Cell & Gene Therapy Products Europe 2025

Basel, Switzerland October 23-24 2025

Genetic Engineering of Hematopoietic Cells: from Lentiviral Gene Replacement to Targeted Gene Editing

Luigi Naldini, MD, PhD

Director, SR-Tiget,
Milan, Italy

SAN RAFFAELE TELETHON INSTITUTE FOR GENE THERAPY
SR-Tiget

OSPEDALE SAN RAFFAELE

FONDAZIONE Telethon

Disclosures

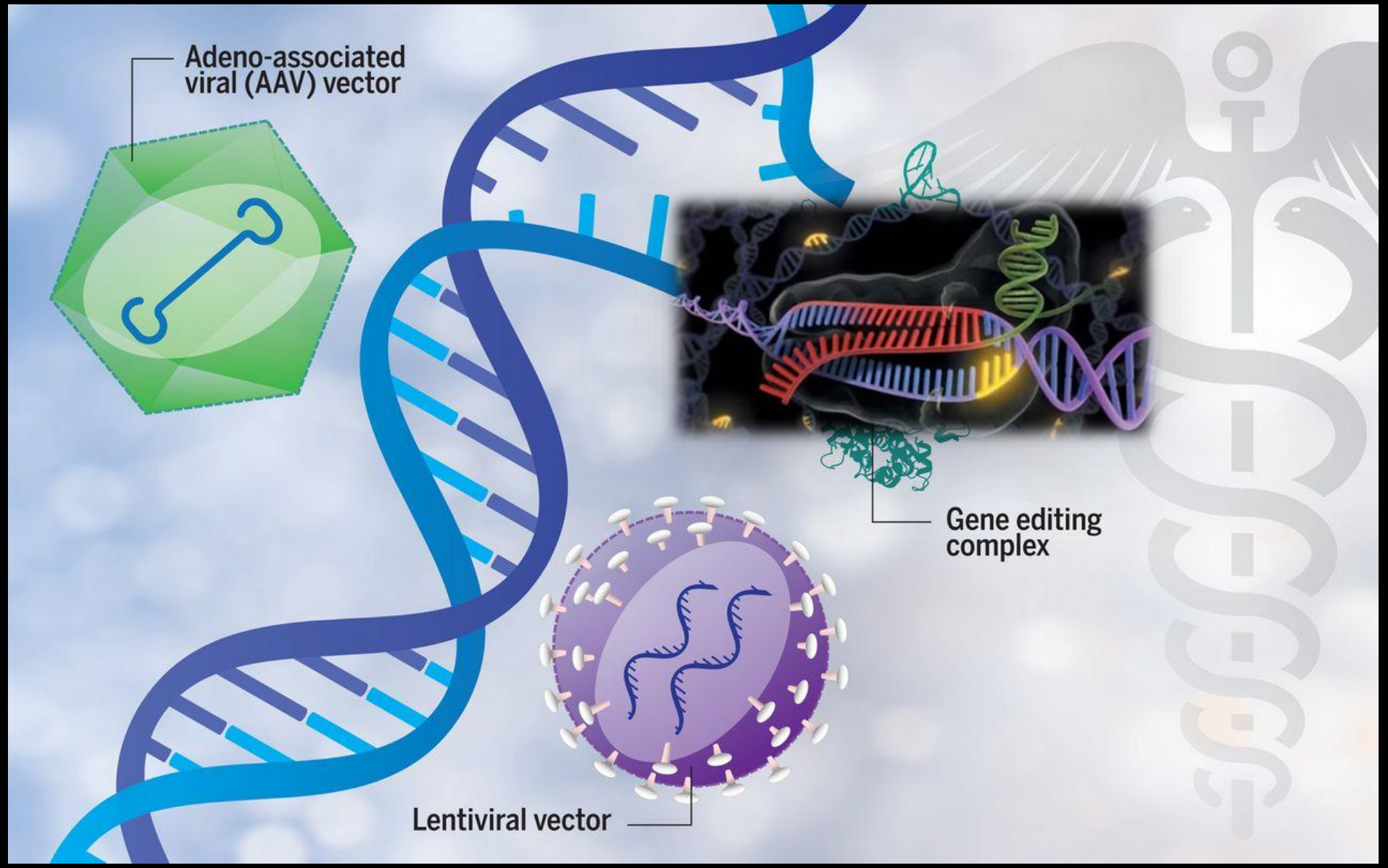
- *Inventor of patents on*
  - *lentiviral vector technology*
  - *targeted genome editing*
  - *HSC manipulation / transplantation*

  owned & managed by Telethon Foundation & San Raffaele Hospital

- *Founder, equity holder, SAB member of*
  - *Genenta Science*
  - *Genespire*
  - *Chroma Medicine*

- *Consultant and SAB member of*
  - *Tessera Therapeutics*
  - *Tr1X*
  - *N'Chroma Medicine*

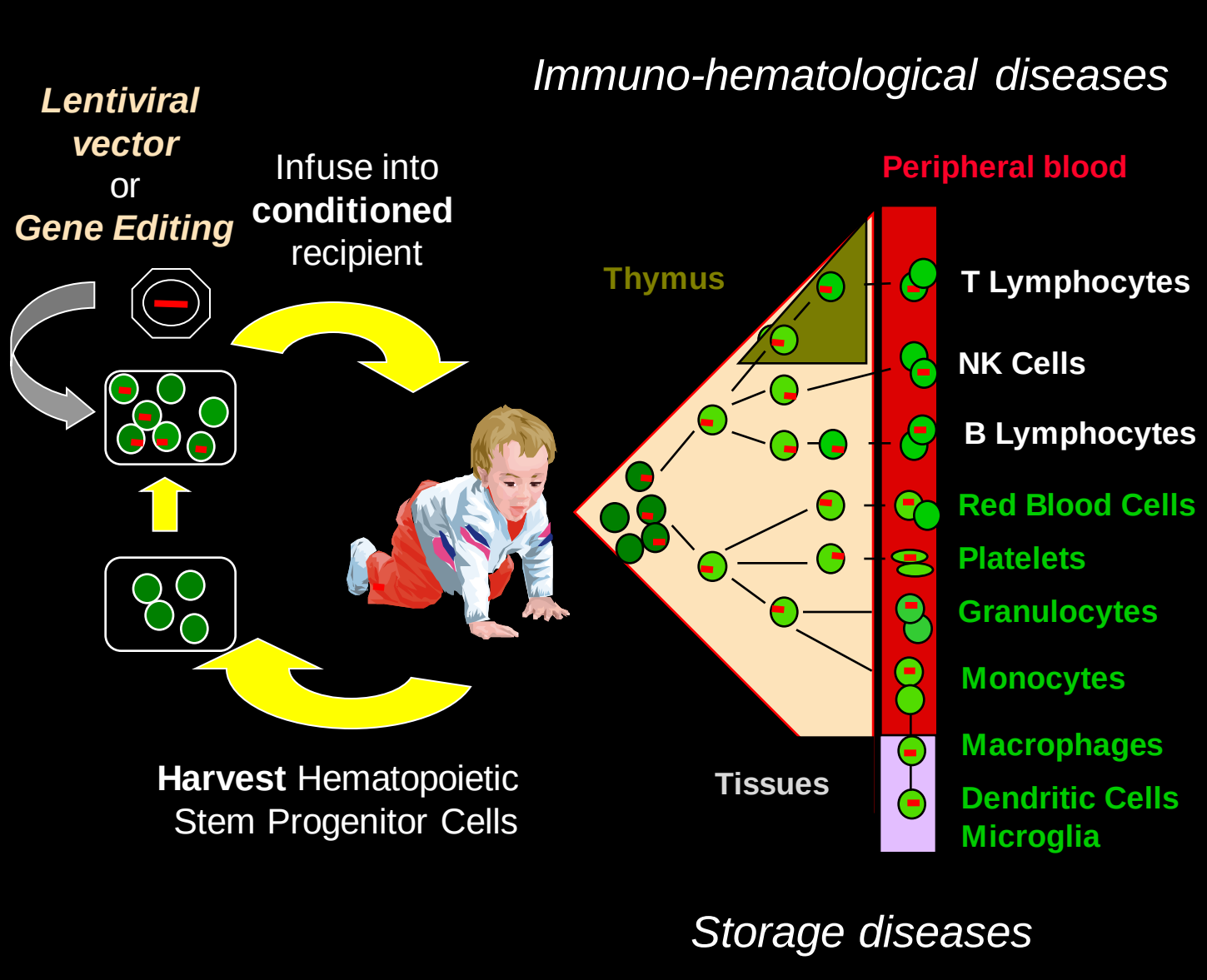
A New Medicine for the 3rd Millenium



A New Medicine for the 3rd Millenium

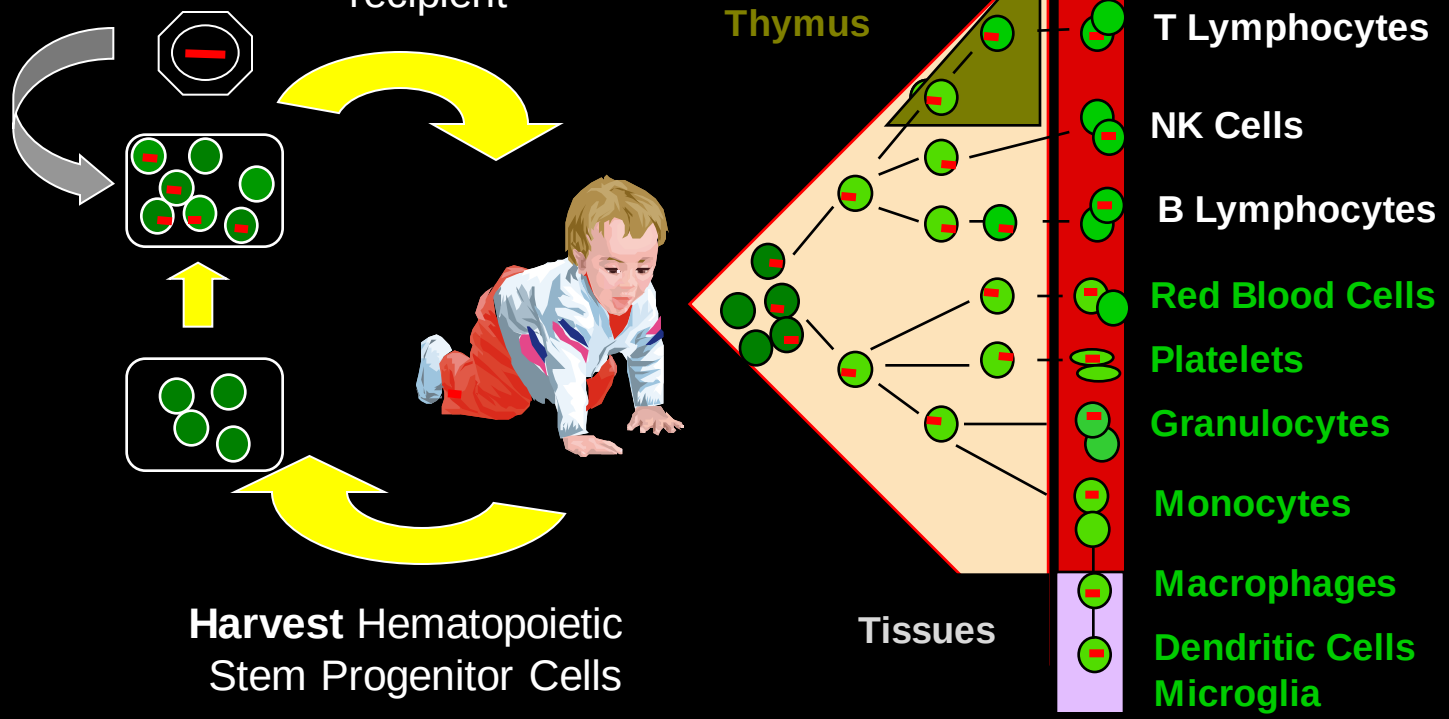
- *New technologies for transferring and editing genes (Gene Therapy)*
- *Effective strategies to isolate and transplant stem cells (Cell Therapy)*
- *Improved manipulation of biological weapons of immunity (Immunotherapy)*
- *Allow to design new therapies for severe to lethal diseases*

HSC Gene Therapy



- *Achieve efficient gene transfer / editing in HSC*
  - Preserving HSC properties
  - Ensure adequate yield
- *Overcome innate cell responses*
  - to incoming viruses & nucleic acids
- *Alleviate risk of genotoxicity*
  - Semi-random vector insertion (*gene transfer*)
  - Off-target DNA breaks, large deletions, translocations, LOH, chromotripsis (*gene editing*)
  - may activate oncogenes or inactivate tumor suppressors
- *Regulate transgene expression*
  - Ectopic, excess or constitutive expression may be toxic

