ABSTRACTS

CRISPR as a research tool in cancer and regenerative medicine

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Oral presentations

Expanding the CRISPR screening toolbox with Cas12a, base editors, and more

John Doench, Genetic Perturbation Platform, Broad Institute of MIT and Harvard, 75 Ames St, Cambridge, MA, USA

We will cover CRISPR technologies that allow genome exploration at both broad scale and fine resolution. For the former, Cas12a allows for facile multiplexing of guide RNAs, simplifying combinatorial perturbations; we will share work mapping synthetic lethal interactions with knockout screens and progress in developing Cas12a for CRISPR activation screens. Additionally, base editor technology enables nucleotide-level manipulation, and we will present screens mapping cancer-relevant genes and pathways.

Linking transcriptome to genotype in single cells identifies mutation-induced transcriptional heterogeneity of IFNγ response

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Abstract text: Pooled genome engineering methods combined with single-cell transcriptomic readouts have enabled a range of experimental designs that can shed light on transcriptional diversity caused by different genetic perturbations.

However, a current limitation of state-of-the-art gene editing approaches is accurately predicting editing outcomes. For example, it is important to accurately verify gene KO to infer gene function, or verify the successful installation of SNPs to infer variant function. Furthermore, unintended edits may occur and contribute to changes in gene expression.

To address these limitations, we developed scSNP-seq; an approach that accurately links genotype and transcriptome information using a barcoding strategy coupling targeted DNA sequencing and RNA sequencing modalities in single cells.

We apply scSNP-seq to study IFNγ response across different genotypes in colon cancer cells using effective base-editors targeting *JAK1*, and demonstrate the accuracy of our experimental approach and bespoke suite of computational tools by linking genotype with transcriptional phenotype in 9,908 cells for scDNA-seq and 18,978 cells for scRNA-seq, with 233 barcodes encompassing 97 unique genotypes with low error-rates for known genotype-phenotype relationships. We identify transcriptional heterogeneity of IFNγ response across groups of *JAK1* missense mutations, which underlines the potential of scSNP-seq for systematically classifying variants of uncertain significance. Our approach has potential for improving genomics-based patient stratification for cancer immunotherapies and for patients with immunological disorders.

Noninvasive assessment of gut function with transcriptional recording sentinel cells

Randall J. Platt, Department of Biosystems Science and Engineering, ETH Zurich, Basel, Switzerland

Transcriptional recording by CRISPR spacer acquisition from RNA endows engineered *E. coli* with synthetic memory, which through Record-seq reveals transcriptome-scale records (Schmidt et al, 2018, *Nature*). Using microbial sentinels that record the bacterial transcriptome as they traverse the gastrointestinal tract, we show how the engineered bacteria recovered from the feces report on a wide range of interactions with the host along the length of the intestine, including quantitative shifts in the molecular and chemical environment resulting from alterations in the host diet, induced inflammation, and microbiome complexity (Schmidt et al, 2022, *Science*). We also improve the efficiency of Record-seq by several fold and demonstrate multiplexed recording, facilitating reconstruction of transcriptional histories of different strains within a single taxon in vivo. Intestinal Record-seq therefore provides a scalable noninvasive platform for interrogating microbial mutualism throughout the length of the intestine without manipulations to host physiology and can determine how single microbial genetic differences alter the way in which the microbe adapts to the host intestinal environment.

Target Discovery - Arrayed & Pooled CRISPR Screening to Drive Drug Discovery

Douglas Ross-Thriepland, AZ and CRUK Functional Genomics Centre, Cambridge, UK.

The SciLifeLab CRISPR Functional Genomics unit.

Bernhard Schmierer, SciLifeLab and Karolinska Institutet, Department of Medical Biochemistry and Biophysics

The Cell and Gene Therapy Core at Lund Stem Cell Centre

Pia Johansson, Cell and Gene Therapy core, Lund University.

Fine-tuning of transcription factor expression dosage uncovers non-linear effects in regulatory networks

Tuuli Lappalainen. Department of Gene Technology, KTH Royal Institute of Technology and New York Genome Center.

Understanding the molecular and cellular consequences of genetic variation in humans requires integration of approaches from population genetics and experimental perturbations, including the CRISPR toolkit. Here, I will discuss recent work on linking gradual changes on transcription factor dosage to single cell transcriptome phenotypes.

Improved methods for CRISPR HDR research.

Jennifer Stott, Integrated DNA Technologies.

Description: CRISPR-based homology-directed repair (HDR) is an invaluable tool to facilitate the introduction of specific mutations into a genomic region of interest. Achieving these precise changes remains a challenge, particularly for large knock-ins. This presentation will provide recommendations for HDR experimental design as well as the use of chemically modified repair templates and small molecule or peptide enhancers for optimizing knock-in efficiency.

Arrayed CRISPR Screening and Custom Engineered Cells for Cancer Drug Discovery

Zofia Świątczak, Synthego.

Synthego has developed automated platforms for synthesizing CRISPR sgRNAs, at both RUO and GMP scales, and for engineering cell lines at scale using the CRISPR-Cas technology. In our gene knockout approach, guide RNAs are multiplexed to produce a high likelihood of gene disruption. Here we show how libraries of gene knockout guide RNAs can be used in an arrayed CRISPR screening approach to identify genes associated with cell survival and death, which could be candidates for molecularly targeted drugs. In addition, we show that cells pre-engineered with gene knockouts are valuable for validation studies when identifying genes associated with cancer drug resistance.

Better genome editing by listening to the cells.

Jacob Corn, Department of Biology, ETH Zurich, Zurich, Switzerland.

Light induced expression of gRNA allows for optogenetic gene editing of T lymphocytes in vivo

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Abstract text: Spatial transcriptomics is revolutionizing our knowledge of cellular states in complex tissues. However, to fully understand the role of individual genes we need methods for precise spatiotemporal gene perturbations. Recently, optogenetic CRISPR has emerged as a promising method for this, but due to its current shortcomings it has not been broadly implemented. To solve this, we have developed a conceptually new optogenetic platform (BLU-VIPR) allowing for efficient optogenetic CRISPR in vivo based on blue-light induced expression of gRNA. The BLU-VIPR optogenetic platform is based on combining a new potent light-responsive transcription factor (VPR-EL222) with ribozyme-flanked gRNAs. The transcription factor VPR- EL222 ensures robust transcription induced by blue light, while the ribozyme-flanked gRNA design ensures precise excision of multiple gRNAs from the resulting mRNA transcript. We demonstrate that this design allows for multiplexed and orthogonal optogenetic gene editing, and simultaneous light-induced expression of gRNAs and proteins (e.g. fluorescent reporters), making the system very versatile. Since the light-induced gRNAs can be combined with different Cas proteins off-the shelf, the BLU-VIPR platform allows for optogenetic control of different CRISPR functionalities, including: Cas9 mediated knockouts, CRISPR activation (CRISPRa) and base editing. Furthermore, since the BLU-VIPR platform is compact and genetically encoded, it can be delivered into primary cells using viral vectors. Indeed, we have for the first time achieved optogenetic gene editing of T lymphocytes in vivo, thus paving the way for spatiotemporal dissection of immune responses in vivo with high precision.

