas9 system for genome editing of mammalian cells (*i.e.*, HEK293) and model T cells (*i.e.*, Jurkat cells) demonstrates a valuable approach in terms of improved delivery of the CRISPR-Cas9 complex, and potential decrease of undesired modifications due to the controlled assembly and activity of the Cas9 nuclease. Ongoing improvements to the current system include a remote and tightly controlled activation of the system (*e.g.,* magnetic induction) for a non-invasive and more precise regulation of the assembly and genomic editing processes.

115 - How the Danish Medicines Agency facilitates the entry into the regulatory landscape for advanced therapy medicinal products

Lotte Dahl Nissen[1]

[1]Danish Medicines Agency

As the EU regulatory landscape for the advanced therapy medicinal products is evolving the Danish Medicines Agency is becoming more transparent about the work done to facilitate the entry into clinical trials and the way towards a centralized marketing authorization. The homepage has been updated with a central entry for the regulation of advanced therapy medicinal products in the EU with regulatory information and relevant contact points for the i.e.

scientific and regulatory advice, classification, GMP certification and clinical trials.

Regulation of advanced therapies in the EU (ATMP) (laegemiddelstyrelsen.dk)

Lotte Dahl Nissen will talk about recommendations for the format and content of the regulatory documentation for applications for clinical trials (under the new clinical trial regulation).

114 - High Throughput Single Cell Analysis Workflow for Accurate Measurement of Genotoxicity Arising From Gene Editing Experiments

Ozcan Sahin[1], Saurabh Gulati[1], Chieh-Yuan Li[1], Saurabh Parikh[1], Benjamin Schroeder[1], Shu Wang[1]

[1]Mission Bio

Advances in cell and gene therapy (CGT) are transforming the way we treat and potentially cure certain diseases including cancer and rare genetic disorders. Precise gene editing technologies such as CRISPR-Cas9, TALENs, and ZFNs have transcended traditional boundaries, offering novel approaches to rectify genetic anomalies at their source. Despite these advancements, the editing process yields heterogeneous populations where some cells may have undesired outcomes that bear the risk of genome toxicity. Notably, these adverse outcomes include the introduction of structural variants, copy number alterations, or chromosomal translocations. Therefore, the development of efficacious gene therapies hinges on the ability to accurately measure and understand these events. Furthermore, since “cells” are the functional units of gene editing products, it is prudent to measure the co-occurrences of editing results and potential genotoxicity events in a single-cell context.

Here, we demonstrate a microfluidics and multiplex PCR based single-cell technology that simultaneously measures the co-occurrence and zygosity of on-target edit, off-target edits, translocations between predicted edit sites, as well as the genomic CNV landscape in over 10,000 cells in parallel. Due to the single-cell resolution, the technology offers a comprehensive view of the heterogeneous editing profile of gene edited products for a proper and fast evaluation of editing outcome and potential malignant events.

To evaluate the robustness of this workflow we used a variety of samples with varying levels of adverse effects occurring from multiple gene editing experiments. These samples all had orthogonal results cross validation using bulk NGS (DNA-seq) and rhAMPseq. Using a targeted sequencing panel we measured on and off target editing activity, presence of structural variations, gene editing induced translocation events and genome wide CNVs at a single cell level in these samples. The resulting data was analyzed using a novel bioinformatics pipeline which was developed to accurately measure all of these modalities simultaneously.Our high-throughput single-cell NGS assay represents a significant advancement in gene editing analysis, measuring off-targets, translocations, and genome stability in over 10,000 cells per assay. This technology has proven effective in accurately detecting a diverse range of INDELs from small (1bp) to large genomic alterations (114kbp) in as few as 0.1% of cells. Additionally, we were able to identify translocation events, pinpointing exact breakpoint coordinates even when they occur in less than 0.5% of the cell population.

Alongside these capabilities, we were able to simultaneously measure allelic on-target CRISPR editing activity in each cell, providing a comprehensive view of both intended and unintended genetic modifications. This dual functionality not only aids in optimizing gene editing protocols but also offers critical insights for understanding genotoxicity caused due to gene editing experiments and the development of safe, efficacious cell-based therapeutics.

113 - Optimized shRNAmir screens unveil cancer vulnerabilities in the essential gene space.

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Loss-of-function genetic screens facilitate the unbiased identification of genes required for cell proliferation and survival across various cell types. Several technology platforms based on RNAi (Achilles and DRIVE) and CRISPR/CRISPRi (DepMap and Project Score) have delineated essentiality maps in hundreds of cancer cell lines and serve as discovery engines for the identification of new targets for therapy development. RNAi-based genetic screens offered the first highly parallel interrogation of many genes simultaneously. However, they suffered from various technical limitations, such as off-target effects and limited efficacy under single-copy conditions. Over the past years, screening efforts have largely shifted from RNAi to CRISPR-Cas9, due to its superior efficacy and specificity. Screens based on conventional CRISPR/Cas9 mutagenesis result in complete gene knockout and most accurately capture essential genes. However, many relevant cancer dependencies and candidate or established drug targets, such as BRD4, CDC7, HDAC3, PRMT5, ATR, XPO1, and others are pan-dependencies in CRISPR screens, while their cancer-specific functions depend on gene dose and thus evade detection. Moreover, hypomorphic phenotypes resulting from RNAi more closely mimic the incomplete target inhibition that can be achieved with small molecule inhibitors and allow the exploration of gene dosage-dependent interactions, providing a highly complementary approach to CRISPR/Cas9. Advancements in our understanding of microRNA biogenesis pathways and the derivation of optimized shRNA design rules and expression systems, have significantly enhanced the suitability of microRNA-adapted shRNAs (shRNAmirs) for large-scale screening. Here, we have combined latest advancements in shRNAmir and miRNA-scaffold design to construct a next-generation shRNAmir library targeting 5119 genes identified as general or context-specific dependencies in CRISPR/Cas9-based screens. Compared to previous tools, benchmarking screens in several cancer cell lines demonstrated a substantially improved dynamic range in the drop-out of shRNAmirs targeting core essential genes (e.g., in MOLM13 -1.77 vs. -1.08 in DRIVE; in THP1 -0.93 vs. -0.35 in DRIVE). In first applications, we have used our improved shRNAmir library to revisit synthetic-lethal vulnerabilities associated with loss-of-function mutations of STAG2, a core component of the cohesin complex that is recurrently mutated in a variety of human cancers. Previous CRISPR screens in isogenic cell lines did not reveal prominent synthetic-lethal interactions beyond STAG1, the paralog of STAG2. Our shRNAmir-based screens not only confirmed STAG1 as synthetic-lethal partner of STAG2, but also detected previously reported synthetic-lethal interactions the DNA repair factors XRCC5 and XRCC6, which were missed in previous CRISPR screens. In addition, our screen pinpointed several other essential genes involved in RNA processing, mitosis, and transcriptional repression, as vulnerabilities in

STAG2-deficient cancers. Taken together, our results illustrate the utility and value of advanced shRNAmir screens as a highly complementary approach to CRISPR screens. These screens are positioned to more precisely identify gene dose-dependent cancer vulnerabilities and potential drug targets, offering promising avenues for the development of targeted cancer therapies.

112 - Efficient detection of CRISPR/Cas9 bulged off-targets with CRISPRoff2

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Predicting off-targets (OTs) in CRISPR-Cas9 systems is crucial for ensuring the precision and safety of genome editing. Although many in silico methods have been developed for detecting and predicting genome-wide CRISPR off-target profiles, few are capable of quantifying the off-target activities with insertions or deletions between target DNA and guide RNA (gRNA) sequences. However, several recent studies have shown that insertions and deletions (indels) resulting in bulges also play a key role for CRISPR off-target assessment. To address this gap, we present CRISPRoff2, a novel tool designed to search and score potential off-targets not only with mismatches, but also insertions and deletions, which is based on a revised binding energy model for the Cas9-gRNA-DNA complex from the original CRISPRoff method. In contrast to other methods, CRISRPoff2, integrated with RIsearch2, uniquely identifies off-targets within a given mismatch or indel threshold by leveraging a gRNA-DNA binding energy score, dynamically removing improbable off-targets. CRISPRoff2 demonstrates enhanced capability in detecting off-target sites with indels, as validated by CHANGE-seq, a recent in vitro experimental method for off-target site detection. Compared to the software employed in original analysis of the CHANGE-seq data, CRISPRoff2 can detect more off-targets (~21000 OTs) from the raw CHANGE-seq data by using our energy score rather than using normal mismatch and indel based approach which detected ~70,000 off-target, as shown in Figure 1a. Examples of bulged off-targets are shown in Figure 1b. In assessing off-targets from CRISPRoff2, our energy-based score outperforms other deep learning based methods like CRISPR-Net in detecting validated off-targets for 101 gRNAs from CHANGE-seq, with higher AUROC values, as shown in Figure 1c. Our results highlight that CRISPRoff2 offers superior coverage of off-target sites with a rational configuration and a reasonable score, outperforming existing methods, including those based on Cas-OFFinder.

111 - Enabling CRISPR-Cas associated research through guide RNA manufacturing solutions

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[1]Integrated DNA Technologies

As CRISPR-based editing moves into therapeutic and clinical applications, it is critical to screen, select, and confirm activity of CRISPR guide RNAs (gRNA). This includes a feature to minimize unintentional genomic alterations from factors like manufacturing errors, cross-contamination, and other genotoxic effects. Leveraging experience in gRNA synthesis and manufacturing, we have done substantial work to ensure efficient on-target editing while reducing unintended events and toxicity. We have developed a method to characterize genome editing levels below

0.1% that are potentially attributable to low levels of cross contamination during manufacturing events. At the outset, our custom arrayed synthetic guide RNA libraries allow for accelerated guide screening and selection. Coupled with our chemically modified gRNAs, which include 2’-O-methyl and 2'-fluoro RNA nucleotides, gRNA libraries are an efficient way to execute CRISPR knockout screens. We enable a wide selection of guide formats and purifications, including HPLC-purified guides. Scale-up synthesis with HPLC-purified guides provide a stage-specific improvement over our current RUO sgRNAs which provide improved on target editing with a reduction in truncated products of synthesis. To support research and developmental stage-specific needs, we offer large scale RUO gRNAs, engineering run gRNAs, as well as cGMP guide RNAs manufactured in an ICH Q7 compliant facility. Our workflows and services include solutions for all applications, with CRISPR support, expertise, and documentation to meet the needs of the genome editing community target therapeutic applications.

110 - Gene therapy for neurological diseases - In vitro model of a gene therapy for SynGAP1 syndrome using base editing.

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Neurodevelopmental disorders (NDDs) have a major impact on the lives of patients and their families and are associated with severe phenotypes and a lifelong burden. Current treatments focus on the symptoms instead of curing the underlying mechanism of the disorder. Many NDDs are due to monogenic mutations affecting gene regulation and upstream pathways, disrupting neuronal development and function, and could therefore be reverted by gene therapies.

Base editors provide a CRISPR/Cas9-mediated tool for the correction of point mutations that have been developed and evaluated for the treatment of some diseases. They have several advantages over conventional CRISPR/Cas9-associated methods, including - but not limited to - high editing efficiency and specificity, activity in non-dividing cells, and avoidance of disadvantageous double-strand breaks. However, the applicability of base editors in post-mitotic cells of the central nervous system (CNS) has not been shown in humans yet.

Therefore, we are investigating next-generation base editors for the correction of G to A point mutations that result in stop codons and premature termination of the translation of the Synaptic GTPase-activating Protein 1 (SYNGAP1) gene. SynGAP1 is one of the most abundant proteins found at the postsynaptic density (PSD) of excitatory synapses and plays a critical role in neuronal function. Mutations in this gene result in an autosomal dominant NDD called SynGAP1 syndrome and have been linked to intellectual disability, autism spectrum disorders, and schizophrenia.

In this study, we generated an induced pluripotent stem cell (iPSC) line from a SynGAP1 syndrome patient, as well as its isogenic control, and a healthy control iPSC line wherein the patient stop-mutation was introduced hetero- and homozygous using CRISPR-Cas9. In addition, we introduced another clinically relevant SynGAP1 syndrome stop-mutation hetero- and homozygous by Cytosine Base Editing into the healthy control iPSC line.

The iPSCs were then differentiated into excitatory cortical neurons by overexpressing Neurogenin2 (Ngn2) and phenotypically analyzed using real-time quantitative PCR, Western-Blot, and Immunofluorescent Staining. A reduction and complete depletion of SynGAP1 mRNA and protein levels was observed for the heterozygous clone and the homozygous clone, respectively, when compared to the healthy control.

For the delivery of base editors into iPSC-derived neurons, eVLPs with VSV-G glycoprotein, MMLV-gag- pol, and MMLV fused ABE8e-SpCas9 ribonucleoprotein were tested on a target locus with established high editing. Treatment of 30-day-old iPSC-derived neurons with eVLPs containing ABE8e-SpCas9 resulted in dose-dependent editing and peaked at 50% editing efficiency. The efficiency could be further increased to 100% at the highest dose by the addition of polybrene.

In the next step base editing will be applied during neuronal development using various delivery methods (rAAVs, eVLPs, LNPs), the precision, correction efficiency, as well as optimum time point for the intervention, will be determined for the respective mutation and cells will be phenotypically compared to an unedited and isogenic control.

109 - Engineered VLPs facilitates effective delivery of adenine base editor to correct SNPs in neurons.

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NEDAMSS (Neurodevelopmental Disorder with Regression, Abnormal Movements, Loss of Speech, and Seizures) is a recently discovered, ultra-rare, progressive, neurodevelopmental, and neurodegenerative fatal disorder caused by a broad range of heterozygous nonsense and frameshift mutations in the intron-less IRF2BPL gene encoding polyA/polyQ tract. Recently, de novo heterozygous mutations of this protein have been detected in several patients suffering from neurodegeneration or delayed neurodevelopment, among other characteristics. The phenotypic spectrum of patients with IRF2BPL mutations is highly heterogeneous and ranges from developmental delay to severe neurodegenerative courses with developmental and seizure-related encephalopathies. The only curative treatment for patients suffering from this disease is gene therapy. Still, efficient gene editing in post-mitotic cells such as neurons and astrocytes continue to be challenging due to inefficient delivery to the targeting cells, unwanted immune responses, and limited packing capacity of gene-editing tools in the most applied delivery system based on adeno-associated viruses (AAVs). As a considerable array of patients contains a single nucleotide polymorphism (SNPs) in the form of transition from cytosine (C) to Thymine (T), the CRISPR-Cas9-based adenine base editor (ABE8e) was applied to reverse the

single-nucleotide adenine (A) to guanine (G) on the minus strand in order to achieve the correction of the stop codon, leading to the translation of the full-length IRF2BPL protein. The ABE8e was therefore fused to MMLV and packed as ribonucleoprotein into engineered virus-like particles (eVLPs) coated with different fusion glycoproteins composed of parts of rabies virus glycoprotein (RV-G) and vesicular stomatitis virus glycoprotein (VSV-G), MMLV-gag-pol to target the gene of interest in *in vitro* iPSC-derived lentiviral ngn2-induced neurons, leading to a gene editing efficiency of 50% without Polybrene and 100% with Polybrene added for 7 days with the highest concentration of eVLPs (pilot experiment, further data will be generated in the meantime). Polybrene is therefore functioning as an effective transduction agent, however, it also inducing toxicity in the neurons, resulting in neural death in the *in vitro* culture, so further optimization will be prioritized to get similar gene editing results without addition of transduction reagents. In order to better recapitulate the complex disease progress of NEDAMSS in human neurodevelopment, cerebral organoids were generated. These will further be transduced with eVLP to evaluate the gene editing efficiency and to estimate time point of intervention and percentage of cells needed to rescue observed phenotypes compared to isogenic controls generated from the application of ABE8e to four patient iPSC lines.

108 - Integrating RNA structure and attention mechanisms for accurate CRISPR-Cas12a system gRNA efficiency prediction

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In the field of genome editing, the CRISPR-Cas12a system is a key technology. Its success heavily depends on the efficiency of guide RNA (gRNA), making the development of reliable prediction tools for gRNA activity important. Although numerous computational tools have been proposed, they often solely rely on sequence-based data, focusing on localized sequence information but overlooking the complex structural information of gRNA. However, previous research has indicated that gRNA spacers with lower Gibbs free folding energy often show reduced efficiency, due to higher structural stability, which means the structure of a gRNA spacer and its nucleotide interactions could play a crucial role in its effectiveness. The limitations of sparse data and the short output of gRNA sequences further exacerbate the issue, as sequence-based methods struggle to capture the higher-level structural features essential for accurate predictions. Therefore, incorporating structural information holds the potential to enhance prediction accuracy. Consequently, we introduce a deep learning-based gRNA efficiency prediction method that integrates a spacer secondary-structure-enhanced attention module, which is built based on the self-attention layer of Transformer. We propose to apply base pairing probability matrix to incorporate structure and base relative distance information to enrich gRNA representation. We first conducted down sampling on a large-scale dataset containing 15,000 gRNA sequences related to AsCpf1 activity to generate our training set with a more uniform distribution of efficiency. This results in a reduction to 11848 gRNAs. Our model was then evaluated on three widely used test sets, achieving Spearman's correlation scores of 79.2, 79.2, and 58.8, respectively, including gRNAs similar to the training set, as shown in Figure 1. The numerical experiments indicate that our model outperforms all previous methods in the field of CRISPR-Cas12a gRNA prediction. Compared to the benchmark model Seq-deepCpf1, our improvements in Spearman's correlation were 2.7, 4.7, and 1.0, respectively. Moreover, we removed similar gRNAs from the test sets that had five or fewer mismatches compared to the

training set, further proving the model's robust generalization capability. Additional ablation studies on external test sets indicate that by adding the structure-enhanced attention module, our model is a more universal tool, and can better capture the structural stability information of gRNA. To our knowledge, we are the first to apply the base pairing probability matrix to the challenge of predicting gRNA efficiency in CRISPR systems. Furthermore, our model is not limited to the CRISPR-Cas12a system, it has the potential to be widely applicable across various CRISPR-Cas systems.

107 - Investigating the interplay between CRISPR-induced double-strand breaks and recombinant AAV Integration in vivo

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The use of paired Cas9 nickases has been proposed as a safer alternative to Cas9 nucleases for therapeutic gene disruption. In our investigation, employing adeno-associated viruses (AAVs) for CRISPR delivery, we demonstrated that utilizing paired D10ASaCas9 nickases for targeted *Hao1* gene disruption resulted in comparable editing efficiency to its nuclease counterpart when combined with the same guide RNAs (gRNAs). Notably, paired Cas9 nickases-induced double-strand breaks (DSBs) are repaired through microhomology-mediated end-joining (MMEJ) in a significant percentage, leading to lower rates of vector sequence insertion into CRISPR-mediated DSBs compared to Cas9 nucleases, and show no observable off-target activity. Remarkably, at low doses of an AAV vector carrying all the elements necessary for Cas9 nickase-mediated gene editing this system maintained editing efficiency, with nearly undetectable AAV integration events, and resulted in a significant therapeutic effect. These findings collectively endorse the use of paired Cas9 nickases for *in vivo* gene disruption, providing a potential avenue to mitigate off-target risks and decrease AAV insertion frequencies. Nevertheless, the *in vivo* application of paired Cas9 nickases necessitates further research, to understand the factors influencing the choice of staggered double-strand break (DSB) repair pathways. The goal of this study is to optimize the methodology of amplicon sequencing data analysis for a better characterization of the genomic modifications introduced by paired Cas9 nickases, putting special attention on large insertions like AAV sequences, and shedding light on the relationship between AAV integration frequency and the choice of DSB repair pathway. We performed a benchmark of different gene editing analysis tools and an optimization of alignment parameters to enhance the detection of AAV insertions. A reanalysis of short-read amplicon sequencing data from various publications in which different CRISPR-Cas systems delivered by AAVs were used revealed a negative correlation between MMEJ repair frequency and AAV insertions. Interestingly, enzymes generating staggered ends, such as those created by paired Cas9 nickases and the type V CRISPR family, resulted in the highest MMEJ repair frequencies and the lowest AAV insertion frequencies. Conversely, in the case of enzymes that create blunt ends, like Cas9 nucleases, indel outcomes - including AAV insertions - likely depended on the sequence context or other variables such as cell type or cell cycle stage. *Ad hoc* experiments with selected guides with high or low predicted MH scores will confirm whether the frequency of AAV insertions depends on the staggered-end formation and/or the pathways involved in the repair

of DSBs.

106 - GeneAbacus: A novel PCR-free assay for validation of CRISPR-Cas gene editing efficiency with single nucleotide precision

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The rapid development of CRISPR/Cas-based genome engineering technologies has revolutionized biomedical research in areas such as gene therapy and drug discovery. Confirmation of successful gene targeting for pool analysis and clone selection is critical for the success and speed of gene editing workflows. Methods used routinely for this are PCR-based which often requires lengthy optimization for primer design and PCR conditions. Moreover, they also lack single nucleotide resolution, thus requiring sequencing for deeper analysis.

Countagen is directly addressing this pressing need by developing analytical products tailored to the gene editing market. GeneAbacus kit is a bundle of reagents, consumables and software to validate successful genetic targeting, therefore providing a seamless 'plug-and-play' solution, to streamline current time-intensive validation workflows from days or even months to 5 hours.

The novel PCR-free technology employs padlock probes and Rolling Circle Amplification (RCA) chemistry to quantify gene editing efficiency with single nucleotide and single molecule resolution. Padlock probes are designed to recognize and differentiate edited and parental sequences in a sample. Reacted probes are clonally amplified by RCA to generate discrete DNA nanoballs that after fluorescence labeling, are subsequently enriched into a small volume and imaged by standard fluorescence microscopy. Automated microscopy image quantification and calculation of gene editing efficiency is performed by a companion software.

To demonstrate the performance of GeneAbacus, we benchmarked it against amplicon-based next-generation sequencing (NGS) by analyzing a set of 26 CRISPR edited one-cell stage mouse embryos. This resulted in a high correlation (R^2=0.9485, *n*=26), whereas digital droplet PCR on a subset of samples showed poor concordance (R^2=0.5339, *n*=15) against the NGS results. Furthermore, to highlight the specificity of the assay, we performed reactions with padlock probes completely matching or containing single base mismatches to targeted sequences. The resulting signal from single base mismatching probes was equivalent to noise levels, underlining the unique specificity provided by Padlock probes and RCA. Finally, to highlight the robustness of GeneAbacus to sequence context, we analyzed a set of target gene regions of 66-84% GC content. The quantification with GeneAbacus also resulted in high concordance with reference methods (R2=0.9955, *n*=10) without any assay optimization required. Overall, these results demonstrate the ability of GeneAbacus to robustly detect and quantify precision edits with high accuracy within 5 hours turnaround time, thus making it a unique tool to swiftly and effectively move to functional analysis and accelerate gene editing workflows.

105 - EDSpliCE effectively corrects mis-splicing caused by an exonic variant in ABCA4

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Bi-allelic pathogenic variants in *ABCA4* are causative of **Stargardt disease** (**STGD1**), an autosomal recessive retinal disorder, characterized by progressive loss of central vision and typically presenting during childhood. The onset of the disease is attributed to the accumulation of cytotoxic products in photoreceptors resulting from dysfunctional ABCA4.

The mutational spectrum of *ABCA4* is diverse, with the **exonic c.768G>T variant** being a severe and frequent variant underlying STGD1. This variant results in missplicing by inducing a 35-nucleotide elongation of exon 6 retained in the mature mRNA transcript. This is due to the weakening of the canonical donor splice site and the presence of a strong cryptic splice donor site located 36 nucleotides downstream. Consequently, a frameshift occurs, leading to the formation of a truncated protein, p.(Leu257Valfs∗17). **CRISPR/Cas-mediated genome editing** strategies have been shown to effectively correct splicing defects and restore proper mRNA processing and protein translation, providing a potential and permanent solution for genetic defects caused by splicing-affecting variants. However, to date, no CRISPR/Cas9-mediated strategies have been established to correct faulty splicing due to pathogenic variants located in close proximity to exon/intron boundaries.

By implementing a novel gene editing platform, namely **Enhanced-Deletion Splicing Correction Editing (EDSpliCE) platform**, we describe the effective correction of the splicing defect induced by the exonic *ABCA4*:c.768G>T variant in minigene assay experiments. The employed EDSpliCE molecule is an AAV-packable chimeric RNA-guided endonuclease able to induce enhanced and directional deletions at the targeted sequence **using a single gRNA.** This allows for effective perturbations of the sequences involved in mis-splicing, thereby leading to **effective splicing correction**. Notably, the same gRNA coupled to the parental Cas9-ortholog did not show any splicing rescue.

By providing these proof-of-concept results, the versatile EDSpliCE platform not only offers a potent means of modulation splicing through skipping of pseudoexons but also presents a flexible solution for correcting mis-splicing caused by exonic variants. This versatility positions EDSpliCE as a promising and cutting-edge gene editing approach with significant potential for **therapeutic applications**, especially in addressing splicing variants within *ABCA4* and beyond.

104 - CRISPR-Cas9-Mediated WBP2 Knockout Modulates Tamoxifen Resistance Estrogen Receptor Positive Breast Cancer Cells via the Hippo Pathway

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Tamoxifen (Tam) is a key estrogen receptor modulator widely used in treating estrogen receptor-positive (ER+) breast cancer. Despite its common use, there is a need to address adverse effects and develop strategies to overcome emerging drug resistance. The WW domain binding protein 2 (WBP2) enhances ERα/PR transactivation through hormone-dependent interaction, suggesting a role in therapy. Understanding the interplay between the Hippo pathway and Tam resistance (TamR) is crucial for devising strategies to overcome resistance in breast cancer.

The clinical study encompassed a cohort of 50 patients who were subjected to a daily regimen of 20 mg Tam treatment. RNA extraction was performed on paraffin-embedded tumors and normal tissues for cDNA synthesis. In parallel, the *in-vitro* study involved establishing WBP2-OE-MCF-7 and WBP2-KO-TamR-MCF-7 cell lines utilizing CRISPR/Cas9 technology. RNA isolation from these cell lines was carried out for subsequent cDNA synthesis. The expression levels of WBP2, YAP, and TAZ genes were quantitatively assessed through RT-qPCR. Statistical analyses, including normality tests, t-tests, X2-tests, and correlation analyses, were applied to evaluate the acquired quantitative data.