HSC Gene Therapy: Results with Lentiviral Gene Transfer



Immuno-hematological diseases

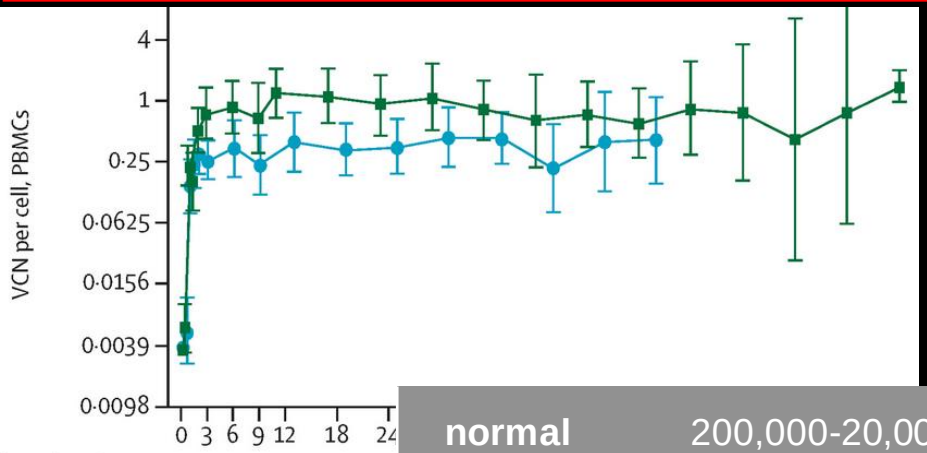
Storage diseases

Mostly safe with substantial & durable benefits in >500 pts, up to 14 yr follow-up

- Wiskott-Aldrich Syndrome, X-SCID, CGD Artemis, LAD, RAG-1 deficiency
- Adrenoleukodystrophy, Metachromatic Leukodystrophy, MPS-I, MPS-IIIA,
- β-Thalassemia, Sickle Cell Disease, Fanconi's Anemia, PKD…
- Gaucher, Fabry's, Osteopetrosis, Cystinosis…

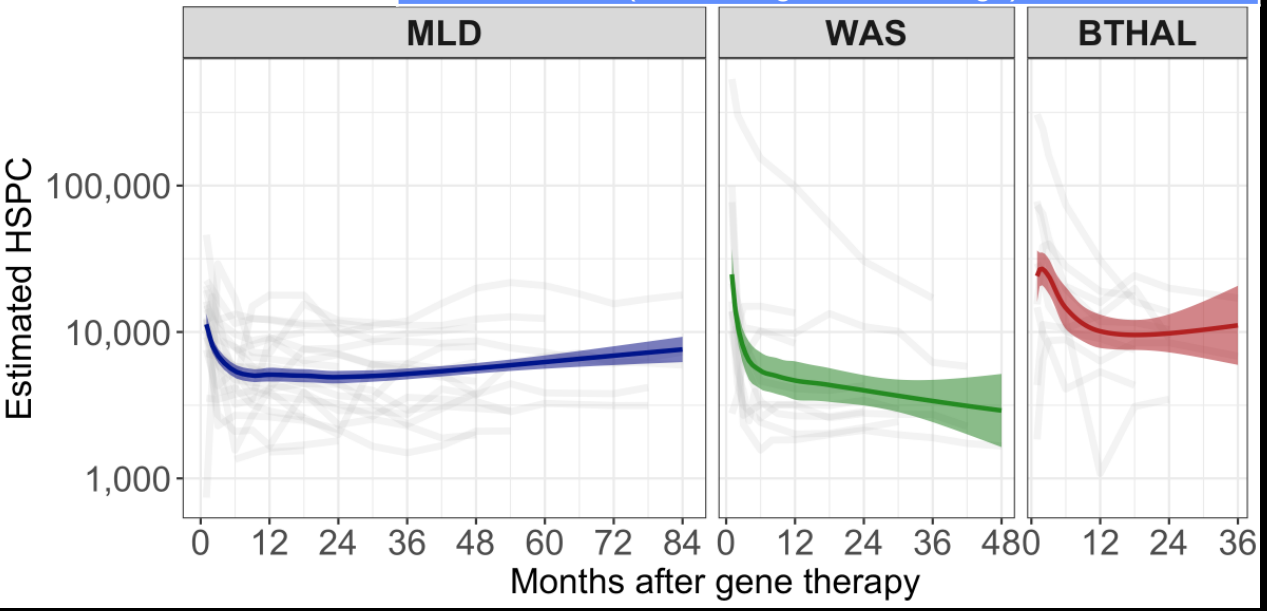
- *Approved therapies*
  Zynteglo (Bthal), Skysona (ALD),
  Lyfgenia (SCD)
  Libmeldy / Lenmeldy (MLD)

Stable Long-Term Polyclonal Reconstitution by LV-transduced HSC



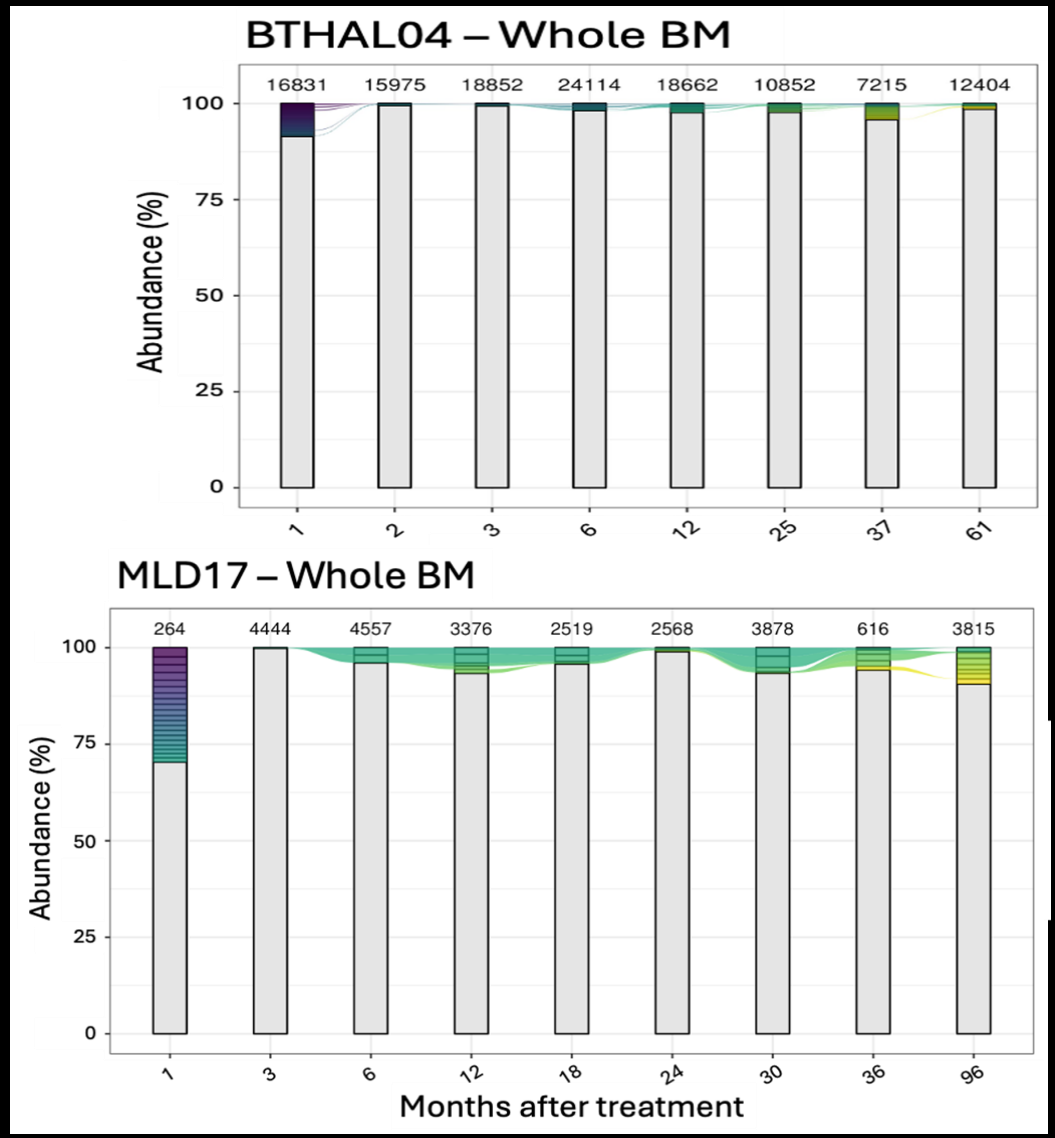
Fumagalli, Calbi & Aiuti
Lancet 2022

normal	200,000-20,000	*Mitchell et al., Nature 2022*
Allo-HCT	30,000-5,000 (decreasing with donor age)	*Chapman et al., Nature 2024*



Calabria… & Montini, Nature, 2024

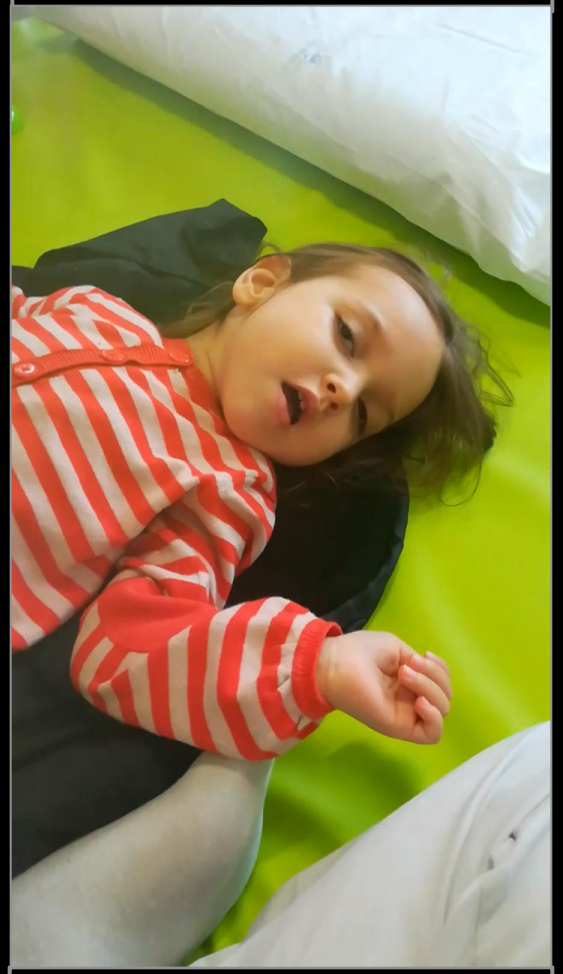
Clonal contribution



Grey area comprises clones contributing ≤1% in MLD, ≤0.1% in β-Thal

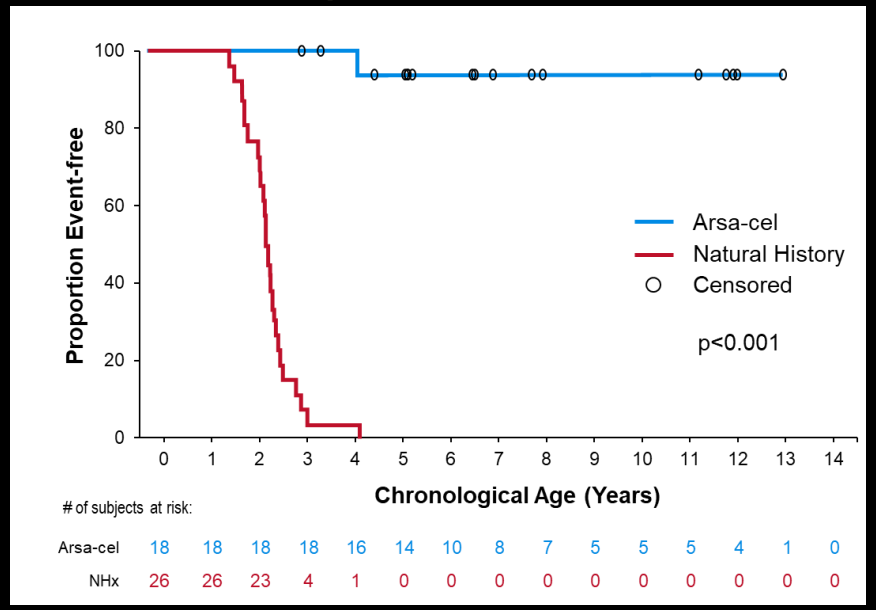
MLD Gene Therapy by Arsa-cel: Clinical Benefits