CRISPRa as a tool to characterize mechanisms of resistance associated with p53 pathway reactivation in diffuse midline glioma

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Abstract text: Diffuse midline gliomas (DMG) are highly aggressive and universally fatal brain tumors occurring in children, and are inoperable given their location within the brainstem. Over 50% of these gliomas retain wild-type p53, resulting in potential susceptibility to molecules that disrupt the interaction between p53 and its negative regulators such as MDM2 and PPM1D. MDM2 inhibitors are currently being evaluated in clinical trials for the treatment of adult brain tumors, but the mechanisms behind patient relapse have not yet been characterized. Here, we have leveraged genomic and transcriptional approaches to map the mechanisms of resistance to MDM2 and PPM1D inhibition in DMG. First, we performed genomic and transcriptomic analyses in patient-derived cell lines to better characterize sensitive tumors and identify putative biomarkers of drug response. Dose response curves and growth assays showed that tumors with inactivating p53 mutations are highly resistant to treatment, but those that retain wild-type p53 exhibit various degrees of drug sensitivity. This suggests that other factors, in addition to p53 mutational status, mediate response to MDM2 and PPM1D inhibition in these brain tumors. By conducting a genome-wide CRISPR activation screen in a patient-derived DMG cell line model, we have identified putative cell death, cell differentiation state and metabolic programs that modulate drug response in the absence of p53 inactivating mutations. Ongoing efforts are focused on validating putative drivers of resistance to MDM2 and PPM1D inhibition in additional cell line and animal models, as well as identifying combination therapies for the upfront treatment of these tumors.

New Frontiers in Pooled Screens

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I will present recent work from our lab on two topics: engineering more persistent T cell therapies using genome-scale overexpression screens and high-throughput approaches to identify causal noncoding variants and their target genes from biobank-scale human genetic data.

The engineering of autologous patient T cells for adoptive cell therapies has revolutionized the treatment of several types of cancer. However, further improvements are needed to increase response and cure rates. Here we identify positive regulators of T cell functions through overexpression of around 12,000 barcoded human open reading frames (ORFs). The top-ranked genes increased the proliferation and activation of primary human CD4+ and CD8+ T cells and their secretion of key cytokines such as interleukin-2 and interferon-γ. In addition, we developed a single-cell genomics method (OverCITE-seq) for high-throughput quantification of the transcriptome and surface antigens in ORF-engineered T cells. Our results provide several strategies for improving next-generation T cell therapies by the induction of synthetic cell programs.

Most variants associated with complex traits and diseases identified by genome-wide association studies (GWAS) map to noncoding regions of the genome with unknown effects. Using ancestrally diverse biobank-scale GWAS data, massively parallel CRISPR screens, and single cell transcriptomic and proteomic sequencing, we discovered 124 cis-target genes of 91 noncoding blood trait GWAS loci. Using precise variant insertion via base editing, we connected specific variants with gene expression changes. We also identified *trans*-effect networks of noncoding loci when *cis* target genes encoded transcription factors or microRNAs. Networks were themselves enriched for GWAS variants and demonstrated polygenic contributions to complex traits. This platform enables massively-parallel characterization of the target genes and mechanisms of human noncoding variants in both *cis* and *trans*.

Uncovering novel dependencies in the DNA repair space with CRISPR/Cas12a screening

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Abstract text: Defective double strand break (DSB) repair is a hallmark of several oncology indications, including breast, ovarian, prostate, and pancreatic cancers. Targeted therapies aim to exploit these repair defects, as in the use of PARP inhibitors to treat BRCA-deficient cancers. PARPi are also used to treat an expanded panel of homologous recombination-deficient (HRD) tumors, but with variable success. Advances in functional genomics have provided a wealth of data about genetic dependencies in hundreds of cell lines. The DSB repair gene mutations that are commonly found in patient tumor samples are underrepresented in cell lines, however, impeding the search for synthetic lethality with these biomarkers. The CRISPR/Cas12a system allows simultaneous knockout of multiple genes in the same cell, enabling pairwise screens of biomarkers and putative targets. Here we use the CRISPR/Cas12a system to screen DSB repair loss for novel dependencies on ATPases. We find dozens of novel genetic interactions between ATPases and DSB repair factors. Two of these interactions were selected for orthogonal validation. These unique, validated targets, one in the context of ATM deficiency and the other interacting with the RAD51 paralogs – RAD51B and RAD51D – demonstrate the power of pairwise CRISPR screens.

Genome-wide CRISPR Screens in Primary Human T Cells Reveal Key Regulators of Immune Function

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Human T cells are central effectors of immunity and cancer immunotherapy. CRISPR-based functional studies in T cells could prioritize novel targets for drug development and improve the design of genetically reprogrammed cell-based therapies. However, large-scale CRISPR screens have been challenging in primary human cells. We developed a new method, single guide RNA (sgRNA) lentiviral infection with Cas9 protein electroporation (SLICE), to identify regulators of stimulation responses in primary human T cells. Genome-wide loss-of-function screens identified essential T cell receptor signaling components and genes that negatively tune proliferation following stimulation. Targeted ablation of individual candidate genes characterized hits and identified perturbations that enhanced cancer cell killing. SLICE coupled with single-cell RNA sequencing (RNA-seq) revealed signature stimulation-response gene programs altered by key genetic perturbations. SLICE genome-wide screening was also adaptable to identify mediators of immunosuppression, revealing genes controlling responses to adenosine signaling. The SLICE platform enables unbiased discovery and characterization of functional gene targets in primary cells.

Uncovering key resistance mechanisms to cancer immunotherapy

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Triple-negative breast cancer (TNBC) is an aggressive disease and presents a substantial unmet medical need. In recent years, immune checkpoint blockade (ICB) therapy against the PD-1/L1 axis shows promise in several cancer types including TNBC. However, challenges remain as cancer cells can evade immune-mediated attacks through multiple intrinsic mechanisms. Here, we performed genome-wide CRISPR screens in a unique human Tumor-Immune co-Culture System (TICS), in order to reveal intrinsic pathways that improve killing of TNBC cells in response to ICB therapy. We identified gRNAs targeting the protein neddylation pathway among the most depleted in TNBC cells in response to an approved ICB drug. Genetic deletion of genes regulating protein neddylation by CRISPR/Cas9 in human TNBC cell lines significantly altered global protein expression, including proteins related to antigen presentation. When co-cultured with primary human lymphocytes in TICS, we observed that CRISPR KO human TNBC cell lines significantly enhanced immune activation in response to clinically approved blocking antibodies against the PD-1/L1 axis. In immunocompetent mice bearing established EO771 wild type breast cancer tumors, PD-1 blockade was unable to delay tumor growth. Moreover, disruption of protein neddylation using CRISPR/Cas9 in EO771 cells did not result in growth delay *in vivo*. Strikingly, PD-1 blockade elicited curative effects in CRISPR KO EO771 tumors that required the presence of CD8+ T cells. Altogether, our work provides new evidence that the protein neddylation pathway in TNBC cells is a key cancer vulnerability to ICB therapy.

Deletion of the TMEM30A gene allows leukemic cell evasion of NK cell cytotoxicity

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Abstract text: Adoptive natural killer (NK) cell therapy is a promising therapeutic strategy in various malignancies, and thus more studies of structures that regulate NK cell recognition and killing of cancer cells are warranted. In this study, we used a genome-wide CRISPR screen to identify genes that provide protection or susceptibility to NK cell cytotoxicity. Several genes with documented roles in NK cell regulation showed up as top hits in the screen, such as genes encoding proteins involved in interferon-γ signaling and antigen presentation, the NKp30 ligand, B7-H6, but also CD58, the ligand for the co-stimulatory receptor CD2. Knock-out (KO) cell lines with depleted B7-H6 and/or CD58 displayed reduced NK cell responses with regards to degranulation, cytotoxicity and cytokine production. Additionally, the integrity of the gene *TMEM30A*, encoding CDC50A - the beta-subunit of the flippase shuttling phospholipids to the inner leaflet of the plasma membrane, was shown to be important for NK cell killing. Accordingly, *TMEM30A* KO leukemia and lymphoma cells displayed increased surface levels of phosphatidylserine (PtdSer), triggered less NK cell degranulation, cytokine production and displayed lower susceptibility to NK cell cytotoxicity. Blockade of PtdSer by Annexin-V or blocking its inhibitory receptors TIM-3 or IRp60/CD300a on NK cells restored killing of *TMEM30A*-deficient cells. Notably, *TMEM30A* is commonly mutated in diffuse large B cell lymphoma and this may serve as an escape mechanism for NK cell immunosurveillance. Our study highlights the potential role for agents targeting the interaction between PtdSer and its receptors in cancer immunotherapy.