An increase in the expression levels of WBP2, YAP, and TAZ was observed in comparison to normal tissue, revealing a robust correlation between the expressions of WBP2 and YAP, and TAZ ($p<0.001$). In patients exhibiting TamR development, a significant correlation was particularly discerned with YAP and WBP2 ($p<0.05$). Additionally, YAP expression levels demonstrated correlations with necrosis ($p=0.041$), perineural invasion ($p=0.048$), and tumor size ($p=0.035$). Substantial variances were also unveiled in TAZ expression concerning necrosis ($p=0.031$), tumor grade ($p=0.017$), and venous vascular invasion ($p=0.014$).

According to expression results, fold-change values of WBP2, YAP, TAZ genes were -1.522, -1.345, and -1.725 in WBP2-KO-TamR-MCF-7cell line, respectively ($p<0.05$), and in MCF-7-OE cell line, they were 0.076, 0.849, 0.565, respectively ($p<0.05$). According to the clonogenic assay results, an increase in the proliferation of WBP2-OE-MCF-7 cells was observed compared to WBP2-KO-TamR-MCF-7 cells. After treatment with Tam resulted in a reduction in the percentage of apoptotic cells in MCF-7 and WBP2-OE-MCF-7 from 6.00% to 43.25% and 24.53%, respectively. Conversely, in MCF-7 TamR, as well as WBP2-KO-TamR-MCF-7, the percentage of apoptotic cells increased from 2.90% to 18.24% and 32.35%, respectively. Flow cytometry analysis discerned that the overexpression of WBP2 exerted a significant inhibitory effect on G1 phase proliferation, concurrently inducing an increase in cell proliferation during the S phase. Conversely, WBP2 inhibition was found to prolong the G1 phase.

The deletion of WBP2 demonstrated significant regulatory effects on the G1/S transition in ER+ breast cancer. This genetic intervention resulted in heightened apoptotic events, reduced proliferation, and induced cell cycle arrest upon Tam administration. Additionally, marked alterations in the expression patterns of genes associated with the Hippo pathway and amplification were observed. The inefficacy of Tam in restraining the growth and proliferation of WBP2-induced breast cancer suggests a potential role for WBP2 upregulation in the development of acquired TamR. These findings underscore the potential of WBP2 knockout as a promising prognostic factor for overcoming complete TamR.

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103 - Nomination of Genome-Wide CRISPR-Cas9 Cleavage Activity using rhAmp Technology

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[1]Integrated DNA Technologies

CRISPR-Cas9 nucleases have transformed the genome editing space because of their ease of use and programmability to create targeted double-stranded breaks. The technology has been widely adopted for basic research, in particular for loss-of-function screening applications. In experiments and applications beyond basic research, characterizing and understanding the genome-wide off-target effects (OTEs) becomes increasingly important and remains a significant hurdle. To interrogate off-target activity, many methods have been developed to empirically nominate off-target editing sites, each one with certain advantages and disadvantages. *In cellulo,* tag-based assays, such as GUIDE-seq, provide an advantage in OTE nomination due to a lower false-positivity rate compared to competing *in vitro* nomination methods. To complement GUIDE-seq, we implemented a cellular tag-based assay rooted in our RNaseH2-dependent amplification technology. The rhAmp nomination strategy allows for a high throughout workflow in 96-well plate format, increases OTE amplification, and shortens total hands-on time by implementing single-tube amplification using rhAmp technology. To compare our rhAmp nomination approach to literature precedent, published guides were used with our workflow and compared to sites nominated by GUIDE-seq. Upon identification of OTEs, rhAmp nomination was able to nominate the highest number of OTEs, which enables the design of rhAmpSeq multiplex targeted amplification pools for downstream confirmation studies. We use this opportunity to report the comparison of methods through identification of sites driven by our rhAmp nomination and confirmation of editing with rhAmp amplicon sequencing methods.

102 - Enzymatic synthesis of ultra-pure long single-stranded DNA to enable Cell and Gene Therapies at scale

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The past decade has seen a genomic revolution, resulting in the development of gene-based technologies and therapies across various industries including pharma, agriculture, diagnostics and computer science. The success of these technologies and therapies is dependent on a steady supply of high-quality synthetic DNA.

Current DNA synthesis methods, based on chemical processes, suffer from several limitations including short DNA length, high error rates, and low production quantities. These limitations hinder the potential of existing gene-based technologies and new ones.

Moligo Technologies has developed a proprietary enzymatic DNA synthesis technology that overcomes these limitations, producing single stranded DNA (ssDNA) molecules with 5 unique selling points:

Purity: With a guarantee of 100% sequence-verified molecules and a purity level of over 99.9%, our technology meets the rigorous standards required for cell and gene therapies and technologies. Our high-fidelity enzymes and sequence-verified production templates ensure the highest level of purity.

Length: Our DNA synthesis technology allows for the production of ssDNA over 10,000 bases in length, which has the potential to revolutionize the treatment of genetic diseases caused by long, defective genes.

Scalability: Our in vitro technology is based on isothermal reactions, making it easy to scale, and we have already achieved multi-milligram production levels. Our high-yield enzymatic reactions will make it possible to produce hundred grams of ultrapure DNA at competitive prices, democratizing access to gene therapies and technologies.

Complexity: Our technology can synthesize any DNA sequence complexity without sacrificing purity. Our approach has been validated through the successful synthesis of complex ssDNA, including telomeric G-quadruplex repeats, strong secondary structures, high GC content sequences and long inverted repeats. DNA sequences with high complexity are very common in human genes and highly represented in constructs used for gene therapies.

Multifunctionality: Our technology enables the synthesis of ssDNA molecules containing multiple modifications in high density, which can be fine-tuned during production. This allows us to fine-tune the properties of our ssDNA molecules for various downstream applications, making them less immunogenic, more permeable to membranes and tissues, and more stable in physiological conditions. This unique advantage will be leveraged to develop new DNA therapeutics, vaccines, gene-based technologies, and DNA-based biomaterials with multiple applications.

This study not only provides comprehensive data on our DNA synthesis capabilities but also encompasses investigations into the successful application of enzymatically synthesized single-stranded DNA (ssDNA) as donor templates for the engineering of various cell types, such as T-cells and Hematopoietic Stem/Progenitor Cells (HSPCs).

Our long single-stranded DNA donor templates and gene vectors, in addition to demonstrating remarkable knock-in efficiency and minimal off-target events, exhibit no adverse effects on cell viability. This finding paves the way for the advancement of non-viral cell and gene therapies centered around extended functional genes.

101 - Insights to Cas9-induced host immune activation

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CRISPR-Cas9-based gene editing brings hope for treatment of rare diseases. Nonetheless, overshadowing the expectations reports of host immunity to the prokaryotic Cas9-endonuclease challenge the feasibility of therapeutic gene editing.To better understand Cas9 immunogenicity we studied lymphocyte response to Cas9-stimuli. Post-stimuli, we observed ex vivo and in vivo secretion of pro-inflammatory cytokines and activation of cellular immune responses, indicating priming and activation of Cas9-specific cytolytic memory T-cell response.In therapeutic context, the formation of cellular host immune response against the core component of therapeutic modality likely results in rejection of the targeted cells, potentially compromising the treatment outcome. Hence, a thorough comprehension of Cas9 immunogenicity and ways to mitigate it are crucial to ensure a versatile utility of therapeutic gene editing.

100 - Harnessing the diversity of CRISPR-Cas proteins for genome editing

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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 cells. 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.

99 - CRISPR associated substrate-linked directed evolution (CaSLiDE) for evolving highly efficient and specific miniature CRISPR-Cas systems

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The development of novel CRISPR-Cas genome editing tools continues to drive significant advances in the life sciences. Particularly, compact CRISPR-Cas such as Cas12f (composed of 400-600 amino acids) or Cas12j (composed of 700-800 amino acids) are promising candidates. Nevertheless, these CRISPR-Cas systems are hampered by their limited editing efficiencies in eukaryotic cells. To pave the way for broader applications in basic research and therapeutic applications, it is important to improve their editing capabilities, specificity and expand their

targeting scope.

In this study, we introduce the concept of CRISPR associated substrate-linked directed evolution (CaSLiDE) as a method to evolve CRISPR-Cas systems with increased efficiency and precision. We applied the CaSLiDE approach to evolve different Cas12f proteins, the ABE8e TadA domain and miniature base editors, thereby improving their utility. Our findings compellingly illustrate the potency of the CaSLiDE approach in the directed evolution of genome editing enzymes, underscoring its significance for future applications.

98 - Deciphering layers of innate immune regulation by genome-wide CRISPR/Cas9 screens

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Type 1 interferons (IFNs) represent a central part of the innate immune system and are key to controlling viral infections. Different pattern recognition receptors (PRRs) act as sensors of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). These predefined motifs initiate signaling cascades, which ultimately induce transcription of type 1 IFNs. Numerous genes encoding proteins that are critical for activation of the type 1 IFN responses have been characterized. However, genetic variation in a network of genes supposedly serve to modulate early IFN responses mediating a balance between immune alertness and excessive inflammation. Here, we employed genome-wide CRISPR/Cas9-based libraries for dissection of signaling pathways leading to expression of IFNb from the *IFNB1* gene. To facilitate high-throughput screening of *IFNB1* expression phenotypes, we developed a cellular reporter system (*IFNB1* expression reporter or IBER) based on lentiviral insertion of gene expression cassette consisting of the *IFNB1*-derived promoter, the d2eGFP gene, and the *IFNB1* 3' untranslated region. In THP1 cells carrying this vector, the destabilized nature of d2eGFP ensured a rapid decline of the fluorescence signal mimicking thus the rapid induction and termination of *IFNB1* expression. We initially performed screens in THP-1 IBER cells treated with cGAMP, an agonist of the cGAS/STING pathway. By combining CRISPR/Cas9-based gene perturbation with tracking of *IFNB1* expression by FACS based on d2eGFP expression, we were able to select for cells with an altered *IFNB1* expression signature and link the phenotype to a network of genes. Among a stringently defined subset of identified genes, 56 genes (17.8% of the candidate genes) matched with genes that have previously in the literature been linked to STING-induced *IFNB1* expression, whereas the remaining 258 genes were not previously linked to immune regulation through STING. By including similar screens mapping the response to M8, a strong RNA RIG-I agonist, we aim at unveiling a complete network of genes controlling IFNb production during virus infection.

97 - Highly efficient and precise gene expression regulation using miniaturized CRISPR-Cas systems

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The CRISPR activator/interference (CRISPRa/i) system stands as a potent technology for modulating target gene expression levels without inducing DNA double-strand breaks.

Nevertheless, existing CRISPRa systems have limitations in accommodating AAV viruses optimized for delivery into human cells due to their substantial gene size. To overcome these constraints and facilitate efficient delivery into target cells through AAV system, we have developed an effective CRISPRa system based on the *Candidatus Woesearchaeota* Cas12f (CWCas12f) effector, known for its highly compact Cas protein. Our optimized CWCas12f-based gene expression regulator, achieved through the engineering of the CRISPR-Cas module, fusion of activation domains, and exploration of various linker and NLS (nuclear localization sequence) combinations, enables precise and efficient regulation of gene expression, surpassing existing CRISPRa system. The compact CWCas12f-VPR system developed in this study holds immense promise in effectively regulating the transcription of endogenous genes over extended periods within living organisms. Building upon this system, our ultimate goal is to develop a long-term memory-directed gene expression regulator by inducing further DNA methylation modifications. This involves further conjugating a specialized domain with the miniaturized CRISPRa system and CWCas12f-VPR system optimized in this study possibly serves as a miniaturized platform for AAV delivery. In this study, our result shows that CWCas12f based activation or interference system will become a foundational platform for future gene therapy and advances in gene functional research.

96 - On- and off-target effects of paired CRISPR-Cas nickase in primary human cells

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Undesired on- and off-target effects of CRISPR-Cas nucleases remain a challenge in therapeutic genome editing. While the use of Cas9 nickases has been shown to minimize off-target mutagenesis, their use in therapeutic genome editing has been hampered by a lack of efficacy. To overcome this limitation, we and others have developed double nickase-based strategies to generate staggered DNA double-strand breaks that mediate gene disruption or targeted knock-in with high efficiency. However, the impact of paired single-strand nicks on genome integrity has remained largely unexplored. Here, we developed a novel CAST-Seq pipeline, D-CAST (dual CAST), to characterize chromosomal rearrangements induced by paired CRISPR-Cas9 nickases at three different loci in primary keratinocytes derived from epidermolysis bullosa patients. CAST-Seq identified 18 off-target mediated translocation (OMT) events upon *COL7A1* editing with either of the two Cas9 nucleases. On the other hand, the concomitant application of two Cas9 nickases preserved high on-target activity but did neither result in OMTs nor off-target indel formation. Similarly, targeting *COL17A1* or *LAMA3* with Cas9 nucleases caused previously undescribed chromosomal rearrangements, while no chromosomal translocations were detected following paired Cas9-based nickase editing. Of note, CAST-Seq and long-read

sequencing revealed that the double nicking strategy induced large on-target aberrations within a 10 kb region surrounding the target sites at all three loci, similarly to those observed for the corresponding nucleases in both frequency and length. However, the chromosomal on-target aberrations in the double nickase samples were qualitatively different from those induced by nuclease editing and included a high proportion of insertions. Taken together, our data indicate that double-nickase approaches combine efficient editing with greatly reduced off-target effects, but still leave substantial chromosomal rearrangements at on-target sites.

95 - Engineered baculoviral protein and DNA delivery platform for large DNA cargo integration and enhanced gene editing in human cells

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Simultaneous delivery of multiple genetically encoded elements is one of the most challenging roadblocks for efficient CRISPR interventions.

Baculovirus vectors (BVs), can deliver large (>45 kbp) synthetic DNA payloads while being integration and replication-deficient in mammalian cells. By engineering a modular DNA assembly platform, we have generated all-in-one BVs for efficient delivery of complex CRISPR toolkits to mammalian cells, with a focus on large DNA knock-in and precise gene editing.

By encoding Cas9, sgRNA, and donor DNAs on a single, rapidly assembled baculoviral vector, we achieved whole-exon replacement in the intronic β-actin (ACTB) locus with up to 30% efficacy, including site-specific docking of very large DNA payloads (up to 18 kbp). We used our approach to rescue wild-type podocin expression in steroid-resistant nephrotic syndrome (SRNS) patient-derived podocytes.

Additionally, we demonstrated that all-in-one BVs could efficiently deliver single and multiplexed prime-editing toolkits (with up to 4 pegRNAs and nicking sgRNAs), achieving up to 100% cleavage-free trinucleotide insertions in the absence of detectable indels.

More recently, we demonstrated that BVs can also be effectively used to stochastically package heterologous proteins and, upon transduction, protein cargoes can be delivered to target mammalian cells while retaining their functionalities. Protein-loaded baculoviruses (pBVs), can quickly and effectively deliver high doses of Cas9 or base editors proteins, resulting in high editing efficiencies in HEK293T.

By leveraging on this property, we implemented a selective protein loading strategy to increase protein delivery efficiency through a reversible, chemically inducible heterodimerization system of protein cargoes (Cas9) and pseudotyping modules.

Using spBVs we achieved high levels of multiplexed genome editing in a panel of human cell lines. Importantly, spBVs maintain high editing efficiencies in the absence of detectable off-target events. Finally, by exploiting Cas9 protein and template DNA co-delivery, we demonstrate up to 5% site-specific targeted integration of a 1.8 kb heterologous DNA payload using a single spBV in

a panel of human cell lines.

Altogether we have developed a highly modular and flexible baculovirus platform that enables a broad range of genome editing applications in human cells, with a particular focus on delivery efficiency and specificity.

94 - Implementation of TRuC T cells for the treatment of prostate cancer

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Finding new effective treatments for prostate cancer, a leading cause of cancer-related mortality in men, is crucial. While adoptive T cell therapy, like chimeric antigen receptor (CAR) T cell, has shown good results in lymphoid malignancies, it has been still ineffective against solid tumors. The promising results obtained with T cells equipped with T cell receptor fusion constructs (TRuCs) might change this paradigm. TRuCs comprise a single-chain antibody fragment (scFv) fused to one of the T cell receptor (TCR) subunits and combine effective elimination of cancer cells with physiological T cell signaling in preclinical models. However, TRuC generation currently relies on retroviral delivery of the transgenic TCR subunit that competes with the corresponding endogenous subunit for TCR assembly. We have developed a strategy that uses precise genome editing techniques for the targeted integration of a specific scFv coding sequence in-frame with the TCR ε-chain gene (*CD3E*). To this end, we have used the prostate-specific membrane antigen (PSMA) to selectively target and eliminate prostate cancer cells that express this membrane protein. This scar-less scFv knock-in retains the endogenous gene regulation of the ε-chain and simultaneously reduces the competition in TCR assembly, so contributing to the physiological-like features of the TRuC platform. Using an optimized genome editing protocol based on low dose CRISPR-Cas12a in conjunction with a short scFv-encoding DNA fragment, we achieved viral vector-free knock-in of the PSMA-targeting moiety in 13-34% of T cells isolated from three different donors. CAST-Seq analysis and targeted amplicon sequencing confirmed the high specificity of the *CD3E*-targeting CRISPR-Cas12a nuclease with no detectable off-target effects. *In vitro* functional assays showed comparable cytotoxicity against PSMA-positive cancer cells when comparing canonical CAR T cells to TRuC T cells generated either via the knock-in strategy or the viral vector-mediated overexpression of the transgenic TCR subunit fusion. Yet, and importantly, TRuC T cells presented with a subdued pro-inflammatory cytokine-release profile, which is associated with a lower risk of cytokine release syndrome. Upcoming *in vivo* experiments will determine whether our revised TRuC T cell platform enhances anti-tumor efficiency and safety in a preclinical solid tumor model.

93 - CRISPR/Cas9-mediated gene correction of Wilson disease H1069Q point mutation in iPS cells

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Wilson disease (WD) is characterized by an autosomal recessive gene defect in the copper transporting protein ATPase7B that leads to cytotoxic copper concentrations in the body, prominently in the liver. The most frequent mutation in western populations is the point mutation H1069Q (replacement of a histidine by a glutamine at position 1069). In this study, the H1069Q mutation was targeted by using the clustered regularly interspaced short palindromic repeats (CRISPR) associated nuclease 9 (Cas9) technology in WD specific induced pluripotent stem cells (iPSCs), followed by differentiation into hepatocyte-like cells (HLCs). One aim of the study was to correct the H1069Q mutation in WD iPSCs and to assess the ability of H1069Q-corrected HLCs to regain Cu resistance, indicating the functionality of ATP7B protein. Another aim was to figure out whether a CRISPR/Cas9 treatment may interfere with or inhibit the potential of iPSCs to differentiate into HLCs due to possible off-target effects. Epithelial cells from freshly donated urine obtained from a WD patient, carrying the compound heterozygous mutation H1069Q/N1270S, were collected and reprogrammed into iPSCs. WD iPSCs were transfected with the plasmid PX459.H1069Q plus a set of single-stranded oligo DNA nucleotides (ssODNs) for homology-directed repair (HDR). Single iPSC clones were analyzed by Sanger sequencing. H1069Q corrected iPSC clones were differentiated into HLCs and analyzed for hepatocyte-specific marker genes via real-time RT PCR. Using MTT assays, the cell viability of ATP7B corrected and uncorrected HLCs were determined after incubation in toxic copper concentrations. After CRISPR/Cas9-mediated genome engineering, 46 % of the cell clones indicated a gene correction of the H1069Q point mutation. The second mutation N1270S was not affected demonstrating the high specificity of the methodology. Corrected iPSCs could be differentiated to HLCs. The HLCs showed an upregulation of hepatocyte-specific marker genes as well as an improved resistance to high copper concentrations. This study demonstrates that genome engineering has a remarkable therapeutic potential to efficiently correct the ATP7B gene, thus further contributing to novel therapeutic approaches for WD specifically and rare monogenetic diseases in general.

92 - Precision targeting tumor cells using cancer-specific genetic alterations with CRISPR/Cas

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An ideal cancer therapeutic strategy involves the selective killing of cancer cells without affecting the surrounding normal cells. However, it is still challenging due to the many shared features between cancerous and normal cells. Here, we introduced a novel therapeutic strategy to induce cancer cell death selectively using the CRISPR/Cas system, named the cancer-specific insertions-deletions (InDels) attacker (CINDELA). CINDELA utilizes the original function of the CRISPR/Cas system in bacteria to cut out a foreign DNA of bacteriophage to target cancer-specific genetic elements which is not available in the normal host cells. Although CRISPR/Cas is widely used in genome engineering, the idea of introducing CRISPR-mediated DNA double-strand

breaks (DSBs) in a cancer-specific fashion to facilitate specific cell death is unexplored.

As a proof of concept, we first demonstrate that CINDELA selectively kills human cancer cell lines, xenograft human tumors in mice, patient-derived glioblastoma, and lung patient-driven xenograft tumors without affecting healthy human cells or altering mouse growth. Although we could achieve cancer-specific cell death with the cocktail of 20~30 CRISPR guide RNAs in these original studies, it is still challenging to apply in the clinical setting because of the issues of multiple guide RNA delivery and regulations for clinical trials. Recently, we improved this original method to reduce the number of required guide RNAs for cancer-specific cell death to less than five, which is feasible to deliver with currently available gene therapy vehicles. Furthermore, we even prove the special case that the single guide RNA can introduce cell death in cancer cells so it can move forward to the clinical trial based on current regulations of in vivo CRISPR therapy methods. The novel concept proposed in this study may become a potential approach for personalized cancer treatments.

91 - Highly efficient gene editing in human primary T cells using novel CRISPR effectors from the human microbiome

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The recent approval of the first CRISPR-based therapeutic represents a milestone for the gene editing field, demonstrating its high translational potential. However, to fully exploit the capabilities of the CRISPR technology, remaining limitations need to be further addressed to deliver the promise of efficient and specific editing at any site in the genome, both in vivo and ex vivo. Importantly, regulatory agencies raised concerns about the serious side effects generated by genotoxic stress in CAR-T cell products highlighting the need for highly precise editing tools to engineer next-generation ex vivo therapies. Thus, improving the safety of Cas nucleases as well as broadening their editing range represent clear unmet needs in the field.

To generate an improved CRISPR editor toolbox, we interrogated a large human microbiome dataset (above 1 million bacterial genomes, >100000 Cas9 loci) to identify novel Cas9 proteins characterized by various molecular sizes, low homology to previously described nucleases, and diverse PAM requirements, as determined by a highly efficient proprietary *in silico* tool.

Selected Cas9 were initially screened for their editing activity in human cell lines using an EGFP reporter, identifying more than 15 nucleases showing comparable levels of EGFP knock-out to *Sp*Cas9 (≥80% editing), thus supporting the efficiency of our selective platform. The editing activity of the best-performing editors was confirmed at endogenous loci of clinical relevance, including *TRAC*, *PD1*, and *B2M*, showing efficient gene modification (≥40% of editing after plasmid-based transfection).

To generate a proof-of-concept in the context of allogeneic cancer immunotherapy, we tested in human primary T lymphocytes seven novel nucleases showing the best activity profile, diverse

PAM specificities and different molecular sizes. Our Cas9 candidates were *in vitro* transcribed (IVT) and selected guide RNAs were easily synthetized by commercial providers due to their compact size and low sequence complexity. All shortlisted candidates demonstrated high editing activity at least on one of the three targets, with more than half showing between 60% and 90% KO efficiency at all three loci, comparably to the *Sp*Cas9 benchmark. The activity profile of the best-performing nucleases was also tested in multiplexing experiments including up to three targets. Furthermore, we are thoroughly investigating the genome-wide specificity profile of these best candidates as well as their propensity to generate translocations between simultaneously cut on-target sites.

Here we present a new set of Cas9 nucleases which not only are highly active in human primary T cells but also show a favorable safety profile, showcasing the strength of our discovery platform. Overall, our novel gene editor portfolio is characterized by high activity in human cells and diverse PAM requirements, opening new targeting possibilities and contributing to the advancement of genome editing-based therapeutics.

90 - Epigenome editing as a novel and safe strategy to control multiple immune checkpoints expression in CAR T cells

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T cell inhibition is a major obstacle to the success of CAR T cell immunotherapy. Mechanistic insights indicate that ligand-dependent activation of various inhibitory receptors coupled to different but synergistic intracellular pathways are associated to this ineffective state, creating an opportunity for novel therapeutic strategies. Genome editing has been used to selectively inactivate genes encoding for inhibitory receptors with encouraging results. However, when scaled up to address multiple targets simultaneously, side effects tend to be magnified due to the increase in genotoxicity related to multiple DNA breaks within the cell. Epigenome editing may be more suitable for multiplexing, as it allows to control gene expression by changing epigenetic marks needless of DNA sequence disruption. Here we report on the use of designer epigenome modifiers (DEMs) to specifically silence two key inhibitory receptors (*PDCD1* and *LAG3)* through targeted deposition of *de novo* DNA methylation at their promoters with a hit-and-run multiplexed approach in prostate cancer specific CAR T cells. Importantly, simultaneous delivery of the two DEMs did not affect the epigenome editing capabilities. Silencing of PD-1 and LAG-3 receptors remained stable throughout the long-term *in vitro* culture and upon multiple cycles of antigen-specific CAR T cell activation. Moreover, edited CAR T cells retained all the conventional functional features during an extensive *in vitro* characterization. Despite these remarkable results, epigenome editing shares with genome editing the risk for unspecific interactions that could cause off-target effects. Therefore, we performed a thorough specificity study based on a comparative transcriptome analysis. We identified a total of three significantly deregulated genes that could be explained by the engineering procedure itself as the corresponding *in silico* predicted DEM off-target binding sites were far from their *cis*-regulatory regions. Our study confirms the feasibility and safety of epigenome editing, and encourages its exploitation to modulate multiple T cell inhibitory pathways to provide improved CAR T cell products.

