EMA / CAT Workshop on Gene Editing

September 16th 2025

Uncovering and Mitigating Heterogeneity of Genome Editing Outcome at ON and OFF target sites

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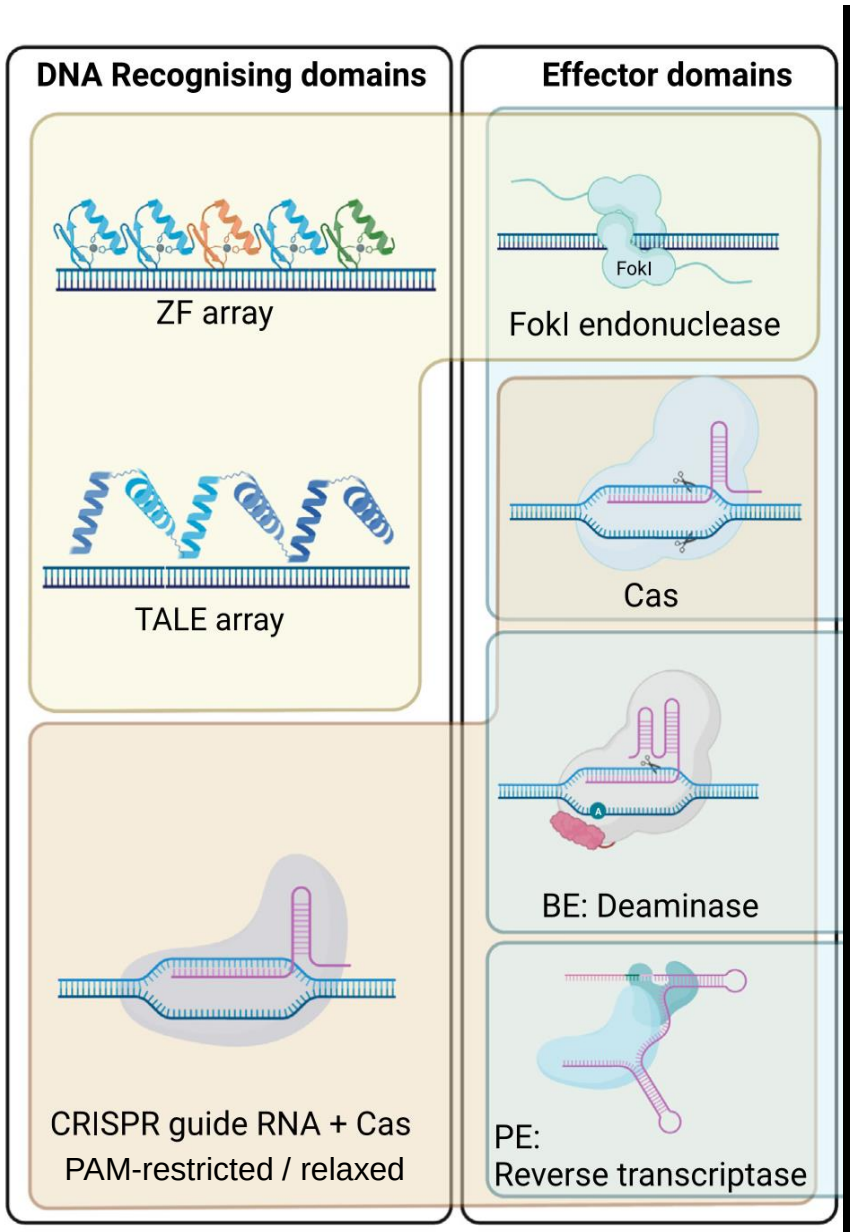
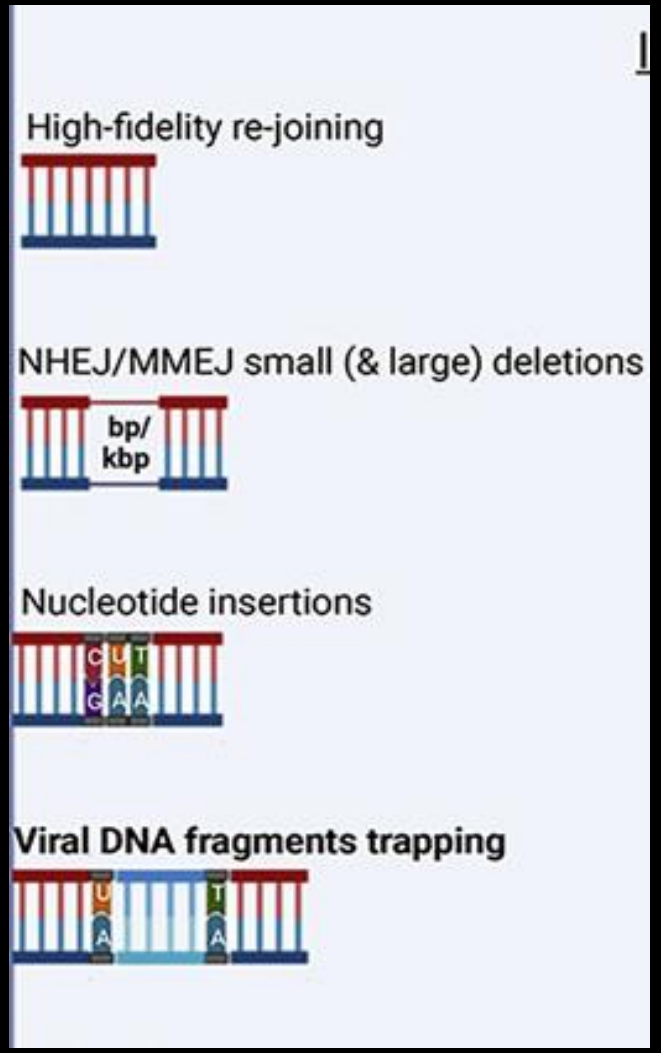
The Gene Editing Toolbox