Identification of novel factors controlling non genetic cell plasticity in Chronic Myeloid Leukemia

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Abstract text: In cancer cell communities gene expression noise can lead some cells to transiently acquire drug resistant phenotypes. In example, the K562 chronic myeloid leukaemia (CML) cells were shown to constantly transition between a CD24- and a drug-resistant CD24+ phenotype. We leveraged the CD24 to track these phenotypic switches in K562 cells to identify the genes regulating state transition by CRISPR screening.

Firstly, to characterize the CD24+ phenotype we reanalysed public scRNA-Seq data of K562 cells. We found that the CD24 mRNA levels inversely correlated with those of several genes involved in oxidative metabolism. By extracellular flux analysis we confirmed a reduced metabolic activity in the CD24+ cells. To identify genes regulating the phenotypic switch, we developed a genome-wide CRISPR-KO screening line in K562 cells and studied the distribution of KO events in the cells changing their phenotype. We leveraged two different approaches to isolate the switching cells. Firstly, we isolated CD24+ and - cells and cultured them for 24h. Afterwards, we sorted again the two lines based on the CD24 expression levels isolating those cells changing their phenotype over the time in culture. The second approach was instead based on the temporal delay between mRNA and protein levels variations upon both gene overexpression and repression. Briefly, we measured CD24 mRNA and protein levels in the same cells by combining mRNA fluorescence *in situ* hybridization (FISH) and immune staining. Those cells showing an inconsistent level of mRNA and protein were identified as transitioning cells. In the two screenings we identified 49 genes regulating state transition that were functionally involved in the regulation of cell energetic homeostasis and cell proliferation.

In conclusion, we identified a set of genes regulating cell plasticity in CML models. Our results suggest a link between cell plasticity and the regulation of cell energetic metabolism.

Posters

1. **Enabling high-content phenotyping in pooled CRISPR screens by in situ guide RNA readout**

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Abstract text: Classical pooled CRISPR screens are limited to cell phenotypes that can be physically separated from each other, such as live-dead screens, or sortable cell phenotypes. We are developing a method using *in situ* guide RNA sequencing directly on a microscope slide, allowing complex phenotyping of cells followed by determination of the gene perturbation they harbour. Briefly, cells containing a pooled CRISPR library (one guide copy per cell) are grown on a slide, imaged, phenotyped, and their position on the slide is recorded. In a second step, the identity of the single CRISPR guide RNA present in each individual cell is determined directly on the microscope slide by barcoded padlock ligation and cycles of hybridization, imaging and stripping. Thus, both phenotype and genetic perturbation of the individual cells in a population can be assessed microscopically. The method combines the advantages of pooled CRISPR screening (**scalability and low cost**) with those of arrayed screens (**complex phenotypic readouts**), and allows CRISPR screens in 3D (**organoids or tissue sections**).

2. A bioreducible polymer nanoparticle delivery system for CRISPR-Cas genome editing

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Abstract text: INTRODUCTION: Widespread adoption of CRISPR-Cas methods has sparked a revolution in genome engineering and precision medicine. Scientists can now precisely insert, delete, rewrite, and regulate specific gene sequences. The major barrier to further progress is the inability to efficiently deliver Cas proteins and single guide RNAs (sgRNAs) into cells. To meet this need, we have developed a bioreducible polymer nanoparticle delivery system that outperforms existing gold-standard reagents for delivery of CRISPR-Cas ribonucleoproteins (RNPs).

METHODS: We determined the optimal nanoparticle formulation conditions using gel retardation assays, dynamic light scattering (DLS), and cryo-TEM. We then used the optimized system to generate two human reporter cell lines (SK-N-BE(2) neuroblastoma and hTERT-RPE1 retinal pigment epithelium) bearing a green fluorescent protein (GFP) fused to the ribosomal protein RPL24. These cells enabled quantitative comparison of gene editing efficiencies with the current gold-standard lipoplex reagent CRISPRMAX. We also transduced both cell types with a lentiviral reporter system to characterize prime editing, wherein a three amino acid segment of GFP is mutated to disrupt its fluorescence. Repair of the mutated bases by RNP-mediated prime editing restores the fluorescence signal. We also characterized the intracellular trafficking behaviour of the delivered RNPs within the endolysosomal pathway using super-resolution STORM microscopy.

RESULTS: We consistently generated nanoparticles with diameters of 95 ± 11 nm that fully complex both types of RNPs and release them efficiently under physiological reducing conditions mimicking the intracellular environment. With the bioreducible polymer nanoparticles, we achieved NHEJ gene editing efficiencies as high as 95%, up to 40% better than existing lipid-based delivery methods. HDR and prime editing efficiencies also equalled or surpassed that achieved with existing reagents. STORM analysis revealed high concentrations of RNPs within hypertrophic perinuclear lysosomes, which was not observed with CRISPRMAX.

3. A genome-scale CROP-seq screen reveals mediators of T cell signaling

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Abstract text:
CROP-seq/ Perturb-seq screens combine CRISPR perturbation with single-cell RNA sequencing. Briefly, cells are transduced with a pooled sgRNA library and transcriptomic profiles of each cell are recorded using conventional single-cell RNA sequencing platforms. Currently, these screens are performed at small scale (~1000 genes) because of high cost among other factors. Here, we performed a genome-scale CRISPR screen in Jurkat cells within an econimcal budget. We focused on T cell activation pathway to leverage published knowledge and benchmark the performance of our platform.

We utilized a CRISPR interference setup that delivers multiple sgRNAs for more perturbations per individual cell. We targeted 18,595 human genes with four sgRNAs in dCas9-KRAB-expressing Jurkat cells combined by stimulation with anti-TCR and anti-CD28 antibodies to activate T cell signaling. We processed 1,000,000 cells in one go, using the 10X Genomics Chromium X platform. Following single-cell library preparation, we amplified a selected set of 374 transcripts and could sequence the whole screen on one single NovaSeq S4 flowcell.

We confirmed that the perturbation of 374 marker genes led to the downregulation of the cognate targets, suggesting that the CRISPR perturbation workflow is functional. We assessed whether T cell activation could be recapitulated from the chosen markers and found that activated cells can be distinguished from their unactivated counterparts using the signature in question. Finally, we assessed the phenotypes of gene knockdowns introduced at genome-scale. Of the 18.595 genes perturbed, a set of 70 genes affected T cell activation, partitioning to 55 activators (diminished signaling upon knockdown) and 15 inhibitors (enhanced signaling upon knockdown). Of note, our screen recovered known key signaling nodes proximal to the TCR including LAT, LCK, ZAP70, CD3E, ITK, RASGRP1 and VAV1.

Overall, the screen presented here will catalyse a paradigm shift for genome-scale CRISPR screens combined with a scRNA-Seq workflow.