89 - CRISPR-Cas9 Patent Battles in US

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The CRISPR-Cas9 system, introduced in 2012, has revolutionized the gene-editing tool. Its groundbreaking impact was further acknowledged with the awarding of the Nobel Prize eight years later.

The rapid progression of this technology sparked a complex legal battle over patents. In the United States, the Broad Institute swiftly secured CRISPR-Cas9 patents through an expedited patent examination, causing friction with entities such as the CVC group and ToolGen. These disputes are particularly unique due to the United States' adherence to a 'first-to-invent' patent system until 2013, setting it apart from other countries' 'first-to-file' systems.

This presentation aims to provide a comprehensive overview of the ongoing CRISPR-Cas9 patent disputes in the United States. It is designed to offer both strategic insights and actionable advice for those engaged in the development of human therapeutics utilizing CRISPR-Cas9 technology.

88 - CRISPR/Cas9 Based Knock-In/Knock-Out of RAG2 Gene in Jurkat Cells as Model for Genome Editing Screening

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The RAG2 gene which encodes recombinase protein is responsible for a critical step in the development of B and T cells. Mutations in the RAG2 gene lead to variety of immune disorders, such as severe combined immunodeficiency (SCID). The severity of these immune-related diseases can vary depending on the RAG2-specific mutation and its impact on V(D)J recombination. The best treatment option for primary immunodeficiency is bone marrow transplantation or gene therapy. The Gene therapy for RAG2 deficiency is still in its early stages and recent advancements in CRISPR/Cas9-based genome editing technologies offer more precise and efficient ways to insert the functional RAG2 gene while minimizing potential off-target effects to correct target protein expression. Although $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) are target of genome editing, the major challenge is the lack of sufficient $CD34^+$ HSPCs in these patients. Using cell lines expressing the RAG2 gene could be a substituted candidate for pre-clinical approaches to develop CRISPR/Cas9 platforms. Based on this background, in this research, we used Jurkat cells to investigate the HDR approach of CRISPR/Cas9 and insert the codon-optimized RAG2 cDNA in the RAG2 gene location. With this strategy, we aimed to knock-in the designed RAG2 sequence downstream of endogenous RAG2 promoter versus knock-out the endogenous RAG2 through frameshift occurrence. The sgRNA for double-strand breaking of DNA was designed for exon2 of the RAG2 gene without any SNP in its target PAM and 20nt DNA sequence. Then the sgRNA was cloned into pX458 vector with Cas9 sequence and GFP sequence

as selection marker. The dsDNA donor for insertion of optimized 1584 bp RAG2 cDNA was designed with 400 bp symmetrical homology arms and silent mutations, next ordered to clone it into the pTwist vector. In the next step, the sgRNA and dsDNA donor vectors were transfected into Jurkat cells in 1:1 ratio with Lipofectamine 3000. The flow cytometry for GFP-positive cells was done for the determination of transfection efficiency. The GFP-expressed cells were collected and the DNA extraction was done for these cell populations. Two sets of junctional PCR primers were designed for screening of targeted insertion of optimized RAG2 in desired loci with correct 5′ to 3′ direction. One of the PCR primer pairs products was used for sequencing and analyzing the data with the Inference of CRISPR Edits (ICE) tool. After the confirmation of optimized RAG2 entrance in pooled cells, the clonal selection was done with limited serial dilution. Finally, the DNA of single clones was analyzed with mentioned PCR primers. The transfection efficiency with flow cytometry was above 50%. The results showed that the rate of insertion in pooled cells was more than 30% with the ICE analyzing tool. Also, the single clones with targeted insertion were selected for the NGS investigation as our next plan. Till this step we confirmed that optimized RAG2 gene can substitute with mutated gene which is better than editing of precise mutation. Checking of new gene expression and off-target screening will be our final steps in this model cells.

87 - Prime editing-mediated correction of the FANCA gene in primary patient cells

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Fanconi anaemia (FA) is a rare genetic disorder caused by mutations in one of the 23 genes involved in the FA DNA repair pathway. The hallmark manifestation of FA is bone marrow failure (BMF), which most FA patients undergo already in childhood. Currently, the only curative option for BMF is allogeneic transplantation of hematopoietic stem and progenitor cells (HSPCs) which requires a suitable donor. Gene therapies introducing a healthy copy of the causal gene into patient´s HPSCs are in clinical trials. However, genome-editing strategies that correct causal mutations could represent a more attractive therapeutic strategy for FA patients, particularly techniques such as prime editing, which can introduce precise correction with high specificity. Nevertheless, its potential for the correction of FA mutations needs to be evaluated. Using prime editing, we aimed to correct patient mutations in the *FANCA* gene. We designed pegRNAs and nicking sgRNAs for *FANCA* c.1 A>G mutation shared by two of our FA patients (patient 1: *FANCA* c.1A>G and c.4010+1-c.4010+18del; patient 2: *FANCA* c.1A>G and c. 3788_3790delTCT), and tested combinations of these gRNAs in both plasmid and RNA form in patient-derived fibroblasts. Overall, we tested the PE2, PE3, PE5, and PE5max systems and were able to reach 25% of precise correction 5 days after delivery of PE tools. Moreover, the amount of edited DNA increased over time in culture indicating functional restoration of the FA pathway and the associated proliferation advantage of corrected cells. We were also able to detect the restored, full-length FANCA protein in edited samples. Our results show that the use of prime editing is feasible in FA

patient-derived cells.

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86 - Exploring the functional impact of lncRNAs through CRISPRi screens in CRC models with KRAS and BRAF mutational background

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Colorectal cancer (CRC) ranks as the third leading cause of cancer-related deaths worldwide with metastatic CRC (mCRC) being particularly challenging. Notably, around 10% and 40% patients with mCRC carry BRAF and KRAS mutations, respectively. These mutations correlate with unfavourable prognosis due to resistance to existing therapies which prompts the need for exploration of new druggable targets. Limited understanding of the downstream effects of BRAF or KRAS mutations hinders the development of effective treatments. While previous cancer research has mainly focused on protein coding gene networks, it has overlooked non-coding genes such as genes for long non-coding RNAs (lncRNAs).

LncRNAs, recognized for their oncogenic roles, exhibit cell type-specific expression, making them attractive candidates for precision therapies. Our hypothesis suggests that untapped lncRNAs within the molecular networks downstream of driver genes could serve as novel targets for CRC therapies.

To identify potential CRC lncRNA therapeutics, we devised a screening strategy combining single-cell RNA-seq (scRNA-seq) and CRISPR inhibition (CRISPRi) in both 2D and 3D models. While early-stage cancer studies traditionally rely on human cell lines, the conversion rate to clinical trials is notably low (≤5%). To address this limitation, we included cell-lines derived and patient-derived organoids (PDOs) as our additional models. PDOs are self-assembling, three-dimensional cellular structures which stably retain key characteristics of primary tissue and as such are more physiologically relevant preclinical cancer models.

To select meaningful lncRNA to build our library, a home-made bioinformatic pipeline was set up to deconvolute scRNA-seq from 29 CRC patients to search for strongly enriched in epithelial cells. We curated a list of 996 epithelial lncRNAs (1963 transcripts), from which 523 lncRNA genes (1026 transcripts) were suitable for CRISPRi experiments. Each transcript has been targeted by 7 distinct sgRNAs. Additionally, our library includes 186 positive controls and 500 neutral controls, making a total of 6545 sgRNAs.

We established multiple BRAF- and KRAS-mutated CRC cell lines as well as CRC PDOs stably expressing the ZIM3-dCas9-KRAB fusion protein, that induces robust knockdown without causing

non-specific effects on cell viability and gene expression. From interesting preliminary data, we identified two oncogenic lncRNAs, namely *CASC19* and *LINC00460* and performed proof-of-concept experiments to validate our system. Efficacy of lncRNA knockdown was assessed via RT-qPCR, and the functional impact on viability was measured through diverse assays in cell lines, spheroids and PDOs, including incucyte monitoring and FACS-based competition assays.

Our ongoing research involves screening in a broad panel of BRAF- and KRAS-mutated CRC cell lines and corresponding spheroids. Promising candidates will undergo validation within PDOs using antisense oligonucleotides (ASOs). This work underscores the cell-type specificity of lncRNAs, emphasizing their potential as therapeutic targets, and introduces CRISPR technology into PDOs, enhancing the exploration of lncRNAs in clinically relevant 3D cancer models. The successful integration of CRISPR in PDOs opens new avenues for advanced investigations in the field.

85 - Efficient rAAV6-mediated HDR with low toxicity as a therapy for GATA2 deficiency through allele-specific gene correction in hematopoietic stem cells

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GATA2 deficiency is an autosomal dominant disorder caused by mutations in the gene encoding the transcription factor GATA2, leading to hematopoietic stem cell (HSC) dysfunction. This condition is associated with a reduction in immune cells, known as cytopenia, and a substantially increased risk of developing malignant blood diseases. Our study focuses on developing a novel therapy for GATA2 deficiency through ex vivo allele-specific correction of the *GATA2* variant c.956_962delGTGGCCT based on Cas9/sgRNA RNP delivery via nucleofection, coupled with DNA donor delivery for homology-directed repair (HDR) using rAAV6. Through the screening of multiple sgRNAs, we identified a sgRNA that exhibited no activity against the WT *GATA2* sequence and efficiently facilitated indel formation in the c.956_962delGTGGCCT allele in K562 cells, peripheral blood mononuclear cells (PBMCs), and hematopoietic stem and progenitor cells (HSPCs). In K562 cells engineered to carry c.956_962delGTGGCCT, co-delivery of Cas9/sgRNA RNPs and a rAAV6 vector carrying a DNA donor designed to correct the variant resulted in complete correction (>99% HDR) and fully restored GATA2 protein levels. Off-target analyses using DISCOVER-seq and subsequent high-throughput amplicon sequencing revealed a single bona fide off-target site in the lncRNA gene *AATBC*, which was targeted by HiFi Cas9 RNPs with 5.25% indel frequency in patient-derived PBMCs. Initially, editing of HSPCs using RNP nucleofection and rAAV6 donor delivery at an MOI of 2500, yielding an HDR rate of 71%, resulted in high toxicity and a severe reduction in colony-forming units (CFUs). Modification of medium composition, codelivery of multiple different mRNA transcripts to mitigate toxicity and increase HDR (GSE56, Ad5-E4orf6/7, and i53), and a 10-fold reduction in rAAV6 MOI alleviated toxicity while maintaining an HDR efficiency of 35% with no reduction in viability and CFUs. We evaluated the impact of *GATA2* editing in HSPCs by examining their differentiation through CFU assays, comparing normal HSPCs to those with a *GATA2* knockout (KO). *GATA2* KO increased the proportion of macrophage colony-forming units (CFU-M) at the expense of

granulocyte-macrophage (CFU-GM) and erythroid colonies, along with a marked reduction in cell proliferation. Rescue of the KO through HDR partially recovered differentiation and proliferation in the HSPCs to a level corresponding to the HDR rate. To investigate the potential selective advantage of *GATA2*-corrected HSCs *in vivo*, immunodeficient mice were engrafted with mixed populations of HSPCs containing cells engineered to carry either (i) the GATA2 c.956_962delGTGGCCT variant or (ii) silent mutations in *GATA2*. We found a significant enrichment of non-disruptive mutations after engraftment, suggesting that HSCs with functional *GATA2* outcompeted HSCs carrying defective *GATA2* alleles. Notably, using the optimized editing protocol in patient-derived HSPCs, we achieved 82% and 73% correction of the c.956_962delGTGGCCT allele at MOIs of 500 and 250, respectively. Ongoing studies focus on engraftment of corrected patient-derived HSPCs in immune-deficient mice, exploration of potential unintended large on-target modifications, and further investigation of potential off-target sites in patient-derived HSPCs using DISCOVER-seq.

84 - Gene editing in hematopoietic stem cells by delivery of CRISPR-based genome editing tool kits in lentivirus-derived nanoparticles (LVNPs)

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Development of new transport vehicles for delivery of gene editing tools is crucial to facilitate safe and efficient *in vivo* genome editing. Current *ex vivo* editing protocols are relevant for a small group of monogenic diseases but limited to specific tissues and complicated by the handling and expansion of stem cells. By adapting the properties of lentiviruses to incorporate polypeptides during virion assembly, we have developed a CRISPR/Cas9 delivery technology based on packaging of Cas9/sgRNA as ribonucleoprotein (RNP) complexes in lentivirus-derived nanoparticles (LVNPs). Delivery of RNP complexes limits the duration of Cas9 activity compared to delivery by mRNA or AAV and is thus expected to reduce off-target nuclease activity. By fusing Cas9 to the N-terminus of Gag/GagPol(D64V), Cas9 was found to be packaged into virus particles and released from Gag/GagPol by the viral protease during virion maturation. We also observed that sgRNA expressed from transfected plasmid DNA in the virus producer cells, was incorporated into virions in a Cas9-dependent fashion. Across multiple loci, potent targeted DNA cleavage leading to indel formation was observed in cells treated with such RNP-loaded LVNPs. Indels appeared 8 hours after the first exposure to LVNPs, and activity was not detectable after 4 days. In accordance, under conditions supporting similar on-target indel formation, we observed less off-target activity with LVNP-directed RNP delivery relative to standard RNP nucleofection. In hematopoietic stem and progenitor cells (HSPCs), treatment with LVNPs carrying Cas9/sgRNA targeting the *HBB* gene resulted in indel rates of up to 45%. Moreover, *in vivo* LVNP administration by subretinal injection in mice resulted in indel rates of up to 32% indels in the *VEGFA* gene. Building on this new delivery platform, we explored the capacity of LVNPs to co-package the Cas9/sgRNA RNP complex in combination with an RNA donor sequence

(converted to double-stranded DNA by reverse transcription) for editing of targeted genes by homology-directed repair (HDR). Using such 'all-in-one' LVNPs carrying an entire gene editing tool kit in cells treated with NHEJ inhibitors (M3814 or AZD7648), we were able to efficiently introduce the E6V *HBB* gene variant causing sickle-cell anemia with efficiencies up to 35% in K562 cells, 40% in HUDEP-2 cells, and 29% in HSPCs. Altogether, our findings mark the invention of a new CRISPR delivery tool supporting *in vivo* delivery of gene editing RNPs and *in vitro* HDR-directed genome editing with reduced off-target activity.

83 - EDSpliCE: A Novel Gene Editing Platform Holding Therapeutic Potential for Splicing Modulation in Inherited Retinal Disorders

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Disease-associated variants impacting splicing play a significant role in shaping the mutational landscape of numerous genes critical to human pathology, including in inherited retinal dystrophies. The pathogenic molecular mechanism act by impacting the recognition of canonical splicing signals or by creating new ones, leading to aberrantly spliced transcripts. Their predominant occurrence in intronic regions makes them of high therapeutic interest, as these sequences provide opportunities for gene editing without expected adverse effects on gene regulation and translation.

Current gene editing strategies, including the first in vivo clinical trial (EDIT-101), leverage on the use of multiple single gRNA to induce the targeted excision of intronic sequences harboring the splicing variant. However, such designs introduce (multiple) double-strand DNA breaks, which are of concern for the risk of chromosomal instability, elevating off-target potential, and when multiple gRNAs are used, they require additional elements for simultaneous delivery increasing the size and limiting delivery by AAV).

To address these concerns while ensuring strong wild-type splicing restoration, we have developed the **Enhanced-Deletion Splicing Correction Editing** (EDSpliCE) platform: **AAV-packable chimeric RNA-guided endonucleases** capable of **introducing enhanced** and **directional deletions** at the targeted sequence **using an individual single gRNA**. In terms of effectiveness, targeted perturbation of sequences involved in mis-splicing result in efficient splicing correction, while the high directional nature of these enzymes also enables addressing mutations in close proximity to exon boundaries. Concerning safety, **chromosomal translocations are nearly undetectable**, rendering **EDSpliCE an exceptionally appealing platform for advancing therapeutic development**.

To prove the effectiveness and safety of EDSplice, we have applied it to address pathogenic splicing variants (including exonic variants) relevant in inherited retinal dystrophies. Furthermore, we have explored splicing modulation as an independent therapeutic approach for autosomal dominant variants. Our preclinical experiments, specifically on *ABCA4*, *RHO*, and *USH2A*, demonstrate consistently strong and effective splicing correction, including upon AAV

delivery of EDSplice. Ongoing experiments focus on deep safety characterization and effectiveness in advanced cellular models.

In conclusion, **EDSpliCE represents a therapeutically viable option** for achieving robust splicing modulation across various inheritance patterns and disorders, **while effectively tackling the primary safety concern associated with the generation of double-strand DNA breaks**.

82 - Prime editing for correction of GATA2 deficiency in human CD34+ hematopoietic stem cells

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Ex vivo gene editing of autologous human hematopoietic stem cells is being developed as a treatment for inborn errors of immunity (IEIs). However, the current gene editing platform relying on Cas9-directed DNA cleavage and the use of rAAV6 as a donor for homology-directed repair is challenged by indel formation, off-target DNA cleavage, and toxicity in hematopoietic stem cells. Thus, there is a need for improved genome editing strategies in hematopoietic stem cells that do not require delivery of a DNA donor template and act without generating DNA double-strand breaks (DSB). Here, we present work using the DSB-independent prime editing technology to correct a patient-derived 7 bp deletion in the *GATA2* gene causing GATA2 deficiency, which can cause immunodeficiency, bone marrow failure, and increased risk of leukemia. We screened several prime editing guide RNAs (pegRNAs) as well as nicking sgRNAs (ngRNAs) to efficiently correct the 7 bp deletion, and we found that editing efficiencies were highly dependent on the ngRNA, resulting in prime editing rates in model K562 cell lines that varied between 20% and 80%. We then aimed at establishing efficient prime editing in CD34+ hematopoietic stem and progenitor cells (HSPCs). Initial attempts using *in vitro*-transcribed PEmax mRNA in combination with synthetic pegRNAs and ngRNAs resulted in low editing efficacies. However, using enhanced pegRNAs (epegRNAs) as well as optimizing nucleofection conditions, mRNA structure and epegRNA:ngRNA ratios, we were able to achieve efficient prime editing of up to 60% of *GATA2* alleles in CD34+ HSPCs from healthy donors as well as in patient-derived HSPCs. Furthermore, we investigated the fitness of the CD34+ HSPCs following editing using either prime editors or conventional HDR-based gene editing. While prime editing generally resulted in less editing than conventional HDR-based gene editing, prime-edited HSPCs showed superior viability and proliferation. Furthermore, as opposed to RNP nucleofection and AAV transduction, which led to a 2-fold decrease in colony forming units (CFUs) compared to mock-treated HSPCs, prime-edited HSPCs showed no significant decrease in CFU potential. We are currently investigating the potential of prime-edited patient-derived CD34+ HSPCs to engraft in immunodeficient mice. We are also investigating off-target editing as well as potential adverse on-target genomic alterations using long-read sequencing. Together, our preliminary results indicate an increased fitness compared to traditional CRISPR-based gene editing approaches,

supporting the further exploration of prime editing to treat IEIs.

81 - CRISPR/Cas9-mediated gene editing delivered by a single AAV vector inhibits viral reactivation of HSV-1 in a latent rabbit keratitis model

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80 - Efficient large knockins in mice and cells validated using an all-in-one, multiplexed long-read sequencing assay

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Large knockins, such as for gene humanization, cDNA overexpression, replacement, reporters and conditional knockins**,** are usually a challenging type of gene editing in animal and cell model creation, compared to simple knockout (KO) and introduction of single-nucleotide polymorphism (SNPs) and small tags. Donor plasmid construction is progressively more difficult with increased size. The use of recombinant Adeno-associated virus (rAAV) donors for manipulating single-cell mouse embryos, circumventing the need for microinjection, greatly improved both birth rates and insertion efficiency. The maximum payload in a single rAAV vector limits the size of insert. To overcome the size limitation, we delivered multiple rAAVs together before RNP electroporation, allowing sequential targeted integrations, each insertion introducing a new gRNA target site for mediating the next insertion. In the meantime, larger inserts with multiple rAAV donors create a genotyping dilemma with the need to amplify numerous junctions and some with amplicons spanning several Kb. Here we report over 100 knockin mouse models completed using up to three rAAV donors per project and the development of a universal genotyping assay to analyze on-target, random and off-target integration by combining target capture and long read sequencing. The all-in-one assay is cost effective and high throughput for screening founder animals and clonal cell lines. The method is also powerful to identify insertion locations of random transgenes, such as retroviral transduced CAR-T cells. In addition to validation of the correct insertion in the genome, the method may help shed light on mechanisms for homology-dependent repair (HDR) and off targeting.

79 - Unraveling genome integrity and safety of gene engineered hematopoietic stem and progenitor cells for the treatment of RAG1 deficiency

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CRISPR-Cas9 genome editing (GE) is a promising platform that in the last decade has progressed from being a research tool to a potential therapeutic treatment. We recently established a CRISPR-Cas9 GE approach in human hematopoietic stem and progenitor cells (HSPCs) to treat *Recombination Activating Gene 1* (*RAG1*) defects, causing inborn errors of immunity (Castiello, Brandas, Ferrari, et al. *Sci Transl Med* in press). An effective knock-out/knock-in strategy was developed to disrupt the mutated *RAG1* gene by non-homologous end joining (NHEJ) and simultaneously insert a codon optimized *RAG1* corrective cassette by homologous-directed repair (HDR) process exploiting adeno-associated vector serotype 6 (AAV6) or integrase-defective lentiviral vector (IDLV) as delivery platforms. However, assessment of safety and genome integrity after GE is becoming extremely crucial for the clinical translation of our GE approach. Detection and validation of unintended events at on- and off-target sites in a highly sensitive and comprehensive manner remains a key challenge in the field of GE. It is increasingly recognized that double-strand break (DSB)-based GE triggers activation of a p53-dependent DNA damage response and its processing may lead not only to small insertion and/or deletions (indels) originating from NHEJ and microhomology mediated end joining or by HDR, but also long-range genomic rearrangements which could propagate towards one or both ends of the chromosome. Thus, we firstly optimized a copy number variation (CNV) assay using droplet digital PCR (ddPCR) on *RAG1* edited HSPC-derived colony forming unit to assess undesired deletion events at on-target site by analysing *TRAF6* and *RAG2* genes, respectively located 56kb upstream and 13kb downstream of the cutting site. Data from this analysis showed the occurrence of potential unintended deletions nearby the on-target site, with a higher number of monoallelic deletions in the *TRAF6* gene compared to the *RAG2* locus. For a more unbiased assessment of genomic rearrangements, we evaluated the presence of large structural variants by optical genome mapping (OGM) which points out the presence of insertions and/or inverted duplication upon GE at on-target site. Additionally, Chromosomal Aberrations Analysis by Single Targeted linker-mediated PCR sequencing (CAST-Seq) was performed to discriminate relevant events detected by OGM and ddPCR-based assays. From this analysis, we observed a safe profile of our ribonucleoprotein with the expected presence of on-target aberrations and the absence of detectable off-target sites. Further analysis will be performed to evaluate the presence of possible unintended genomic rearrangements in long-term edited HSPCs. Overall, data from these analyses will be instrumental to shed light on the mechanisms and the impact of GE procedure on HSPCs and further implement *RAG1* HDR-mediated GE before moving to the clinical setting.

78 - Prenatal In Vivo Base Editing for the Treatment of Krabbe Disease

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Infantile Krabbe disease (KD) is an autosomal recessive genetic disorder characterized by a severe and progressive neurodegeneration leading to fatal outcome within the first 3 years of life, caused by mutations in gene encoding for the lysosomal hydrolase galactocerebrosidase (GALC). Clinical manifestations are neurological, due to de-myelination in the central and peripheral nervous systems (CNS and PNS), astrocytic gliosis, and neuroinflammation. Currently, KD remains without a definitive treatment. Hematopoietic stem cell transplantation can delay the onset and progression of the disease thanks to GALC reconstitution in CNS tissues by hematopoietic stem cells-derived myeloid cells and only when performed in pre-symptomatic newborns, highlighting the narrow therapeutic opportunity window. Therefore, an effective and safe therapy that can correct the underlying mutations, prevent early-onset neuropathy, and preserve the developing brain is urgently needed. In this regard, we are developing a prenatal, in-vivo gene editing platform to mediate *Galc* mutations correction in the CNS, PNS and hematopoietic system using adenine base editors (ABEs). *In utero* intervention brings many advantages, including early treatment before disease onset, increased accessibility to the brain and to proliferating cell progenitors, high immune tolerance and reduced BE doses needed. We have generated engineered Virus-Like Particles (eVLPs) containing the ABE8e-NG and a cognate guide-RNA targeting the non-sense point mutation (TG**A**>TG**G**) that introduces a premature stop codon in the *Galc* gene, thus leading to absence of protein expression and the onset of the disease in the KD Twitcher mouse model. The use of ABE to correct point mutations in situ guarantees a permanent therapeutic benefit compared to current strategies based on Adeno-Associated Vector-delivered GALC cDNAs and, when packaged into eVLPs, it ensures a fast, transient, and widespread delivery to many cell types involved in KD. For the initial proof-of-concept, we have developed a reporter system harbouring a portion of the mutated *Galc* gene fused to an mCherry reporter gene, where ABE-mediated correction of the *Galc* mutation activates mCherry expression. Using this reporter system in combination with ABE-eVLPs in 293T cells, we achieved up to 95% correction frequency of the target mutation as assessed by mCherry protein expression by flow cytometry, immunoblotting and enzyme linked immunosorbent assay (ELISA). We then tested the ABE-eVLPs in an immortalized KD Schwann cell line by transduction in a dose dependent manner, to find the best dose capable of effective base editing without compromising cell viability. Analysis of GALC protein reconstitution showed a 2-3-fold increased gene expression in edited KD Schwann cells as compared to unmanipulated controls. We are now progressing to *in vivo* testing of our BE-eVLP platform by their *in utero* injection in pregnant female mice to assess functional gene correction and GALC protein expression, as well as biodistribution of eVLPs in the foetal and surrounding maternal tissues. Our final aim is to develop a safe and efficient *in vivo* BE platform to treat KD, which could ideally serve as a proof-of-concept therapeutic platform for other early-onset lysosomal storage disorders and neurodegenerative diseases.