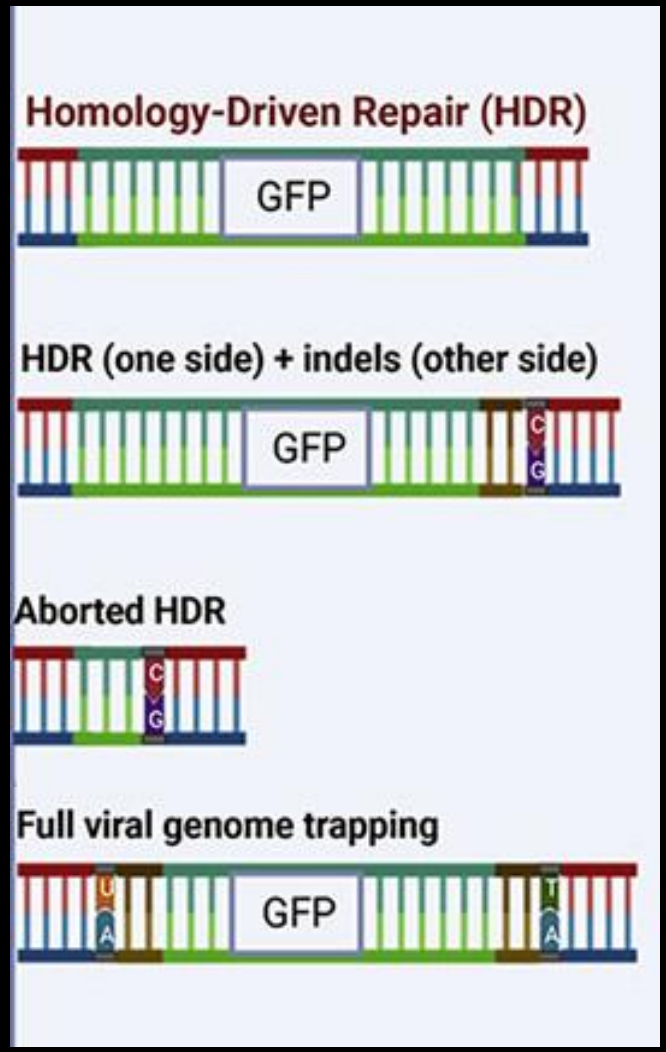


gRNA-dependent Specificity & Genotoxicity Assessment

	Off-target
Standard assays	In-silico (prediction) Digenome-Seq / Circle-Seq / Change-Seq (free DNA) Guide-Seq (*in cellulo*) CAST-Seq (*in cellulo*) (nomination) NGS / rhAMP-Seq (validation)
Exploratory assays	Optical/electronic mapping scDNA sequencing (Tapestri by Mission Bio)
Limitations	Cost & time (low specificity of prediction and nomination, limited positive predictive value) Human genome variation representation **gRNA purity** **functional significance**
Common frequency	<1%

Heterogeneity of Edit Outcome at Target Site





Ferrari, Jacob et al., Cell Stem Cell 2022

gRNA-dependent Specificity & Genotoxicity Assessment

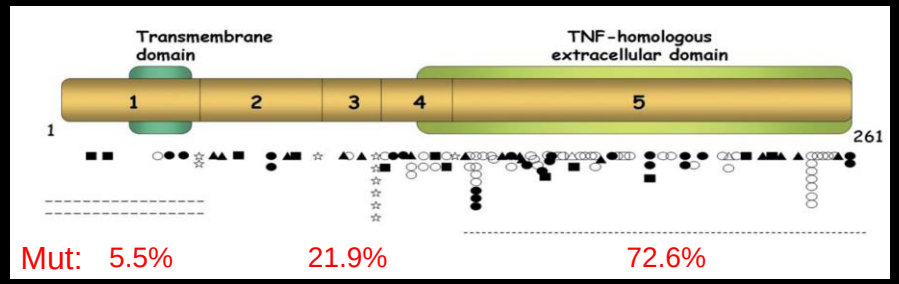
	Off-target	On-target
Standard assays	In-silico (prediction) Digenome-Seq / Circle-Seq / Change-Seq (free DNA) Guide-Seq (*in cellulo*) CAST-Seq (*in cellulo*) (nomination) NGS / rhAMP-Seq (validation)	Surveyor / TIDE (Sanger) ddPCR NGS (short edits) CAST-Seq
Exploratory assays	Optical/electronic mapping scDNA sequencing (Tapestri by Mission Bio)	Tiling by ddPCR Optical/electronic mapping Long read sequencing scDNA sequencing
Limitations	Cost & time (low specificity of prediction and nomination, limited positive predictive value) Human genome variation representation **gRNA purity** **functional significance**	Cost & time **Question bias** Ploidy **functional significance**

A Case Study

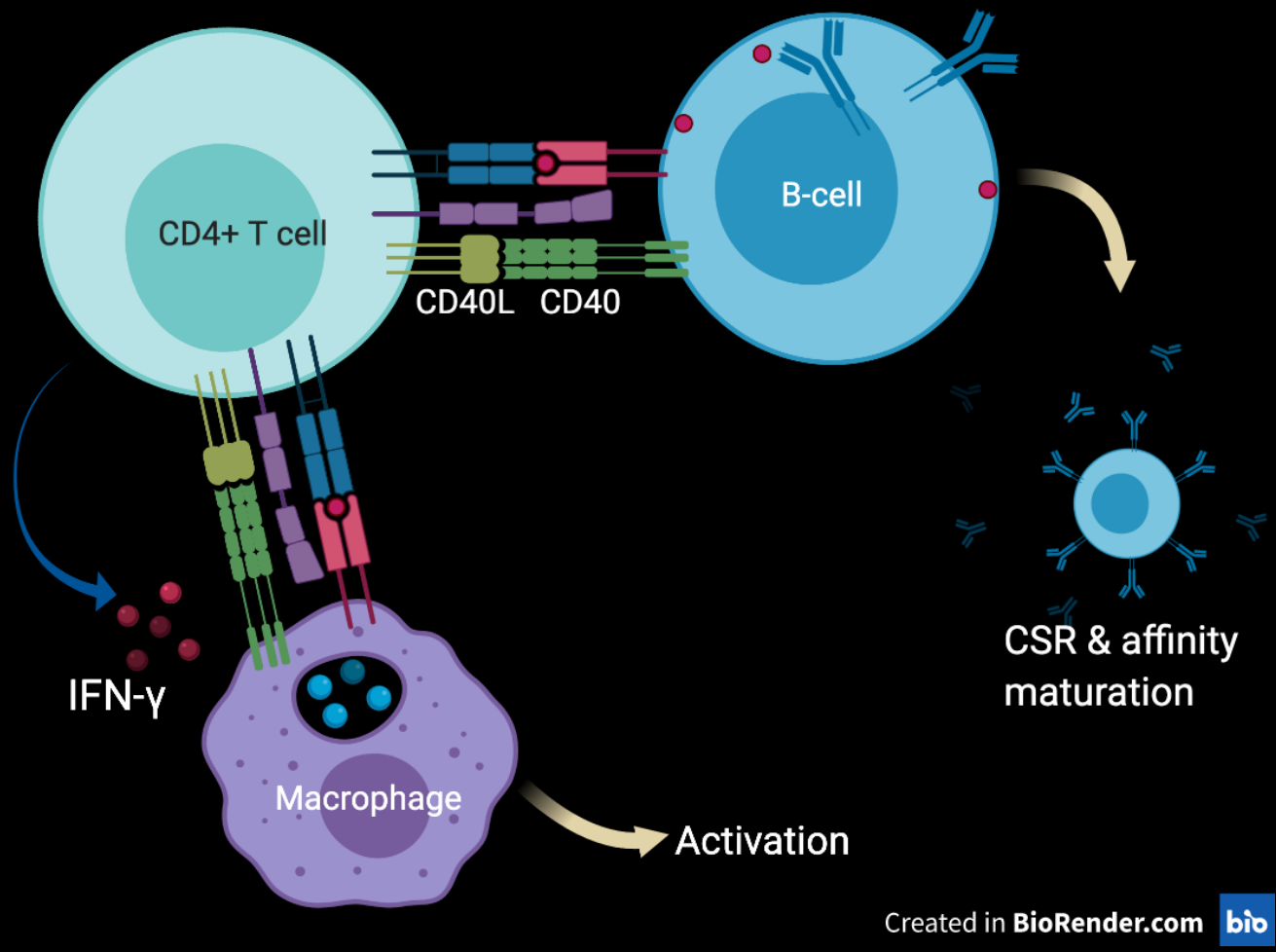
HDR Gene Editing
of CD40 Ligand Gene
in HIGM-1 CD4 T Cells