Untreated LI NHx

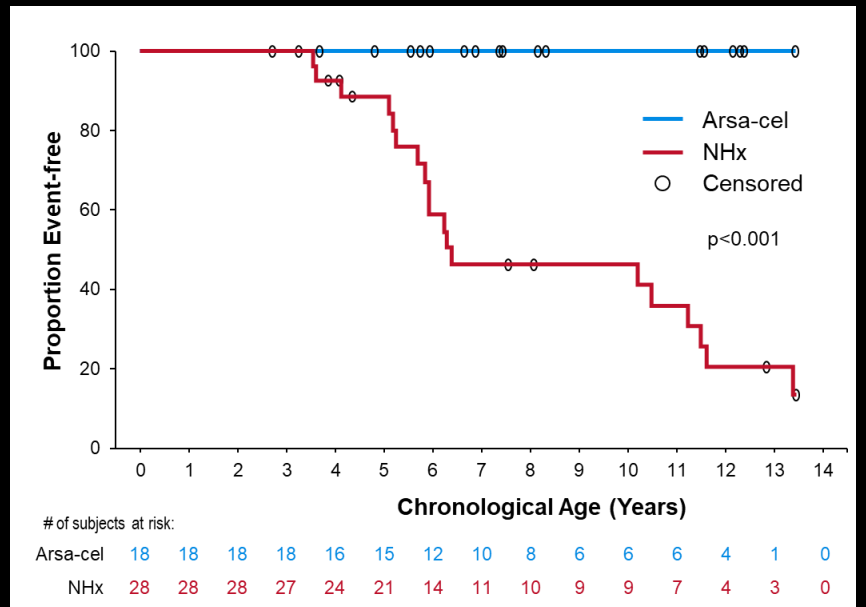


3 years 10 months of age
GMFC-MLD Level 6
2 years 7 months post-onset

Motor Impairment Free Survival



Overall Survival



Fumagalli, Calbi et al. Lancet 2022; Fumagalli et al. N Engl J Med, 2025

The Current Outlook for HSC Gene Therapy

- *May become preferred to allogenic HSC transplant in genetic diseases*
  - Available to every patient
  - Abrogates risk of graft vs host disease and rejection
  - Mixed chimerism sufficient for full benefit
  - Enhanced benefit by increased gene dosage

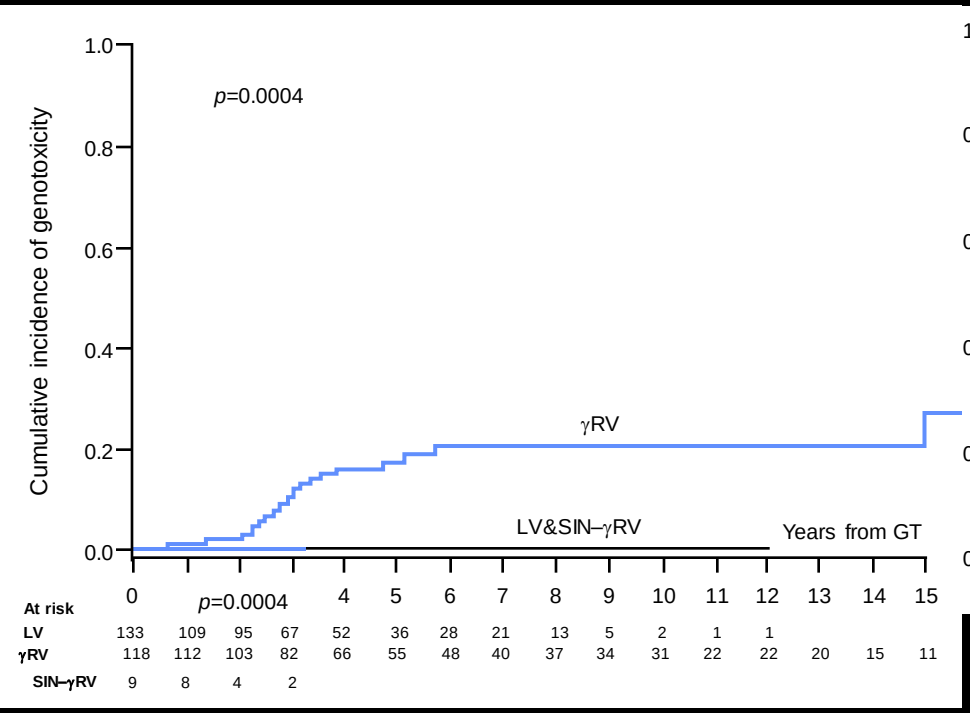
- *Outstanding challenges*
  - Need for toxic conditioning
  - Vector & cell manufacturing

- *Concerns (long-term)*
  - Residual genotoxic risk of engineering
  - Long-term stability and clonal composition of engineered graft

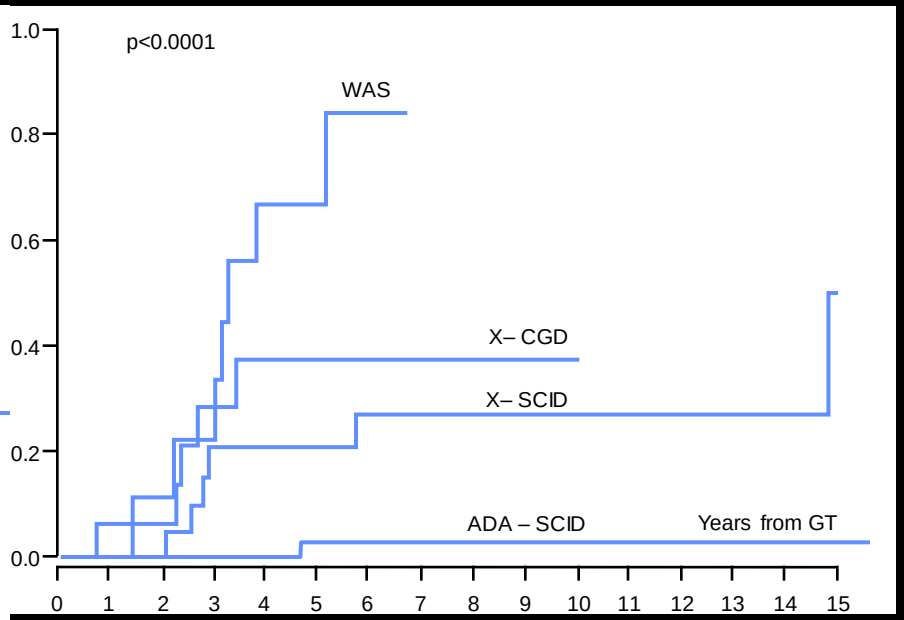
Insertional Genotoxicity May Trigger Leukemia Development

Leukemia incidence in HSC GT

Stratified by vector type



Stratified by disease using γRV



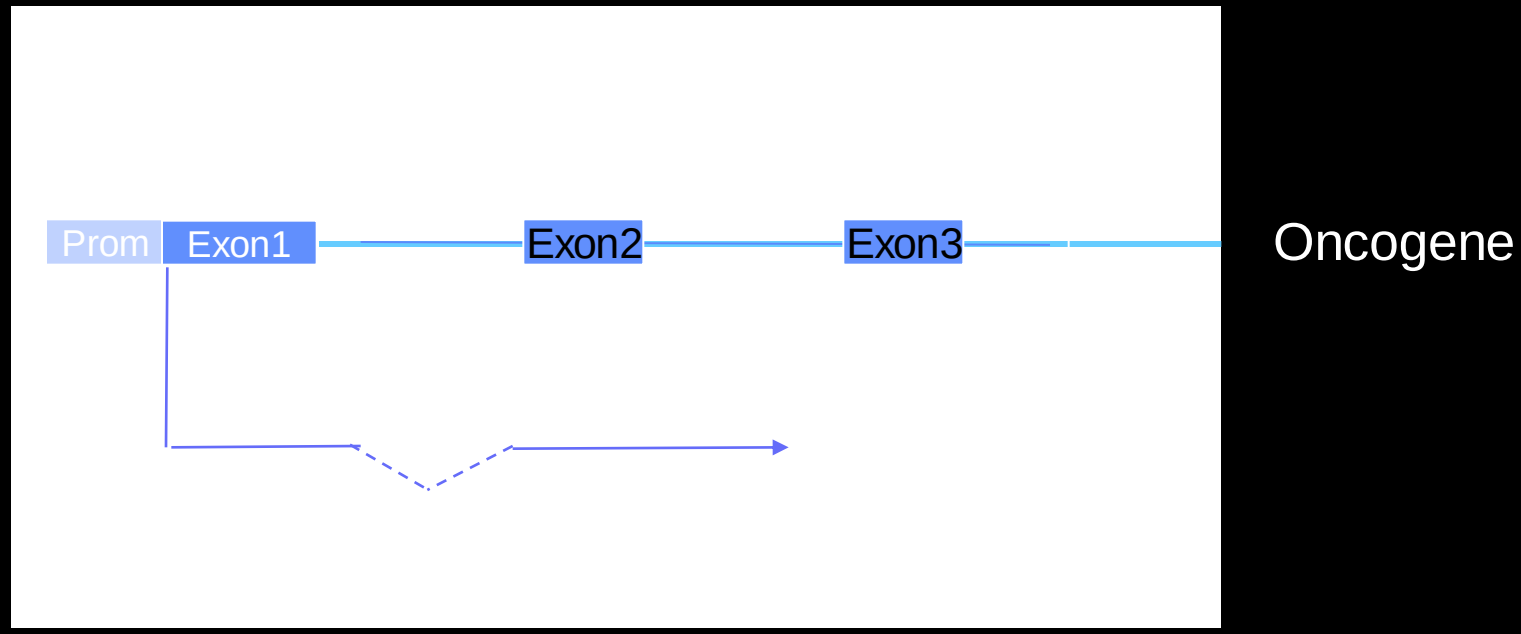
Risk factors

- *Vector type: γRV vs SIN LV*
- *Disease background*
  - *Transgene function*
  - *BM inflammation*

Tucci et al. *Nat Commun* 2022

Insertional Genotoxicity & Leukemogenesis

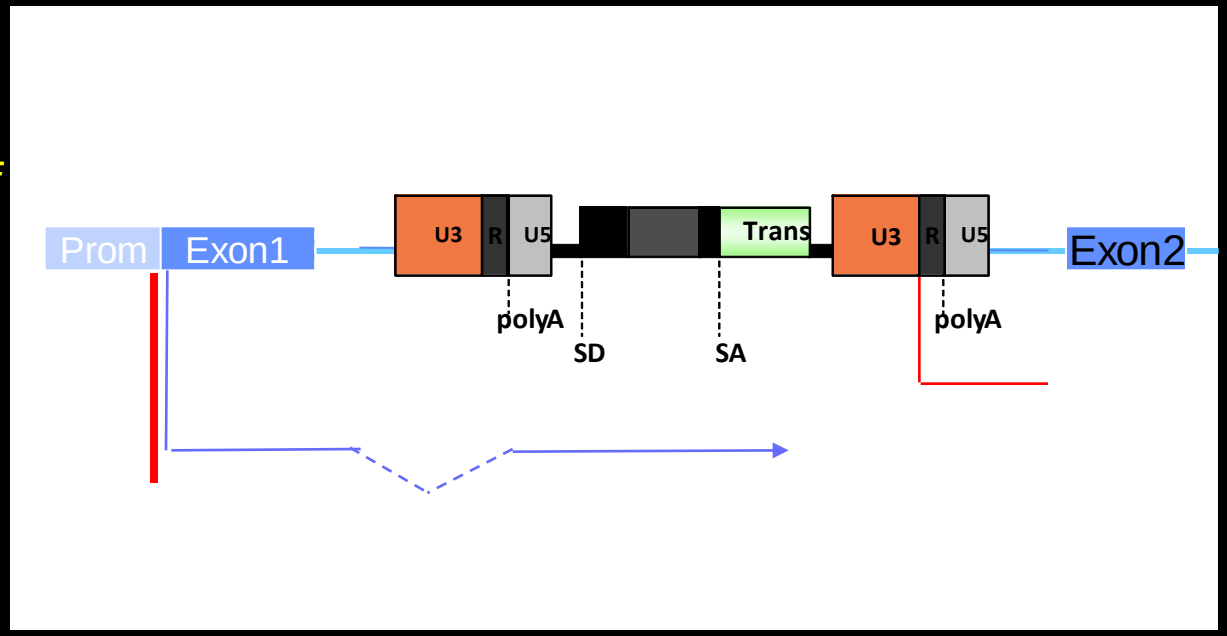
- *Random vector integration near cancer gene may activate its oncogenic potential*