77 - ePsCas9- a CRISPR tool for in vivo therapeutic genome editing at AstraZeneca

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Among the Cas-enzymes found in the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems, *Streptococcus pyogenes* Cas9 (SpCas9) is the most commonly used for therapeutic genome editing. There are known limitations of SpCas9; high promiscuity and off-target activity. Our group has identified a high-fidelity enzyme of the Type II-B subfamily, *Parasutterella secunda* Cas9 (PsCas9), and demonstrated its ability to edit the mouse genome after viral delivery. Analysis of the editing events highlighted fewer off- target events and chromosomal translocations compared to SpCas9. To further improve fidelity and editing efficiency we rationally engineered PsCas9, creating the variant ePsCas9. Here we present editing and safety data from *in vivo* delivery of ePsCas9 to C57BL/6NCrl mice. Delivery of ePsCas9 mRNA and sgRNA encapsulated within lipid nanoparticles induced a high degree of insertions and deletions (indels) within the targeted gene one week after dosing. More specifically, ePsCas9 achieved indels in 60% of reads; this was three times greater than SpCas9 editing. The difference in protein reduction correlated to the degree of editing. The level of editing indicates that the LNP is taken up into nearly all hepatocytes. Investigation of sgRNA modification indicates that ePsCas9 requires only end modification compared to the highly modified gRNA of SpCas9. The immunotoxicology cytokine panel and clinical chemistry revealed neither liver damage nor acute inflammation during the study. Collectively, these results introduce ePsCas9 as a highly efficient engineered enzyme that has high-fidelity and a good genomic safety profile, making it a therapeutically applicable enzyme that has the potential to deliver high quality precision medicines to patients.

76 - Generation of an experimental model for whole genome CRISPR screening to study hypoxia-induced drivers of chemotherapy resistance in muscle invasive bladder cancer

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With ~500,000 cases a year, bladder cancer is the 10th most common cancer worldwide. Chemotherapy resistance (CR) is a problem in muscle invasive bladder cancer (MIBC) due to the presence of intratumoural hypoxia. Whole genome CRISPR screening (WGCS) is a powerful tool for discovering novel genes involved in therapy resistance but has not yet been applied in MIBC. This research aims to characterise and generate an *in vitro* model for future WGCS experiments studying drivers of hypoxia-induced CR in MIBC.

Three routinely used human MIBC cell lines (T24, J82 and UMUC3) were chosen for model development. The suitability of the MIBC cell lines for WGCS was determined by measuring baseline cell viability, hypoxia-induced CR and Cas9 transduction and cutting efficiency.

Cell viability was assessed by flow cytometry using Annexin V/7AAD staining and demonstrated that the baseline viability of T24 (66%) cells was significantly ($p<0.001$) lower than in J82 (90%) and UMUC3 (95%) cells at the 48h timepoint (2-way ANOVA/Tukey's multiple comparisons test). Hypoxia-induced CR to varying doses of cisplatin (0.001μM-100μM) was assessed using trypan blue exclusion using the Countess™ automated cell counter. Our data demonstrates that T24 cells showed significant hypoxia (1%, 0.1% O_2) induced chemo-resistance in a dose dependent manner when compared to cells grown in normoxia (21% O_2) (2-way ANOVA, Tukey's multiple comparisons test). Notably, this resistance was found at 1μM cisplatin (at 48 and 72hrs) which coincides with peak plasma concentrations in patients, highlighting clinical relevance. No cisplatin resistance was found in J82 or UMUC3 cells. These results demonstrated T24 cells were most suitable for our study. Therefore, lentiviral transduction of Cas9 was only performed and functionally validated in this cell line.

T24 cells were transduced with a lentiviral Cas9 expression vector and blasticidin-selected to generate a polyclonal population of Cas9-expressing cells. Cas9 expression levels, assessed by flow cytometry, were found to be 85%. To confirm the Cas9 cutting efficiency, transduction of a sgRNA targeting the AAVS1 gene was performed. Following transduction, genomic DNA was extracted and the AAVS1 gene was amplified by PCR, purified and Sanger sequenced. The ICE analysis tool from Synthego confirmed 90% induction of indels showing successful function of Cas9 within the population.

To confirm that Cas9 expression does not alter the phenotype of T24 cells, growth curves comparing proliferation of wildtype (WT) vs Cas9 expressing T24 cells were conducted under normoxic (21% O_2) and hypoxic (1%, 0.1% O_2) conditions for 5 days. No significant differences were observed between the WT or Cas9 expressing T24 cells (unpaired t-test).

In conclusion, we have developed a suitable *in vitro* MIBC model for conducting WGCS experiments to determine hypoxia-induced drivers of CR. Future work will involve carrying out a WGCS and validation of identified targets in single gene knock-out experiments with an aim to find druggable targets for clinical translation.

75 - Off-targets for good: Mispairing alters the rate and position of AsCas12a cleavage

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AsCas12a, a small CRISPR-associated nuclease, uses a guide RNA (gRNA) to direct sequence-specific DNA cleavage. Cas12a recognizes DNA sequences with a T-rich PAM site and cleaves DNA to produce 5' overhangs. It also tolerates small differences between the gRNA and DNA target sequences, especially in PAM-distal regions. This contributes to 'off-target' activity, where the nuclease may cut additional sequences, causing unexpected and adverse consequences in the genome. Our objective was to elucidate the kinetics and specific cleavage outcomes upon programming Cas12a with a gRNA that mispairs with its DNA target. To understand how such intentional modifications to the gRNA or target DNA influence AsCas12a, we performed *in vitro* cleavage assay experiments with targets and gRNAs containing PAM-distal mispairs. We fluorescently tagged each end of the target and non-target DNA strands. Upon

analysis, our data revealed their cleavage rates, initial DNA cleavage sites, and end trimming rates for each of the DNA strands and ends. We found that differences between the gRNA and target DNA sequences produced mispair-dependent cleavage products of different lengths and related trimming profiles. In conclusion, mispairing affects CRISPR-Cas enzymes' ability to accurately recognize and cut their intended target sites, which impacts their gene editing potential. Intentionally programming AsCas12a with PAM-distal mispairs could even alter gene editing outcomes simply by changing the gRNA sequence.

74 - Base editors provoke non-predictable chromosomal translocations and off-target editing

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Base editing has emerged as a promising alternative to designer nuclease-based genome editing. Despite its promise of reduced genotoxicity, a comprehensive genome-wide analysis of base editing-induced chromosomal rearrangements in primary cells is lacking. To this end, we edited primary human T cells at three previously validated genomic targets (within the *CCR5*, *EMX1* and *FANCF* genes) with either CRISPR-Cas9 nuclease, the adenine base editor ABE8e, one of three cytosine base editors (evoCDA1-BE4max, TadCBEd, CBE-T1.52), or the Cas9-D10A nickase. We used CAST-Seq to identify gross chromosomal aberrations and multiplex amplicon sequencing (rhAmpSeq) to simultaneously determine editing frequencies at the target sites and at the more than 100 nominated off-target sites (OTs). Sequencing confirmed high on-target activity, with 70-78% edited alleles for the nucleases and 44-84% for the base editors. As expected, editing with Cas9-D10A nickases induced insertion/deletion mutations (indels) at near background levels. The percentage of on-target alleles with large (>200 bp) chromosomal rearrangements ranged from 45% for T cells edited with CRISPR-Cas9 nuclease to 2-7% of alleles for CBE, 5% for ABE, and 0,5% for the nickases. Notably, in evoCDA1-BE4max-treated cells the total number of chromosomal translocations exceeded that found in Cas9-nuclease edited T cells by a factor of 3. Furthermore, we observed extensive C to T conversion (up to 99% of alleles) and mutagenesis (up to 7% of alleles) at nominated OT sites. In contrast, T cells edited with the recently developed TadCBEd or CBE-T1.52 showed only marginal OT effects. Remarkably, while ABE8e editing induced few translocations, 17 of the 40 identified OTs showed substantial OT activity with more than 10% of alleles being modified. In conclusion, our study reports for the first time the genome-wide detection of chromosomal rearrangements and OT activity induced by base editors. While OT effects in TadCBEd and CBE-T1.52 edited T cells were low, our data revealed the genotoxic potential of ABE8e and especially of the hyperactive evoCDA1-BE4max. These two base editors induced mutagenic OT editing at sites that were not altered by CRISPR-Cas9 nucleases complexed with the same gRNA. This suggests that the mechanism of

gRNA-dependent OT activity of base editors is different from that of CRISPR-Cas nucleases, and that nucleases cannot be used as a surrogate for identifying OT effects induced by base editors.

73 - Functionalized Lipid Nanoparticles for Hepatic Delivery of RNA-therapeutics

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Lipid nanoparticles (LNPs), used for the delivery of the mRNA-based COVID-19 vaccines, have become the gold-standard delivery system for RNA-based therapeutics. The technological advancements of the multifunctional CRISPR/Cas9 gene editing tool have further opened up avenues for LNP administration of CRISPR therapy. However, the delivery specificity, CRISPR efficacy and risk of potential off-target effects remain important challenges that hinder straightforward clinical translation. Functionalized LNPs hold great promise to facilitate more specific uptake in targeted tissues/cells thereby enhancing editing efficiency while minimizing off-target effects.

This study aims to investigate the functionalization of mRNA-LNPs with targeting peptides (P-LNPs) and identify the best candidate that facilitates uptake in mouse hepatoma cells (Hepa 1-6). Selected P-LNPs are to be validated in mice, quantifying P-LNPs potential enrichment in the liver hepatocytes by multiple independent methods. As a baseline for testing various peptides, the standard Moderna LNP formulation is used. A microfluidics device is employed for the preparation of P-LNPs loaded with firefly luciferase (fLuc) mRNA, followed by standard characterization of physicochemical properties. Each peptide is conjugated to LNPs through maleimide-thiol coupling.

Data confirms high efficiency and quick peptide conjugation to LNPs, i.e., >80% conjugation within 25 minutes measured by HPLC and a fluorometric assay. Hepa 1-6 cells show a **7-to-14-fold increase** in fLuc expression at various doses, 24 hours after delivery of the top P-LNP candidate compared to Moderna LNP. P-LNPs show a similar favorable toxicity profile as the Moderna formulation in the cell studies, which indicates that the effective concentration of peptides is safe to use in LNP formulations. In addition, the increase in bioluminescence was partially retained (5-fold) after incubating the LNPs with bovine serum (15 minutes) prior to transfection.

In conclusion, preliminary data supports that functionalization of Moderna LNPs with peptides can promote increased fLuc bioluminescence in hepatoma cells. This functionality is retained in the presence of serum. Currently, mouse studies with P-LNPs are ongoing to support collected data and monitor changes in fLuc bioluminescence in the main organs after intravenous injection. In addition, experiments will be conducted for quantifying LNP-delivery of ssDNA barcodes to further document that functionalized P-LNPs can be used to direct biodistribution towards certain organ(s) or cells of interest. Further studies are also planned to investigate the functional uptake of P-LNP candidatesin different human hepatocarcinoma cells, in combination with efficacy tests of CRISPR/Cas9 gene editing.

72 - Enhancing CRISPR-Cas9-Mediated Homology-Directed Repair Using Modified sgRNAs

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CRISPR−Cas genome-editing relies on the ability of the Cas endonuclease to induce a targeted double strand break which can then be repaired by one of two DNA repair pathways: homology-directed repair (HDR) or nonhomologous end-joining (NHEJ). Repair by NHEJ is error-prone, consequently, it can be used to inactivate genes by introducing random insertions or deletions. Conversely, repair by HDR is error-free and therefore it is the desired pathway of choice for the insertion of accurate genetic modifications. Yet, this is restricted by the relatively low efficiency of HDR compared to NHEJ. To enhance HDR, researchers have tried a few different approaches such as modifying the protein part of the CRISPR-Cas9 by fusing HDR-inducing protein domains. Or by using modulators of DNA repair pathways, typically, NHEJ inhibitors. In recent years it has become apparent that modifications to the RNA part of the CRISPR-Cas9 influence activity and possibly the mode of repair. In this project we construct and screen a library of modified guide RNAs for their ability to enhance HDR. This was achieved by fusing them to the RNA component of the CRISPR-Cas system. After four rounds of screening, sequencing and bioinformatics analysis, we generated a short list of around 150 candidates. When tested functionally, we found that some of these candidates can enhance HDR.

71 - Enhancing the precision and fidelity of CRISPR/Cas9 genome editing through chemical inhibition of end-joining repair pathways

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Genome editing through CRISPR/Cas9-induced DNA double-strand breaks offers promising opportunities to correct various genetic defects. However, the simultaneous activation of DNA repair pathways to repair the targeted lesion leads to a low efficiency of templated DNA integration and the generation of unwanted mutations, representing major limitations of genome editing applications. To overcome these challenges, we developed pharmacological treatments that modulate DNA repair outcomes and improve precise genome editing efficiency. Specifically, we identified potent and selective compounds that inhibit DNA-dependent Protein Kinase (DNA-PK) and DNA Polymerase Θ (PolΘ), both involved in mutagenic end-joining repair pathways. The combined treatment, dubbed 2iHDR, significantly and robustly boosts homology-directed repair-mediated precise gene editing, resulting in up to 80% efficiency of templated insertions with minimal unintended Insertion-Deletions (InDels). Importantly, this strategy also reduces the risk of Cas9-related off-target effects, improving the performance and safety of this gene editing tool.

70 - Design of highly efficient sgRNA libraries through comprehensive feature analysis

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High-throughput functional genomics using CRISPR/Cas9 has emerged as a transformative technology for unraveling the genetic drivers of disease pathogenesis and thereby accelerating drug discovery. The quality and reproducibility of CRISPR screens are directly linked to a reliable single guide RNA (sgRNA) design for precise targeting of the Cas9 effector molecule to the desired genomic region while minimizing off-target effects. To generate robust libraries of optimal sgRNA sequences, we have developed an algorithm that incorporates several features, including nucleotide sequence, evolutionary conservation, and protein domain organization.

To benchmark the Myllia sgRNA library, we compared its performance with various published libraries, such as GeCKOv2, TKOv3, Brunello, VBC, and MinLibCas9. To this end, we targeted several essential genes and assessed the dropout of the corresponding sgRNAs in a fitness screen. We also included an experimental arm in which we focused on a set of non-essential genes affecting the expression of a cell surface marker that could be analyzed in a FACS-based assay. Data on the performance metrics of the Myllia library, as well as other libraries, will be shared at the conference. Ultimately, such datasets will be useful to improve sgRNA design and potentially reduce the library size to facilitate gene function studies in intricate models where only a limited number of cells are available.

69 - Image-enabled cell sorting and transcriptomic profiling to identify cellular phenotype-transcriptome relationships

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In a clinical context human blood can be used to profile several aspects of human health like metabolite levels, hormone panels or organ function. In addition, the human blood holds different populations of immune cells, in number, morphology, and activation state often directly correlated to certain disease phenotypes. Their accessibility makes them an interesting model for screening therapeutic agents, especially in the context of inflammation driven conditions. However, since inflammatory diseases are quite heterogeneous, the ultimate utility of these models depends on the ability to accurately classify the cells in health and disease.

Technological advances have made single-cell characterisation of diverse cell populations on several levels, including immunophenotyping and transcriptomics a reality. Imaging flow cytometry combines the spatial resolution of fluorescence microscopy with flow cytometric cell sorting and allows the rapid identification and isolation of cells with specific cellular phenotypes.

In this study we are using the BD S8 Imaging Flow Cytometry system for label-free sorting of leucocyte populations (PBMC, PMN) from fresh blood of healthy donors. Using imaging parameters generated by the CellView Technology like lightloss, radial moment and eccentricity we can directly separate aggregates of cells from singlets and live from dead cells. We furthermore apply an in house developed imaged-based deep learning approach (Phenospace) to classify 'normal' leucocyte cell types from healthy donors using lightloss images only. Using known stimuli for cell activation we are inducing disease-relevant phenotypes or activate pathways that stimulate an inflammatory cell response. The multicolour fluorescence imaging part of the S8 is used to confirm pathway activation by protein localisation via spatial correlation of two signals. We quantified the translocation of the NF-κB pathway component p65 from the cytoplasm to the nucleus upon LPS induced TLR4 activation, as detected by an increase in correlation between p65 and the nuclear dye 7AAD. Additional immunoprofiling of CD14+/CD16+ cells successfully confirmed the existence of activated subtypes. This set of data will be used to train our AI model on the recognition of 'disease' phenotypes.

Our aim is to use a combination of Phenospace and transcriptomic profiling to determine whether morphology serves as a direct readout of (mal)functional cell states, including transcriptional expression, and response of cells in a drug or CRISPR screen setting. The data will directly be used to complement with our DiseaseLINX platform to identify novel drug therapies by searching for drug candidates whose molecular effects anti-correlate with disease molecular signatures. We aim to develop a workflow that can be used in a clinical setting to examine disease progression, identify suitable therapies, and give insights on resistance phenotypes of non-responders.

68 - Development of molecular biology assays to measure precision and fidelity of gene insertions

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Gene insertion/replacement holds great promise for the treatment of multiple diseases. There are several approaches to insert genes into the genome, both targeted and randomly. For safety reasons it is important to map transgene integration to evaluate the potential risks associated with genomic locations. We have developed multiple Illumina based assays to detect viral

integrations and off-target nuclease activity. In addition to this, we are using long-read Oxford Nanopore sequencing to monitor the fidelity of on-target integration of large DNA donor constructs. We will present examples on the use of these assays within the development of preclinical gene therapy programs.

67 - Evaluation of CRISPR off-targets in single cells reveals previously unidentified off-targets and gives mechanistic insights in relation to cellular chromatin and gene expression state

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CRISPR-based gene correction has great potential for the curative treatment of genetic diseases, but off-target mutations at unrelated genomic sites pose a potential safety risk for clinical application. In order to mitigate this risk, a better understanding of underlying mechanisms is needed. Particularly, knowledge of how off-target effects might differ between individual cells and different cell types is still lacking to date.

We analyzed CRISPR off-target mutagenesis in mouse embryos at the single-cell stage with RNPs containing SpCas9 together with either a promiscuous or specific *Pcsk9*-targeting sgRNA (previously described in Akcakaya et al., Nature 2018). Our experiments demonstrated unique off-target profiles in individual cells even under the same conditions. To explore mechanisms underlying off-target editing outcomes, we established a workflow assessing single-cell CRISPR off-targets alongside chromatin state and gene expression in mouse embryonic stem cells. Once again we observed distinct off-target profiles in line with our results in embryos. Importantly, integration of the scATAC-seq and scRNA-seq data unveiled a potential influence of chromatin environment and gene expression activity on off-target mutagenesis. These results indicate the outcome of a gene editing medicine could differ greatly between different organs due to the natural differences in gene expression and chromatin landscape. To study the off-target differences in different tissues, we have generated a mouse model with doxycycline-inducible SpCas9 expression and constitutive sgRNA expression in all organs. Preliminary results from this model indicate indeed different editing outcomes in the different organs.

Taken together, our results provide the first mechanistic insights of CRISPR off-targets at the single cell level as well as in different organs *in vivo*. Our findings emphasize the need to consider chromatin and gene expression in order to fully understand and minimize any potential safety risks of therapeutic gene editing.

66 - CRISPRon/off: Combined CRISPR/Cas9 on- and off-target design

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The design of gRNAs for CRISPR is rapidly evolving, trying to keep up with the latest developments in CRISPR. Once the target is selected, the design of optimal gRNAs is a job in two parts. The first part is the selection of *efficient* gRNAs that can affect the design change, while the second part is the search for potential *off-targets* for the efficient gRNAs. Off-targets of CRISPR/Cas9 mediated genomic edits are of critical importance for both experimentalists and clinicians in determining potential side effects of a CRISPR/Cas9 experiment or clinical treatment. Here we present a webserver with a combined workflow for on/off-target assessment based on our CRISPR/Cas9 methods, **CRISPRon**, which is the current best model for on-target efficiency, and **CRISPRoff**, which is versatile energy model for gRNA off-targets capable of competing with the best available deep learning methods. In the figure, we show the workflow of on-target and off-target assessment on https://rth.dk/resources/crispr

65 - Efficient single-cell cloning and culture of hiPSCs in small-scale fluid-walled cell culture chambers

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Human induced pluripotent stem cells (hiPSCs) represent a significant advancement in disease modeling, drug discovery, and regenerative medicine. When combined with cutting-edge gene-editing technologies like CRISPR-Cas9, hiPSCs offer an unparalleled opportunity for patient-specific disease mechanism studies. However, a major challenge in this domain is the need for high-quality single-cell culture techniques to derive effective cellular models from gene-edited hiPSCs. The sensitivity of hiPSCs to handling and manipulation has been a significant hurdle in generating clonal cultures efficiently.

In this study, we evaluate a novel small-scale cell culture technique that uses fluid walls for both bulk- and single-cell hiPSC cultures. Our assessment focuses on changes in gene expression in bulk culture, and the maintenance of pluripotency, genetic stability, and tri-lineage differentiation in single-cell cultures.

Our findings indicate that bulk-cultured hiPSCs in small-scale environments show a marked increase in mitochondrially encoded transcript expression. This elevation reverses when cells return to standard culture settings. Interestingly, this increase in transcript abundance does not correlate with mitochondrial DNA copy number and is only detectable under specific medium and coating combinations.

Moreover, our refined and automated single-cell cloning workflow, applied to various genetically distinct hiPSC lines after CRISPR-Cas9 editing in these small-scale chambers, achieved cloning efficiencies up to 85%. Quality control analyses, including karyotyping, pluripotency marker expression, and tri-lineage differentiation, confirmed that the derived colonies preserved pluripotency marker expression, genomic integrity, and effective differentiation capabilities into

mesoderm, endoderm, and ectoderm.

64 - MyoPax: the vanguard in cell and gene therapies for muscle disorders

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Muscle disorders affect >20 million people in the EU. Muscle weakness is a consequence in common diseases (cancer, trauma, critical illness) or in genetic muscular dystrophies and may lead to loss of personal autonomy and premature death. For the vast majority of patients palliative care is the only option, with no approved drugs for >95% of muscular dystrophy patients. The Muscle Research Unit of the Charité University Hospital Berlin has developed a proprietary technology to produce highly regenerative, primary human satellite cell-derived muscle stem cells (PHSats, patented EU/US). PHSats are purely myogenic, highly proliferative, safe and can rebuild muscle tissue and replenish the muscles stem cell pool offering a long-lasting autologous treatment for muscle regeneration. Beyond the replacement utilization of these cells to regenerate and replenish muscle in local defects, the Muscle Research Unit of the Charité University Hospital Berlin applied gene editing technologies to patient´s PHSats suffering from genetically caused muscle degeneration. The disease-causing mutations were highly efficiently corrected using mRNA-mediated CRISPR-based gene repair. Consequently, these scientific discoveries led to the initiation of a Charité-sponsored first-in-human clinical trial (Basket) for the treatment of muscular dystrophies to restore essential muscles in these patients. The clinical translation of these scientific discoveries is a long path with multiple challenges. To accelerate this translation and bring this innovative therapeutic platform for muscle disorders to the patients, the spin-off MyoPax of the Charité Universitätsmedizin and the Max Delbrück Center in Berlin has been founded. In 2022 MyoPax joined the biotech spin-off incubator program of the BioInnovation Institute (Novo Nordisk Foundation) and established operational entities in Berlin and Copenhagen. MyoPax has obtained both Rare Pediatric Disease Designation (RPDD) and Orphan Drug Designation (ODD) from the FDA for the Charité-sponsored first-in-human trial (MUST) in which unedited PHSats will be injected into children to restore a prenatal urethral muscle sphincter defect. In preparation for both clinical trials, MyoPax has conducted an audit of the ATMP producing GMP facility for manufacturing of edited and unedited PHSats. In addition, MyoPax performed GAP analysis on the GMP technology transfer process with regulatory experts. Meanwhile, the automation and scale-up of the manufacturing process are ongoing. Furthermore, MyoPax has performed market analysis of several indications and a pricing study to demonstrate marketability. New clinically critical indications are in pre-clinical testing for which public funding was raised from the EU and in Denmark. Currently, MyoPax aims for private fundraising to support future clinical trials and enable industrial scale-up. In summary, building a Startup like Myopax accelerates treatment development in muscle disorders to translate our proprietary technology into the clinic and provide effective, safe, and sustainable muscle regeneration therapies for muscle defects, genetic muscular dystrophies, and acute muscle wasting.

63 - Genome-wide CRISPR screening in postmitotic hepatocytes of murine liver

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As a hub for various biochemical processes, the liver plays a vital role in the human body. The hepatocytes in the adult liver are predominantly post-mitotic, and they undergo proliferation in response to tissue damage. However, the lack of an *in vitro* model with non-proliferating hepatocytes limits our ability to study the pathological processes in the liver, such as non-alcoholic steatohepatitis, hepatitis due to viral infections, hepatocellular carcinoma (HCC) and liver toxicity caused by drug administration. Genetic knockout screening through CRISPR/Cas9 has revolutionized the establishment of unbiased genotype-phenotype associations. However, current CRISPR screening methods are primarily suited for *in vitro* setups, particularly 2D cell cultures of rapidly dividing cancer cell lines. Thus, genetic screening on *in vitro* models typically does not allow for studying terminally differentiated and postmitotic cells. In addition, *in vitro* models fall short of mimicking the physiologic context a cell experiences *in vivo*, for example, cell metabolism and nutrient availability, as well as interaction with neighboring cells, the extracellular matrix, the immune and the vascular system. With these inherent limitations of *in vitro* models, a necessity arises for a screening method for a non-proliferative *in vivo* setup. In this study, we present a new platform to conduct direct *in vivo* CRISPR screening in the mouse liver to study the genetics of post-mitotic cells. Noting that conventionally used hydrodynamic tail vein injection of transposons results in the integration of multiple sgRNAs per cell, thus hampering genetic analysis, we optimized the delivery method of the sgRNA library to hepatocytes and modified CRISPR-StAR, a screening paradigm with internal controls to circumvent noise introduced by misrepresentation of sgRNAs occurring in the *in vivo* setup. Subsequent to the *in vivo* selection of transduced cells, we induced genome editing in adult mice. By conducting genome-wide *in vitro* and *in vivo* screening, we discovered that fewer genes were depleted in the adult liver and with less magnitude compared to the screening from the *in vitro* cell line. We show this is due to the technical limitation of CRISPR/Cas9 to deplete cells in non-proliferative cells. To our knowledge, this is the first genome-wide CRISPR screening performed in post-mitotic liver *in vivo*. This *in vivo* essentialome will allow for subtractive analysis of tumor essentialities, especially in HCC, as well as to rapidly uncover the mode of action for liver toxicity or improvement of regenerative processes in the liver.