X-linked Hyper IgM Immunodeficiency (HIGM-1)

CD40LG Xq26.3



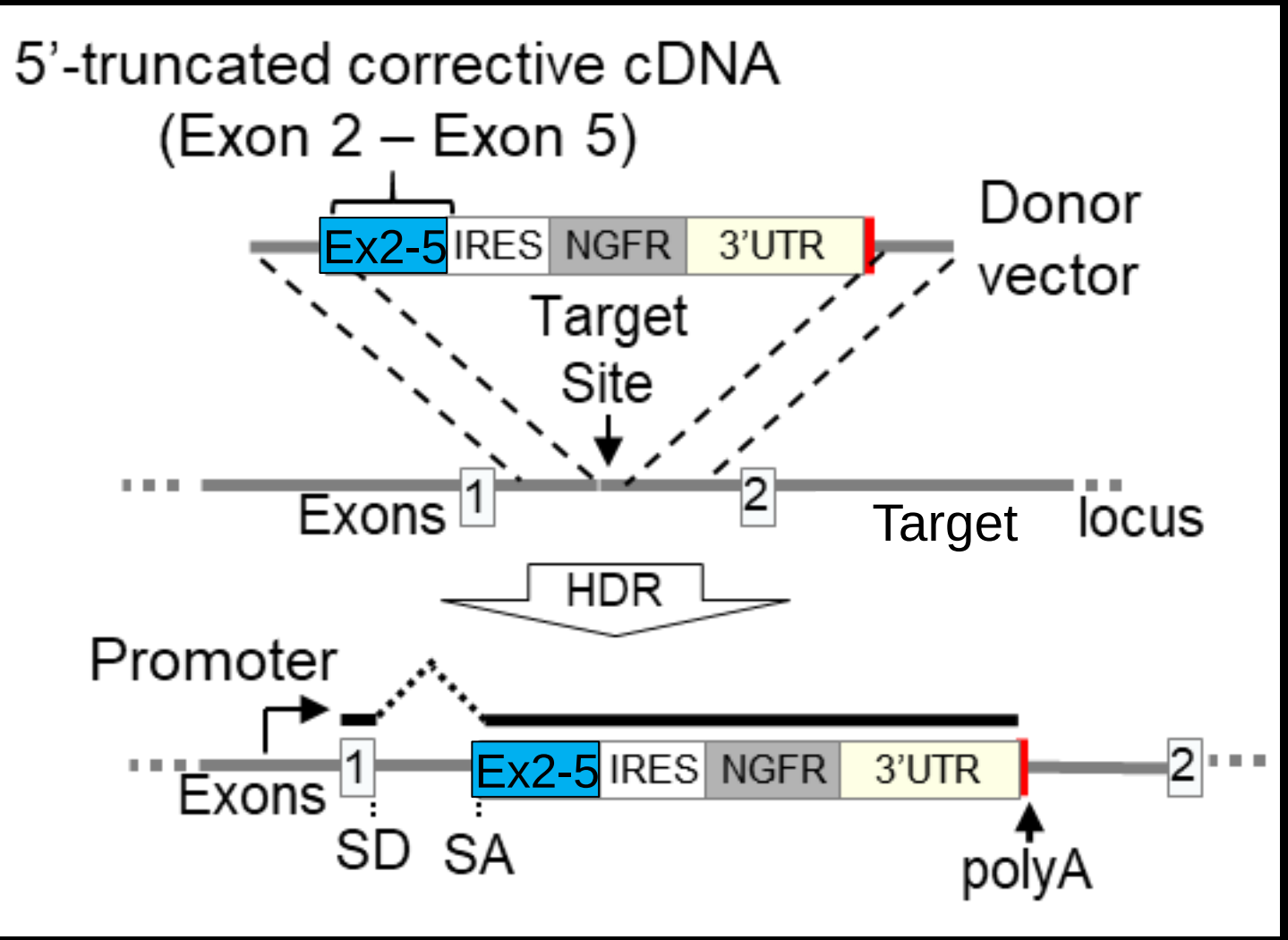
Expressed by activated CD4+ T cells



Gene Replacement studies in HIGM1 mouse model

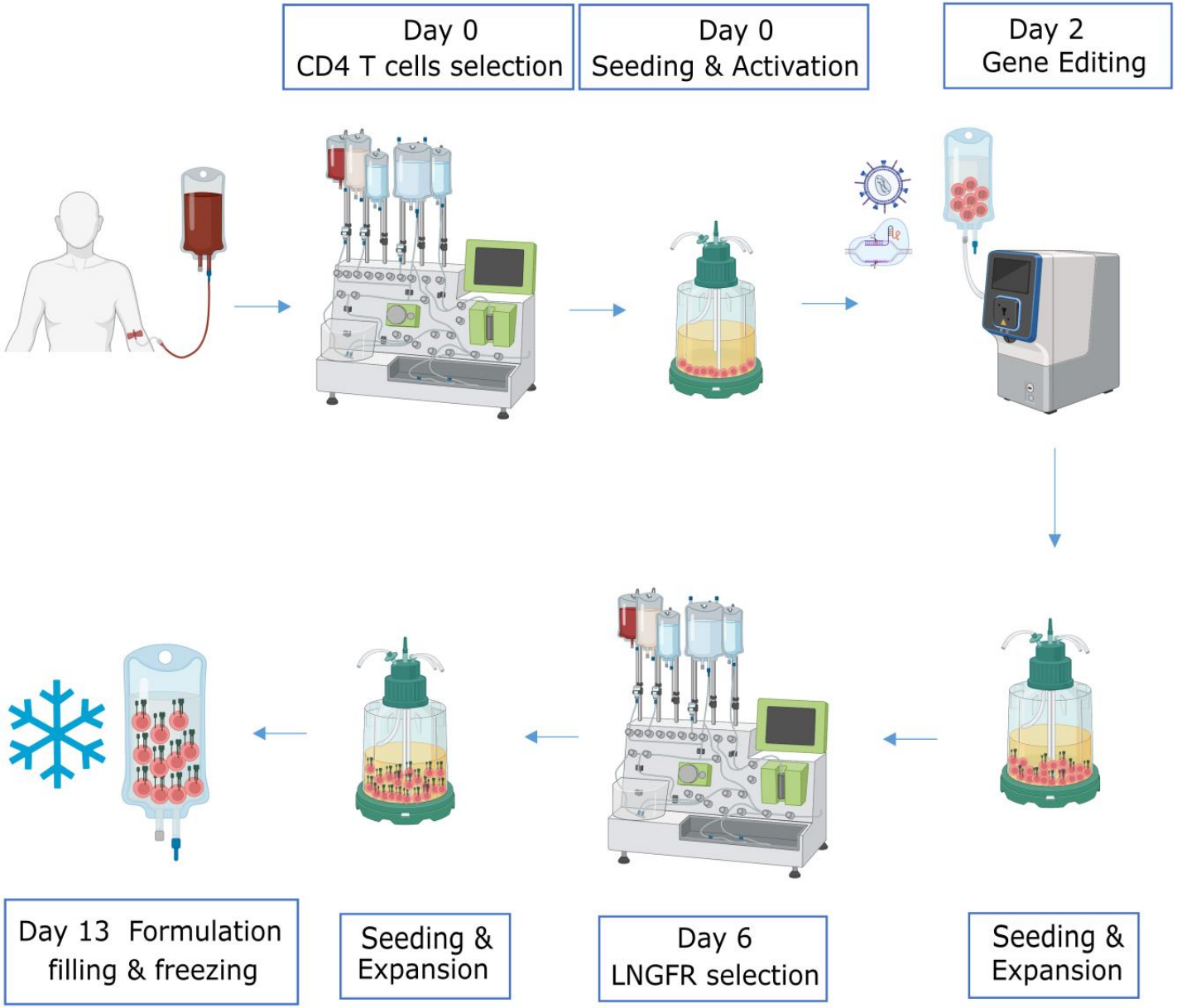
- Partial immune reconstitution from few ex-vivo corrected cells
- Abnormal T/B cells proliferation with constitutive CD40L expression

“One Size Fits All” *CD40LG* Gene Correction Strategy for HIGM-1

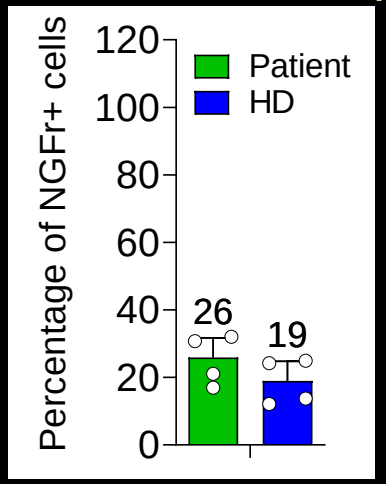


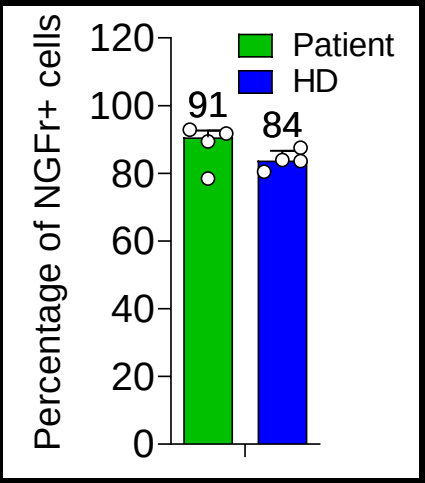
Knock-in of 5’-truncated corrective cDNA downstream endogenous promoter