Insertional Genotoxicity & Leukemogenesis

- *γ-retroviral vector*

Transcriptional trans-activation Upregulation of transcript

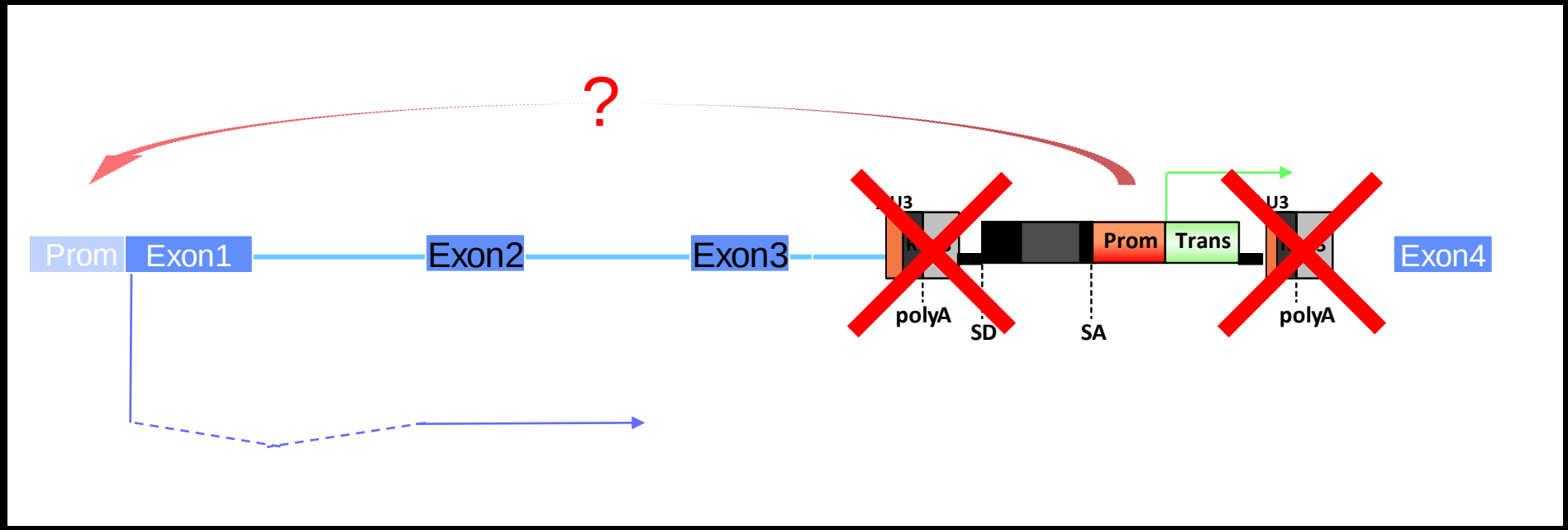


Promoter insertion Splicing capture Upregulation of truncated transcript

Oncogene

- *Insertional bias for promoter & growth-related genes*
- *Strong enhancer promoter in LTR*

Lower Genotoxicity of Lentiviral Vectors



- *Transcriptionally inactive (self-inactivating, SIN) LTR*
- *Insertional bias for body of expressed genes*

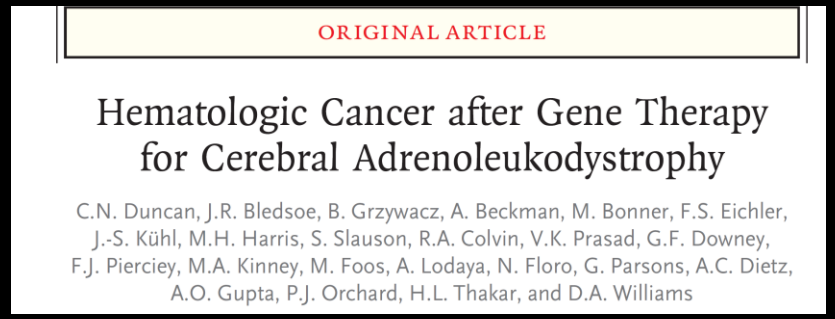
Lower Genotoxicity of Lentiviral Vectors

Table 1. Blood-Cell Diseases Treated with Lentiviral Vectors and Their Transcriptional Regulatory Elements.*

Disease Targeted	Regulatory Element
Cerebral adrenoleukodystrophy	MND retroviral long-terminal repeat
Metachromatic leukodystrophy	Phosphoglycerate kinase gene promoter
β-thalassemia	β-globin locus control region and promoter
Sickle cell disease	β-globin locus control region and promoter
ADA-deficient SCID	EFS promoter
X-linked SCID	EFS promoter
Artemis SCID	*DCLRE1C* gene promoter
Rag-1 SCID	MND retroviral long-terminal repeat
Wiskott–Aldrich syndrome	*WASP* gene promoter
Chronic granulomatous disease	Cathepsin G/FES chimeric myeloid promoter
MPS I (Hurler's syndrome)	Phosphoglycerate kinase gene promoter
MPS III A (Sanfilippo A syndrome)	*ITGAM* gene promoter
Fabry's disease	EFS promoter
Fanconi anemia A	Phosphoglycerate kinase gene promoter
Leukocyte adhesion deficiency I	Cathepsin G/FES chimeric myeloid promoter
Pyruvate kinase deficiency	Phosphoglycerate kinase gene promoter
Cystinosis	EFS promoter
Hemophilia A	*CD68* gene promoter

Currently > 500 patients

- No malignancies reported, except:


ORIGINAL ARTICLE

Hematologic Cancer after Gene Therapy for Cerebral Adrenoleukodystrophy

C.N. Duncan, J.R. Bledsoe, B. Grzywacz, A. Beckman, M. Bonner, F.S. Eichler, J.-S. Kühl, M.H. Harris, S. Slauson, R.A. Colvin, V.K. Prasad, G.F. Downey, F.J. Pierciey, M.A. Kinney, M. Foos, A. Lodaya, N. Floro, G. Parsons, A.C. Dietz, A.O. Gupta, P.J. Orchard, H.L. Thakar, and D.A. Williams

- *7 MDS/AML out of 67 patients in ALD GT (Duncan et al., NEJM 2024)*
  - *Malignancies bear LV insertion in MECOM-EVI1 or PRDM16 leading to transcriptional upregulation of truncated oncogenic form*
  - *Clones bearing insertions in MECOM-EVI1 or PRDM16 enriched in patients with some expanding over time*
  - *Most likely dependent on unique LV design with strong γ-retroviral promoter (MND) within vector (Kohn, Booth, Naldini, N Engl J Med. 2024; Montini et al., Mol Ther 2025)*

Adressing the Genotoxicity Risk of Gene Transfer

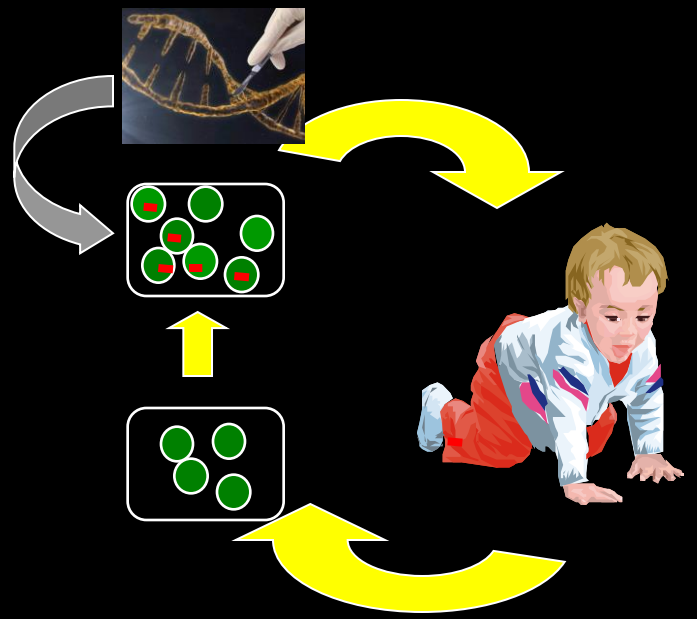
- *Genotoxicity becoming better understood as emerging from interplay of*
  - Insertion site that can unequivocally implicate or exonerate the vector
  - Vector design (promoter strength, cryptic transcriptional signals)
  - Manufacturing process yield
  - Disease background and prior drug exposure
- *Emerging tumorigenesis in HSC gene therapy for SCD and in CART*
  - Mostly ascribed to preexisting mutations in harvested cells
  - May instruct safer deployment (pre-screening for at-risk mutations, i.e. CHIP).
  - No standard pre-clinical test would have captured this
- *We have learned much more form clinical than pre-clinical testing*
  - Start clinical testing with appropriate risk-benefit balance
  - Address emerging risks

Next Generation HSC and T Cell Gene Therapy

Ex vivo Gene Addition

(beyond lentiviral gene transfer)

- Alleviate residual concerns for genome-wide insertional mutagenesis
- Improve transgene expression
  - Consistency
  - Rescue physiological control



Infuse back into patient

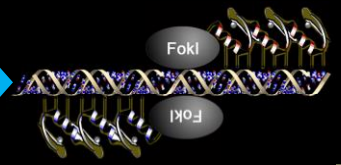
Harvest HSPC

or T cells

(Designer Endo-)Nuclease Mediated Targeted Gene Editing

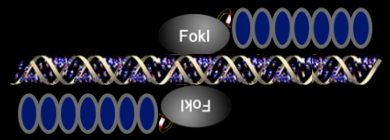
Transient expression by
RNA or RiboNucleoProtein
electroporation
Lipid NanoParticles

ZFNs

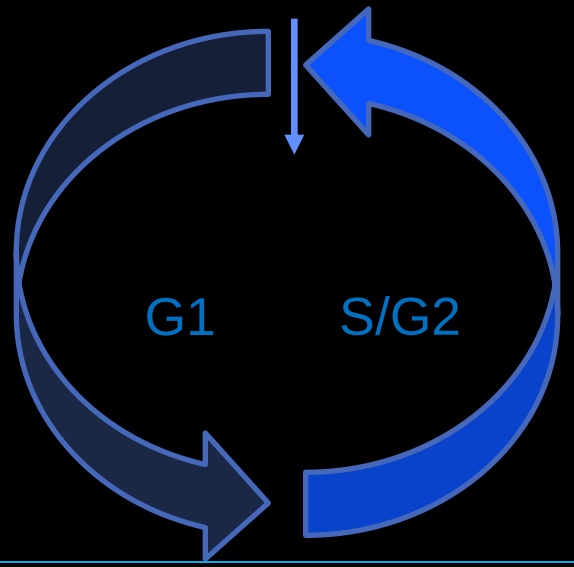


CRISPR/Cas

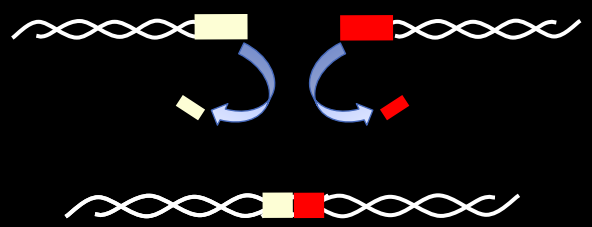
TALENs

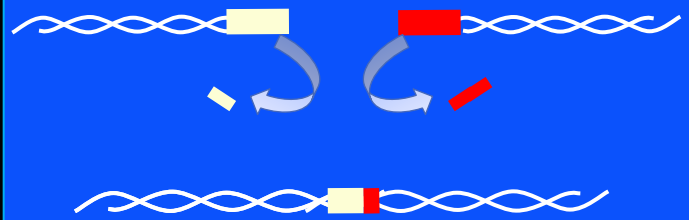


DNA DSB at target site



Repair by *NHEJ*
Non Homologous
End Joining
↓
Loss of Function



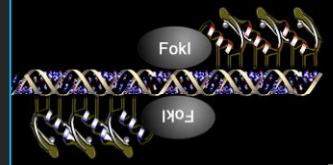


Repair by *MMEJ*
Microhomology Mediated
End Joining
↓
Loss of Function

Clinical Applications of NHEJ-Mediated Gene Disruption

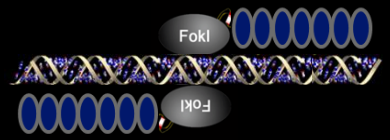
- *Unique application*
- *achieved at high efficiency, also biallelic*
- *multiplexing feasible*
- *DDR impacts cell growth*
- *Genotoxic risk*
- circumscribed to on & off nuclease targets
- deletions, translocations, loss of chromosome arm, LOH, chromotripsis

ZFNs

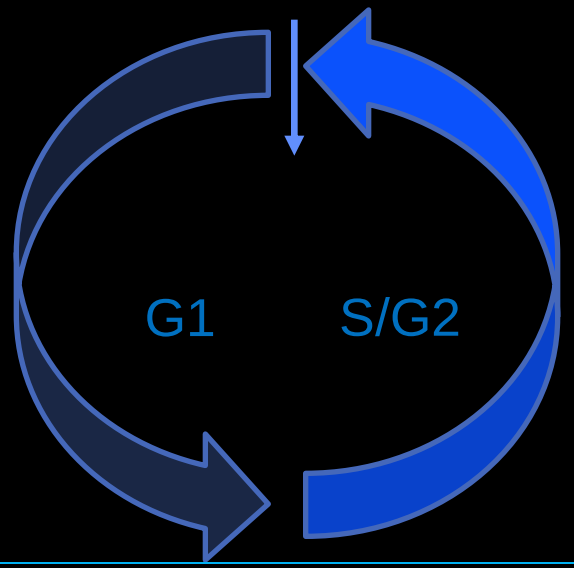


CRISPR/Cas

TALENs



DNA DSB at target site

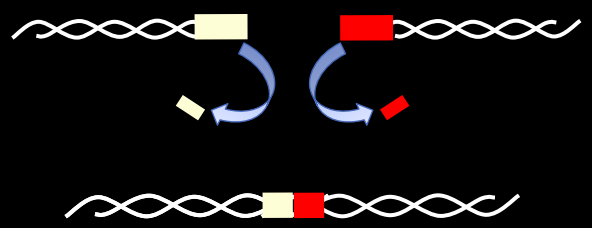


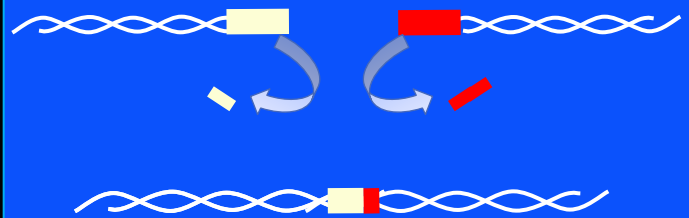
- *Sickle Cell disease, β-thalassemia*

rescue of fetal Hb by disrupting γ-globin repressor expression (erythroid enhancer) (FDA/EMA approved Casgevy by Vertex)