4. AddCell: A Knowledge Base of CRISPR-Edited Cell Lines

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Abstract text: Model cell lines are the heart of molecular biology research in life science. Since its invention in 2012, CRISPR-Cas9 technology has transformed the face of life science by enabling the generation of thousands of cell lines originating from a wide range of tissues and a myriad of transgenic animals recapitulating disease phenotypes. Huge excitement around the revolutionary aspects of CRISPR-based studies and increasing support from funding agencies have translated into the number of edited cell lines vastly growing by every year.
Nevertheless, establishing proper cell lines that would satisfy the scientific goal of research projects consumes time and resources, imposing considerable restraints on the budgets and timelines available to academic teams. The absence of an effective platform for sharing cell lines has led to duplication of efforts, wasted time and money in generating similar cell lines, and introduced variability in the process of generating cell lines. This variability can threaten research reproducibility and lead to inconsistent results from experiments with the same settings.
Sharing edited cell lines between academic groups requires a comprehensive knowledge base to monitor new research publications and collect information on parental cell lines and edited genes. Recent advancements in natural language processing (NLP) and large language models (LLMs) have enabled retrieving relevant information from research articles. In this regard, we introduce AddCell (https://addcell.org/), a knowledge base that provides a comprehensive collection of automatically collected and manually curated information on genetically engineered cells from open-source scholarly articles. Our database presents the collected information in a user-friendly and searchable format. AddCell empowers life-science researchers by providing access to information on CRISPR-edited cells from other academic institutes. This platform promotes sharing between research groups, resulting in savings in time and budget while ensuring reproducibility and sustainability in science.

5. Analysis and validation of target genes related to dysfunction in tumor-infiltrating CD8 T cells.

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Abstract text: T-cell exhaustion is characterized by a progressive loss of functions, impairing the ability of T-cells to kill target cells. Exhaustion develops under conditions of inflammatory signals and/or antigen-persistence, which can occur following chronic infections and cancer. There are many genes associated with T-cell dysfunction in cancer, such as the inhibitory receptors PD-1, and Tim-3, which negatively affect cytokine production, killing activity, and the survival of these cells. Genome-wide transcriptional profiling has pinpointed hundreds of genes that have an altered expression in exhausted CD8 T cells. Despite these characterizations, the function of many genes expressed by dysfunctional CD8 T-cells in cancer is unknown.

We designed an *in vivo* CRISPR-Cas9 screen to scan for putative genes regulating CD8 T-cell function in cancer. We used an OT-1 CD8 T-cells adoptive transfer system into B16-OVA tumor-bearing mice, where OT-1 T-cells become dysfunctional, as evidenced by reduced IFN-γ and high PD-1 expression. We designed a gRNA library targeting 260 genes which was transduced into activated transgenic Cas9-GFP-OT1 CD8 T-cells. These cells were adoptively transferred into mice bearing a melanoma B16-OVA tumor. After 7 days, we sorted the cells from the tumor based on three phenotypes: undifferentiated (IFN-γ^-Eomes$^-$), functional (IFN-γ^+), and terminally differentiated dysfunctional (Eomes$^+$IFN-γ^-). Sequencing analysis identified several genes that may be related to controlling the differentiation of CD8 T-cells into functional and dysfunctional subsets, including Socs1, Cblb, and FoxP1. We selected 18 genes for further analysis.

Using CRISPR-Cas9 technology, we aim to validate these genes and investigate their role in promoting or inhibiting dysfunction in tumor-infiltrating CD8 T-cells. Here, we are creating knockouts by sgRNA/Cas9 electroporation into primary, and unstimulated OT-1 CD8 T-cells before adoptive transfer into B16-OVA tumor-bearing mice. With this study, we expect to provide a step to the molecular understanding of T-cell dysfunction and reveal new therapeutic targets for cancer.

6. Cancer therapies based on nucleolar stress: Who should we treat?

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Abstract text: Upregulated ribosome biogenesis and protein synthesis is a frequent observation in cancer and is thought to contribute to tumor progression. In this context, drugs that target nucleoli and rRNA synthesis are being actively explored for the treatment of cancer. Examples of this are the antibiotic Actinomycin D, or BMH-21, a chemical known to trigger the degradation of the large catalytic subunit of RNA Pol I that is currently in clinical trials. Despite the emerging interest in this approach, we currently lack a full understanding of which mutations modulate the sensitivity to these drugs, and thus which patients are more likely to benefit from -or fail to respond to- these therapies. To systematically address this problem, we carried out a genome-wide CRISPR screen using a lentiviral sgRNA library in human A375 melanoma cells. This allowed us to identify several specific genes and pathways which could potentially impact in the efficacy of nucleolar stressors such as ActD and BMH21 in cancer treatment. Surprisingly, some of these mutations provide resistance to one of the therapies but sensitivity to the other one, which we interpret as the drugs having opposite effects in the cell -despite the supposed target being RNA-Pol I in both cases. We are currently validating a number of our hits and trying to obtain preclinical evidence supporting the usefulness of our discoveries for cancer therapy. Our initial results will be discussed.

7. CDCA2 is a determinant of proliferation rate, cell cycle progression and proteasome inhibitor response in multiple myeloma

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Abstract text: Background: Despite therapeutical advances, multiple myeloma (MM) remains an incurable disease with a median overall survival of 5-7 years due to relapsed/refractory disease. While initiation and disease progression in MM are strongly correlated to mutations in cell cycle genes, the mechanisms between these genes and treatment strategies in MM are not well characterized. The purpose of this study was to determine the role of *CDCA2*, a cell cycle gene, in multiple myeloma as it has been identified as a poor prognostic marker in other cancers.

Methods: CRISPR-Cas9-mediated gene knockout of *CDCA2* was employed on two human MM cell lines, MOLP-8 and RMPI-8226. The effect of indel formation on mRNA and protein expression was investigated by droplet digital PCR and immunoblotting. The clones were characterized functionally by proliferation assay and cell cycle analysis by flow cytometry, and response to the first- and second-generation proteasome inhibitors (PI)s, bortezomib and carfilzomib, respectively, assessed by dose-response screens.

Results: In all monoclonal cell populations, knockout of *CDCA2* resulted in a significant decrease in proliferation rate for both MM cell lines. Cell cycle analysis showed a significant increase in percentage of cells in G1 compared to control. For RPMI-8226, knockout of *CDCA2* conferred resistance towards both PIs, in contrast to MOLP-8 clones that showed increased sensitivity in response to PI exposure, suggesting a difference in underlying disease biology between cell lines – and that the shift in sensitivity in *CDCA2* knockout cells is not just an unambiguous effect of decreased proliferation.

Conclusion: The findings of this study underline the role of *CDCA2* as an oncogene in MM, with impact on cell proliferation, cell cycle progression and shift in response for both bortezomib and carfilzomib. Further studies are needed to fully dissect the mechanisms of action of CDCA2 in MM.

8. Characterization of Multiple sclerosis-associated single nucleotide polymorphisms in oligodendrocytes

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Abstract text: Multiple Sclerosis (MS) is a heterogeneous disease with a strong genetic disposition with autoimmune and demyelinating components. In particular, disease progression is considered to be associated with failure in remyelination from central nervous system (CNS)-resident oligodendrocytes (OLs). However, why OLs fail to remyelinate axons to secure neurological function is largely unknown. We have identified MS-associated single nucleotide polymorphism (SNP) candidates transcriptionally accessible in the OL-lineage and will assess how these SNPs influence OL cell interaction, differentiation and myelination. This is carried out applying screening platforms such as massive parallel reporter assay (MPRA) and CRISPR/Cas9-activation/inhibition to iPSC-derived human OL to identify preferential transcription factor binding and epigenetic alternation in the OL-lineage. In parallel we are also applying CRISPR/Cas9 prime editing (PE) in order to evaluate epigenetic, transcriptional and functional outcomes related to various MS-associated SNPs. We believe this novel approach will provide crucial knowledge to why remyelination is haltered in MS.