GMP-Compliant Scaled Up CD4+ T cell Editing Process

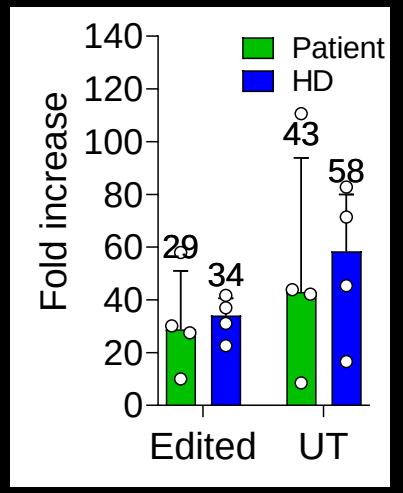


NGFR+ cells pre and post selection

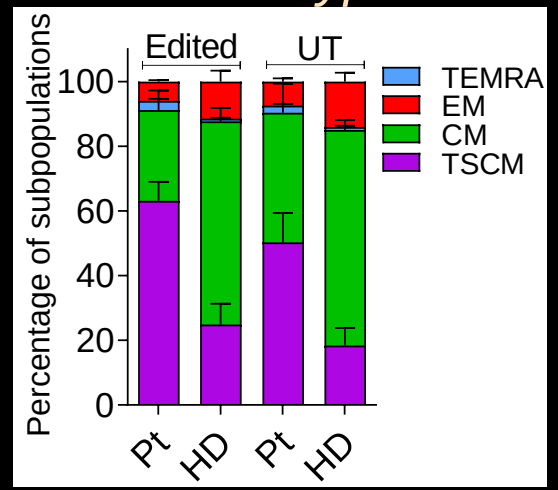




Fold increase



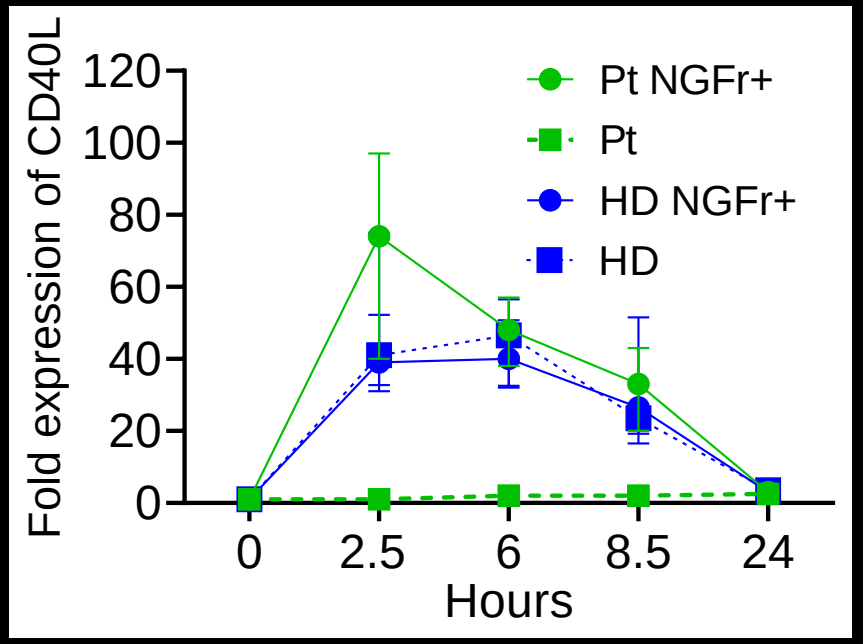
Phenotype



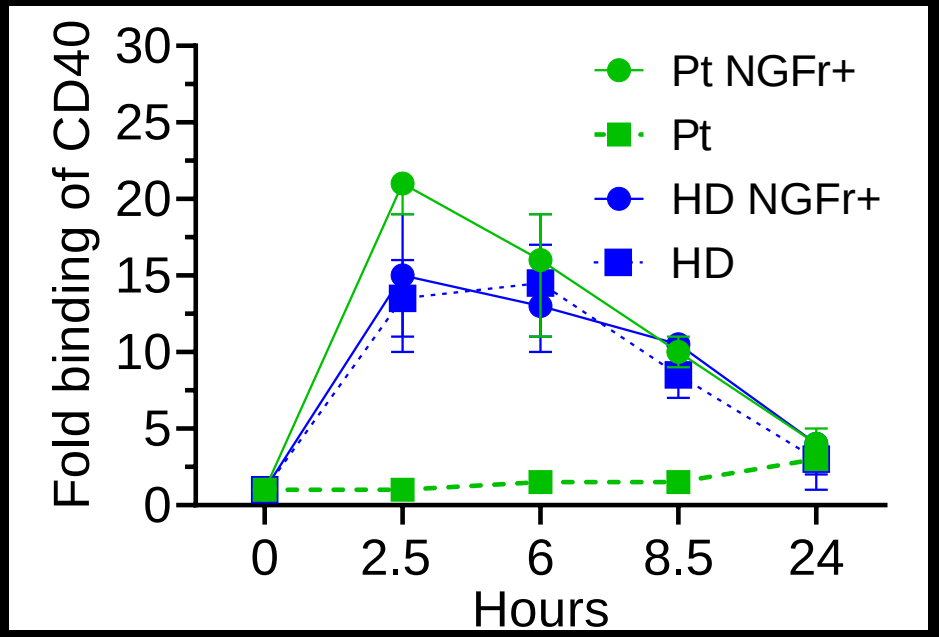
Asperti, Canarutto et al.,
Mol. Ther. Meth. & Clin. Dev., 2023

Rescue of CD40L Regulated Expression & Function in Edited Cells

CD40LG expression



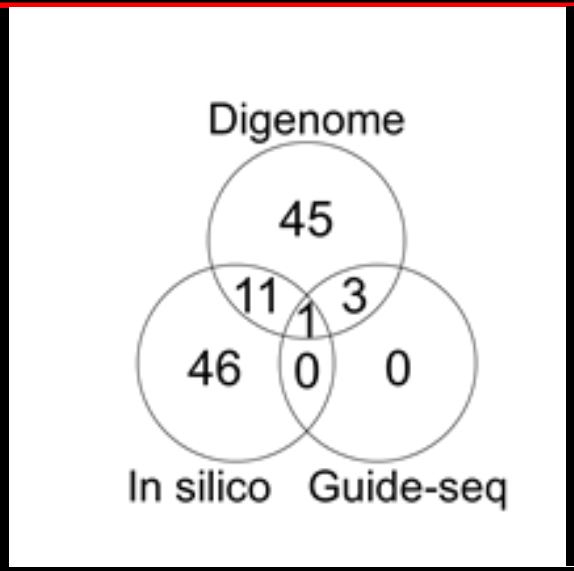
CD40LG binding of CD40



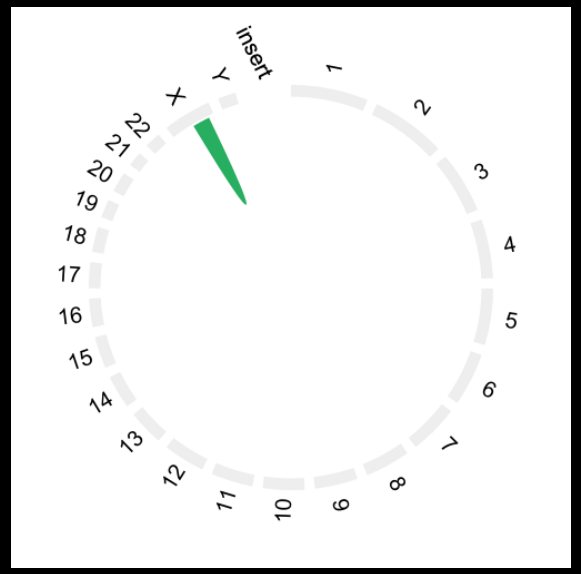
Patient samples courtesy of N. Gonzalez, V. Lougaris, A. Finocchi, Hyper IgM Foundation