>100 pts, up to 4 yr follow-up

Repair by *NHEJ*
Non Homologous End Joining
↓
Loss of Function





Repair by *MMEJ*
Microhomology Mediated End Joining
↓
Loss of Function

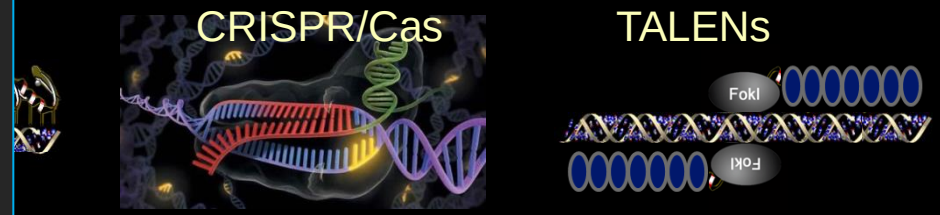
gRNA-dependent Specificity & Genotoxicity Assessment

	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%

Targeted Gene Editing by Homology Directed Repair (HDR)

Uniquely allows gene-size editing

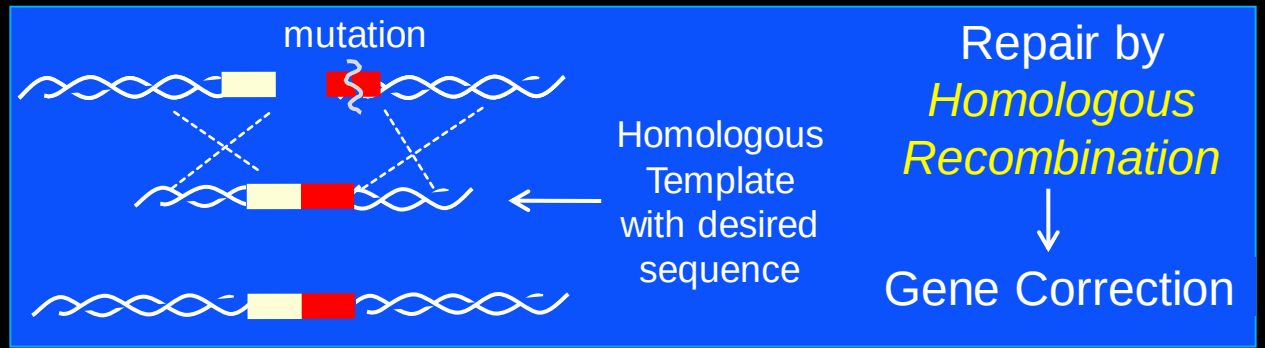
- *In situ gene correction*
  - Restore function and expr. control
- *Targeted integration*
  - of transgene/expression cassette into genomic safe harbour
- *Genotoxic risk*
  - On/Off nuclease targets
  - potential for large deletions, translocations..
  - Trapping of template at induced & spontaneous DNA breaks
- *constrained in HSC*
- *need for template codelivery*



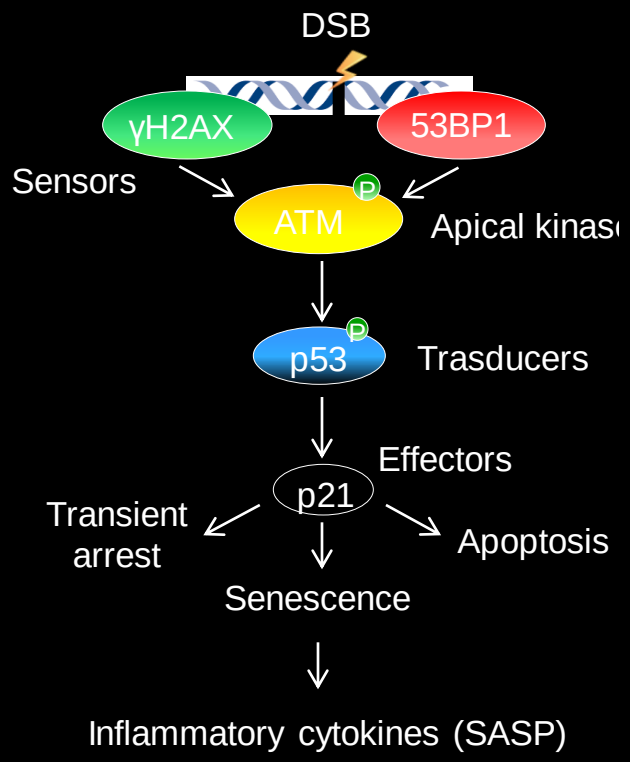
DNA DSB at target site

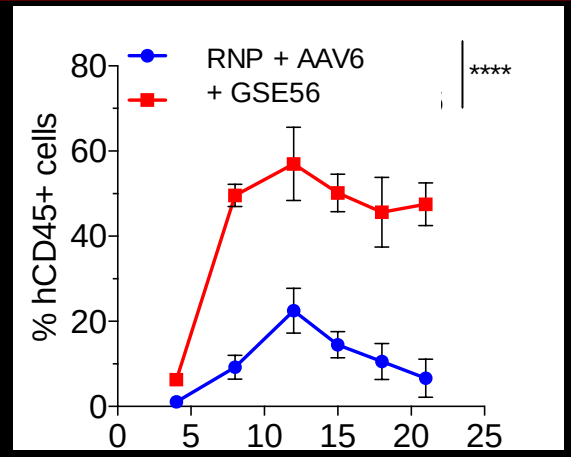
S/G2

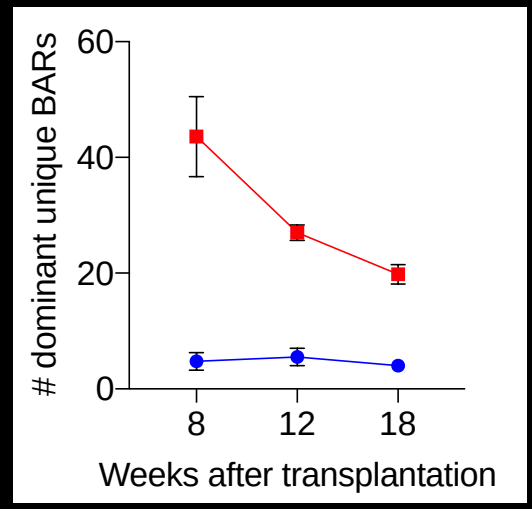
Co-delivery of
- *Donor template by AAV6, IDLV, oligonucleotide*



Optimizing HSPC Gene Editing







Ferrari, Jacob et al., Nat Biotech, 2020

Schiroli & Conti et al, Cell Stem Cell 2019

Cell Stem Cell
Article
CellPress

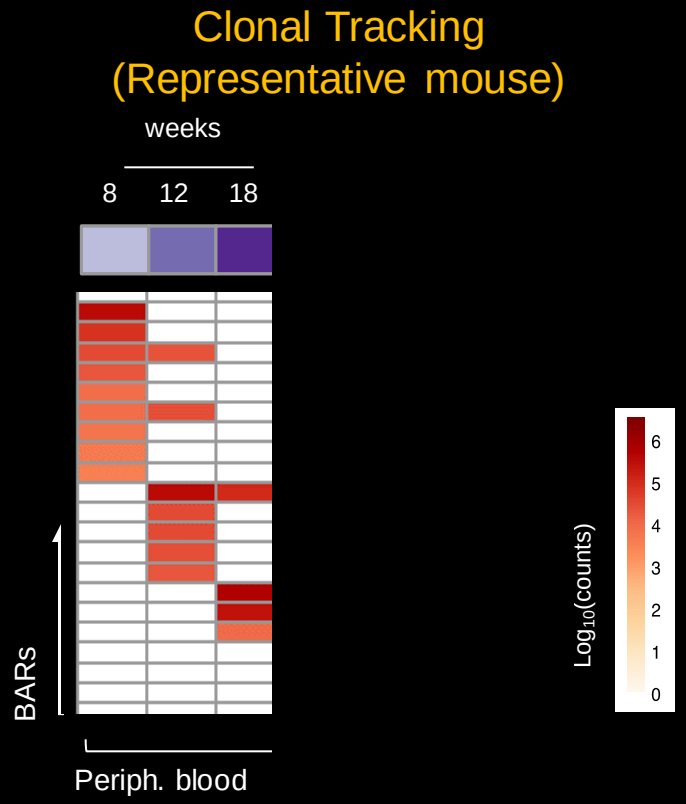
Precise Gene Editing Preserves Hematopoietic Stem Cell Function following Transient p53-Mediated DNA Damage Response

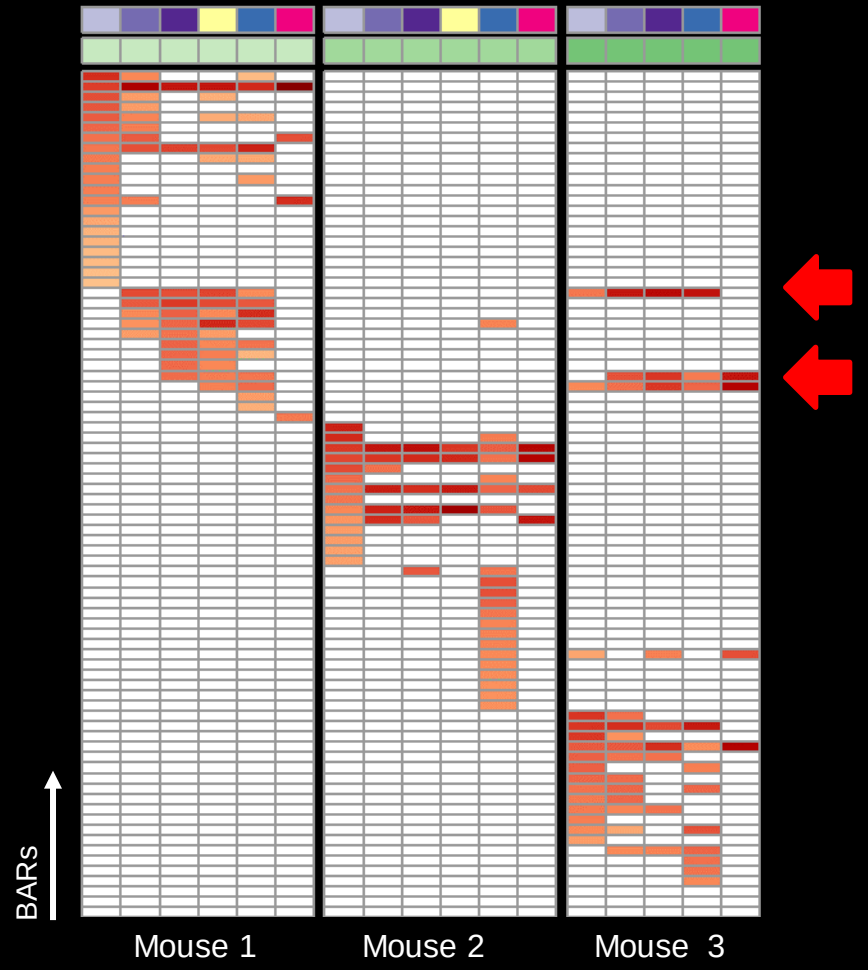
ARTICLES
https://doi.org/10.1038/s41587-020-0551-y
nature biotechnology

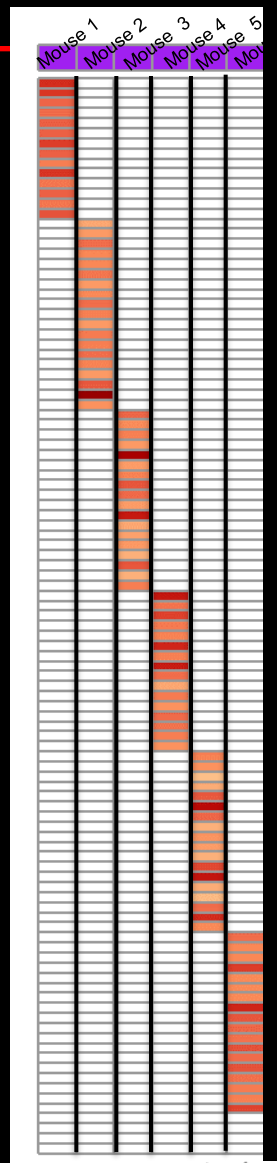
NATURE BIOTECHNOLOGY | VOL 38 | NOVEMBER 2020 | 1298–1308