62 - Enhanced CRISPR base editing design from data generation and deep learning

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CRISPR base-editors (BEs) hold the potential to overcome several obstacles with gene editing first introducing a cleave in the DNA. They enable inducing precise point mutations in the genome, resulting target nucleotides in an editing window of ~8nt inside the DNA target edited with different frequencies. The gRNA design for BEs requires accurate prediction of both gRNA efficiency and outcome frequency. A major challenge is that most datasets come with low efficient gRNAs not useful in constructing a prediction model, more data is needed. Although several machine learning-based methods for predicting BEs efficiency have been developed, there remains potential for improvement, particularly in accurately predicting outcome products observed with high frequency. Furthermore, the current data sets are very diverse. This on one hand gives the challenge of how a machine learning model effectively can exploit the different data sets, while on the other hand raises the need for data set aware prediction, providing a "tailormade" prediction allowing to weight the data set most alike the specific experimental method to be used for the BE-based gene editing. To meet these challenges, we for both adenine-BE (ABE, converting A•T into G•C base pairs) and cytosine-BE (CBE, converting C•G into T•A base pairs) generated a pool of ~11,500 gRNAs using SURRO-seq technology. We thereby increased the pool of publicly available data to ~17,000 gRNAs for ABE and CBE respectively. Upon integration with published datasets, we trained deep neural networks for ABE (CRISPRon-ABE) and CBE (CRISPRon-CBE) respectively, to simultaneously predict gRNA efficiency and outcome frequency while in the input flagging the individual data sets, which enhances the performance on the individual data sets respectively. In addition, outcome frequencies should be considered in the context of gRNA editing efficiency. Therefore, we simultaneously evaluated gRNA efficiency and outcome frequency using a *K*-dimensional Pearson correlation coefficient R_K (*K*=2). After testing on independent test sets, we found that our BE efficiency prediction models outperform the existing publicly available BE-predictors. Our models, CRISPRon-ABE / CRISPRon-CBE based on different combinations and weights of the available public and own data yield substantial prediction improvements over the existing methods. On the Song test set our CRISPRon-ABE models exceed DeepABE, BE-HIVE and BE-DICT in R_2 in the range of 0.09 to 0.44 and similarly our CRISPRon-CBE models exceed DeepCBE, BE-HIVE and BE-DICT in R_2 in the range of 0.04 to 0.52. On our independent test set corresponding enhancements are in the range of 0.10 to 0.44 for ABE and in the range of 0.05 to 0.46 for CBE.

61 - Genome-wide CRISPR-Cas9 knockout screen identifies DNA damage response pathways and BTK as essential for cisplatin response in diffuse large B-cell lymphoma

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Recurrence of diffuse large B-cell lymphoma (DLBCL) after first-line treatment is observed in up to 40% of patients. The current standard of care for these patients is platinum-based treatment prior to autologous stem cell transplantation. The prognosis for refractory or relapsed DLBCL patients is poor, and there is a need to identify genes that sensitize DLBCL cells to platinum-based treatment. In this study, whole-genome CRISPR-Cas9 knockout (KO) screens were performed in DLBCL cells to identify genes affecting cisplatin response in DLBCL.

Whole-genome CRISPR knockout screens were performed in DLBCL cells using the Brunello library, including four sgRNAs per gene, followed by exposure to two doses of cisplatin or saline for 10 days. MAGeCKFlute was used to identify genes and pathways implicated in sensitivity and resistance to cisplatin. Mutation frequencies of the candidate cisplatin-response genes were assessed in both local (n=55 tumor/normal samples) and external (n>1200) DLBCL cohorts. Individual cisplatin-affecting candidate genes were chosen for validation based on positive/negative selection scores or clinical relevance for subsequent functional analysis to investigate cisplatin response in DLBCL cells. Viability was assessed using MTS-based dose-response assays after treatment with platinum-drugs and inhibitors of targeted genes, and drug interactions were calculated using the Bliss Independence Model.

The CRISPR screens revealed depletion of gRNAs targeting 425 genes and enrichment of gRNAs targeting 326 genes, conferring sensitivity and resistance to cisplatin upon knockout, respectively. Depleted gRNAs were enriched for genes involved in DNA damage response (DDR) in the Nucleotide Excision Repair (NER), Mismatch Repair (MMR), and Fanconi Anemia (FA) pathways, and the B-cell receptor activation pathway. Knockout of the NER genes *XPA* and *ERCC6* sensitized DLBCL cells to platinum drugs, irrespective of proliferation rate, increased DNA damage levels, and altered cell cycle distribution. *BTK* knockout also sensitized DLBCL cells to platinum drugs, and chemical inhibition using ibrutinib displayed synergism with platinum drugs at low concentrations. RNA-Seq revealed that higher *XPA* expression at time of diagnosis is associated with better overall survival rates in local DLBCL patients.

In conclusion, this study shows that the NER genes *XPA* and *ERCC6*, in addition to *BTK,* are essential for response to platinum-drugs in DLBCL. Platinum-drug regimens are still the backbone of relapsed DLBCL treatment, and improvements in these therapies are essential for improved patient survival. The findings from this study contribute to our knowledge of genes involved in response to platinum-based drugs.

59 - Improved CRISPR/Cas9 Off-target Assessment based on Guide RNA Binding Energy

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The computational design of guide RNA constitutes a crucial step in employing CRISPR/Cas9 for genome editing. The optimization of the guide RNA design involves determining guides with increased on-target activity while demonstrating reduced off-target effects. Experimental identification and validation of off-target activity is a time- and resource-intensive endeavor, prompting the compelling alternative of computational prediction based on reliable scoring of

various potential off-targets.

Our current off-target method, CRISPRoff, is based on modeling Cas9-gRNA-DNA approximate binding energies and compute scores of the off-targets of a guide RNA. Here, we present an improved scoring scheme by optimizing the contribution of RNA-DNA stacking energy to the off-target score calculation and also weighting the Protospacer Adjacent Motif (PAM) preference of the Cas9. The number and type of the mismatches are tolerated differently at different positions. This information is used to optimize the weights of the binding energy of each position using. The PAM information used as an additional feature during the optimization. The presented CRISPRoff method better explains Cas9 off-target activity through higher correlation between the predicted energy-based score and experimentally obtained off-target activities compared to the previous model. The improved model also contributes to a more comprehensive understanding of the mechanism of Cas9 activity.

58 - Development of a CRISPR-based epigenome editing platform to enhance the long-term engraftment of ex-vivo genetically modified HSPCs

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Autologous transplantation of genetically modified haematopoietic stem and progenitor cells (HSPCs) has emerged as a promising technology for the treatment of human blood disorders, such as primary immunodeficiencies or haematopoietic malignancies. *Ex vivo* HSPCs genetic engineering relies on the isolation of $CD34^+$ cells from the patient`s mobilized peripheral blood followed by the correction of the disease-causing mutation by utilizing gene therapy tools. Once corrected, HSPCs are transplanted back into the patient by intravenous infusion. A successful treatment requires the most primitive HSCs with long-term repopulating potential to home and engraft into the bone marrow niche, where they self-renew and establish a new population of genetically modified cells that pass on a correct copy of the gene to the blood cell progeny. While this approach enables efficient genetic correction *in vitro*, it suffers from poor engraftment of corrected cells *in vivo*, thus limiting the therapeutic potential of HSC-based genetic therapies. To overcome this problem, we devised a CRISPR-based epigenome editing system for the upregulation of genes involved in HSPCs homing and engraftment (engraftment enhancers). We first selected potential engraftment enhancers among regulators of HSPCs migration, stemness and interaction with BM stromal cells and evaluated the impact of the *ex vivo* manipulation on the expression of these genes in the different HSPC subpopulations. Primitive HSCs, multipotent progenitors (MPPs) and $CD38^+$ committed progenitors were isolated, and the expression level of engraftment enhancers was quantified before and after manipulation in each subpopulation. We observed a decrease in the expression of the engraftment enhancers during cell manipulation and HSC differentiation. By exploiting the ability of the CRISPR-epigenome editing system to induce a transient yet robust transcriptional activation of a desired target gene, we upregulated the expression of engraftment enhancers in HSPCs by delivering a catalytically dead Cas9 fused to the tripartite regulator VPR (dCas9-VPR) alongside two guide RNAs targeting the promoter region of the target genes. A time-course analysis revealed a significant peak of gene activation after 16h of the CRISPR system delivery, which returned to baseline expression levels 96h later; this demonstrates that the platform is compatible with the kinetics of HSPCs homing and

engraftment after transplant, while avoiding the potential negative effects of persistent gene expression perturbation in HSPCs. Lastly, we tested the efficiency of this system in providing a homing and engraftment advantage to HSPCs gene-edited with a CRISPR/Cas9 platform for the treatment of Wiskott-Aldrich Syndrome, by measuring their increased migration *in vitro* in response to chemotactic cues. Overall, our data show the feasibility of our epigenome editing-based system for transient upregulation of genes involved in HSPC engraftment. We now aim to integrate this platform to already established gene correction systems to boost gene edited HSPC engraftment upon transplantation and reach sensible gene correction rates *in vivo*, with the final aim of unlocking the full potential of HSPC-based cell therapies for the treatment of blood disorders.

57 - Base editing of hematopoietic stem cells restores immune function in a mouse model of familial hemophagocytic lymphohistiocytosis

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Familial hemophagocytic lymphohistiocytosis (FHL) is an immune-hematologic disorder caused by hyperactivated T cells and macrophages, leading to severe inflammation and multi-organ damage. This uncontrolled immune activation results from impaired lysis of antigen-presenting cells by CD8+ T cells and NK cells. Mutations in the UNC13D locus account for about one third of FHL cases and result in FHL type 3 (FHL3). Curative therapy relies on allogeneic hematopoietic stem cell (HSC) transplantation. However, as HSC transplantation is still associated with high mortality in FHL patients, novel treatment options are urgently needed. As a proof of concept, we developed a cytosine base editing (CBE) strategy to disrupt the disease-causing cryptic splice site in Unc13d intron 26 of Jinx mice, a preclinical model of FHL3. Electroporation of CBE-encoding mRNA and guide RNA into Jinx CD8+ T cells and HSCs resulted in 61-71% of edited Unc13d alleles. Genetic and functional assays confirmed correct splicing of the Unc13d pre-mRNA and restored cytolytic activity of the edited T cells. Furthermore, transplantation of Unc13d-edited HSCs into conditioned Jinx mice demonstrated functional restoration of lymphocyte cytotoxicity and protection against hemophagocytic lymphohistiocytosis in vivo. High-throughput sequencing-based CAST-Seq and rhAmp-Seq analyses to characterize CBE-associated off-target effects revealed unexpectedly high genotoxicity, in particular CBE-induced chromosomal translocations and insertion/deletion mutations at both on- and

off-target sites. Interestingly, off-target activity was different in T cells and HSCs, supporting the notion that off-targeting is cell type specific. Furthermore, translocations persisted in T cells but not in HSCs, suggesting a cell type-specific way of coping with chromosomal rearrangements. Despite the high off-target activity, secondary transplantation of CBE-modified HSCs into 12 mice did not result in graft-related malignant transformation. In conclusion, our results demonstrate successful base editing to reverse the clinical phenotype in a preclinical FHL3 mouse model, but also reveal cell type-specific off-target effects, underscoring that cell type-specific safety studies are essential to properly assess the risk-benefit ratio of these novel technologies.

56 - Initiation of a phase I/II trial of CRISPR-Cas9-mediated PD-1-deficient tumour-infiltrating lymphocyte-based adoptive T-cell therapy in metastatic melanoma

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Blocking inhibitory T cell proteins, such as programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA4), with therapeutic antibodies has proven highly successful. Combining these antibodies with other treatments often surpasses individual therapy outcomes. Adoptive T-cell therapy with in vitro-expanded tumour-infiltrating lymphocytes (TILs) has demonstrated durable responses in metastatic melanoma (MM) patients. Our institution recently participated in a phase III trial confirming the superior efficacy of TIL therapy over ipilimumab (CTLA4-blocking) for MM.

We previously hypothesized that coupling PD-1 disruption in TIL therapy products using CRISPR-Cas9 could enhance outcomes and reduce systemic toxicities associated with PD-1-blocking treatment. Our pre-clinical data showed feasibility, minimal workflow impact, and high efficiency, with no adverse effects on TIL expansion potential, cytotoxicity, or tumour reactivity. Here, we report on the feasibility of scaling our pre-clinical experiments to clinical scale.

PD-1 disruption efficiency of patient-ready CRISPR-Cas9 reagents in expanded TILs was measured and compared to pre-clinical study results. Degradation of patient-ready RNP complexes during the TIL expansion process was evaluated, showing complete cas9 clearance before the end of TIL expansion, thus indicating complete RNP complex clearance. Over 50 potential off-target editing sites were predicted and investigated post-expansion in TIL samples, revealing no significant off-target editing in any loci. PD-1 disruption by patient-ready reagents was comparable to pre-clinical studies at both protein and gene levels.

Our study demonstrates the feasibility of producing a patient-ready, highly PD-1-deficient TIL therapy product using CRISPR-Cas9 with a non-labor-intensive, simple, non-viral workflow. Based on these findings, we have now initiated the first phase I/II TIL therapy trial in MM employing CRISPR-Cas9-mediated PD-1-deficient TILs.

55 - Development of a lipidoid library for Cas9-RNP-delivery

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Development of a lipidoid library for Cas9-RNP-delivery

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) protein 9 system, also known as CRISPR/Cas9 is the third generation of gene editing technology. CRISPR technology directs the Cas9 nuclease by using a guide RNA, thus dramatically simplifying its application. CRISPR/Cas9 technology has shown great potential in the treatment of genomic diseases with the ability to target undruggable targets and cure previously untreatable genetic diseases. Many non-viral CRISPR/Cas9 delivery systems are based on pDNA or mRNA that will finally lead to the assembly of the Cas9/sgRNA RNP complex in cells after transcription and translation of the delivered nucleic acids. In contrast, direct protein delivery has been suggested to have intriguing advantages. Since all components of RNPs are pre-assembled, the RNP complex can immediately function as an editing system in cells upon delivery. In addition, RNPs are cleared more rapidly, reducing off-target editing when the correct dose is administered. However, Cas9/sgRNA RNP delivery systems still suffer from low serum tolerance, poor endosomal escape and limited *in vivo* applicability, which remain to be overcome. Here, we synthesized a small library of different lipid-like substances for RNP lipid nanoparticle (LNP) formulations which were characterised with regard to size distribution, zeta potential, encapsulation efficiency and green fluorescent protein (GFP) reporter gene knockout *in vitro*. The lipidoids were synthesized by epoxidylation in a one-step solvent free reaction and purified via silica column chromatography. Cas9 protein and a sgRNA targeting GFP were assembled into RNPs and encapsulated into LNPs based on the set of different lipidoids. An established LNP formulation containing the ionizable lipidoid C12-200 served as reference and newly synthesised compounds were screened in analog compositions replacing C12-200 at the same N/P ratio. After turbulent pipette mixing and dilution with HBG buffer LNP formation was accomplished for subsequent characterisation and *in vitro* experiments. Knockout efficiency was evaluated in HeLa-GFPtub cells by flow cytometry. All tested formulations showed a clear dose-dependent relationship, and one potent lipidoid was found to have 1.6-fold lower EC_{50} (1.57 nM) GFP knock-out efficiency compared to the commercially available C12-200 (2.53 nM).

54 - CRISPRBITS- Empowering Health Precision

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CrisprBits is pioneering the use of the disruptive CRISPR technology in India, with a focus on affordable and accessible solutions in healthcare. Our long-term vision is to implement cutting edge solutions centered around CRISPR technology, in diagnostics, therapeutic gene editing and microbe editing to mitigate impact of climate change.

A comprehensive diagnostic strategy entails early detection, screening, prevention, and surveillance at affordable price points. CRISPR-based diagnostics, with their unparalleled precision in detecting genetic material by targeting specific DNA or RNA sequences through the CRISPR-Cas system, offer unprecedented accuracy in identifying genetic mutations or pathogens.

This specificity not only enhances diagnostic reliability but also opens new avenues for personalized medicine and swift detection of infectious diseases.

At CrisprBits, we have developed a platform to provide on-the-spot, instrument light *point-of-need* diagnostic solutions, addressing a significant unmet need not only in India but also in many low and middle-income countries. *Point-of-need* clinical diagnostics represents the initial stride towards achieving universal healthcare, and our platform extends its application to impact public health initiatives, as well as environmental and food surveillance. Our platform seamlessly integrates highly specific CRISPR-based detection with isothermal amplification, requiring minimal instrumentation. As a proof of concept, we have successfully combined loop-mediated isothermal amplification with CRISPR detection on lateral flow strips to identify *Salmonella typhi* (Typhoid fever) and *typhimurium* (Salmonellosis), covering the entire assay from sample extraction to the final report, with a remarkable limit of detection of 1 copy of the target DNA. Other applications like detection of Carbapenem resistance markers in ICU, Sickle cell anemia single nucleotide change in high risk population, Methicillin resistant Staphylococcus aureus in the hospital, and the presence of *foot and mouth disease* virus in cattle are in the pipeline.

Moreover, CrisprBits is also at the forefront of utilizing disruptive CRISPR technology in India, emphasizing affordable and accessible healthcare solutions. In a proof of concept study we are currently investigating the use of FnCas9 for correcting a rare mutation in an iPSC line suffering from a patient suffering from Leber Congenital Amaurosis. Besides this we are also exploring site specific integration of a gene of interest using guide directed homologous recombination in several cell lines for better precision fermentation application.

53 - Predicting Off-Target Activity in the CRISPR/Cpf1 System Using Multilayer Perceptron

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Genome editing plays a crucial role in gene therapy by facilitating the precise alteration of specific genomic locations. The discovery of the CRISPR/Cas system has positioned genome editing as a highly efficient alternative for modifying a genome through the design of an optimal gRNA for a target sequence. The 20-nucleotide target sequence within the gRNA is designed for optimal binding to the target location. The mismatch tolerance in specific gRNAs hinders the clinical application of the CRISPR/Cas system by causing significant variations in on-target and off-target binding activities. It has been reported that certain nucleotides at specific positions are more susceptible to mismatches, leading to off-target cleavage. The main aim of this study was to predict potential off-targets along with their cleavage efficiencies and comprehend the significance of features in classifying high-activity (positive) off-target sites from low-activity (negative) off-target sites. The developed pipeline utilizes an alignment-based approach to predict potential off-target sites in the human genome, integrated with a multilayer perceptron to predict the target efficiency of the identified sites. Multiple machine learning models were trained using three different combinations of sequence-based features and energy weights for

mismatches, and base-dependent binding energy features, and the best-performing model was chosen for the pipeline. All the executable Python scripts for off-targets and cleavage efficiency prediction specific to AsCpf1 and LbCpf1 are available for access on **GitHub** (https://github.com/TeamSundar/CRISPR-Cpf1_study). Furthermore, the study also investigated positional preferences of nucleotides, the distribution of mismatches, and classification-dependent feature importance between high-activity and low-activity off-targets. These analyses aimed to comprehend the significance of specific features in off-target binding and cleavage. This study also reveals that Thymine is predominantly disfavored in the seed region of positive off-targets and the position adjacent to PAM, Thymine is mostly disfavored while Guanine is favored in that position. Mismatch distribution analysis revealed that mostly positions 16, 17, 18, and 23 tolerate mismatches more frequently in positive off-targets. It has also been predicted that transition-type mismatches are preferred at these positions. The Multilayer Perceptron interpreted the low to moderate melting temperature of the non-seed region and base-dependent PAM binding energy, as the most significant predictors for high activity off-targets. GC content, some types of 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 LbCpf1 and AsCpf1.

52 - Revolutionizing CRISPR Therapies: A Scalable Manufacturing Platform for eVLPs production

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CRISPR genome editing tools have revolutionized the gene therapy field. Among all genome editing tools developed so far, base editing is one of the most promising as it can precisely and efficiently correct single-nucleotide variants while avoiding double-stranded breaks in the DNA and, therefore, harmful consequences of large indels and chromosomal rearrangements. Despite being such a promising genome editing tool, the expression of CRISPR base editors (BEs) *in vivo* should be transient to limit off-target editing. Engineered virus-like particles (eVLPs) have emerged as safer carriers of such editors, as BEs are delivered as ribonucleoprotein (RNP) complex to cells. The short half-life of the BE RNP complex reduces the off-target effects while maintaining efficient on-target editing. Although these particles preserve the targeting capacity of viral vectors, avoiding viral genome integration, the absence of genetic material in the eVLPs prevents their replication after delivery. Consequently, a high number of particles is necessary to achieve high editing efficiency. Here, we propose to develop a stable producer cell line for eVLPs manufacturing.

To develop a stable producer cell line (PCL), the different genetic cassettes required for eVLPs production (gag-pro-pol, envelope protein) as well as the cargo (base editor) will be successively integrated into 293 cells adapted to grow in suspension cultures. In addition to the base editor, another element needed for base editing is the gRNA, which binds the BE protein, forming the RNP complex. However, as the gRNA sequence varies according to the target genomic site, its coding sequence will not be integrated into the cell's genome and will be delivered subsequently. A T7 RNA polymerase will also be stably integrated into our PCL to catalyse RNA synthesis in the

cytoplasm and improve BE/gRNA RNPs formation during particle production.

The productivity of our eVLPs production system is being evaluated in both transient and stable production. The genome editing capacity of the eVLPs is being evaluated by targeting a cystic fibrosis-causing variant in a cellular model of the disease. Overall, generating a scalable platform to produce CRISPR-based therapies will bring more efficient, safe, and economical therapies to the clinic.

51 - Lipid nanoparticle delivery of dCas9-SAM to primary T-cells: towards a CRISPR activation-based therapeutic to cure HIV

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A cure for human immunodeficiency virus (HIV) remains elusive, resulting in a life-long need for antiviral therapy to suppress virus replication and prevent onset of AIDS. A major hurdle towards curing HIV is the persistence of integrated provirus that remains in a transcriptionally silent, latent state. One strategy to eradicate latent HIV is to activate viral transcription, followed by elimination of infected cells through virus-mediated cytotoxicity or immune-mediated clearance.

We hypothesized that specific activation of HIV transcription through CRISPRactivation (CRISPRa) provides a promising strategy of reversing HIV latency without affecting host-cell transcription. However, the success of such an approach is critically dependent on the efficient delivery of the CRISPRa system to resting primary CD4+ T-cells, the key cellular reservoir of latent HIV. We aimed to develop mRNA-lipid nanoparticles (LNP) to deliver the dCas9-synergistic activation mediator (SAM) system to T-cells, and assess the potency of HIV-specific CRISPRa to activate viral transcription.

LNPs encapsulating mCherry mRNA were incubated for 72hr with CD4+ T-cells from HIV-negative donors. Using the Onpattro LNP formulation, we were unable to transfect non-stimulated CD4+ T-cells, consistent with reports that resting T cells are difficult to transfect. In contrast, our novel, proprietary LNP formulation X was able to transfect a striking 92±2% (mean±SEM) of non-stimulated CD4+ T-cells with minimal toxicity.

We then used LNP X to co-encapsulate the dCas9-SAM machinery, consisting of mRNAs encoding dCas9-VP64 and MS2-p65-HSF1, and a gRNA. We targeted the CRISPRa to CD25, a surface receptor expressed lowly in non-stimulated T-cells. Treatment with these CD25-specific CRISPRa-LNPs induced upregulation of CD25 expression by up to 3.3±0.7-fold over baseline, indicating successful delivery of CRISPRa to primary T-cells.

Next, we produced CRISPRa-LNPs co-encapsulating gRNAs targeting the HIV promotor region between bp -250 to -92 relative to the transcription start site. The potency of these HIV-specific CRISPRa-LNPs was assessed in a Jurkat T-cell line containing a GFP reporter under the control of the HIV promotor. Treatment with the HIV-specific CRISPRa-LNP was found to induce HIV transcription using a range of HIV-targeting gRNAs, reaching up to 88.2±3% GFP+ cells compared with 11.9±5% using a scrambled control gRNA.

We finally assessed the ability of HIV-specific CRISPRa-LNP to activate viral transcription in CD4+ T-cells isolated from people living with HIV on suppressive antiviral therapy. We found that 72hr treatment with HIV-specific CRISPRa-LNPs resulted in a median 2.75-fold increase (IQR 1.63-4.67) in HIV RNA transcripts compared to non-treated control cells, as determined by digital RT-qPCR. No increase in HIV RNA transcripts was observed upon treatment with scrambled control CRISPRa-LNPs. The activation of HIV transcription occurred at minimal reduction in overall cell viability and did not induce generalized cellular activation, as measured by the expression of activation markers CD25, CD69 and HLA-DR.

In conclusion, we developed a novel LNP formulation capable of delivering the dCas9-SAM CRISPRa system to non-stimulated $CD4^+$ T-cells, and showed potency of HIV-specific CRISPRa-LNPs at activating viral transcription in samples from people living with HIV *ex vivo*. These findings warrant further pre-clinical investigation of CRISPRa-LNPs as a potentially curative therapeutic.