Asperti, Canarutto et al., Mol. Ther. Meth. & Clin. Dev., 2023

Off-Target Analysis: Consensus ≥2 Orthogonal Methods



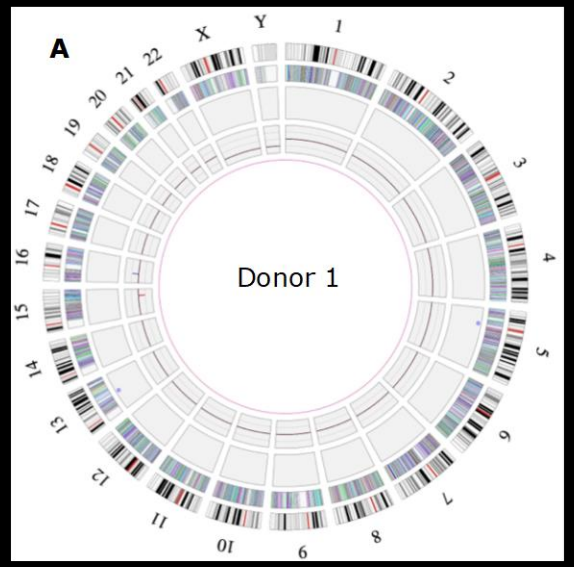
CAST-Seq

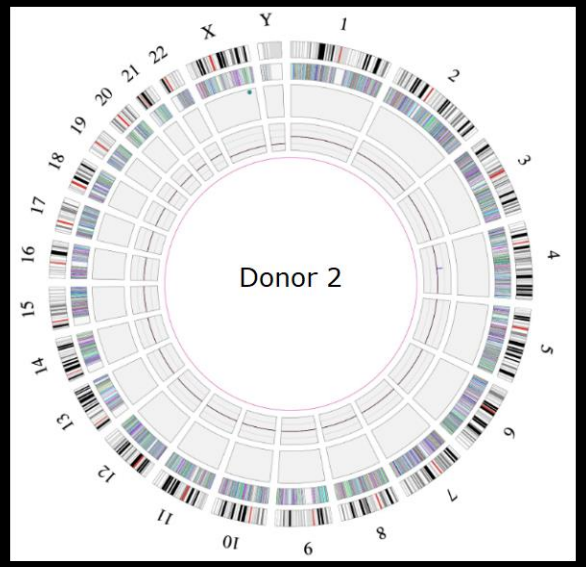


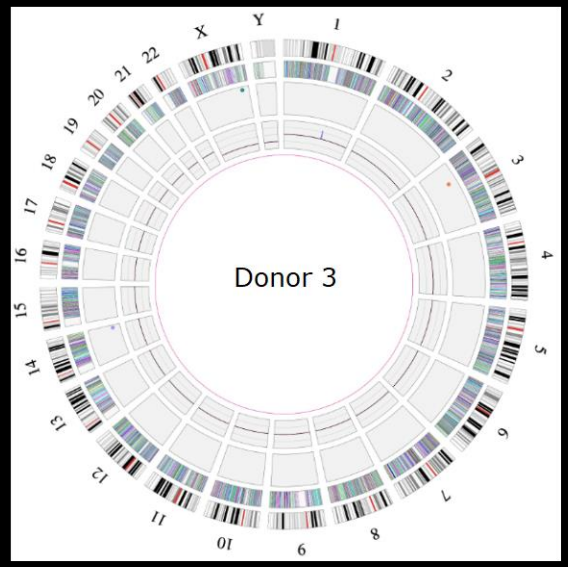
Sensitivity:
Guide-Seq 0.1%
Cast-Seq 0.01%
NGS 0.01%

In collab with Cathomen Lab

Optical Genome Mapping

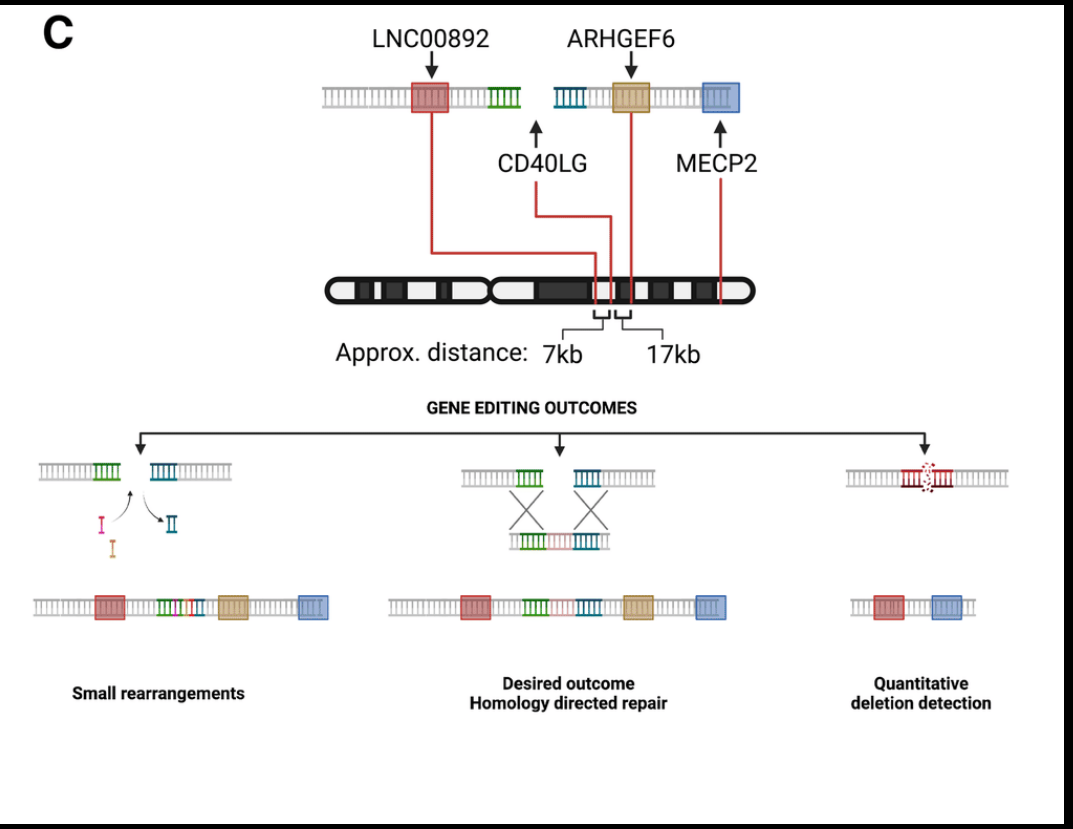






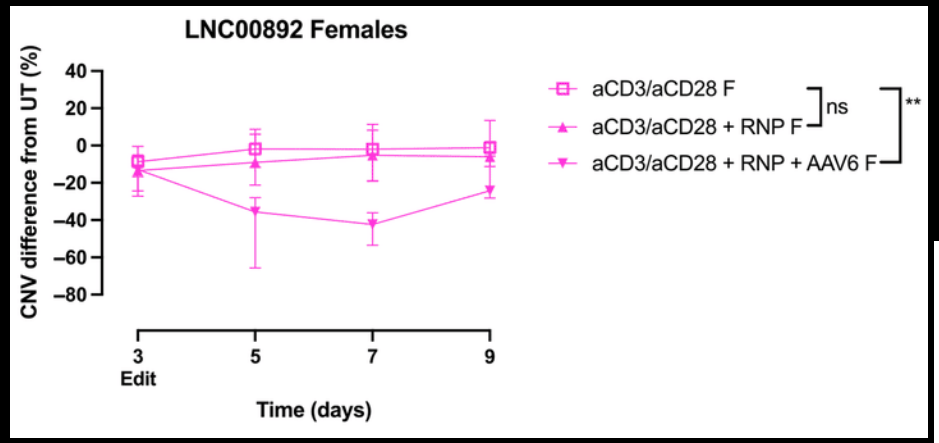
No detectable off-targets with HiFi Cas9