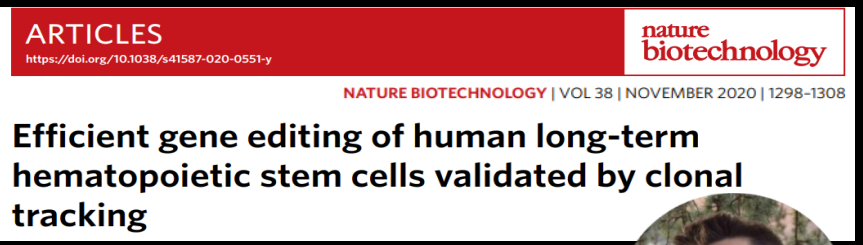
Efficient gene editing of human long-term hematopoietic stem cells validated by clonal tracking

Edited Repopulating HSPC Preserve Symmetric & Asymmetric Division





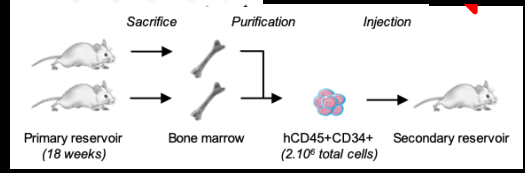




Ferrari & Jacob et al., Nat Biotech, 2020

Evidence for

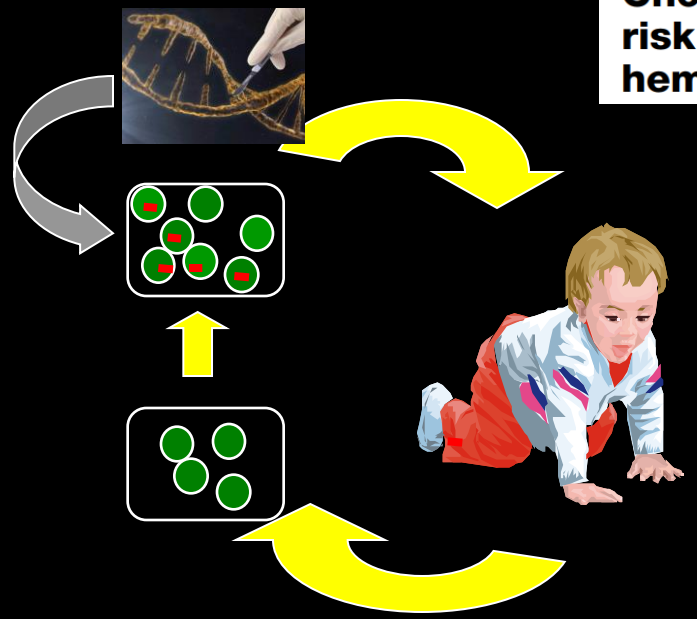
- *ex vivo symmetric* division



Optimizing ex vivo HSC and T Cell Gene Editing

Ex vivo Gene Addition by Targeted gene editing

- *Increase efficiency*
  - Inhibit (transiently) DDR
  - Template choice/delivery
  - Delivery (electroporation vs. LNP)
- *Assess genetic outcome at target site*
  - Occurrence of deletions & translocations
  - Heterogeneity of repair



Harvest HSPC

Ferrari , Jacob et al., Cell Stem Cell 2022

Cell Stem Cell

Clinical and Translational Report

Choice of template delivery mitigates the genotoxic risk and adverse impact of editing in human hematopoietic stem cells

Vavassori, Ferrari et al., Blood 2023

BLOOD

Regular Article

GENE THERAPY

Lipid nanoparticles allow efficient and harmless ex vivo gene editing of human hematopoietic cells

Canarutto et al., EMBO J., 2023

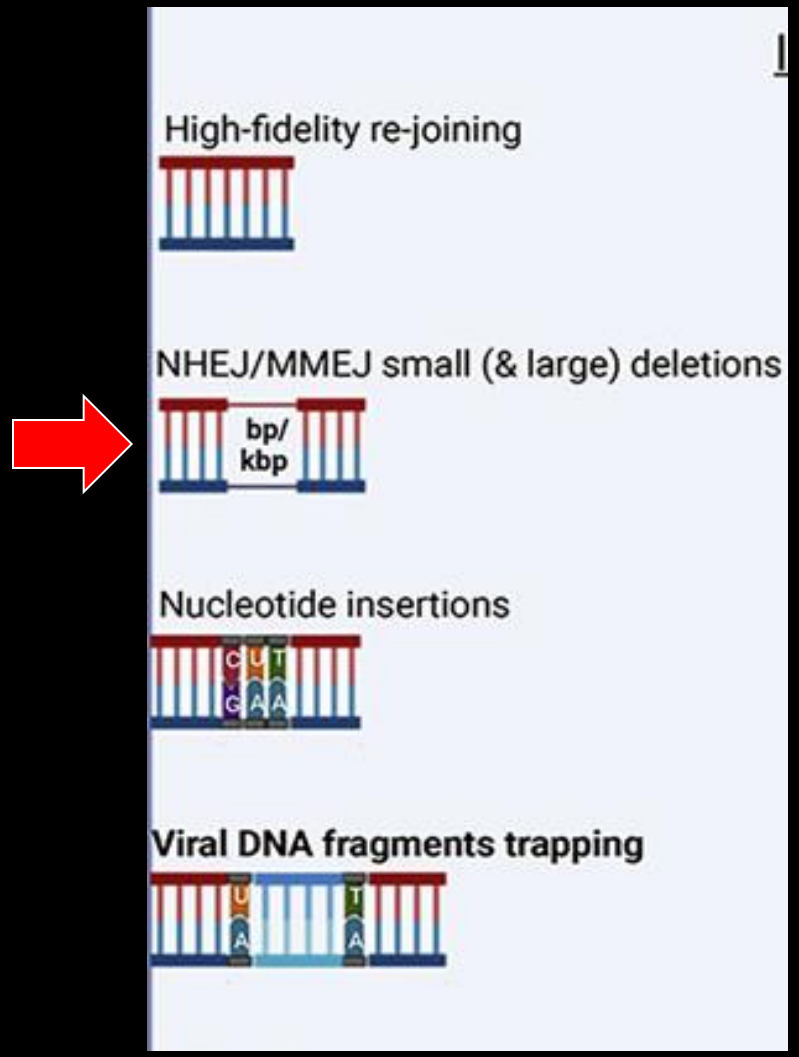
Article

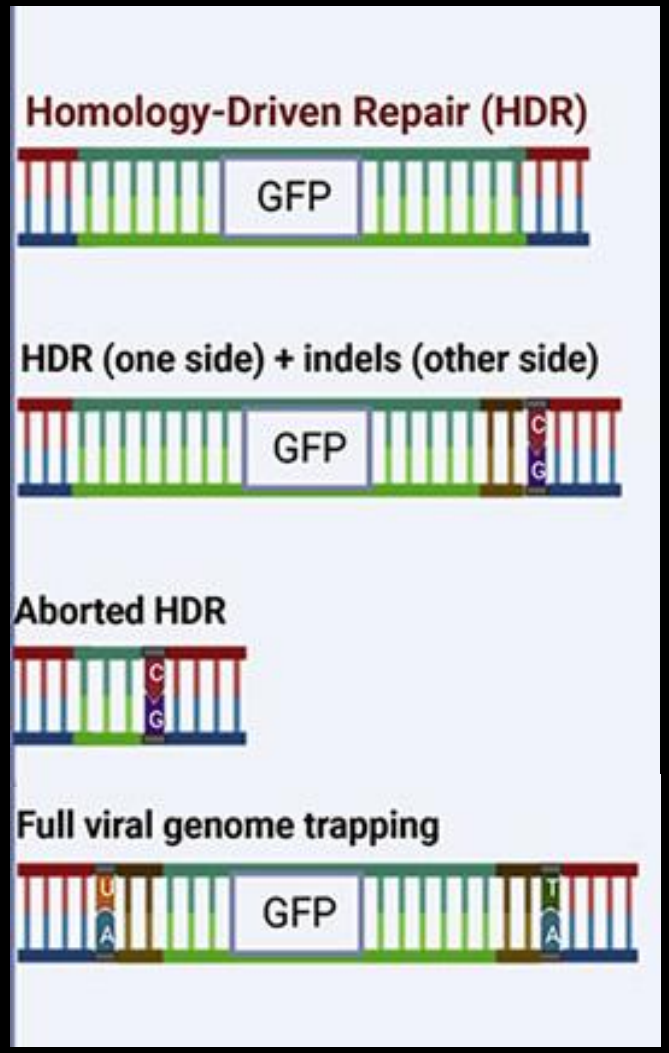
SOURCE DATA | TRANSPARENT PROCESS | OPEN ACCESS

THE EMBO JOURNAL

Unbiased assessment of genome integrity and purging of adverse outcomes at the target locus upon editing of $CD4^+$ T-cells for the treatment of Hyper IgM1

Heterogeneity of Edit Outcome at Target Site



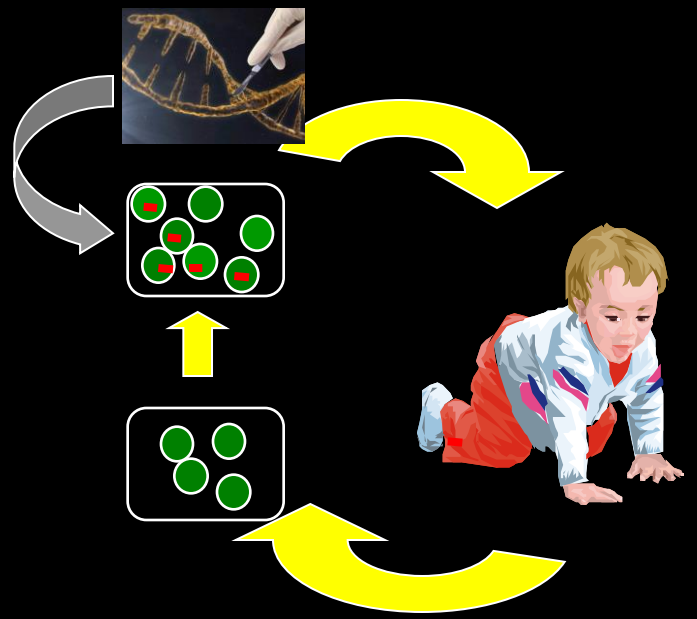


Ferrari, Jacob et al., Cell Stem Cell 2022

Toward Safer HDR-mediated HSC and T Cell Gene Editing

Ex vivo Gene Addition by Targeted gene editing

- *Increase efficiency*
- *Assess genetic outcome at target site*
  - Occurrence of deletions & translocations
  - Heterogeneity of repair
- *Select for intended edit*
- *Purge unwanted outcomes*