9. Creation of genetically modified mouse models using CRISPR/Cas at Karolinska Center for Transgene Technologies

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Abstract text: Karolinska Center for Transgene Technologies (KCTT) is Karolinska Institutet's transgenic core facility for the generation, rederivation, and cryopreservation of genetically modified (gm) mice. Use of CRISPR/Cas has over the last years become the predominant method at our facility to produce new gm mice, whether it concerns knockouts, knockins, conditional alleles, or other types of models. Depending on the type of mutation, the CRISPR/Cas components are delivered into the mouse zygotes either through pronuclear microinjection or electroporation. An important step in the production of a CRISPR/Cas mouse strain is the downstream quality control of the new gene edited mouse. Not only is the first-generation offspring typically genetic mosaics but, in addition, both unwanted on-target and off-target genetic alterations is a factor to consider. Together with Karolinska Genome Engineering (KGE), as fee-for-service, we can offer a full package portfolio from the initial design of the gene editing strategy over the in vivo production and quality control of the gm allele to the breeding of candidate mutant founder mice for germline transmission. Once your newly developed gm mouse strain is established, as a safety backup to protect your valuable research investment, we offer cryopreservation and storage of your strain as embryos or sperm.

10. CRISPR screen identifies DNA damage response pathways, histone linkers and BTK as essential for cisplatin sensitivity in diffuse large B-cell lymphoma

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Abstract text: Background: Recurrence of diffuse large B-cell lymphoma after first-line treatment is seen in up to 40% of cases. These relapsed patients receive platinum-containing salvage therapies including the DNA damaging agent, cisplatin. However, half of the patients receiving salvage therapy experience disease progression and reemerging relapse. To identify genes affecting cisplatin response in DLBCL, a CRISPR knockout screen was performed, and candidate genes were filtered for mutations in clinical cohorts prior to functional validation.

Methods: Whole genome CRISPR knockout screens were performed in DLBCL cells using the Brunello library, followed by exposure to two doses of cisplatin or saline controls for 10 days. MAGeCK analysis was conducted for identification of enriched and depleted genes and pathways. Mutation frequencies of the candidate genes were assessed in both a local cohort with 80 tumor-normal samples and in five external cohorts. Single genes were chosen based on positive/negative selection scores or clinical relevance for subsequent functional analysis to investigate cisplatin's mechanisms of action in DLBCL cells.

Results: We identified 425 significantly depleted genes conferring sensitivity to cisplatin, and 326 significantly enriched genes conferring resistance to cisplatin. As expected, multiple depleted genes are involved in DNA damage repair (DDR) in the Nucleotide Excision Repair (NER), Mismatch Repair (MMR), and Fanconi Anemia (FA) pathways. Other interesting pathways were chromatin organization and B-cell receptor activation. In our local cohort, 19% of the depleted genes and 18% of the enriched genes were mutated in the clinical samples.

Conclusion: This study shows that the DDR pathways; NER, MMR, and FA play essential roles in response to cisplatin in DLBCL, and that gene perturbations affecting cisplatin response are also seen mutated in patients. Further *in vitro* validation and survival analysis regarding platinum treatment response and mutation frequencies of candidate genes in DLBCL patients will be conducted.

11. CRISPR-based studies of pathogenic neutrophil biology

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Abstract text: Rheumatoid arthritis (RA) is a chronic autoimmune disease. It is characterized by inflammation of small joints of hand and feet, resulting in progressive destruction of cartilage and bone. Neutrophils often make up more than 90% of cells found in synovial fluid (SF) of patients with active disease. Animal models support a pathogenic role for neutrophils in autoimmune joint inflammation, showing that neutrophil depletion could be a potent intervention. We hypothesize that neutrophils contribute to the pathogenesis of RA by acting as an amplification loop of local inflammation, by releasing potent bioactive molecules and autoantigens. Limiting neutrophil recruitment and activation at the inflammatory site could thus serve as a novel approach.
Firstly, we use in vitro CRISPR screens to identify potential drug targets affecting neutrophil migration/activation and validate these using in vivo animal models. To study migration, transwell assays are used where the CRISPR-modified neutrophils are induced to migrate toward different chemoattractants. Cells are separated based on whether they migrate. To study activation, the cells will be activated by PMA or IgG immune complexes, and cells will be separated by FACS sorting based on the upregulation of activation markers (eg. CD11b), or binding of RA patient-derived ACPA antibodies. The sgRNA sequence of separated population will be sequenced to identify genes regulating the migration and activation. The prioritized genes will be tested with in vivo animal models.
Secondly, we will identify how SF from RA patients induces neutrophil migration, and if the migration correlates to disease activity. We will focus on blocking experiments using antibodies and inhibitors. Initial candidates include IL-8 and C5a. etc. To study clinical correlates, together with clinical collaborators, we will analyze if any clinical parameter correlates with migration induced by SF. Besides DAS28, VAS, and CRP, we will also measure several factors, including general inflammatory factors (eg. IL-6).

12. CRISPR/CAS9 TECHNOLOGY AS A USEFUL TOOL IN THE STUDY OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Abstract text: Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with various somatic mutations, the most frequent of which targeting *ATM*, *TP53*, *NOTCH1*, *MYD88* and *SF3B1* genes. Their thorough exploration could shed light on the disease etiology, or even lead to discovery of potential novel drug targets. However, patient CLL cells do not proliferate *ex vivo*, thus generation of isogenic cell lines is needed for extensive experiments.

Using CRISPR/Cas9 in CLL-derived HG3 and MEC1 cells, we generated isogenic cell lines carrying disruptive mutations in *ATM* or *TP53*. These cell lines show complete loss of the respective proteins and abrogation of downstream signaling pathways.We also used CRISPR/Cas9-based homology directed repair to obtain HG3 cells with recurrent mutations of *NOTCH1* (P2514fs), *SF3B1* (K700E) and *MYD88* (L265P). Selected cell lines were subjected to CRISPR/Cas9 dropout screening to identify genes, whose deletion is lethal to the introduced mutations. In particular, *SPDYE1* and *LUC7L3* were found to be synthetically lethal with the *NOTCH1* mutation, while *SNUPN* and *UQCRC1* were found to be essential for *SF3B1*-mutated cells. Simultaneously, the cell lines were screened with a library of 859 approved drugs. The screening demonstrated sensitivity of *NOTCH1*-mutant and *SF3B1*-mutant cell lines towards inhibitors of various hormone receptors or inhibitors of 20S proteasome. The knockout models were also used for studies of the performance of anti-CD19 CAR T-cells. We observed different effectiveness at eradicating tumor cells *in vivo* depending on the driver mutation, with *TP53* mutations connected to inferior performance of CAR T-cells. In summary, we generated a panel of isogenic cell lines carrying mutations recurring in CLL patients. These models are indispensable for further studies of the mutations' impact on CLL therapy.

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13. Development of a pooled CROP-seq screen to identify transcription factors required for mDA neuron development

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Abstract text: In our study we aim to identify transcription factors that are crucial for the specification of midbrain progenitors and their differentiation into specific midbrain dopaminergic (mDA) neuron subtypes *in vitro*. Our goal is to perform pooled CRISPR-Cas9-based *in vitro* screening to knockout the function of selected genes identified from single cell RNA-seq data of the human ventral midbrain *in vivo.* Resulting perturbations will be assessed by single cell RNA-seq.

Here we present our technical findings for setting up the CRISPR-Cas9 perturbation screening in progenitor cells of the midbrain dopaminergic lineage. After applying stringent quality control criteria, we were able to develop an optimized Cas9-knockin hESC line (HS980-Cas9-KI) that had high levels of Cas9 inducibility at the stem cell stage. We also overcame differentiation-associated *AAVS*1 silencing to retain Cas9 inducibility in mDA neuronal progenitors derived from our HS980-Cas9-KI line.