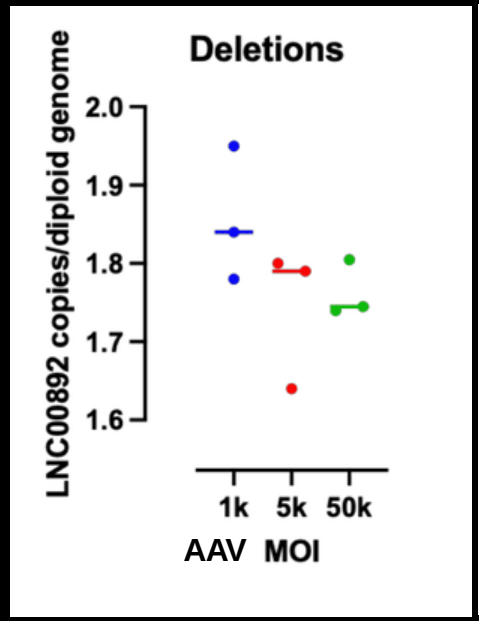
On-Target Assessment

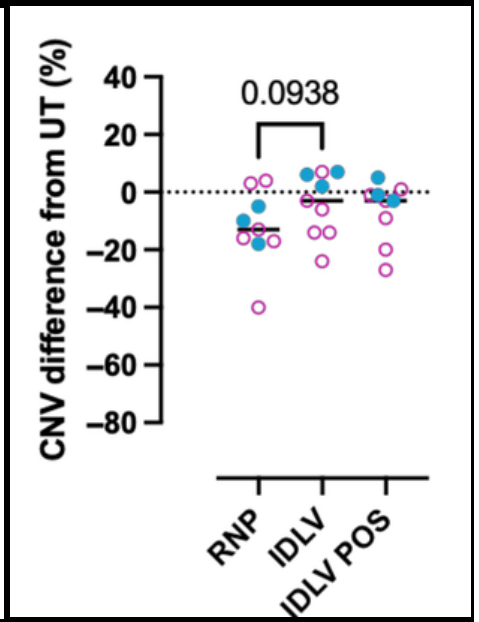


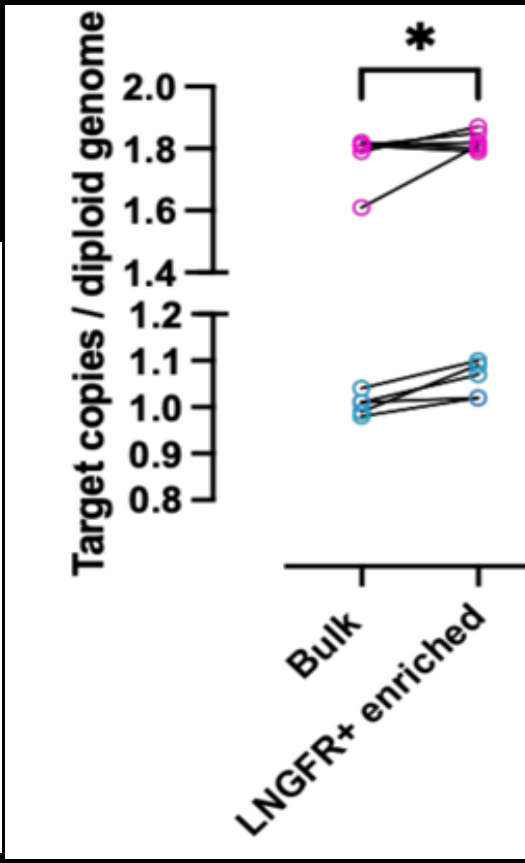
Tiling assays on closest annotated gene(s)