50 - Gene-edited primary muscle stem cells rescue dysferlin-deficient muscular dystrophy

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Dysferlin deficiency due to loss-of-function mutations in the *DYSF* gene underlies a panoply of yet untreatable genetic muscular dystrophies characterized by progressive muscle weakness and atrophy. Skeletal muscle is endowed with a tissue-specific population of adult stem cells, which are responsible for muscle regeneration and thus stand out as a very attractive source for cell replacement therapies. We targeted a frequent founder frameshift mutation in *DYSF* exon 44 that results in a complete absence of dysferlin protein and progressive dystrophy. mRNA-mediated delivery of SpCas9 in combination with a mutation-specific sgRNA to primary muscle stem cells from two homozygous patients consistently resulted in >90% on-target editing with >60% exon 44 re-framing, rescuing a full-length and functional dysferlin protein. To assess the *in vivo* therapeutic potential, we generated a new mouse model harboring a humanized *Dysf* exon 44 with the founder mutation, hEx44**mut**, and a corresponding control harboring the wild-type human exon 44 (hEx44**wt**). hEx44**mut** mice recapitulated the patients' phenotype and

an identical re-framing outcome in primary muscle stem cells after mRNA-based editing. Finally, gene-edited murine primary muscle stem-cells were able to regenerate muscle, rescued full-length dysferlin and repopulated the stem cell niche when transplanted back into hEx44mut hosts in a close-to-autologous setting without any immune modulation. These findings are the first to show that a CRISPR-mediated therapy can ameliorate dysferlin deficiency. We suggest that gene-edited primary muscle stem cells could exhibit utility, not only in treating dysferlin deficiency syndromes, but also perhaps other forms of muscular dystrophy.

49 - Novel characterization assays enable efficient GMP manufacturing of CRISPR RNP complexes

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The CRISPR-associated gene editing system has emerged as a highly advantageous method to develop new gene and cell therapies. A proven technique for effective *ex* vivo genome editing in a variety of cell types is to deliver a CRISPR-ribonucleoprotein (RNP) complex consisting of single guide RNA (sgRNA) and Cas nuclease. RNP delivery has become a widespread approach for gene-editing clinical trials, but drug developers face significant challenges when initiating their clinical programs, including the design of the target-specific guides, the production of reliable, high-quality nucleases, and the ability to characterize the RNP complex for the efficient and safe delivery of a genomic therapy. Aldevron has developed a suite of novel characterization assays measuring specific activity of the RNP complex *in vitro*, free (unbound) guide RNA, and free Cas nuclease. These analytical data can support in the quality release process and stability studies for a GMP-manufactured RNP complex. Aldevron, in partnership with Integrated DNA Technologies (IDT), can help to accelerate new CRISPR-based therapies to support genomic medicine via manufacturing support for key GMP CRISPR components, including Cas nucleases, custom sgRNA, and RNPs.

47 - Development and validation of a novel PD-1/PD-L1 and PD-L2 co-inhibition assay for CRISPR-screening in T cells

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Programmed death 1 (PD-1) and its ligands PD-L1 and PD-L2 are key immune checkpoints that mediate coinhibitory signals inhibiting T cell activation and function. Checkpoint blockade of the PD-1/PD-L1 and PD-L2 axis has revolutionized cancer immunotherapy, but the pathways downstream of PD-1 signaling are still not well characterized. In addition, the response rates of patients to current anti-PD-1 or anti-PD-L1 therapies are relatively low. Thus, understanding pathways involved in PD-1-mediated negative regulation of T cells is critical to developing novel immunotherapies.

To investigate the signaling pathways mediated by PD-1 engagement with its ligands, we established a new model of co-inhibition based on engineered K562 cancer cell lines. We generated a novel K562 cancer cell line expressing the potent anti-CD3 clone, OKT3, on the cell surface as a membrane-bound ScFv antibody fragment ($OKT3^+$ K562). The $OKT3^+$ K562 cancer cell

line induced a potent polyclonal activation of +90 % of T cells in cocultures and was then further modified to express either PD-L1 or PD-L2 (K562 OKT3$^+$ PD-L1$^+$ and K562 OKT3$^+$ PD-L2$^+$). We assessed the ability of these cancer cell lines to inhibit T cell growth in a 4-day CFSE proliferation assay. When co-cultured with T cells, the OKT3$^+$ K562 cell lines expressing PD-L1 inhibited T cell proliferation. To validate our findings, we performed a CRISPR-mediated PD-1 knock-out (KO) in T cells and challenged them with K562 OKT3$^+$ K562 OKT3$^+$ PD-L1$^+$ and K562 OKT3$^+$ PD-L2$^+$. PD-1 KO was confirmed by decreased expression of PD1 on the T cell surface, and PD-1 KO counteracted PD-L1/PD-L2 mediated coinhibition.

This novel model of co-inhibition will be used for a genome-wide pooled CRISPR screen. T cells will be transduced with the lentiviral pooled Brunello library and Cas-9 will be electroporated. The transduced cells will then be challenged by the engineered cell lines, sorted for proliferation and sequenced for sgRNA enrichment. The CRISPR screen will allow us to identify novel regulators of T cell survival and proliferation under PD-1 co-inhibitory stimulation.

Overall, we have generated a valuable assay for studying T cells *ex vivo* that simulates stimulatory and co-inhibitory signals for T cells. This assay will aid the study of PD-1 mediated signaling upon the engagement with its ligands. In addition, this model of co-inhibition can be further applied to investigate other receptor/ligand interactions that mediate coinhibitory signals in T cells.

46 - Next-Level Insights: High-Resolution Analysis of Chromosomal Aberrations with NHEJ Inhibition

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Therapeutic gene editing strategies, which typically use designer nucleases to incorporate a donor template into specific genomic sites, often face challenges due to unwanted indels that arise through non-homologous end-joining (NHEJ) repair pathways. To counteract these suboptimal integration efficiencies, researchers are redirecting the cellular repair preference toward the homology-directed repair (HDR) pathway by utilizing compounds that inhibit NHEJ. The broader implications of such systemic repair inhibition, especially regarding the repair outcomes of off-target double-strand breaks, have yet to be fully understood. In our study, hematopoietic stem and progenitor cells (HSPCs) edited with CRISPR-Cas9 were treated with a cocktail of end-joining repair inhibitors. Our application of CAST-Seq and digital PCR (dPCR) revealed that before removing the inhibitors, up to 60% of the alleles at known off-target sites contained double-strand breaks. More strikingly, following inhibitor treatment, we documented a 15-fold increase in both total translocations and those involving the on-target locus. The absolute frequency of three specific translocations, quantified by dPCR, increased from 0.24% to 1.6% of total alleles. While integrating donor sequences remains a promising avenue for corrective gene therapy, our findings highlight the critical role of precise sgRNA design. More importantly, our results advocate for the novel use of repair-inhibiting compounds not just to enhance integration efficiency, but as pioneering tools to enhance the resolution of existing techniques for detecting off-target effects and aberrations. This approach could mark a new frontier in the discovery and development of designer nucleases, providing unprecedented clarity and control over genomic

editing processes.

45 - The epigenome as a novel player in CRISPR safety

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Over the past few years, the gene editing field has been revolutionized by the arrival of CRISPR-Cas9 (Jinek et al., 2012). This new editing technique is now the tool of election of experimental therapies treating a wide number of diseases, some of them recently approved by regulatory bodies across the globe. While studies suggest a correlation between the epigenetic profile and CRIPSR editing efficiency (Daer et al., 2017) very little is known regarding how epigenetics may impact CRISPR toxicity. As the epigenome is known to influence overall genome stability (Putiri and Robertson, 2011) and CRISPR editing is known to cause rearrangements and increased DNA instability (Leibowitz et al., 2021) it is likely that an interaction may occur. The human epigenome is highly variable within the population therefore it is important to understand the implications of this interaction so we can accurately predict and mitigate potential toxicity on a patient-by-patient basis.

Here we determine whether the epigenome has a role in determining CRISPR-induced toxicity by characterising adverse outcomes. By using high throughput imaging technologies to investigate the genotoxic impacts of CRISPR editing under different epigenetic conditions we were able to determine that DNA methylation may play a role in regulating genome stability. Furthermore, by optimising bespoke sequencing technologies to analyse the occurrence of double strand breaks we hope to understand the influence of epigenetics features on Cas9 off-target binding.

44 - Antigen-scaffold-mediated expansion of CRISPR-Cas9 knockin CAR- and TCR-T cells generates highly enriched, efficacious T-cell products

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The engineering of autologous T cells using CRISPR/Cas9 is a promising treatment option for cancers. Expression of chimeric antigen receptors (CAR) can induce profound clinical responses in hematologic malignancies, whilst T cell receptor-engineered T (TCR-T) cells have led to durable responses in early clinical trials. Previously, most of these therapies have applied viral delivery and expression of the CAR or TCR of interest. However, lentiviral engineering is associated with challenges regarding the production and functional characteristics of the engineered cells.

Non-viral delivery of TCRs using electroporation of CRISPR/Cas9 and homology-directed repair templates (HDRT) solves several of the problems associated with viral delivery. Most importantly, it allows for a more uniform expression of the TCR or CAR than viral delivery. In this study, we investigated novel HDRT types for the delivery of synthetic receptors using CRISPR/Cas9. We

further optimized protocols for effective knockin (KI) of TCRs, using closed-circular DNA as HDRT and small molecule inhibitors that increase KI efficacy, resulting in ~20% KI of a NY-ESO-1-specific TCR (diagram in Figure).

Following knockin, we adapted an existing protocol for specific expansion of antigen-specific CRISPR TCR-T and CAR-T cells using antigen-presenting scaffolds (Ag-scaffolds) that co-present peptide MHC (pMHC), IL2, and IL21 for TCR-T cell expansion, and CD19 antigen for the anti-CD19 CAR-T cells. Applying this expansion technique, we could achieve >90% antigen-specific cells after 14 days of *in vitro* culture. The expanded TCR-T cells could effectively kill peptide-presenting cancer cells and showed favorable phenotypes as determined by flow cytometric analysis. Similarly, antigen-specifically expanded CRISPR CAR-T cells effectively killed target CD19-expressing Jeko-1 cancer cells even after multiple recurrent challenges. We further compared Ag-scaffold expanded lentiviral engineered T cells with Ag-scaffold expanded CRISPR engineered T cells. We found that both lentiviral and CRISPR-engineered T cells were effectively expanded using Ag-scaffolds and were more effective at killing target cells than cells expanded using conventional cytokines.

This framework of engineering T cells using CRISPR (or lentivirus) followed by Ag-scaffold expansion provides an effective tool for generating T cells with antigen receptors of interest for TCR-T and CAR-T cell therapy.

43 - He-RASE: a fast cellular model to screen CRISPR/Cas systems editing efficiency and specificity for dominant mutations

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The CRISPR/Cas system is a promising approach for treating a variety of genetic disorders and it can potentially address autosomal dominant mutations by disrupting mutant alleles via the Non-Homologous End Joining (NHEJ) DNA repair pathway. However, the low specificity of the Cas nuclease poses a challenge as many single-nucleotide dominant mutations cannot be targeted due to the inability to distinguish between mutated and wild-type alleles. Thus, developing a highly specific Cas nuclease capable of efficient allele-specific single-nucleotide discrimination is critical to broaden the range of dominant diseases that can be treated. Although many new and/or engineered Cas nucleases have been identified, targeting the mutations responsible for dominant genetic diseases remains challenging due to the rarity of some diseases and limited availability of affected patients' cells. To address this issue, we propose **He-RASE** (**He**k293T **R**eporter-based **A**llele-**S**pecific **E**diting), a cell line based on HEK293T that mimics the "biallelic context" of the dominant disease. Our cellular model precisely mimics the heterozygosity of dominant mutations and replicates the biallelic context. To test this, we are using a rare genetic dominant disease caused by TP63 heterozygous missense mutations, known as Ectrodactyly-Ectodermal Dysplasia-Cleft lip palate (EEC). EEC patients' TP63 loci enable us to screen the engineered nucleases effectively and functionally. This screening allows us to identify the most promising candidates for the editing efficiency and the allele-specificity on the mutations of interest. He-RASE can be extended to many genes that cause autosomal dominant diseases and allows a quick and reliable system to screen many new Cas nucleases.

42 - Mitigating the risk of T cell receptor mispairing in CRISPR-T cell receptor-engineered human T cells

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Engineering human T cells to express a predefined T cell receptor (TCR) specific to a pathogenic or oncogenic target is a compelling therapeutic avenue. Yet, mispairing of the endogenous and introduced TCRαβ chains may result in unforeseen TCRs with unwarranted peptide-MHC recognition. Here, we present a method for stepwise CRISPR/Cas9 mediated generation of human TCR-engineered T cells devoid of endogenous TCRαβ chains. To avoid multiple rounds of electroporation, the endogenous TCRβ chain is knocked out in activated human T cells by amphiphilic peptide delivery of the Cas9-gRNA complex. Next, the $CD3^{negative}$ cells are purified by magnetic-activated cell sorting. Finally, the cells are subjected to TCR knock in using electroporation-mediated delivery of the homology-directed repair template and the Cas9-gRNA complex targeting the TRAC locus. Altogether, this represents an advantageous and safe approach to CRISPR-engineering of human TCR T cells by mitigating the risk of mispairing between the endogenous and inserted TCR chains.

41 - CRISPR/dCas9 gene editing for overexpressing apolipoprotein AI, paraoxonase 1 and cholesterol transporter ABC1 in hepatocytes - a promising tool to obtain functional HDL

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Alteration of lipid metabolism as well as the oxidative and inflammatory stress are critical factors in atherosclerosis progression, the main cause of cardiovascular diseases. A promising therapeutic strategy to reverse the atherosclerotic process is to improve the quality and quantity of high-density lipoproteins (HDL), the athero-protective lipoproteins synthesized mostly by the liver. CRISPR/Cas9 system has gathered enormous interest for therapeutic applications due to the high efficiency, versatility, specificity and stability of the gene-editing process. The aim of the present study was to use CRISPR/dCas9 gene editing to obtain functional HDL by activating the transcription of the endogenous HDL major proteins, apolipoprotein AI (ApoAI) and paraoxonase 1 (PON1), and the hepatocytes’ ATP binding cassette A1 transporter (ABCA1). To this purpose, human hepatocytes (Huh7 cell line) were transfected using commercially available CRISPR activation plasmids for ApoAI, PON1 or ABCA-1. After the transfection, the expression level of the targeted proteins was measured in the cellular lysate or in the conditioned medium (CM), in normal or pro-inflammatory condition (cells exposed to TNFα). The expression of proteins regulating the hepatic cholesterol level: ABCG8 transporter, cholesterol 7 alpha-hydroxylase (CYP7AI), scavenger receptor BI (SR-BI) and nuclear liver X transcription factor (LXR) was measured in the lysate of cells overexpressing ApoAI, PON1 or ABCA1. The oxidative status of transfected Huh7 exposed to TNFα was evaluated by measuring the total intracellular reactive oxygen species (ROS) or mitochondrial ROS. In addition, the functionality of the secreted APOAI and PON1 was evaluated by measuring the effect of CM from transfected Huh7 on

TNFα-activated endothelial cells (EC) as ROS levels, and expression of pro-oxidant p22phox and inflammatory vascular cell adhesion molecule (VCAM-1). The obtained results showed that**:** (i) the transcriptional activation of ApoAI and PON1 upregulated their cellular expression and secretion in hepatocytes' CM both in normal and TNFα-activated hepatocytes; (ii) ABCA1 and APOAI overexpression stimulated ABCG8, SR-BI, and CYP7AI expression; (iii) LXR level was increased after the transcriptional activation of PON1, APOAI or ABCA1; (iv) ApoAI and PON1 transcriptional activation resulted in the decrease of the oxidative stress in TNFα-exposed Huh7; (v) CM from transfected Huh7 reduced ROS levels and decreased the protein expression of p22phox and VCAM-1 in TNFα-activated EC lysates. In summary, the present study shows that CRISPR/dCas9 system successfully stimulated the cellular expression and secretion of APOAI, PON1 and ABCA1 in cultured human hepatocytes. We demonstrated that the overexpressed proteins are functionally active, having the capacity to (i) modulate proteins involved in the homeostasis of hepatic cholesterol; (ii) reduce the oxidative stress in TNFα-activated hepatocytes and (iii) decrease the inflammatory and oxidative stress in TNFα-activated EC. All these results suggest that CRISPR/dCas9 can be a future promising tool for obtaining hepatic functional HDL.

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40 - A new CRISPR-mediated Apc Knockout Allele leads to Pyloric Gland Adenoma-like Gastric Polyps in the Mice with C57BL/6; FVB/N Mixed Background

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Mouse models of colorectal cancer (CRC) are essential to understand the pathogenesis of the CRC and they are widely used by the researchers working in this field. With the increase in the knowledge of genetic alterations causing CRC, a myriad of genetically engineered mouse models (GEMMs) of CRC has been produced by different genetic manipulation techniques. The advent of the CRISPR/Cas9 system has revolutionized the field of genetic engineering and facilitates the production of new GEMMs. In this study, we aimed to generate a novel Apc knockout mouse model by CRISPR/Cas9 system and evaluate the phenotypic effects of this new allele in two mouse strains. For this purpose, we targeted the exon 16 of mouse Apc gene by using CRISPR/Cas9 system and chose the mouse carrying an Apc frameshift mutation at codon 750 (Δ750) as the founder. Then, we backcrossed the mutant FVB-ApcΔ750 mice with wild type C57BL/6 mice and evaluated the phenotypic effects of knockout allele on F8-FVB-ApcΔ750, F4-B6; FVB-ApcΔ750 and F1-B6; FVB-ApcΔ750 with macroscopic and microscopic examination of the gastrointestinal system. As a result, the polyp number, the distribution of polyps in the gastrointestinal system and the survival of mice showed significant variation between F8-FVB-ApcΔ750 and F4-BL6; FVB-ApcΔ750 mice. Additionally, it was observed that F1-BL6; FVB-ApcΔ750 mixed background mice developed gastric polyps which morphologically resemble pyloric gland adenoma of humans. In conclusion, we characterized a new CRISPR-mediated Apc knockout allele by using two mouse strains and showed that this allele can exert a strain-specific effect on the mice phenotype and it can be associated with gastric polyp formation.

39 - Engineering CRISPR nucleases for allele-specific editing of dominant genetic diseases

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Ectrodactyly-Ectodermal dysplasia-Cleft (EEC) syndrome is an autosomal dominant genetic disease caused by point mutations in the TP63 gene, characterized by a triad of ectrodactyly, ectodermal dysplasia, and facial clefting, along with several other clinical manifestations such as corneal blindness. Although surgery can improve some aspects of the patient's life, the biggest unmet medical need is blindness caused by corneal opacification as a result of the progressive deficiency of limbal stem cells.

We identified three EEC syndrome-causing mutations that form a common 5'-TATG-3' PAM and identified Cas12a-RVR as a potential candidate to be improved for targeting these genetic alterations. The 5'-TATV-3' PAM recognized by Cas12a-RVR is too broad to ensure high specificity of the nuclease. Therefore, we rationally engineered CRISPR/Cas12a (formerly Cpf1) to target mutations that cause EEC syndrome by identifying the amino acid residues that interact with the PAM and substituted them with other residues that form a more specific bond with the desired 5'-TATG-3' bases. We conducted energy fold change analyses to ensure that the structure remains stable and increases specificity towards the desired PAM. We selected two amino acids as the best candidates and inserted these modifications into plasmids carrying the sequence for Cpf1-RVR, either as a single or in combination.

Engineer CRISPR/Cas12a variants showed a high editing efficiency on the mutated target gene and a low off-target activity on the WT sequence. Since EEC syndrome is sporadic, we validated our engineered CRISPR system in a biallelic EEC syndrome cellular model. This cellular system enables us to rapidly evaluate through fluorescence the gene editing efficiency on both the mutated and the wild-type allele.

Overall, this rational approach opens the door for the potential development of more engineered Cas proteins to target almost any dominant mutation.

38 - Generation of heterozygous and homozygous NF1 lines from human induced pluripotent stem cells using CRISPR/Cas9 to investigate bone defects associated to neurofibromatosis type 1

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Neurofibromatosis type 1 is one of the most common genetic disorders caused by heterozygous germline *NF1* mutations. NF1 affects many systems, including the skeletal system. To date, no curative therapies are available for the skeletal manifestations such as scoliosis and tibial dysplasia, mainly due to the lack of knowledge on the mechanisms underlying this process. By using CRISPR/Cas9 mediated gene editing in human induced pluripotent stem cells to minimize the variability due to genetic background and epigenetic factors, we generated isogenic heterozygous and homozygous *NF1*-deficient hiPSC lines to investigate the consequences of neurofibromin inactivation on osteoblastic differentiation. Here, we demonstrate that loss of one or both copies of *NF1* does not alter the potential of isogenic hiPSCs to differentiate into mesenchymal stem cells (hiPSC-MSCs). However, *NF1* (+/-) and *NF1* (-/-) hiPSC-MSCs show a defect in osteogenic differentiation and mineralization. In addition, we show that a mono-allelic deletion in *NF1* in an isogenic context is sufficient to impair cell differentiation into osteoblasts. Overall**,** this study highlights the relevance of generating isogenic lines, which may help for genotype-phenotype correlation and provides a human cellular model to understand the molecular mechanisms underlying in NF1 and thus discover new therapeutic strategies

37 - Direct delivery of Cas9 or base editor protein and guide RNA complex enables genome editing in the retina

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Genome editing by CRISPR-Cas holds promise for the treatment of retinal dystrophies. For therapeutic gene editing, transient delivery of CRISPR-Cas9 is preferable to viral delivery which leads to long-term expression with potential adverse consequences. In the eye, successful delivery of Cas9 protein and its guide RNA as ribonucleoprotein (RNP) complexes has been reported in the retinal pigment epithelium *in vivo* but not into photoreceptors, the main target of retinal dystrophies. We investigate the feasibility of direct RNP delivery to photoreceptors and RPE cells. We show that RNPs composed of Cas9 or adenine-base editor and guide RNA, without addition of any carrier compounds, induce gene editing in the photoreceptors at variable rates depending on the guide RNA efficiency and on the locus. But Cas9 RNP delivery at high concentrations leads to outer retinal toxicity indicating a need to improve delivery efficiency for future therapeutic use.

36 - Genome-wide CRISPR-Cas9 screen for the elucidation of novel mediators of cytotoxicity and cytokine production in natural killer cells

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Introduction of NK cells in the clinic have widened immunotherapeutic approaches in hematological malignancies. Despite the strides made, mechanisms of their cytokine and cytotoxic responses have not yet been fully characterized. CRISPR-Cas9 screens employ a genome-wide approach to identify key genetic factors in response to stimuli both in cell lines and primary cells. To further elucidate the network of NK cell cytokine and cytotoxic responses, we performed a genome-wide CRISPR-Cas9 screen in NK-92 cells. $Cas9^{+}$ NK-92 cells were transduced with an in-house optimized mCherry-encoding vector carrying the human Brunello sgRNA library at a low multiplicity of infection (MOI) to achieve 30% transduction efficiency. Subsequently, the cells were cultured and expanded for 7 days, upon which they were stimulated with K562 in a 4-hour *in vitro* responsiveness assay. The cells were stained for viability, degranulation (CD107a) and interferon gamma (IFNg) production. Gating on mCherry positivity distinguished the transduced cells which were then bulk sorted on four populations according to CD107a and IFNg positivity on BD FACS Aria Fusion. DNA was harvested from the resulting cells and subject to next generation sequencing.

Following in-depth bioinformatic analysis, the data was stratified into hypo- and hyper-responders to K562, according to sgRNA enrichment or depletion, and validated through gene set enrichment analysis. Initial readouts confirmed the differential expression in key genes necessary in these pathways such as IFNG, TBX21, LAMP1, NCR3, UNC13D and RAB27A, emphasizing biological significance. In depth data refinement and filtering revealed the presence of essential intracellular cytotoxic signaling mediators, including CD247, GRB2, LAT, DAP12 and SHP-1/2 in the top hits while also uncovering new targets.

Following the identification of novel genetic mediators driving the cytokine and cytotoxic responses, the hits will be confirmed and validated in primary cells, which will widen our understanding of NK cells and their involvement in antitumor responses. A genome-wide overview of this complex mechanism has the potential to change the landscape of immunotherapeutic approaches by revealing targets in central pathways for improving the potency of NK cell-based therapies.

35 - Studying the pre-existing CRISPR/Cas9 immunogenicity in Icelanding population and circumventing the immunogenicity barrier

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Therapeutic genome editing (TGE) with CRISPR-Cas9 holds great promise to cure rare and genetic diseases but recent finding describing immunity against the endonuclease Cas9 puts its utility in question. The Cas9 isolated from *Streptococcus pyogenes* (SpCas9), is known to trigger host immunogenicity. The continuous cytosolic presence of exogenous proteins can increase antigen presentation via MHC-I. Evading the cellular immunosurveillance could therefore represent a major advancement to broaden the clinical applications of CRISPR. Here, we rationally engineered AZ proprietary fdCas9 (Fast degrading SpCas9) with a single amino acid substitution to introduce a chaperone-mediated autophagy (CMA) binding moiety. This mutation directly target proteins for lysosomal degradation and reduced induction of immune cells. We observed Cas9 specific pre-existing IgA levels significantly lower in children than in adults in the Icelandic population (n= 65, children= 35, Adults= 30). Moreover, healthy donor peripheral blood monocyte cells (PBMCs) and whole blood treated with either SpCas9 or fdCas9 showed reduced T-cell responses in fdCas9 treated group compared to SpCas9. We further demonstrated how the same individual's PBMCs responded differently and IFN-γ release correlated with toxicity and immune activation. We also observed that fdCas9 endonuclease induces significantly lower T cell proliferation in healthy donor whole blood. Taken together, the novel version of SpCas9 that circumvents activation of host immunity, making it an attractive enzyme for therapies that require *in vivo* administration of CRISPR agents to patients.