Using the candidate HS980-Cas9-KI line, we designed a screening pipeline by optimising conditions for sgRNA library delivery and selection, Cas9 induction and target cell enrichment, to achieve a suitable assay endpoint. We obtained good levels of sgRNA/Cas9 double positive cells at the post-CRIPSR-induction stage, and our preliminary data indicate an observable CRISPR loss-of-function survival phenotype at assay endpoint.

We are now in the final stages of carrying out the pooled CRISPR screen with droplet based scRNA-seq readout and hope to report our findings in due course. Those transcription factors that are found to be important for generating mDA neuron subtypes will be validated and used to improve differentiation protocols of human pluripotent stem cells (hPSCs) and develop future cell therapy strategies for Parkinson's disease.

14. Development of an experimental model for CRISPR screening in therapy resistant muscle invasive bladder cancer cells

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Abstract text: Introduction
Bladder cancer (BC) is the 10th most common cancer worldwide. Cisplatin resistance (CR) is a problem in muscle invasive BC (MIBC) due to intratumoural hypoxia. Whole genome CRISPR screening (WGCS) allows discovery of novel genes driving CR. This research characterised a cell model for WGCS experiments studying hypoxia-upregulated genes driving CR in MIBC.

Method
Cell death assay: MIBC cell lines (T24, J82 & UMUC3) baseline cell death was determined using Annexin V/7AAD flow cytometric staining at 24, 48 and 72h (n=3).
Cisplatin dose curves: T24(n=4), J82 and UMUC3 (n=2) cells were cisplatin treated (0.001µM–100µM). Cell viability was determined at 24, 48 and 72h under normoxic (21% O_2) and hypoxic conditions (0.1%, 1% O_2).
Growth curves: T24 wild type (WT) and T24 cells transduced with a lentiviral Cas9 expression vector (T24 Cas9) were incubated under normoxic (21% O_2) and hypoxic conditions (0.1% and 1% O_2) for 5 days (n=3). Cell counts were taken daily and cell viability determined by trypan blue exclusion.

Results
Cancer cells can have high death rates. Establishing accurate seeding densities accounting for this is essential prior to WGCS. Cell viability of T24 (66.2±2.2) cells was lower ($p<0.001$) than in J82 (90±0.3) and UMUC3 (94.9±0.8) cells at 48h timepoint (unpaired t-test). T24 cells showed cisplatin resistance ($p<0.05$) at 1µM cisplatin (peak plasma concentration in patients) at 1% (61.5±8) and 0.1% O_2 (53.7±3.6) compared to normoxia (31.8±5.7). No resistance occurred in J82 or UMUC3 cells. Expression of Cas9 had no effect on T24 cell growth in all conditions and timepoints.

Conclusion
Three widely used MIBC cell lines were screened and a robust workflow was developed to characterise the suitability of these cell models for use in future WGCS experiments to study the genetic drivers of CR in MIBC. T24 cells were the most suitable for our study.

15. Evaluation of the N6-methyladenosine (m6A) RNA modification pathway as a driver of tumor proliferation via high-throughput CRISPR screening

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Abstract text: The methylation of RNA adenosines into N^6-methyladenosine (m^6A) is the most abundant internal mRNA modification in mammalian cells. It is regulated by writers (e.g., methyltransferase-like 3 (METTL3)) and erasers, while readers mediate its molecular effects at the level of different biological processes (e.g., RNA translation and decay). Consequently, m^6A affects multiple cellular functions. Recently, a CRISPR-Cas9 knock-out (KO) screening determined that AML proliferation depends selectively on the enzymatic activity of METTL3. This recent discovery opens new questions regarding the mechanisms by which readers and eraser proteins contribute to the function of m^6A in AML, which may be investigated with CRISPR-base editing.
In this technology, cytidine or adenine deaminases fused to a nicking Cas9-UGI (uracil glycosylase inhibitor) nuclease perform C->T and A->G transitions respectively, allowing programmable nucleotide changes. These enable the study of m^6A at increased resolution and precision, if compared to classic genetic knockouts.

The aim of this project is to study m^6A in AML cell proliferation by means of a pooled CRISPR-base editing screening. We will develop a CRISPR-base editing platform for the high-density mutagenesis of the genes that are active in the m^6A-pathway as well as AML-driver genes. Enrichment analysis of the sgRNAs will determine the association between specific single-nucleotide mutations and the proliferation phenotype.

Our project will 1) study the m^6A pathway without the confounding factors related to the removal of whole genes; 2) highlight domains associated with proliferation phenotype and that might represent druggable sites; 3) assess the function of variants of uncertain significance (VUS). The study will investigate the role of m^6A writers, erasers and readers in AML proliferation and expect to provide molecular insights for the development of novel drug treatments.

16. Genome-wide CRISPR-Cas9 screen for the elucidation of novel mediators of cytotoxicity in natural killer cells

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Abstract text: Clinical applications of NK cells have widened immunotherapeutic approaches in hematological malignancies. Despite the strides made, mechanisms of their cytokine and cytotoxic responses have not yet been fully characterized. Genome-wide CRISPR-Cas9 knockout screens have elucidated numerous 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 were transduced with the Brunello sgRNA library cloned into an in-house optimized mCherry-encoding vector at an MOI of 3 to achieve 30% transduction. After a 7-day expansion, the cells were stimulated with K562 in a 4-hour *in vitro* responsiveness assay. Viable $mCherry^+$ cells were FACS-sorted into four subgroups according to degranulation (CD107a) and IFNg positivity. Harvested DNA from the resulting cells was subject to next-generation sequencing.

Following analysis, the data will be stratified into hypo- and hyper-responders to K562, according to sgRNA enrichment or depletion, and validated through gene set enrichment analysis. Advanced bioinformatic analyses will be performed to confirm known, and potentially identify novel genetic mediators driving the cytokine and cytotoxic responses. Upon confirmation and validation in primary cells, the identified hits will widen the 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.

17. Genome-wide CRISPR/Cas9 knockout screening revealed genes involved in CD20 regulation in rituximab-resistant cells

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Abstract text: CD20 antigen has been used as a target of monoclonal antibodies (mAb) such as rituximab (RTX) in the therapy of B-cell malignancies for more than two decades. However, malignant B cells downregulate CD20 on their surface, resulting in mAb resistance and therapy failure. Therefore, it is crucial to investigate the CD20 regulation to enhance the efficacy of anti-CD20 mAb. This project aimed to perform CRISPR/Cas9 knockout screening to identify genes whose disruption restores CD20 surface expression.

To create a model mimicking the situation in patients who have developed resistance to mAb therapy, we generated RTX-resistant CD20-low B-cell line by chronic exposure to rituximab. These cells were transduced by the GeCKO lentiviral library to obtain a collection of single-gene knockouts. After 2.5-week cultivation, the top 5% of cells with the highest expression of CD20 were sorted out. Using next-generation sequencing, we identified gene knockouts responsible for CD20 upregulation.

CRISPR/Cas9 screening revealed several genes whose disruption increased CD20 surface expression. *CSK*, encoding a negative regulator of Src kinases, as well as *PTEN*, a well-known tumor suppressor, were among the top hits. These two genes are involved in the B-cell receptor (BCR) pathway – an essential pathway in B cells. Interestingly, we identified four genes *SSR1-4*, encoding all four subunits of the TRAP complex, an endoplasmic reticular complex involved in protein translocation across ER membrane. *STT3A*, encoding the catalytic subunit of oligosaccharyltransferase, was another ER-associated gene revealed by the screening. These results indicate that both BCR signalling and ER play an important role in CD20 regulation. Selected genes were validated, and the mechanism of their function is being investigated. The understanding of underlying mechanisms could provide a way for a potential enhancement of anti-CD20 mAb therapy.