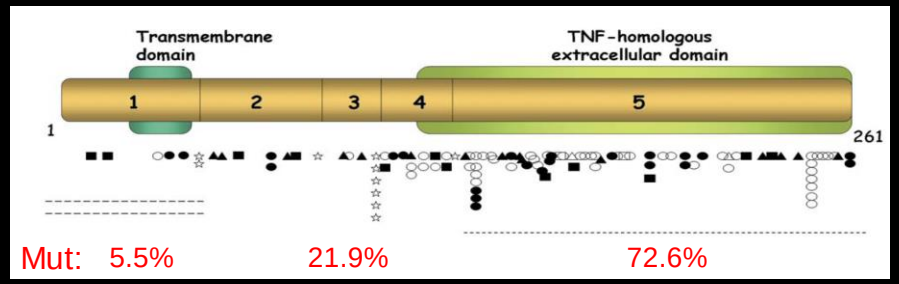
Harvest HSPC

A Case Study

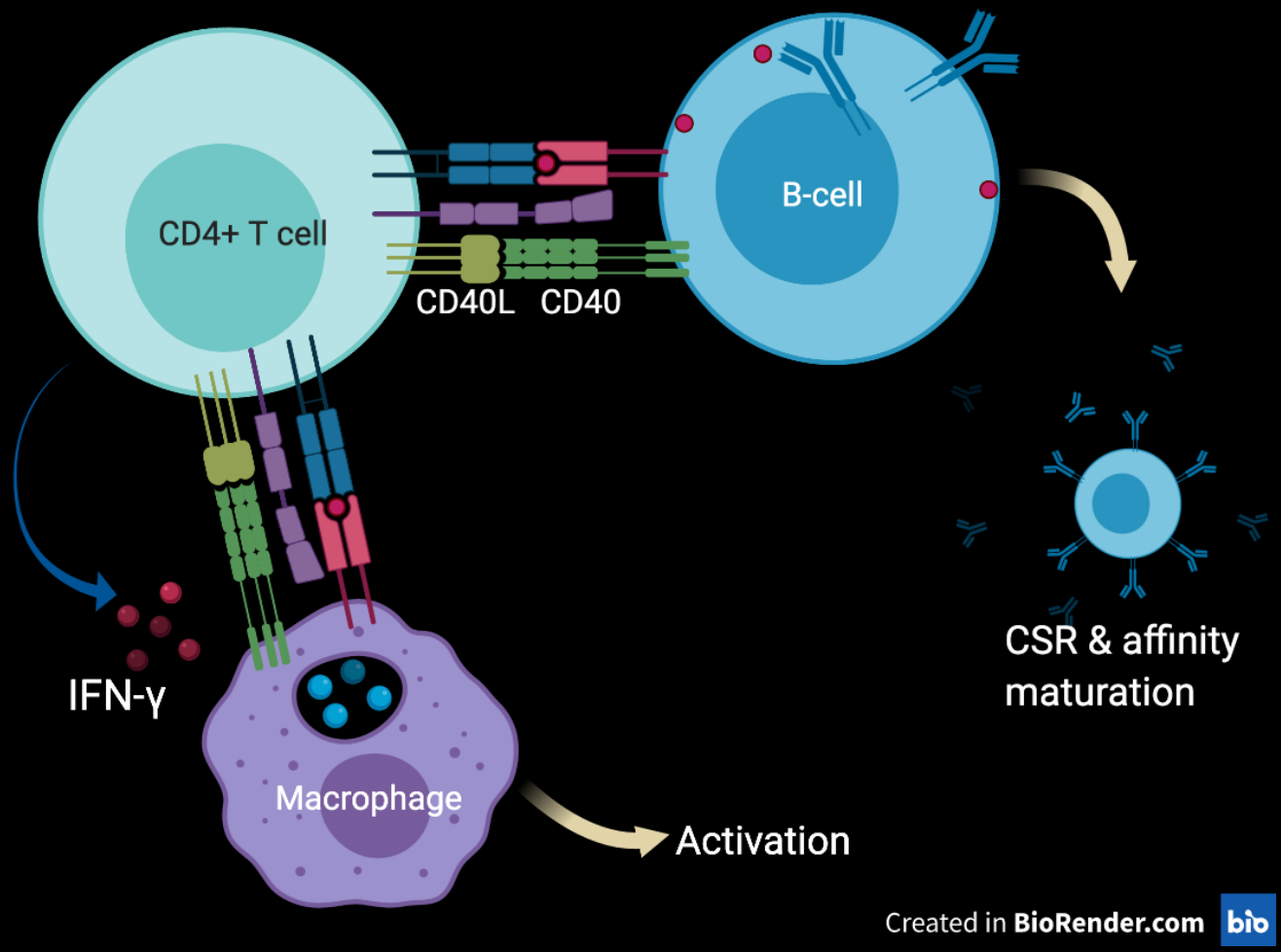
HDR Gene Editing
of CD40 Ligand Gene
in HIGM-1 CD4 T Cells

X-linked Hyper IgM Immunodeficiency (HIGM-1)

CD40LG Xq26.3



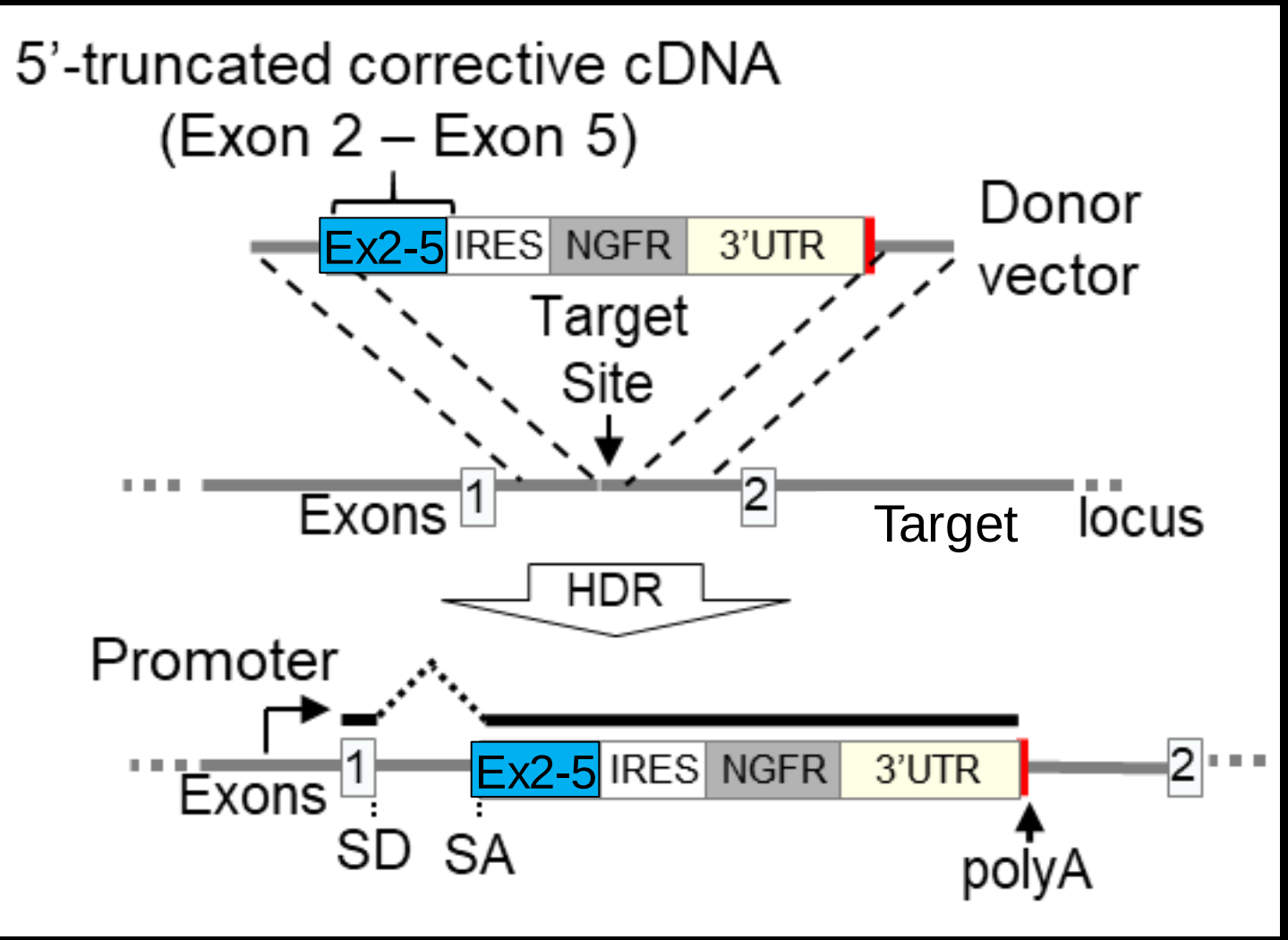
Expressed by activated CD4+ T cells



Gene Replacement studies in HIGM1 mouse model

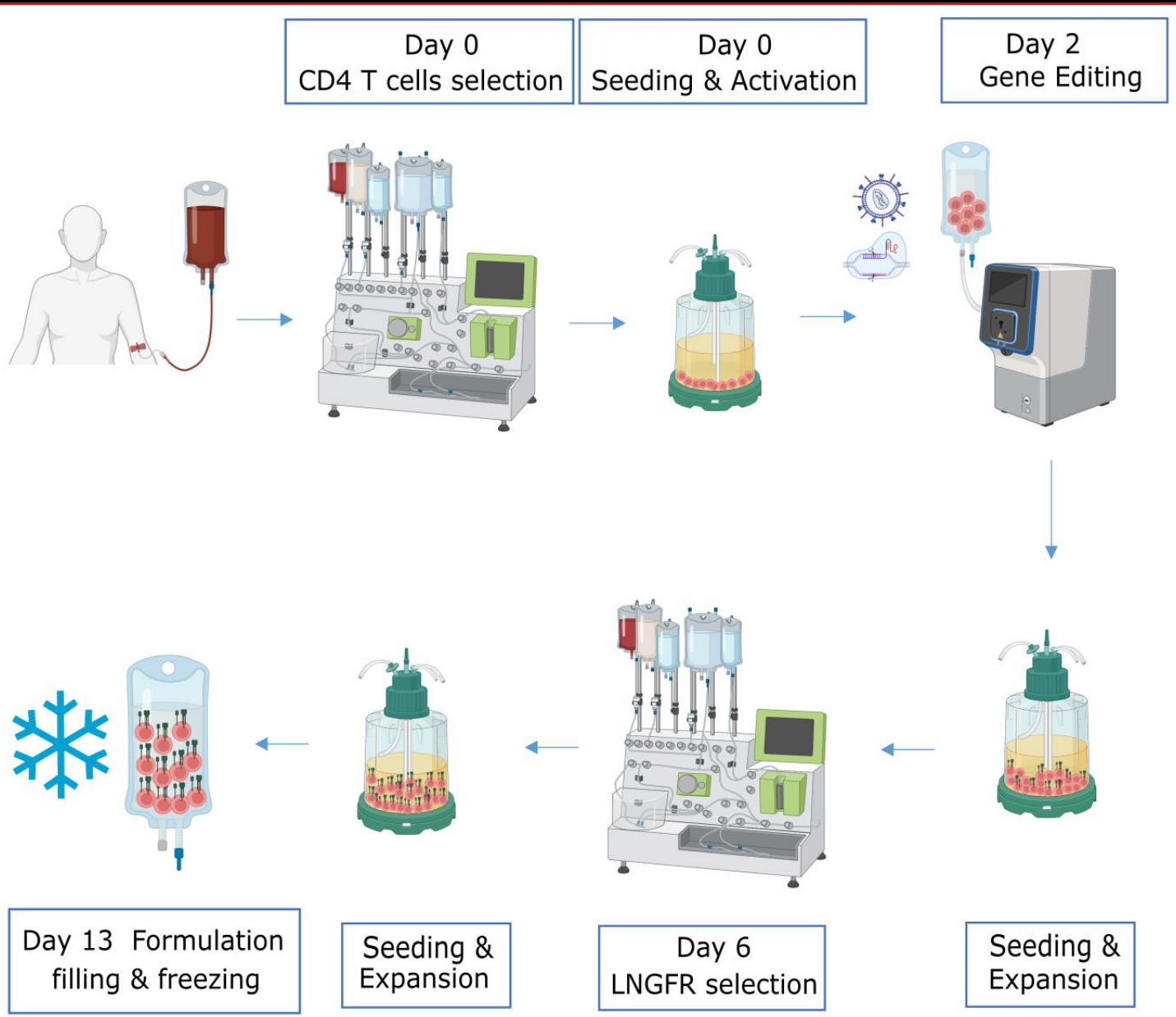
- Partial immune reconstitution from few ex-vivo corrected cells
- Abnormal T/B cells proliferation with constitutive CD40L expression

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

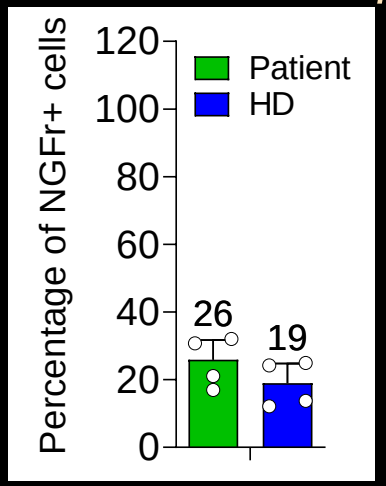


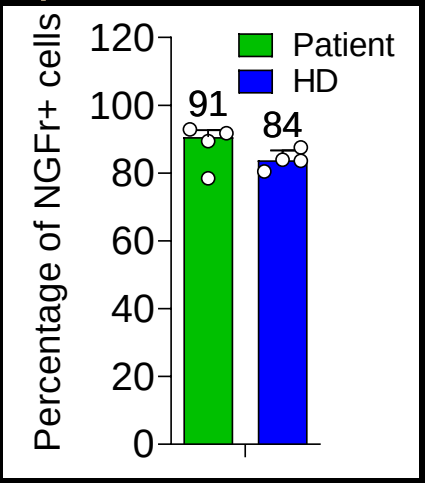
Knock-in of 5’-truncated corrective cDNA downstream endogenous promoter