34 - CRISPR based transcriptional monitoring system

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BCL-6 is a major regulator of GC B-cell development and has an established role in the etiology of the subset of non-Hodgkin's lymphoma (NHL) with a large-cell component. It is located at chromosome 3q27, which is a common site of translocation in NHL. Mice that were deficient in BCl-6 have normal B-cell development, but lack the ability to develop Germinal Centers and perform affinity maturation. This demonstrate the crucial role of BCl-6 in GC B cell proliferation and genomic remodeling, such as SHM and class-switch recombination. Interestingly, It was observed that resting B cells and activated B cells have comparable levels of BCL6 mRNA, but protein expression was much higher in germinal center B cells than in resting B cells. This suggests that BCL-6 protein levels are controlled by translational or posttranslational mechanisms but yet to be find out.

CRISPR monitoring system which is comprised of CRISPR-activator-associated reporter—Suntag-P65-HSF1 (SPH) and optimized miniCMV-mCherry (SPH-OminiCMV). This system can be integrated into the 3'UTR of a gene and the expression of that gene can be readily monitored. We have modified the reporter system and Fast fluorescent timer (Tocky FT) was incorporated. Benefit of this system is the transition of blue fluorophore to red which occurs after 6 hours, that can give direct estimation of gene transcription. Both of these transcriptional monitoring systems were stably established in A20 cell line (that resemble germinal centre B cell physiology) and expression profile of housekeeping beta-actin and BCL6 gene were observed.

Both these systems were tested by blocking the transcription and translation of the cells with Actinomycin D and Cycloheximide. We have found that the systems were behaving properly when the result of the fluorescence were compared with qPCR. Then, transcriptional profile of house keeping beta actin gene was tracked with mCherry based system, which revealed that trasncription can be tracked at longer time intervals (for instance, after 24 hours). As mCherry has a longer half life and it is not possible to track smaller changes at shorter time points. That is why for BCL6 transcription monitoring, Tocky FT system was designed. This system was tested by stimulating the cells with anti-IgG. Upon stimulation, transcription and translation was down regulated. The results also indicates that this system can also be used for transcription monitoring at longer time points and not shorter time points. The reason is, that upon stimulation, we speculate some phenotypic changes in the cells which increase the blue fluorescence at 4 and 8 hours despite the fact that mRNA was going down. But after 24 hours, down regulation can be in the fluorescence can be seen. Secondly, the dynamic range of this system is of factor 2, which is a small change in fluorescence so tracking a very small change might not be feasible.

33 - p38 MAPK fuels proliferation stress and DNA damage impairing the functionality of genetically engineered hematopoietic stem and progenitor cells

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Ex vivo activation and cell cycle progression of hematopoietic stem and progenitor cells (HSPCs) is a prerequisite to reaching adequate levels of genetic engineering by homology-driven repair (HDR) for clinical applications. Here, we show that shortening *ex vivo* culture mitigates the p53-mediated DNA damage response initiated by the concomitant exposure to nuclease and template delivery vectors and endows edited HSPCs with greater hematopoietic reconstitution capacity upon transplantation. However, this comes at the cost of a lower HDR-mediated gene correction, rendering *ex vivo* culture an unavoidable step, although detrimental. Mechanistically, we uncovered that *ex vivo* activation triggers a p38 MAPK-mitogenic ROS axis that fuels proliferation stress and heightens DNA damage burden across HSPC subsets. p38 inhibition prior to genetic engineering delayed G1/S transition, expanded transcriptionally-defined hematopoietic stem cells (HSCs), and increased multi-lineage output at single-cell resolution. Accordingly, *in vivo* analyses coupled with clonal tracking revealed superior engraftment, persistence throughout serial transplantation, and enhanced polyclonal repertoire of p38 inhibitor-treated gene-edited HSPCs. Altogether, our data point to proliferative stress as a driver of human HSPC dysfunction with fundamental implications for advancing precise gene correction into clinical practice.

32 - Cas9-mediated precise and template-free gene editing of a muscular dystrophy founder mutation: From single editing and off-target analysis to double editing and clinical translation

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Limb Girdle Muscular Dystrophy type 2A/R1 (LGMD2A/R1), caused by mutations in the Calpain 3 gene, is a severe muscular dystrophy leading to progressive loss of the hip and shoulder girdle musculature with no treatment available. With thousands of patients worldwide, *CAPN3* c.550delA is the most frequent mutation in LGMD2A/R1. We genetically corrected this founder mutation in primary human muscle stem cells (PHSats) from patients. PHSats can be isolated highly efficient without contaminating fibroblasts from patient muscles and expanded in *in vitro*.

Mutation-specific targeting with a synthetic sgRNA and Cas9 mRNA yielded highly efficient and precise correction of *CAPN3* c.550delA. The single cut generated by Cas9 results in a 5' staggered overhang of one base pair, which triggers an overhang-dependent base replication of an A:T at the mutation site. This precise reframing recovers the open reading frame and the *CAPN3* DNA sequence is repaired template-free to wild type, which leads to CAPN3 re-expression and functional recovery. Off-target analysis using amplicon sequencing of 43 targets nominated by GUIDE-Seq and *in silico* prediction did not show any editing demonstrating the safety of this approach. This is also true for the translocation analysis confirming the safe and precise repair for *CAPN3* c.550delA.

Additionally, we evaluated the potential of editing two patient mutations in one cell. Most of the patients with *CAPN3* c.550delA are compound heterozygous and carry a second mutation in *CAPN3*. We can target two mutations even with different gene editing tools. In this regard, we show the successful editing of *CAPN3* c.550delA with Cas9 mRNA in combination with base editing of the second mutation *CAPN3* c.1468C>T using ABE8e mRNA in compound heterozygous patient PHSats.

Resulting genetically corrected PHSats are a valuable tool for autologous transplantation back into patients to recover muscle structure and function. To test if corrected PHSats from *CAPN3* c.550delA patients have the potential to form functional muscle tissue, we transplanted repaired cells into immunocompromised *Capn3*-Knock-out mice. Gene-corrected PHSats contribute to the mouse muscle, repopulate the stem cell niche and express CAPN3.

Currently, we are planning a First-in-human clinical trial to transplant repaired PHSats into patient muscles. The trial is prepared to launch in 2025.

31 - CRIPSR screen identifies UBE2A loss-of-function to confer bortezomib sensitivity in diffuse large B-cell lymphoma

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Diffuse large B-cell lymphoma (DLBCL) is the most frequent type of malignant lymphoma, accounting for 30-40% of all newly diagnosed non-Hodgkin lymphoma cases. Since standard treatment only cures 60% of patients, numerous clinical trials have attempted to improve the first-line R-CHOP treatment regimen through addition or substitution of drugs. Addition of the proteasome inhibitor bortezomib to R-CHOP have been shown to improve clinical outcome of molecularly classified high-risk DLBCL patients, yet improved understanding of molecular determinants of bortezomib response are needed for future targeted treatment approaches.

To identify genes affecting bortezomib response in DLBCL cells, we perform genome-wide CRISPR knockout screens selected by bortezomib, and address the clinical relevance of identified genes by inclusion of mutational data of DLBCL patient cohorts. DLBCL cells were screened using the Brunello library in two independent replicates and with bortezomib at a low (5ng/mL) and high (112ng/mL) concentration or saline as control for 8 days. MAGeCK analysis identified depletion of gRNAs targeting 230 and 280 genes upon low and high bortezomib exposure, respectively, of which 4% were in common. Conversely, 7.5% of enriched gRNAs were shared between low and high bortezomib concentrations with 87 and 85 gRNAs enriched, respectively. Enriched gRNAs reflect genes in which CRISPR/Cas9-induced loss-of-function make cells more resistant towards bortezomib, whereas depleted gRNAs constitute genes where loss-of-function confer sensitivity. We applied MAGeCK-Flute to identify affected pathways among the identified sensitizing and resistance-inducing knockouts. Apoptosis and P53 signaling where identified as resistance involved pathways in both bortezomib concentration screens. In contrast, different pathways of sensitivity were observed between bortezomib doses, of which mTOR signaling and proteasome assembly appear to be most relevant.

As we aim for identification of potential predictive molecular markers of bortezomib sensitivity, mutation analysis of identified response-affecting genes were performed in our local cohort of 55 primary DLBCL patients. The ubiquitin conjugating enzyme E2 A (*UBE2A*), identified to confer sensitivity upon loss-of-function in the bortezomib low dose screen, is mutated in 9% of our cohort and in 6.4% in the publicly available TCGA DLBCL cohort (n=47). In addition, *UBE2A* mutations are included in genetic subclassification of DLBCL and based on its clinical relevance, single gene *UBE2A* knockout studies were performed to examine the functional impact of UBE2A in DLBCL. Knockout of *UBE2A* was conducted by CRISPR/Cas9 RNP delivery using nucleofection in the DLBCL cell lines RIVA and HBL1 and confirmed by indel analysis and western blotting. *UBE2A-KO* increased sensitivity to bortezomib over a range of concentrations in MTS-based dose-response studies without impacting DLBCL cell proliferation, validating findings of the genome-wide CRISPR screen. Additional functional analysis of UBE2A including combinatory drug studies, apoptosis, proteasomal activity among others are currently ongoing. In conclusion, this study shows that loss-of-function of UBE2A confer bortezomib sensitivity in DLBCL cells, which is of clinical relevance as missense, nonsense, and splice site mutations are observed in 6-9% of DLBCL patients.

30 - REMAX: Innovative REframing Strategies to MAXimize Dystrophin Restoration in DMD

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Duchenne muscular dystrophy (DMD) remains a devastating disease with no effective cure. However, CRISPR technology has emerged as a promising therapeutic avenue, particularly through DNA cleavage approaches that restore the frame of the disease allele ("reframing"). To achieve a successful therapeutic effect, it is imperative that reframing is highly efficient. In this study, we introduce REMAX, an innovative strategy designed to maximize the efficiency of reframing in treating DMD. We designed REMAX to target pivotal exons—53, 51, and 45—enabling a highly effective gene-editing therapy that addresses approximately 30% of DMD mutations. In initial experiments, we screened CRISPR candidates in HEK293T cells to identify candidates with optimal REMAX editing activity. We employed commonly used SpCas9 and other CRISPR variants such as high-fidelity Cas9s, SaCas9, and SpCas9 VQR. Subsequently, the best-performing candidates and designs were tested in DMD human immortalized primary myoblasts with deletions in exon 52, exon 48-50, or exon 44. Upon transfection of REMAX components into these myoblasts, a significant reframing activity at the genomic DNA level was demonstrated. Moreover, upon differentiation into myotubes, robust dystrophin restoration, higher than the standard strategies, was observed, supporting the therapeutic potential of REMAX. To validate these findings in vivo, we generated a novel humanized DMD knockout transgenic mouse model, containing a single copy of human DMD transgene harboring exon 50 deletion. This model was specifically designed to assess the effectiveness of REMAX targeting exon 51. Notably, all REMAX components successfully fit into a single viral vector, offering the advantage of minimizing the required viral dose. Ongoing efforts include injecting the viral vector carrying the REMAX components into the humanized DMD mouse model to assess REMAX performance in vivo. The results of this ongoing study hold promise for advancing CRISPR-based therapies and bringing us closer to a more effective treatment for DMD.

29 - Disruption of Epidermal Growth Factor Binding Site by Sequential Knock in CRISPR/Cas9 Genome Editing of Epidermal Growth Factor Receptor and Evaluation of Introduced Mutations in Cervical Cancer Cells

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Cervical cancer is the fourth most frequent cancer in women with an estimated 604 000 new cases and 342 000 deaths in 2020. according to World Health Organization. About 90% of the analyzed cervical tumors share a common characteristic: overexpression of the epidermal growth factor receptor (EGFR). EGFR signaling is activated by ligand binding and controls cell proliferation, differentiation, motility and survival. **Epidermal growth factor (EGF) is one of the major EGFR ligands and this interaction is a frequent point of interest in the development of new cancer** therapeutics. After binding to the extracellular region of EGFR, EGF induces receptor dimerization and autophosphorylation triggering numerous downstream signaling pathways. Activated EGFR internalizes and translocates to the nucleus mostly by clathrin

mediated endocytosis. EGF binds to the domain I of EGFR protein via β loop. Hydrophobic interactions between Leu-14, Tyr-45, Leu-69, and Leu-98 of domain I of EGFR and Met-21, Ile-23, and Leu-26 of EGF play a major role in ligand binding. We hypothesized that mutations of Leu-14 and Tyr-45 would prevent EGF-EGFR binding and tested this by generating a mutant EGFR cDNA expression plasmid. Interaction between fluorescently labeled EGF and mutant EGFR was tested in cells with low native EGFR expression transfected with mutant EGFR cDNA. After confirming that mutant EGFR had a disrupted EGF binding, we conducted genome editing in cervical cancer cell line ME180 with high level of native EGFR expression. Using "knock in" CRISPR/Cas9 strategy and homology directed repair (HDR), we introduced mutation(s) of selected amino acids into one or both alleles of EGFR in ME180 cells. Because both mutations were not obtained in any of the clones, we have performed an additional round of CRISPR/Cas9 using the isogenic homozygote clone with Tyr-45 mutation. The second round of CRISPR/Cas9 conducted to knock in a Leu-14 mutation was less precise. In most of the successful secondary clones with both desired mutations, one of the EGFR alleles was lost. Successful modification of EGFR alleles and absence of off-target mutations were confirmed with whole genome sequencing. Disrupted EGF binding in CRISPR/Cas9 single cell clones was confirmed by immunocytochemistry. In addition, cell proliferation, cell cycle and downstream EGFR/PI3K/AKT signaling were evaluated in several mutant ME180 cell clones with disrupted EGF binding. As anticipated, presence of EGF did not induce phosphorylation of EGFR either in ME180 clones with homozygous Tyr-45 mutation or clones with a single functional allele with both mutations. Only in cells expressing wild type EGFR addition of EGF caused its phosphorylation. Interestingly, while phosphorylation of PI3K kinase, the immediate target of EGFR, showed no response to EGF addition in cells with mutant EGFR, addition of EGF led to phosphorylation of AKT 1, 2, 3 in wild type cells and clones alike. Experiments are underway to explore the EGF dependent AKT signaling independent of EGFR function.

27 - CRISPR/Cas9-based pipeline to introduce custom edits in patient T cells

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Emerging CRISPR/Cas9 gene therapy trials demonstrate the therapeutic potential of correcting mutations in patient cells. However, as these trials are largely focused on common hematologic diseases, such as sickle cell disease, little is known about how to optimize CRISPR for other monogenic mutations. To address this challenge, we developed a CRISPR/Cas9-based T cell editing pipeline that streamlines CRISPR tool discovery for highly efficient and safe correction of mutations in *ADA2*, *AIRE,* and *RMP* loci in patient T cells. Our data demonstrate a rapid and selective expansion of CD3+ T cells from patient and healthy donor peripheral blood mononuclear cells (PBMC) after 8-day cytokine stimulation. CRISPR guide-RNAs (gRNA) for *ADA2*, *AIRE,* and *RMRP* were screened directly in patient T cells, and off-target safety of best-performing gRNAs was assessed using genome-wide, unbiased identification of double-strand breaks enabled by sequencing (GUIDE-seq), where no gRNA off-targets were identified for any of the

loci. Finally, we tested multiple strategies to increase precise homology-directed repair (HDR) outcomes and developed an HDR-optimized T cell pipeline that can achieve as high as 80% mutation correction levels in patient T cells. In conclusion, our work demonstrates that the CRISPR/Cas9 T cell pipeline is robust and suited for creating small custom edits, such as mutation correction, in patient T cells. Furthermore, it has the potential to be converted into a GMP-compatible workflow and scaled up to fit clinical gene therapy needs.

26 - Standardization and harmonization of gene editing in human induced pluripotent stem cells: tagging of a lysosomal protein using CRISPR/Cas9

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The discovery of human induced pluripotent stem cells (iPSCs) and the development of CRISPR/Cas9 gene editing technology have revolutionized biomedical sciences, beginning a new era of both research and medicine. However, due the sensitive nature of iPSCs, gene-editing in these cells presents specific challenges regarding evaluation, implementation, and standardization. In recent years, core facilities have emerged as pivotal players, harmonizing procedures and offering expertise in the swiftly advancing fields of iPSCs and gene editing. Here we present our streamlined pipeline for the generation of CRISPR/Cas9-mediated-tagged endogenous loci in human iPSCs, followed by a comprehensive quality assessment of successfully edited clones. Our approach involves lipid-mediated transfection of iPSCs with a donor plasmid containing the fluorescent tag of interest, CopGFP, along with homology arms flanked by single guide RNA (gRNA) recognition sequence. Subsequently, nucleofection of cells with Cas9/sgRNA ribonucleoprotein complexes (RNPs) facilitates the targeted tag integration into the specific genomic loci, such as the constitutive exosome marker shown here. Following clonal expansion and identification of successfully edited clones, we perform a series of quality controls that includes testing for mycoplasma, confirmatory STR analysis, evaluation of the undifferentiated state of the cells, morphology and growth pattern analysis, assessment of genome stability through molecular karyotyping and G-banding, and examination of potential off-target effects. Altogether, by presenting our refined approach, we contribute to the ongoing discourse on the practical implementation of CRISPR/Cas9 technology in human iPSCs, emphasizing the crucial role of core facilities in advancing the standardization and application of these cutting-edge techniques in biomedical research.

25 - Genome-wide CRISPR activation and interference screens for decoding the molecular signature of induced pluripotent stem cell to T cell differentiation

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Induced pluripotent stem cells (iPSC) are a promising avenue for the production of next-generation adoptive cell therapies (ACT). They serve as a potentially unlimited source of cells from which to differentiate any immune cell of interest. Specifically in T cell-based ACTs, this approach allows the customization and personalization of the final T cell product and the generation of an off-the-shelf, allogeneic product. The differentiation of iPSCs to T cells poses great challenges, mainly associated with the low efficiency of the process and protocol variability.

Therefore, decoding the molecular signature of this process is of significant importance for advancing this type of therapy. In this regard, CRISPR activation (a) or interference (i) screens offer a comprehensive and systematic approach to unravel the complexities of gene function and regulate the differentiation process.

In this project, we will perform CRISPR/Cas9 activation (CRISPRa) and interference (CRISPRi) screens. For these screens, we will generate iPSCs constitutively expressing dead Cas9 (dCas9) fused to either the VP64 transactivating domain (or other transactivating domains) for CRISPRa or the transinhibiting domain KRAB for CRISPRi screens. For the CRISPRa screen, the dCas9-VP64 iPSCs will be transduced with the whole-genome CRISPRa Calabrese library (containing between 4 to 6 gRNAs targeting each gene). For CRISPRi, the dCas9-KRAB iPSCs will be transduced with the whole-genome CRISPRi Dolcetto library. We will then follow the enrichment or dropout of gRNAs in differentiated populations to evaluate which genes regulate the differentiation at different stages of the process.

We are currently developing these assays and setting up these systems in iPSCs; a challenge of these experiments is related to the lentiviral constructs being very large (~14kb), but we have shown in preliminary experiments that we can obtain >90% transduction efficiency of cell lines, suggesting this will be feasible in T cells and iPSCs. We anticipate that the knowledge obtained from the screens will not only enhance the understanding of T cell development but will also provide opportunities for more precise manipulation of gene expression, which will lead to the generation of more robust protocols for iPSC-derived T cell products.

Keywords: CRISPR screens, iPSC, T cell, CRISPR activation, CRISPR interference.

24 - A CRISPR-Cas9 double-hit strategy mitigates on-target aberrations and chromosomal translocations

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CRISPR-Cas nuclease-based genome editing aims to precisely modify the human genome. The introduced DNA double-strand breaks (DSBs) are typically repaired by non-homologous end-joining (NHEJ). It is well known that imperfect repair can lead to small insertions and deletions (indels) at the target site, and it has been speculated that large on-target aberrations are due to repeated cleavage of the target site by the programmable nuclease. Furthermore, DNA repair of multiple DSBs, e.g., a target site and an off-target site, may result in chromosomal translocations. We hypothesized that preventing repeated re-cleavage of the target site would reduce on-target aberrations as well as the frequency of translocations. To test this hypothesis, we targeted different loci in the human genome with a "double-hit strategy", i.e. we designed two CRISPR-Cas9 nucleases that targeted each locus (BCL11A, CD40L, CSF2) within 100 bp. To assess the extent and the frequency of chromosomal rearrangements, we used CAST-Seq and rhAmpSeq. Our data indicate that the use of two CRISPR-Cas9 nucleases to excise a ~80 bp DNA fragment reduced the frequency of large on-target deletions at e.g. the BCL11A locus by a factor

of two and the average deletion size from 780 to 430 bp. Furthermore, the number of chromosomal translocation events between two target sites, e.g. CD40L and CSF2, was lowered 3-fold when each locus was hit twice instead of once. In conclusion, our study shows that large on-target aberrations and the frequency of chromosomal translocations can be mitigated by applying a CRISPR-Cas double-hit strategy.

23 - Xential: universal toxin-based selection for precise genome engineering

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CRISPR technology enables precise and efficient genome editing. Originally discovered CRISPR system for creating indels was re-imagined by scientists all over the world and turned into a versatile instrument allowing to edit nucleotides, control gene expression, change epigenome profiles and insert DNA sequences, all that with unprecedented accuracy and precision. Thus, this technology holds immense potential in various fields, from medicine to agriculture. However, editing efficiency, especially in primary cells, is one of the major challenges of CRISPR technology, where it can be as low as few percent of the total population. In this work, we present a method for rapid and robust generation of a homogenous population of cells with bi-allelic integration of a DNA cassette without the need for clonal selection. To this end, we utilized mutations in Diphtheria Toxin (DT) receptor, which make cells insusceptible to death induced by DT. Selecting for cells with edited DT receptor allows for co-selection of a second editing event in an independent locus, such as nucleotide editing, gene editing or insertion. We demonstrated efficacy of this method in variety of human cells, including cancer cell lines, human induced pluripotent stem cells (hiPSC) and primary T-cells, as well as *in vivo* in humanized liver in mice.

Currently Xential system is used in many projects in the AstraZeneca portfolio. It is used for engineering of cell lines in applications as diverse as cellular reporters, monitoring protein folding or speeding up lineage differentiation programs.

22 - Precision Gene Editing Corrects Phenotypic Effects of Dominant Keratin 6 Mutation in Pachyonychia Congenita Patient Cells.

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Pachyonychia congenita (PC) is a rare hereditary skin disorder characterized by autosomal dominant inheritance. PC is caused by mutations in genes encoding the hyperproliferation-related keratins K6, K16, or K17. Individuals affected by PC suffer from

debilitating palmoplantar keratoderma associated with distressing blistering that severely limits their ability to walk. Current therapeutic strategies focus on pain management through the use of emollients to alleviate hyperkeratosis and measures to minimize friction and trauma to the feet, warranting the search for therapies that lead to improvements in quality of life. When considering a gene therapy strategy for PC, the primary goal should be to disable the pathogenic allele that exerts a dominant negative influence on the healthy allele. For this reason, advances in gene editing technology, particularly CRISPR tools, open up possibilities for gene therapy protocols in pachyonychia patients. Our investigation specifically identified the c.513C>A (p.Asn171Lys) mutation in the human keratin 6 gene KRT6A, which generates a novel PAM motif (VTTT) for the Cpf1/Cas12 nuclease. To address this mutation, we designed a gRNA targeting the sequence adjacent to this PAM and evaluated its efficacy and allelic specificity in vitro. Electroporation of keratinocytes from patients carrying this mutation with CRISPR/Cas12 RNPs with this guide results in a highly efficient and specific disruption of the pathogenic allele, leading to the restoration of the intermediate filament cytoskeleton in these cells. As a first step toward clinical translation, we are introducing a mouse model with humanized skin derived from corrected keratinocytes to evaluate its efficacy in correcting the characteristic hyperkeratinization phenotype associated with this disease. Future efforts will focus on the vectorization of CRISPR/Cas12 for in vivo delivery in humanized patient skin models.

21 - Conquering gene editing off-target effects with Gibco™ CTS™ HiFi Cas9 Protein

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Gibco™ CTS™ HiFi Cas9 Protein is a GMP-manufactured, high-fidelity Cas9 protein with exceptional specificity to support its use in cell and gene therapy development programs. The Cas9 protein is designed to minimize off-target edits while maintaining high on-target editing efficiency. With this new protein, we are now offering customers working in a clinical environment a low off-target solution for their genome editing projects.

The CRISPR-Cas9 genome editing system has seen exponential growth from fundamental research to applications in therapeutics. Continuous efforts are being made to improve the use and effectiveness of the enzyme. For therapeutics applications, the major concerns are GMP compliance and off-target activity, which is characterised by Cas9 activity outside of the targeted area creating unwanted indels, mutations, inversions, or translocations, which can result in undesired phenotypes or loss of functional gene activity.

Gibco™ CTS™ HiFi Cas9 Protein, has been developed as part of our Cell Therapy Systems or CTS™ product line for clinical use, and designed for low off-target effects. CTS products are provided with a Drug Master File (DMF) (or Regulatory Support File depending on jurisdiction) to support your IND submissions. CTS-branded products undergo stringent QC and functional testing and are already used in numerous clinical trials and commercial cell and gene therapy manufacturing processes.

Here we compare the on-target activity and off-target effects with another supplier's GMP grade high fidelity Cas9 by performing knock-outs in and knock-ins in iPSCs and primary human T cells (including TCR knock-outs and CAR knock-ins). We show that the Gibco™ CTS™ HiFi Cas9 Protein

significantly reduces off-target effects when compared with the competitor's Cas9, while maintaining high on-target activity as compared to CTS™ TrueCut™ wild-type Cas9 protein.

20 - Base editing mediated correction of severe β0 thalassemia mutations.

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β-thalassemia is a highly prevalent monogenic recessive disease caused by mutations affecting the synthesis of the hemoglobin β-chains. Mutations in the β-globin gene (*HBB*) locus reduce (β^+) or abolish (β^0) the production of β-chains, β^0/β^0 being the most severe genotype. The imbalance between α- and β-globin production leads to the precipitation of uncoupled α-globins, which causes erythroid cell death, ineffective erythropoiesis and severe anemia. Transplantation of autologous, genetically modified hematopoietic stem/progenitor cells (HSPCs) is an attractive therapeutic option with two recently FDA approved gene therapy products. However, current gene therapy strategies based on the use of lentiviral vectors or CRISPR/Cas9 nuclease are not equally effective in all patients and/or might raise safety concerns. Base editing, a CRISPR/Cas9 derived genome editing tool, allows the introduction of point mutations (e.g. A>G by adenine base editors or ABEs) at precise locations within the genome.