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18. Identifying Novel Regulators of Hemogenic Reprogramming With CRISPR/Cas9-based Knockout Screening

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Abstract text: Hematopoietic stem cells (HSCs) self-renew and continuously produce mature blood cells. For these reasons, HSC transplantation is the only curative treatment for a variety of hematologic malignancies. However, the expansion of HSCs *in vitro* for clinical application remains challenging. The generation of patient-tailored HSCs by direct cellular reprogramming has the potential to overcome the limitations. We have previously shown that the ectopic expression of the transcription factors (TFs) GATA2, GFI1B, and FOS in fibroblasts generates hematopoietic stem and progenitor cells (HSPCs) through a dynamic endothelial to hematopoietic transition. Nevertheless, a comprehensive understanding of the molecular regulators underlying this dynamic process in humans is needed to improve the efficiency and fidelity of the process. Here, we optimized a CRISPR/Cas9 knockout (KO) screening toolbox to map genes encoding for positive and negative regulators of hemogenic reprogramming. We fined-tuned the conditions for efficient KO with a constitutive Cas9 and a sgRNA library targeting 116 genes implicated in HSC self-renewal and expansion. In parallel, we established the delivery of the hemogenic TFs to human fibroblasts using a polycistronic lentiviral vector allowing antibiotic-mediated selection of transduced cells. We tested 6 polycistronic constructions and showed that the order of GATA2, GFI1B, and FOS which is translated to elevated levels of GATA2 and GFI1B, results in the highest reprogramming efficiency measured by the activation of the early hemogenic markers CD9 and CD49f. After 15 days of inducing reprogramming of edited fibroblasts, we purified reprogrammed (CD9+CD49f+) and non-reprogrammed (CD9 CD49f-) populations and performed deep sequencing. We ranked the lists of hits and identified candidate genes that may function as barriers or facilitators of hemogenic reprogramming including CD44 and STAG2, respectively, for future exploration. Overall, our findings provide the foundation for CRISPR/Cas9 screening to define drivers of human hematopoietic reprogramming and ultimately provide an efficient source of patient-specific HSCs.

19. Immuno-CRISPR (iCR) mouse model, a quick model for verifying druggable targets in vivo affecting hematopoietic system

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Abstract text: Background: Rheumatoid arthritis (RA) is an autoimmune disease predominately affecting the joints, which is strongly associated with the activity of autoreactive CD4 T cells. *DNMT3A* mutations are one of the most common somatic mutations found in patients with rheumatoid arthritis. However, the role of DNMT3A in CD4 T cells, especially follicular T helper cells (Tfh), is poorly understood. KRN+ mice have autoreactive CD4+ T cells that cause arthritis in mice expressing the I-A^{g7} MHC class II molecule.

Methods: To understand the function of DNMT3A in CD4 T cells in the autoreactive setting, KRN+ *Dnmt3a* iCR mice were generated by knocking out *Dnmt3a* in KRN+ I-A^{b} bone marrow cells, subsequently transferred into irradiated *Tcrb*$^{-/-}$ I-A^{b} mice. Mature T cells developing in the KRN+ *Dnmt3a* iCR mice were further transferred into *Tcrb*$^{-/-}$ I-$A^{b/g7}$ recipient mice, resulting in arthritis. Twelve days later, Tfh and non-Tfh were sorted by flow cytometry, and the insertion and deletion (Indel) percentages in *Dnmt3a* were analyzed by sequencing. The function of *Dnmt3a* was further analyzed using *Cd4*^{cre} *Dnmt3a*$^{fl\text{-}R878H/fl\text{-}R878H}$ conditional knockout mouse model.

Results: The Indel percentage of *Dnmt3a* in Tfh was significantly lower compared to in non-Tfh. In a mixed bone marrow chimeric setting, *Cd4*^{cre} *Dnmt3a*$^{fl\text{-}R878H/fl\text{-}R878H}$ has a selective disadvantage compared to WT cells in the development of Tfh´s.

Conclusion: Immuno-CRISPR (iCR) mouse model is a rapid model for identifying the role of selected genes in *in vivo* disease models. DNMT3A is important for Tfh differentiation and can be a potential drug target affecting Tfh.

20. In situ detection of genomic cis-interaction by proximity ligation assay using oligonucleotide-labelled Cas9

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One prominent question in cell biology is the functional implication of higher-order chromatin structure. While chromatin conformational capture assay has greatly advanced our understanding of chromosome organization, it remains challenging to understand the interplay between the chromatin structure and cell states due to the loss of cellular contexts. Here we introduced an *in situ* technique to directly visualize the chromatin interaction between targeted loci by leveraging the chromatin labelling efficiency of Cas9 and the specificity of proximity ligation assay. We first generated nucleotide-conjugates of Cas9 and validated nuclease activity of Cas9 conjugates and rolling circle amplification reactivity. Using a DNA origami-patterned platform with controllable distance between a pair of targets, we characterized the detection efficiency and spatial resolution of the assay. We next validated reaction efficiency *in situ* by targeting repeated genomic loci in fixed cells. Furthermore, we demonstrated the feasibility of detecting the cis-interaction by targeting the well-characterized β-globin locus control region.

21. PRCISR™ CRISPR: Vivlion's CRISPR-enabled discovery platform

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Abstract text: Exploring the enormous space of the human genome with CRISPR-based target identification strategies is challenging, particularly for large scale combinatorial approaches that aim to edit multiple genes in a single cell. PRCISR™ CRISPR is Vivlion's proprietary monogenetic and combinatorial CRISPR-based discovery platform. It combines expert knowledge and proprietary library generation technology to facilitate the application of uniformly distributed single and combinatorial CRISPR libraries at scale. PRCISR™ CRISPR is unique in maximizing gene editing efficiency and hit-retention rates, enabling powerful screen parallelization for the identification of combinatorial phenotypes by enhancing throughput and innovative target identification. PRCISR™ CRISPR begins with expert consultation on study design, followed by the generation of highly uniform CRISPR libraries, and their screening in different contexts. PRCISR™ CRISPR then delivers sample sequencing and state-of-the-art computational analyses. Our PRCISR™ CRISPR pipeline is optimized and validated to find true positive hits, enabling the discovery of robust candidates for accelerated validation and mechanistic follow-up.

22. Primary immunodeficiencies and cancer: Targeting actin regulation in T cells

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Abstract text: Primary immunodeficiencies (PIDs) constitute a group of early-onset monogenetic diseases affecting the immune system. PID patients have increased risk of developing malignancies of poor prognosis. A substantial number of PIDs are caused by mutations in regulators of the actin cytoskeleton (actin regulators), leading to impaired adhesion, migration, proliferation, intracellular signaling, and genomic instability. Although malignancies are a common comorbidity or PIDs, their association with abnormalities of the actin cytoskeleton remains to be determined. In this study, we aim to understand the relationship between adhesion and malignancies in T cells. To achieve this, we will perform a custom CRISPR-screen targeting actin regulators in both primary human T cells and Jurkat T cells, a human acute lymphoblastic leukemia (ALL) cell line. More specifically, a library of actin regulator knockouts (KOs) will be generated by lentiviral transduction and stimulated by activating antibodies (anti-CD3 & anti-CD28) coated on glass coverslips to initiate cell adhesion. By quantifying the relative single-guide RNA (sgRNA) abundance between non-activated (unbound) and activated (bound) T cells, both healthy and cancerous, we will shed light into the importance of actin regulators in T cell activation and adhesion, while narrowing down possible molecular mechanisms of malignancy in PIDs.

Keywords: Primary immunodeficiencies, cancer, cytoskeleton, actin regulators, T cells, adhesion, targeted CRISPR-screen, sgRNA.

23. Probing the consequences of CADASIL mutants on Notch3 using overexpression constructs and CRISPR/Cas9 gene editing of the endogenous gene.