Sensitivity 5% on bulk





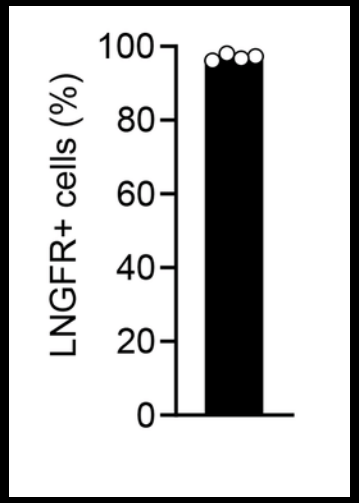




Large deletions purged spontaneously and by selection

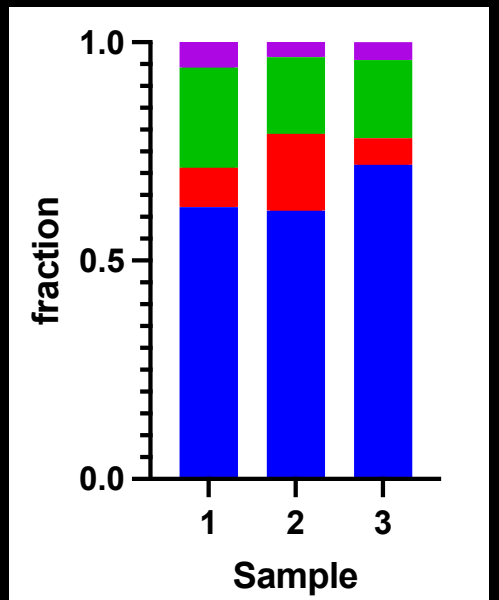
Canarutto et al., EMBO J., 2023

On-Target Assessment



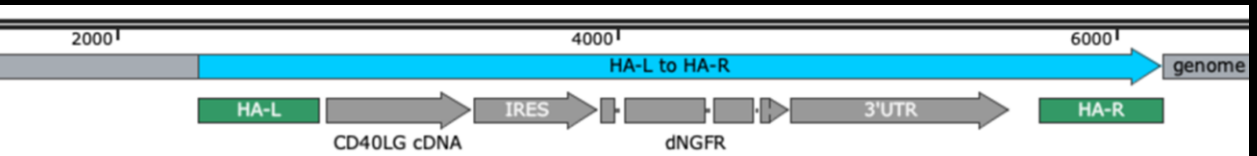
In CD40L+ pts edited cells

Long-Read Sequencing of Target Locus by Nanopore

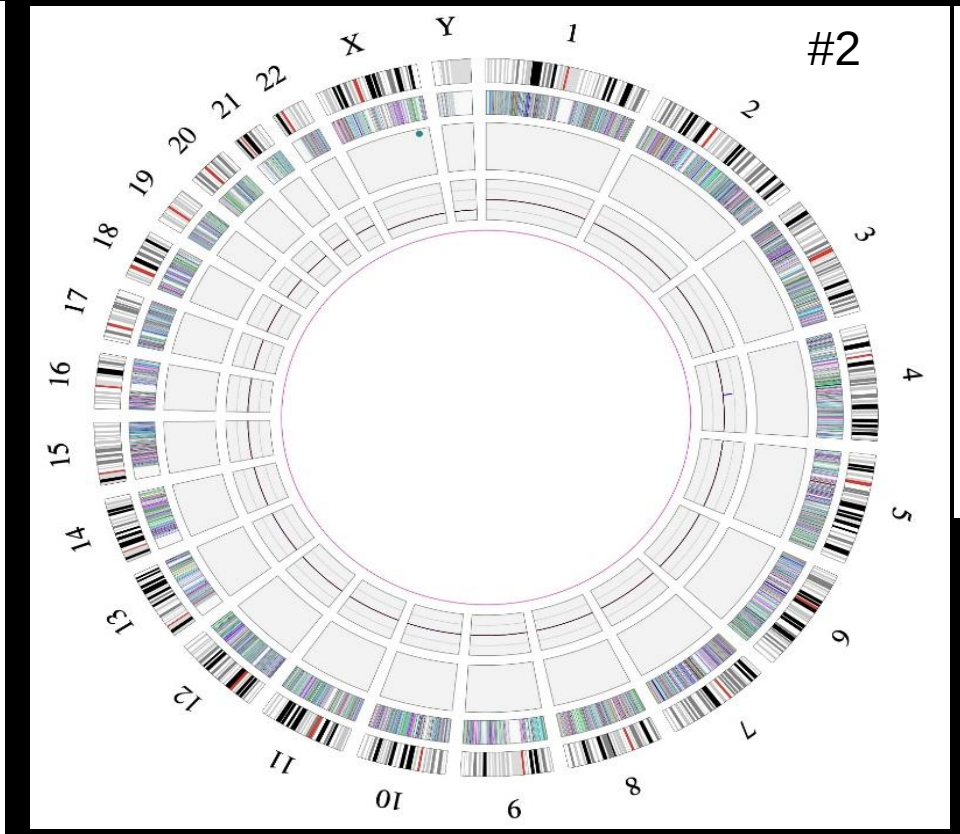


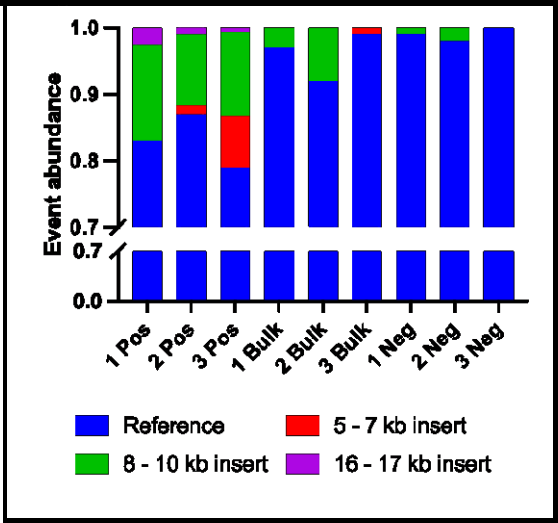
- Native, indels<50bp
- True HDR (bilateral and 0.5x monolateral HDR)
- Imprecise presumably functional HDR (concatamers + imprecise editing. Half reads counted half)
- Other

Precise bilateral HDR



Optical mapping (n=3)





Some (15%) ON Target template trapping

Few private events unrelated to Cas9 putative off-targets

Canarutto et al., EMBO J., 2023

Summary: Clinical Development of HIGM-1 Gene Correction

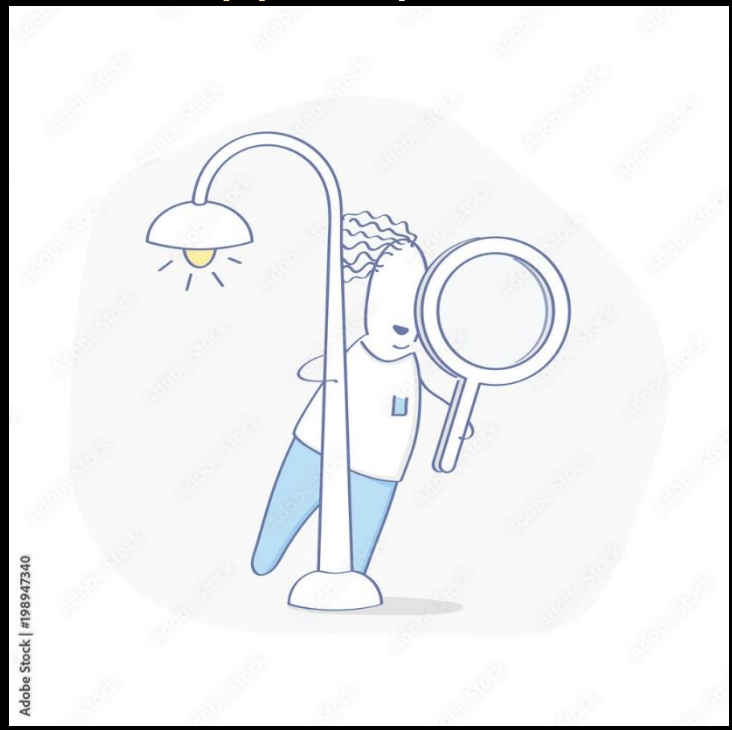
- *Development of GMP-compliant full scale process for HDR-editing of T-cells*
  - *In situ* gene correction of most *CD40L* mutations
  - Rescue of CD40L function and regulated expression
  - Selection of cells carrying intended edit coupled to purging adverse on target outcomes
  - Some heterogenous outcomes at target site albeit functional
  - Preserved genome integrity by genome-wide unbiased analysis
  - Clinical trial expected to start early next year

Canarutto et al EMBO 2023; Asperti, Canarutto et al Mol Ther Meth and Clin Dev 2023

Nuclease-based Gene Editing: Where We Stand Today

- *(guide-dependent) Off-target genotoxicity*
  - Assays established but high burden / low gain
- *On-target genotoxicity*
  - Significant question bias from each assay
  - Relevance of genomic neighborhood, type of editing reagents & target cell for safety