The two β^0 mutations CD39 (CAG>TAG) and IVS2-1 (G>A) and the β^+ mutation IVS1-110 (G>A) are among the most common and severe β-thalassemia mutations in the Mediterranean area and Middle East. We have previously reported an efficient base editing strategy to correct the IVS1-110 mutation. Here, we evaluate the efficacy and safety of β^0 mutation correction approaches (CD39 and IVS2-1).

First, we designed specific ABEs/gRNAs combinations allowing gene correction with efficiencies of up to ~90% in HSPCs from β-thalassemia patients. This led to high β-globin levels and improved α/non-α globin ratios in red blood cells (RBCs) in vitro differentiated from edited HSPCs. The delayed erythroid differentiation typically observed in β-thalassemic cell cultures was corrected by our treatment.

To evaluate the safety profile of our approaches, we nominated off-target sites based on sequence homology by in silico prediction, and GUIDE-seq. Overall, we did not identify off-target mutations with anticipated clinical relevance. Importantly, while the homologous *HBD* gene was detected as an off-target site in the CD39 mutation correction strategy, the base editing approach preserved the integrity of the β-globin locus as evaluated by long-read sequencing of the region encompassing these adjacent on- and off-target sites. Moreover, RNA-seq comparison

of control and edited β-thalassemic HSPCs showed no discernible impact of the editing procedure at the transcriptomic level.

Finally, corrected HSPCs were transplanted in immunodeficient mice to evaluate the engraftment and differentiation capability of edited HSCs. We observed no differences in the engraftment and differentiation potential of edited and control HSCs, as measured by the frequency of human $CD45^+$ cells and the analysis of lineage-specific markers. The gene correction efficiency reached up to ~70% in the bone marrow human cells of transplanted mice. Editing was similar in the different lineages, showing no skewed hematopoiesis. Importantly, we detected high β-globin levels in bone marrow derived erythroid cells of treated mice and an increased frequency of circulating human RBCs, confirming the correction of the β-thalassemic phenotype in vivo.

Overall, our study demonstrates that gene correction using base editing is safe and efficient in bona fide HSCs, thus paving the way for the clinical development of these base editing approaches for the treatment of β-thalassemia.

19 - Universal CAST-Seq: Off-target detection of CRISPR nucleases and base editors

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Genome editing can be associated with genotoxicity, such as mutagenesis at off-target sites and chromosomal translocations. This risk must be carefully evaluated before gene-edited products can be used clinically. We have recently described CAST-Seq, a diagnostic assay for the genome-wide detection of CRISPR-Cas-induced chromosomal aberrations in clinically applied cells. The method is based on the detection of chromosomal rearrangements resulting from the simultaneous cleavage of the on-target and an off-target site. While CAST-Seq is able to identify CRISPR-Cas nuclease-induced chromosomal aberrations with high sensitivity, the sensitivity to detect off-target activity of base editors or prime editors is lower because these editing platforms do not rely on the formation of DNA double-strand breaks. In addition, in rare cases, designing effective CAST-Seq primers for a specific target site in GC-rich regions of the genome can be tedious. To overcome these limitations, we developed universal CAST-Seq (U-CAST), a method for detecting off-target activities of any CRISPR-based editing platform without the need for primer optimization. U-CAST is based on the fact that any DNA double-strand break, inter alia one introduced by a highly specific CRISPR-Cas12a nuclease in the *CYBB* locus, can serve as an "anchor" to detect off-target events triggered by any genome/base/prime editor. Performing U-CAST with CRISPR-Cas9 nucleases targeting reference genes, such as *VEGFA*, confirmed that the results obtained with U-CAST are comparable to those obtained with conventional CAST-Seq with minimal compromise in terms of sensitivity: some ultrarare translocation events were missed. Using U-CAST to identify the off-target activities of CRISPR-Cas9 nuclease and base editors, all targeting the same site in the EMX1 gene, revealed that the evoCDA1-BE4max base editor generated more off-target mutagenesis than Cas9 nuclease, while the CBE-T1.52 base editor

effectively mitigated off-target effects. Because the method can be performed with already established U-CAST primers, U-CAST saves time and costs, and is particularly well suited for screening genome and base editors in early development.

18 - Studying the role of TRIM28 and transposable elements dysregulation in neurodevelopmental disorders using CRISPRed in vitro models

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Neurodevelopmental disorders (NDDs) are caused by different perturbations during early brain development. Many NDDs have a genetic origin, but the underlying genetic alterations remain unknown for most of them. The study of these disorders has been challenged by the limited access to human brain tissues at early development time-points and the incomplete recapitulation of patients' phenotypes in animal models. In this project, we use state-of-the-art *in vitro* models coupled to cutting-edge gene editing and sequencing techniques to study the effects that mutations in TRIM28, a master regulator of transposable elements (TEs), have in transcriptional regulation and their involvement in neurodevelopmental phenotypes. Our lab previously demonstrated that TRIM28 is required to silence specific TEs during brain development in mice and human neural progenitor cells. However, to date, a link between TRIM28 and brain disease in humans has not been demonstrated. Our aim is to prove that mutations affecting TRIM28 cause neurodevelopmental phenotypes and to understand how TRIM28 dysfunction impairs neurodevelopment and leads to disease.

Four *de novo* heterozygous missense mutations affecting TRIM28 were identified in patients with moderate intellectual disability through whole exome sequencing analysis. These mutations affect relevant protein domains and are predicted to impair TRIM28 function. To test their pathogenicity *in vitro*, we generated CRISPR-edited induced pluripotent stem cells (iPSCs) lines carrying two of these mutations, which affect a relevant well-characterized functional domain of the protein (TRIM28-mutant). Using CUT&RUN and RNA-seq, we found that the mutant cells show a loss of the histone mark H3K9me3 coupled with overexpression of genes and TEs, predominantly endogenous retroviruses (ERVs), an abundant type of TE. Then, to evaluate if such variants have a loss-of-function effect, we generated a *TRIM28* knock-down iPSC model using a lentiviral-based CRISPRi approach (TRIM28-silenced) and performed the same sequencing experiments. The transcriptomic changes observed in the TRIM28-silenced cells highly overlaps with those previously observed in the TRIM28-mutant, pointing towards a loss-of-function effect caused by the mutations. To model the effects of this chromatin dysregulation in neurodevelopment and test if they also affect the differentiation potential to neural cell types, we differentiated the TRIM28-mutant iPSCs into monolayer forebrain neural precursors (fbNPCs) and unguided neural organoids, which also show epigenetic and transcriptional changes that

affect both TEs and protein-coding genes.

These results show that dysregulation of the TRIM28-mediated epigenetic mechanism and downstream aberrant TE and gene expression can be related to neurodevelopmental phenotypes. Our findings highlight the critical importance of regulating TEs during human brain development and will ultimately lead to the description of a novel monogenic neurodevelopmental disorder caused by mutations in TRIM28.

17 - CRISPR-Mediated Inactivation of Faah and Magl in Peripheral Nociceptive Neurons: Towards Modulating Endocannabinoid Levels for Pain Control.

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Inactivation of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), the enzymes that modulate endocannabinoid levels, is a promising approach to pain relief. To achieve loss of function of Faah and Magl in mouse cells, third-generation adenoviral vectors expressing CRISPR/Cas9 were designed with guides targeting the coding regions of these genes. Initial validation through electroporation of CRISPR/Cas9 RNPs with corresponding guide RNAs into mouse embryonic fibroblasts (MEF) demonstrated effective generation of indels in the intended regions consistent with loss of function mutations. RT-PCR analysis confirmed a significant decrease in Faah and Magl expression in electroporated cells. To investigate the functional effect of gene editing mediated mutagenesis, we assessed endocannabinoid levels in treated cells by HPLC-MS analysis and discovered changes in the endocannabinoid profile in accordance with the functional loss of each hydrolase. After construction and production of the CRISPR-carrying adenoviral vectors, their efficacy in inhibiting Faah and Magl expression was confirmed by MEF infection. Moving towards inactivating Faah and Magl in nociceptive neurons of dorsal ganglia, intrathecal injections of CRISPR adenovirus preparations were performed in mice. The response to mechanical stimulation, measured with an automated Von Frey aesthesiometer, showed a slight delay in the paw withdrawal response in mice injected with adenoviral vectors compared to sham-treated controls, suggesting some reduction in tactile sensitivity at the plantar surface. Dorsal root ganglia dissections and individual genotyping of each ganglion were initiated four weeks post-injection. Sanger sequencing unambiguously revealed gene editing in a small fraction of genotyped ganglia, with varying editing levels observed between animals. Further analysis by NGS sequencing will provide a more precise characterization of editing efficiency. The observed changes in the endocannabinoid lipid profile in lumbar and thoracic dorsal root ganglia pools align with functional loss of each enzyme. This study, the first to showcase in vivo gene editing in dorsal root ganglion cells albeit with limited efficacy, sets the stage for exploring the therapeutic potential of gene editing to modulate

endocannabinoid levels in the peripheral nervous system for pain management.

16 - Sequence specific depletion of cancer cells using the dsDNA collateral activity of a novel CRISPR/Cas nuclease

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Since the discovery of the CRISPR-Cas9 editing technology, numerous new Cas variants have been identified with the ambition to create alternatives or superior treatment approaches for various diseases including cancer. However, one major hurdle to overcome is to enable highly selective killing of cancer cells without affecting surrounding healthy tissue.

Here we report that our proprietary CRISPR nuclease termed G-dase E, capable of RNA-triggered DNA and RNA destruction, can elicit programmable and sequence-specific elimination of cancer cells expressing a target transcript without affecting the viability of the surrounding (non-target) cells.

In order to demonstrate target specific depletion of human cells G-dase E RNP complexes were maturated with target-specific gRNAs and cell depletion was determined by quantitative fluorescence imaging and cytotoxicity assays.

In control experiments, a spacer sequence targeting the mRNA of the housekeeping gene GAPDH was used and non-differential cell death could be triggered in all cell types tested including head neck and cervix cancer cell lines.

Next, we addressed the potential of G-dase E RNPs to specifically target clinically relevant oncogenes in cancer cells using cervix carcinoma and head neck cancer cell lines expressing the human papilloma virus oncogenes E6 and E7. Several gRNAs were designed to match different regions of the clinical relevant HPV16 E6/E7 (head neck) and HPV18 E6/E7 (cervix carcinoma) mRNAs. These gRNAs were assembled with G-dase E into functional RNPs and delivered to cell cultures via electroporation. Strikingly, we succeeded in the selective killing of HPV positive cells by transfecting RNPs that target the HPV18-E6/E7 or HPV16-E6/E7 mRNA, whereas HPV negative cells that do not express the mRNA of these oncogenes remained viable even in co-culture experiments.

In summary, we provide first evidence that G-dase E can be programmed to induce selective cell ablation 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 an innovative and novel therapeutic tool.

15 - Polyplex-based CRISPR/Cas9 In Vivo Mutagenesis for Efficient Lung Cancer Modeling in Rodents

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In recent years comprehensive genomic studies of human tumors have revealed thousands of genetic mutations linked with cancer. We have to find out a way to sift through this massive information to assess the role of these cancer-gene candidates. In this scenario, conventional transgenic technologies are extremely laborious with costly and exceedingly long model generation times. Here, we suggest a platform for lung mutagenesis using CRISPR/Cas9 RNPs that enables the introduction of a group of mutations in genes chosen for their potential to cause tumors into lung tissue. We use synthetic biomaterials for the delivery of the CRISPR/Cas9 reagents to adult lung epithelial cells.

First, we have evaluated a family of poly(β-aminoesters (PBAE) polymers for their capability to serve as carriers of CRISPR RNPs in the respiratory airways using a TdTomato reporter system and identified the cell types targeted as basal cells from trachea and bronchi; and Clara/club, neuroendocrine and alveolar type 1and 2 cells from lungs. Then, we have validated the effectiveness of this system by targeting a group of tumor suppressor genes, specifically *Rb1*, *Rbl1*, *Pten*, and *Trp53*, chosen for their potential to cause lung tumors, namely Small Cell Lung Carcinoma (SCLC). The disruption of these genes by means of in vivo gene editing leads to the development of SCLC. The SCLC obtained and their metastases show the same histologic, inmunohistochemical and genomic characteristics of the SCLC arisen in GEMMs models and human patients.

In conclusion, our CRISPR/Cas9 RNP delivery system utilizing cationic polymers offers an efficient approach for modeling lung tumorigenesis by simultaneously inactivating a set of tumor suppressor genes, in a manner independent of the mouse genetic background and using readily available reagents that do not require mouse germline manipulation or custom viral vector production.

This innovative strategy holds promise for faster and more cost-effective cancer modeling

14 - Revolutionizing CRISPR/Cas9 Gene Therapy with Targeted Chemical Modulation

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The global CRISPR market is projected to achieve a valuation of USD 7.1 billion by 2032, exhibiting an estimated Compound Annual Growth Rate (CAGR) of around 15.6% starting from 2023. Investments in this sector are propelled by the potential of gene editing to pioneer innovative therapies for genetic disorders, personalized medicine, and applications in agriculture and industrial biotechnology. Leading this domain are pharmaceutical giants, such as CRISPR Therapeutics, Vertex Pharmaceuticals, and Editas Medicine, actively advancing diverse gene editing products. Despite its transformative potential, the CRISPR/Cas9 technology encounters significant challenges, encompassing concerns about editing efficacy, accuracy, precision, and safety in genome editing. These challenges originate from the Cas nuclease's activity during in vivo or ex vivo editing, leading to off-target effects, unforeseen large deletions, complex rearrangements near the target site, cellular toxicity, and immunogenicity. Tackling these issues is crucial to ensure the secure and responsible evolution of genome editing applications.

The notable diversity and purifying selective pressure on CRISPR-Cas systems, coupled with the co-occurrence of multiple CRISPR/Cas system types in many genomes, imply a dynamic co-evolutionary struggle for survival between prokaryotes and parasitic MGEs. This inspires the exploration of naturally occurring Cas protein modulators.

We instituted a high-content thermofluor assay, enabling the effective identification of chemical binders for the Cas9 protein from diverse chemical libraries. Our exploration yielded several clusters, some displaying high binding affinities at concentrations in the picomolar range. Subsequently, we devised an in vitro cleavage assay, revealing a remarkable surge in on-target editing efficiency, exceeding 50-fold enhancements across various target sequences. Simultaneously, there was a substantial reduction in Cas9 off-target editing by more than 20-fold. In HEK293 cells, treatment resulted in over a 2-fold increase in CRISPR/Cas9 knockout efficiency. In Jurkat cells, the induced CRISPR/Cas9 GFP knockin efficiency reached 26%, nearly 4 times higher than the control condition.

Collectively, a market void exists for chemical modulators capable of enhancing the precision and efficiency of CRISPR editing. Our innovative small molecules showcase the ability to modulate the activities of Cas variants, encompassing Cas9, nCas9, dCas9, and Cas12, providing an opportunity to fill this void. Through the utilization of our compounds, CRISPR editing can attain greater precision, efficiency, and specificity, accompanied by reduced off-target effects. These compounds are designed for seamless integration into existing manufacturing processes and harbor the potential for application in the development of consumer products. This includes direct sales of modulator-Cas9 complexes to research laboratories, partnerships with commercial distributors, or licensing agreements with pharmaceutical companies. We are seeking funding to support our research and development endeavors, paving the way for these groundbreaking advancements to lead in the clinic and shape the future of medicine.

13 - CD4 T cell targeting lipid nanoparticles in the search for a genetic HIV cure.

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Until today, there is no definite cure for human immunodeficiency virus (HIV), the retrovirus that causes acquired immunodeficiency syndrome (AIDS) by replicating in CD4+ immune cells. Current therapies inhibit virus replication, but cannot eliminate the viral reservoir, i.e. viral DNA copies integrated in the genome of long-living CD4+ cells. To address this problem, our genetic cure strategy aims to eliminate integrated HIV DNA copies using CRISPR Cas9 gene editing technology, delivered to HIV target cells (i.e., CD4+ cells) by modified lipid nanoparticles (LNPs).

Overall, previous studies provide a strong proof-of-concept that permanent viral elimination is possible by gene editing. Still, achieving efficient delivery to HIV-infected cells is the main hurdle that impedes the development of an effective therapy. AAV-mediated delivery is most reported, but it is a non-targeted method and large scale AAV production is costly and requires large biomass. In addition, AAVs have limited cargo capacity and come with risks of liver toxicity and immunogenicity, impeding redosing. LNPs are interesting alternative delivery vehicles, as their production is inexpensive and easy to upscale. This was recently demonstrated by the global rollout of Moderna and Pfizer/BioNTech COVID-19 vaccines. In addition, by 'clicking' LNPs to an anti-CD4 nanobody (Nb), targeted delivery to HIV target cells only is achieved. This involves bio-orthogonal click chemistry between a tetrazine (Tz)-modified nanobody and a trans cyclooctyne (TCO)-functionalized LNP.

First, we showed that anti-CD4 nanobody clone 3F11 specifically and selectively binds different T cell lines (i.e. SupT1, Jurkat) as well as primary human CD4+ cells at nM concentrations. Moreover, the specificity and selectivity of the nanobody were retained after modifying it with a tetrazine functional group by Sortase A mediated transpeptidation. In parallel, we screened a LNP library to identify the optimal formulation, as it is known that the composition of LNPs impacts their tropism. Here, we identified a LNP formulation with optimal particle size, charge, and mRNA encapsulation efficiency, that enabled LNP binding to and transfection of up to 100% of Jurkat T lymphocytes. Moreover, the latter formulation successfully transfected up to 25% of primary human CD4 T cells, that are known to be notoriously hard to transfect.

Next, we combined the optimal LNP composition with Nb functionalization by incubating CD4+ SupT1 cells with Tz-nanobody, followed by transfection with TCO-modified LNPs. A twofold higher transfection efficiency was seen for CD4+ cells that were pre-targeted with Tz-Nb, compared to cells that were not incubated with Tz-Nb.

To conclude, this data demonstrates that click chemistry can be applied to target LNPs towards CD4+ T cells. Our ongoing research focusses on loading the CD4-targeted LNPs with CRISPR Cas9 components, in this way contributing to an efficient delivery platform for the HIV cure of the future.

12 - A CRISPR-based approach using deadCas9-sgRNA to detect SARS-CoV-2

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Rapid, highly specific, and robust diagnostic kits to detect viruses and pathogens are needed to control disease spread and transmission globally. Of the many different methods proposed to diagnose COVID-19 infection, CRISPR-based detection of nucleic acids tests are among the most prominent. Here, we describe a new way of using CRISPR/Cas systems as a rapid and highly specific tool to detect the SARS-CoV-2 virus using the *in vitro* dCas9-sgRNA-based technique. As a proof of concept, we used a synthetic DNA of the M gene, one of the SARS-CoV-2 virus genes, and demonstrated that we can specifically inactivate unique restriction enzyme sites on this gene using CRISPR/Cas multiplexing of dCas9-sgRNA-*BbsI* and dCas9-sgRNA-*XbaI*. These complexes recognize and bind to the target sequence spanning the *BbsI* and *XbaI* restriction enzyme sites, respectively, and protect the M gene from digestion by *BbsI* and/or *XbaI*. We further demonstrated that this approach can be used to detect the M gene when expressed in human cells and from individuals infected with SARS-CoV-2. We refer to this approach as dead Cas9 Protects Restriction Enzyme Sites, and believe that it has the potential to be applied as a diagnostic tool for many DNA/RNA pathogens.

10 - Detection of sgRNA via SHERLOCK for CRISPR-related gene doping control purposes

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Contributing to the protection of athletes' health is a key objective in the field of anti-doping research. Communicating the risks of doping and developing new analytical detection methods is not only important to maintain the integrity in competitive sport, but also to protect athletes from various health risks via the resulting deterrent effect that can prevent potential doping attempts. Especially the invention and highly dynamic progress of CRISPR-based gene editing techniques is a development, that needs to be monitored and addressed in this context as it provides a potential platform that facilitates the abuse of CRISPR in order to achieve performance enhancements among athletes, defined as *gene doping*. However, the expanding CRISPR toolbox has not only created apprehensions about potential gene doping attempts, but also provides a new opportunity for its detection via biosensing of nucleic acids. Hence, pursuing on a previous study, an analytical method was optimized for the direct detection of sgRNA associated with *Streptococcus pyogenes* in serum via successive reverse transcriptase-recombinase polymerase amplification (RT-RPA) and qualitative nucleic acid detection by means of Specific High Sensitive Enzymatic Reporter UnLOCKing (SHERLOCK). The performed method characterization confirmed the methods' specificity and increased the detection sensitivity to a concentration of 1 fM sgRNA in 100 µL serum. Furthermore, the successful detection of sgRNA in all anticipated post-administration samples from an *in vivo*

mouse model indicated a detection window exceeding 24 h, which supports the applicability of the proposed doping control strategy. Therefore, this study contributes to advancing the protection of human health by establishing preventive measures against the misuse of CRISPR-based gene therapeutic techniques in the context of gene doping via the detection of CRISPR utilizing CRISPR.

9 - In vivo correction of human phenylketonuria variants via prime editing and base editing: A gateway to equitable treatment of inborn errors of metabolism.

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Phenylketonuria (PKU), an autosomal recessive disorder caused by pathogenic variants in the phenylalanine hydroxylase (*PAH*) gene, results in the buildup of blood phenylalanine (Phe) to neurotoxic levels. Current dietary and medical treatments are chronic and reduce, rather than normalize, blood Phe levels. The most frequently occurring *PAH* variants in PKU patients are c.1222C>T (R408W), c.1066-11G>A, c.782G>A (R261Q), c.728G>A (R243Q), c.1315+1G>A, and c.842C>T (P281L), varying widely across populations. R408W is the most prevalent variant in the U.S. and parts of Europe. Using real-world data from a PKU cohort managed at a Metabolic Specialty Clinic, we found that most patients with at least one R408W allele (n = 36) experience chronic, severe Phe elevations and do not meet Phe monitoring guidelines—highlighting the high unmet medical need arising from the challenges of lifelong adherence to dietary and medical therapy.

Motivated by these findings, we are developing “one-and-done” CRISPR editing therapeutics for PKU. We have established a pipeline to rapidly screen and optimize prime editing or base editing strategies in vitro and deploy them in vivo with either lipid nanoparticles (LNPs) or adeno-associated viral (AAV) vectors on a timescale of months. In initial work, we created six humanized PKU mouse models with the aforementioned *PAH* variants and, in parallel, prime-edited variant-bearing hepatocyte cell lines. In homozygous or compound heterozygous PKU mice, we observe complete and long-term durable normalization of blood Phe levels (>90% reduction) as soon as 48 hours after treatment, with whole-liver corrective *PAH* editing as high as >50% with either prime editing or base editing. We additionally subjected our lead gRNAs to comprehensive off-target screening analyses and verified several genomic sites with bona fide off-target mutagenesis in hepatocytes. To reduce the off-target editing at these sites, we explored the use of hybrid gRNAs in which certain positions of the spacer sequence are substituted with DNA nucleotides—a strategy compatible with the use of mRNA-LNPs for in vivo delivery. Comprehensive and variant-aware specificity profiling of hybrid gRNAs revealed dramatically reduced off-target editing and reduced bystander editing in vitro and in vivo. We have developed therapeutic leads for R408W and P281L to permanently normalize blood Phe levels and definitively treat PKU in these patients—on track for early-phase clinical trials within a few years—and have promising candidates for the other *PAH* variants.

Despite these encouraging results, we are cognizant of the risk of “mutational discrimination” in unduly focusing on high-prevalence variants skewed towards specific ancestry groups. Accordingly, we have extended our pipeline to develop and validate a corrective therapeutic for

any variant in *any* patient with a hepatic inborn error of metabolism. We have begun to apply this workflow in real time to patients with devastating, ultra-rare inborn errors of metabolism such as urea cycle disorders and organic acidemias, tackling any variant—even n-of-1 variants—identified via universal newborn screening. Our goal is to be able to rapidly devise and deploy a personalized editing treatment for any patient in need.

8 - The Indel Code and its relevance to clinical development of CRISPR-directed gene editing in cancer

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The fundamental activity of many CRISPR/Cas systems is to cleave double-stranded DNA with precision and efficiency. While this seemingly simple reaction should be enough to disable transcriptionally active genes with similar effectiveness, it is not. Instead, the composition of the genetic footprint left behind actually determines whether the gene has been functionally disabled. We are using CRISPR to genetically knock-out NRF2, a master regulator of stress response, known to promote tumor cell survival in numerous cancers including lung, esophageal and head and neck. NRF2 protects the tumor cell against external stress, including cancer therapy, by disassociating from its partner, KEAP1, and activating a cascade of pro-carcinogenic reactions such as angiogenesis, inflammation, invasion, and metastasis. More importantly, it concurrently empowers drug resistance. As we advance our clinical protocol through the US FDA approval process with a goal of maximal *effective* disruption of NRF2, we have been successful restoring chemosensitivity in a variety of cancer types. We have now extended this platform approach by demonstrating the restoration of sensitivity to targeted therapies including BRAF inhibitors in tumor cells. During this experimental workflow, we have established that maximal end point editing is not a predictive index of clinical potential. Effectiveness depends on the heterozygous state of sequence alterations created by the natural enzymatic action of CRISPR/Cas. Within the diversity of genetic outcomes of human gene editing lies an *Indel Code,* composed of a hierarchy of indel pairs with varying impact governing the execution of functional changes in targeted human cells. We will discuss our understanding of this code and demonstrate the impact it has on the clinical application of CRISPR-directed gene editing. We will also discuss how the Indel Code activates or deactivates the genetic rearrangement known as exon skipping, an under-represented off target event induced by the action of CRISPR/Cas. The heterozygous indel code is a predictive index of the effectiveness of functional genetic knockout driven by CRISPR/Cas and should be considered in the design and execution of clinical protocols.