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Abstract text: Human Notch3 regulates vascular smooth muscle maturation and missense mutations are linked to a dominantly inherited vascular dementia disease CADASIL, characterised by accumulation of the separated extracellular domain (ECD). The pathological mechanism is unclear because different CADASIL mutants can have opposite effects on ligand-dependent Notch3 signalling, which involves the proteolytic separation of ECD and subsequent release from the membrane of the intracellular domain (ICD). Notch3 is overexpressed in cancers and an undefined ligand-independent but Adam 10-dependent activation mechanism regulates breast cancer stem cells. Full-length Notch3 is endocytosed and undergoes separation of ECD and ICD in the early endosome. Endogenous Notch3 in the MCF7 breast cancer line, and in human vascular smooth muscle cells (VSMCs) differentiated from embryonic stem cells, also displayed separate subcellular localisation of the ECD and ICD in the endosomal pathway, and this separation is suppressed by ADAM10 inhibition. To verify Notch3 antibody specificity for endogenous Notch3, a N3KO-MCF7 line was generated using CRISPR-Cas9 and verified by western blot. Immunofluorescence data on the N3KO-MCF7 line has shown that, ECD and ICD antibodies were specific to Notch3. Different expressed CADASIL mutants show increased ligand-independent activation even when ligand-induced signalling is disrupted, suggesting that a common underlying mechanism may stimulate ECD release in CADASIL, following ECD separation in the endocytic pathway. A CADASIL mutation C455R has been introduced into MCF7 cells by CRISPR-Cas9 to verify the endocytosis behaviour of the mutant at the endogenous level and heterozygous MCF7 Notch3 C455R mutant line generated mimicking the CADASIL genotype. Work is in progress to generate homozygous C455R mutant line to enable mutant protein localisation to be studied without a background of WT Notch3. Having validated the guide RNA, future work will introduce this mutation into embryonic stem cells to differentiate into VSMCs to investigate Notch3 localisation in a disease relevant cell type.

24. Same-day genome editing analysis with Countagen's GeneAbacus kit

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Abstract text: Introduction
The rapid development of CRISPR/Cas-based genome editing technologies has opened many possibilities to advance research and development. Assessing the editing outcome is the first critical step in determining the success of the genome editing experiment. However, current methods to analyze editing efficiency and specificity can be expensive and time-consuming.

Method
We have developed a same-day, in-house solution, free from the risk of PCR amplification bias, to identify desired edits and precisely quantify efficiency of an editing technique. Gene editing efficiency and specificity can be determined faster and in a more precise manner than with sequencing based methods and digital PCR, respectively. Our technology is based on Rolling Circle Amplification (RCA) using padlock probes (PLPs) that specifically convert target DNA sequences to single RCA amplicons, thus enabling digital quantification. To achieve maximum quantification, all amplicons are imaged with a 20x magnification field-of-view and subsequently analyzed using Countagen's proprietary image analysis software (GAIA). Overall, the workflow takes less than 5 hours, using only standard laboratory equipment.

Results
Our technology offers single nucleotide precision and high sensitivity in determining editing efficiency in CRISPR edited pools and clones. The technology was successfully demonstrated in extracted DNA samples from various cell types and organisms. Compared to amplicon sequencing, our assay showed excellent concordance (Pearson correlation of r^2=0.9524), whereas digital PCR showed poor concordance (Pearson correlation of r^2=0.5338). Furthermore, our assay shows consistent performance across varying GC contents underlying its robustness.

Conclusion
Overall, we have developed a rapid, sensitive and precise method to enable same-day genome editing analysis in order to accelerate the path towards functional analysis experiments. Our technology shows excellent concordance with sequencing-based methods at a fraction of the time. Our first product to be launched, GeneAbacusTM, is currently being validated by external beta testers.

25. The SciLifeLab CRISPR Functional Genomics Unit: Fantastic tools and where to find them

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Abstract text: CRISPR-Cas systems can be used for a variety of molecular research applications, including gene knockout, introducing small alterations, and transcriptional modulation. At CRISPR Functional Genomics (CFG) we are specialists in using CRISPR-Cas technology, and it is our mission to facilitate and accelerate research by providing flexible and affordable precision gene-editing, as well as massively parallel perturbations in our customer's cell line of choice.

CFG focuses mainly on pooled CRISPR-Cas screening approaches from planning to data analysis. Using different modalities of pooled screens, thousands of genes can be interrogated in parallel to study cell proliferation and viability, drug sensitivity or resistance, signal transduction, host-pathogen interactions (bacteria or viruses), cell differentiation, organoid formation, immune response, etc. CRISPR-KO screens are complemented by CRISPR inhibition loss- and CRISPR activation gain-of-function screens. Novel advanced methods such as Perturb-Seq and base-editing saturation mutagenesis screens to study drug-target or protein-protein interactions are also available.

In addition, we provide CRISPR editing services for specific genes or genetic elements. Examples include the generation of knockout cell lines and the introduction of point mutations, as well as protein tags to label or inducibly degrade proteins. We mainly work with cell lines but can also help in creating genetically modified induced pluripotent stem cells (iPSCs) and mouse strains in collaboration with other KI core facilities. We constantly implement novel methodologies, such as base- and prime editing, to improve the efficiency of our edits.

26. Using CRISPR-Cas9 mutagenesis in Drosophila to investigate functional redundancy between Suppressor of Deltex and DNedd4 during Notch endocytosis

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Abstract text: The Notch Signalling Pathway is one of the most significant mechanisms within embryogenesis, possessing numerous functions that maintain health and cellular organisation as well as in post-developmental mechanisms. However, the setting of Notch activity is not a simple matter of stimulus and response but remains an emergent property of the regulatory networks in which the core signalling pathway is embedded and, with ligand-independent signalling, dependent on Notch endocytic flux. Understanding the mechanisms that process Notch during endocytosis can provide accurate knowledge of the molecular systems behind numerous human diseases including cancers and Alzheimer's disease. In *Drosophila*, Notch is down-regulated by Suppressor of Deltex (Su(dx)) and DNedd4, both members of the NEDD4 family of E3 ubiquitin ligases which function in endocytic trafficking. However, little is known whether the Su(dx) and DNedd4 proteins act via similar or different regulatory mechanisms towards Notch activity. The effect of DNedd4 itself on Notch was found to be isoform specific as only the long version of the isoform notably down regulates Notch activity in both *Drosophila* and *in vitro* when overexpressed. Preliminary work studying combinations of existing *Drosophila Su(dx)* and *DNedd4* mutant alleles indicates some functional overlap, as combined mutants have stronger phenotypes in wing development. However, to understand the full biological functions of DNedd4 and Su(dx), it is important to study null mutations of each gene separately and in combination. A null allele of *Su(dx)* is already available, but existing mutations of *DNedd4* leave open the possibility of some retention of biological activity and/or some dominant negative activity. Therefore, CRISPR/Cas9 mutagenesis, via embryonic injection, is currently being used to generate a defined null for *Drosophila DNedd4 in vivo* to better understand the nature of this particular component of Notch signalling.

27. Linking transcriptome to genotype in single cells identifies mutation-induced transcriptional heterogeneity of IFNγ response

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28. Light induced expression of gRNA allows for optogenetic gene editing of T lymphocytes in vivo

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29. CRISPRa as a tool to characterize mechanisms of resistance associated with p53 pathway reactivation in diffuse midline glioma

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30. Uncovering novel dependencies in the DNA repair space with CRISPR/Cas12a screening

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31. Deletion of the TMEM30A gene allows leukemic cell evasion of NK cell cytotoxicity

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32. Identification of novel factors controlling non genetic cell plasticity in Chronic Myeloid Leukemia

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