  - Most events biologically neutral or preserve intended outcome
- *Unbiased genome integrity assesment*
  - Low resolution

Avoid the looking under the lamppost paradox



Nickase-Based Editing

- *Base Editing*
  - reduces (vs. Nuclease) but does not abolish DNA DSB and adverse consequences
    - variably affected by deaminase type & expression level and
    - interaction with endogenous repair pathways (BER)
  - detectable impact on exome mutational landscape by ultra-deep WES
    - likely through inhibition / saturation of BER (CBE)
    - engagement of alternative error-prone DNA repair
  - guide-dependent Off-target activity likely to be higher that nuclease-based tools
  - development of improved versions may alleviate some of above concerns


nature biotechnology
Article
https://doi.org/10.1038/s41587-023-01915-4
Genotoxic effects of base and prime editing in human hematopoietic stem cells

Addressing Genotoxic Risk of Gene Editing

- *Plethora of emerging new tools in constant evolution*
- *Human genome sequence variation and unbalanced representation of regional differences in available databases*
  - Limits off-target prediction by testing a generic / individual donor human genome
  - Animal models useless (except for guide-independent effects)
  - Clonality tracking in patients challenging (except with NHEJ or lymphocytes)
  - Patient-specific emerging toxicity to be addressed in the clinic
- *Complexity of interaction with cellular DNA and repair machinery*
  - Requires novel *ad-hoc* testing (avoid the “looking under the lamppost” paradox)
- *Hit-and-run process makes difficult to trace eventual adverse events*
- *Multiplexing increases combinatorially heterogeneity of outcomes*
- *Confidence on safe use to be built for specific guide and platform*