GMP-Compliant Scaled Up CD4+ T cell Editing Process

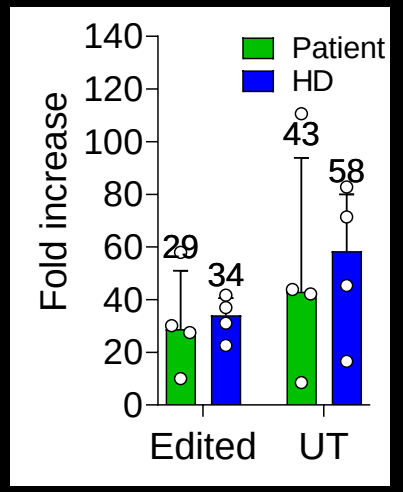


NGFR+ cells pre and post selection

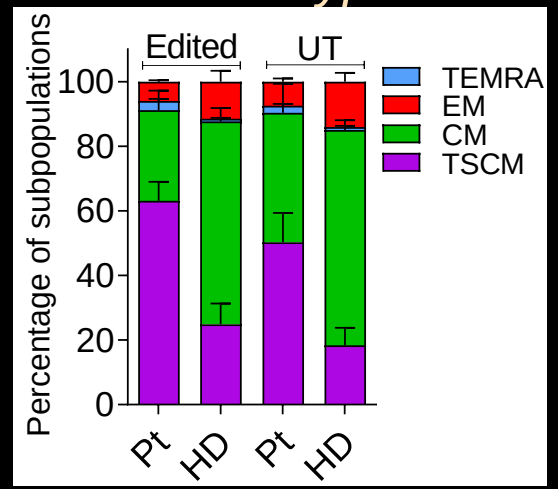




Fold increase



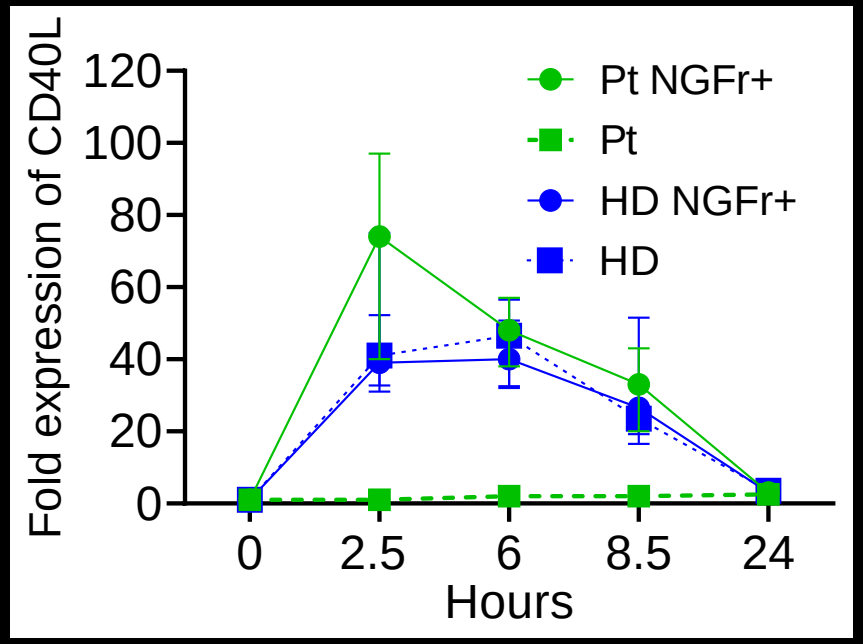
Phenotype



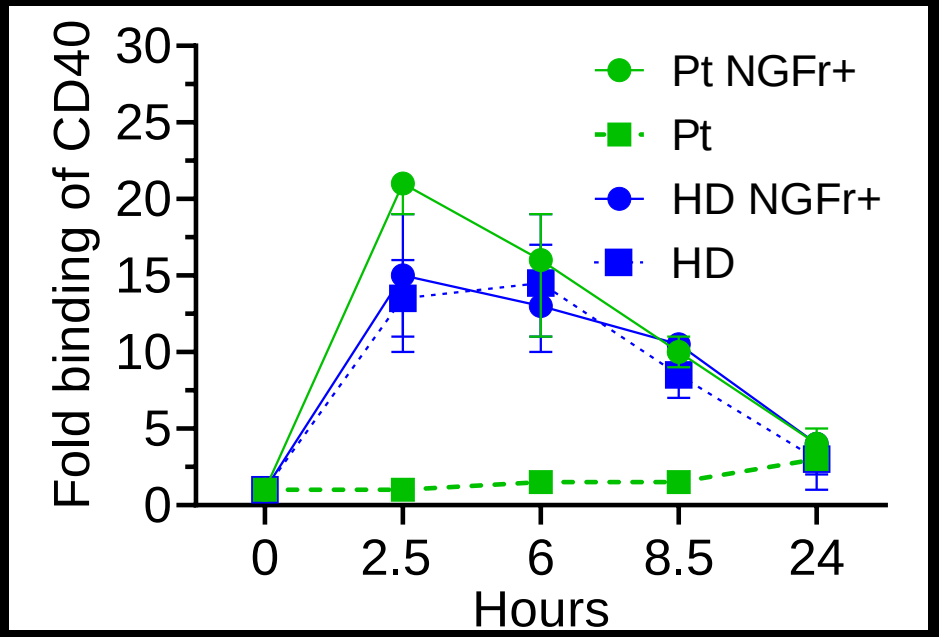
Asperti, Canarutto et al.,
Mol. Ther. Meth. & Clin. Dev., 2023

Rescue of CD40L Regulated Expression & Function in Edited Cells

CD40LG expression



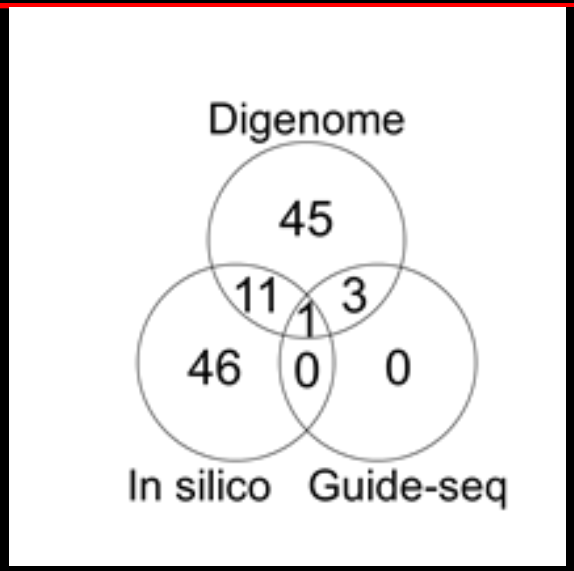
CD40LG binding of CD40



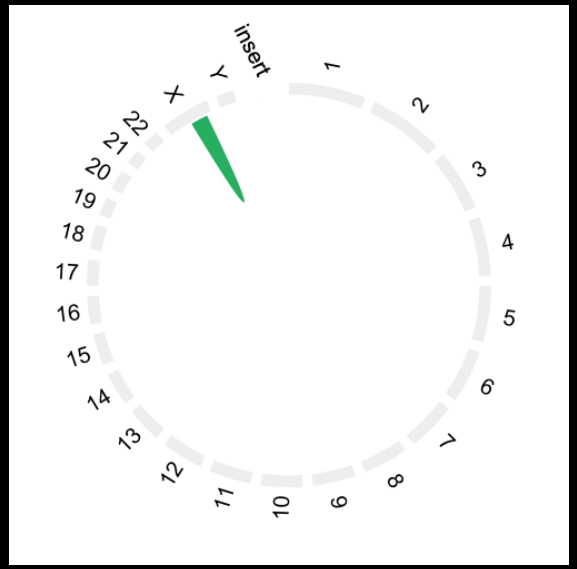
Patient samples courtesy of N. Gonzalez, V. Lougaris, A. Finocchi, Hyper IgM Foundation

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

Off-Target Analysis: Consensus ≥2 Orthogonal Methods



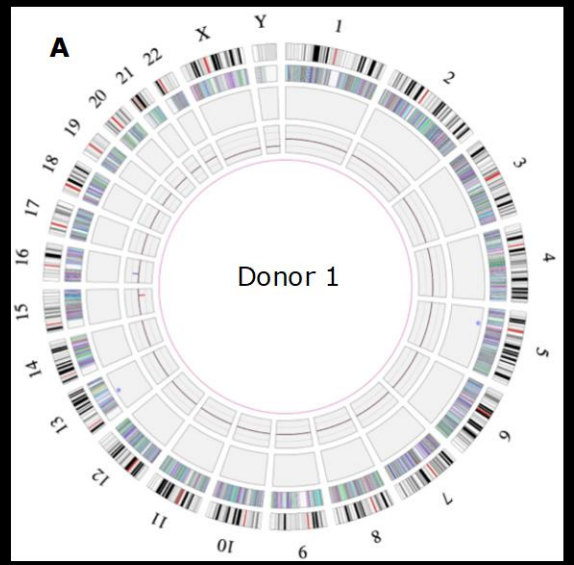
CAST-Seq

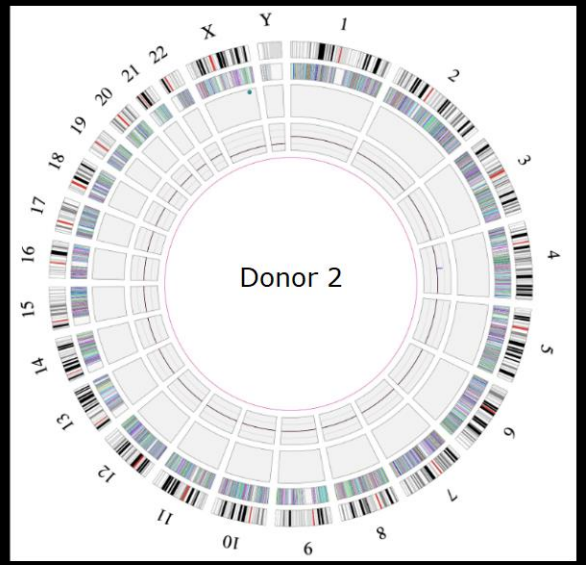


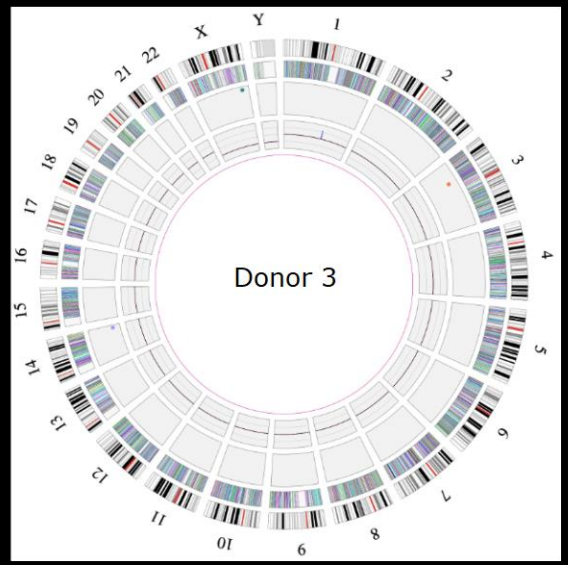
Sensitivity:
Guide-Seq 0.1%
Cast-Seq 0.01%
NGS 0.01%

In collab with Cathomen Lab

Optical Genome Mapping

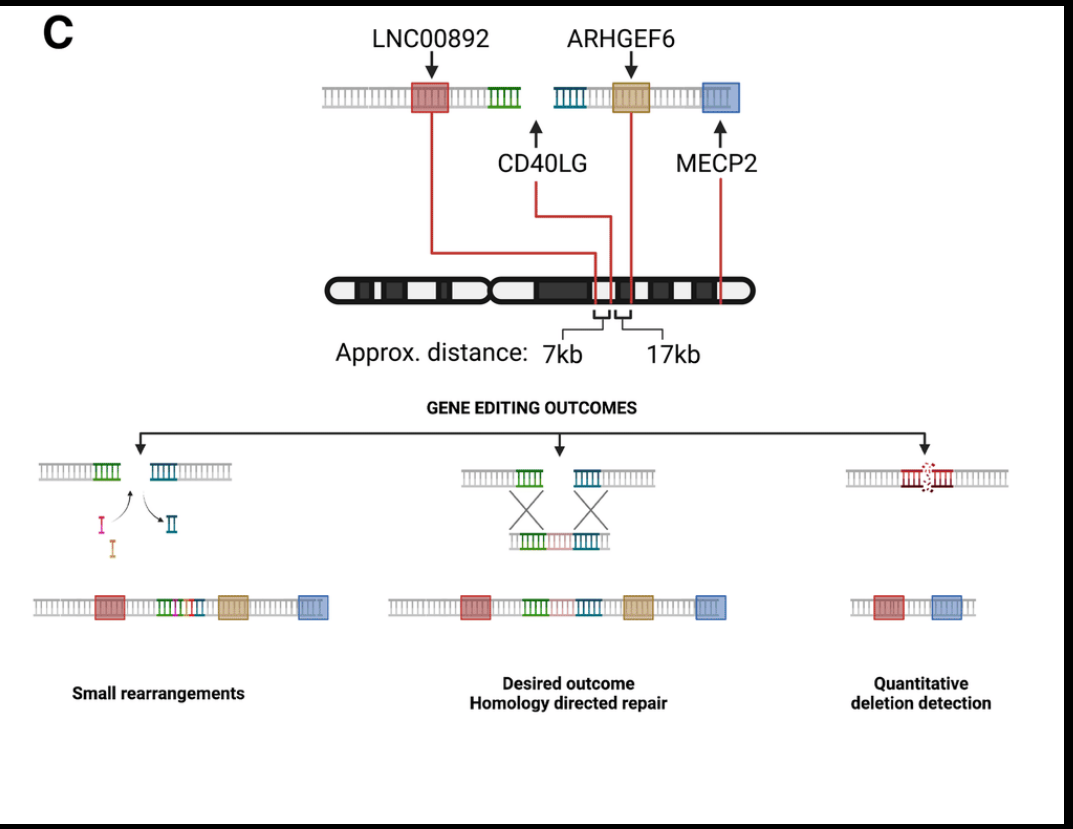


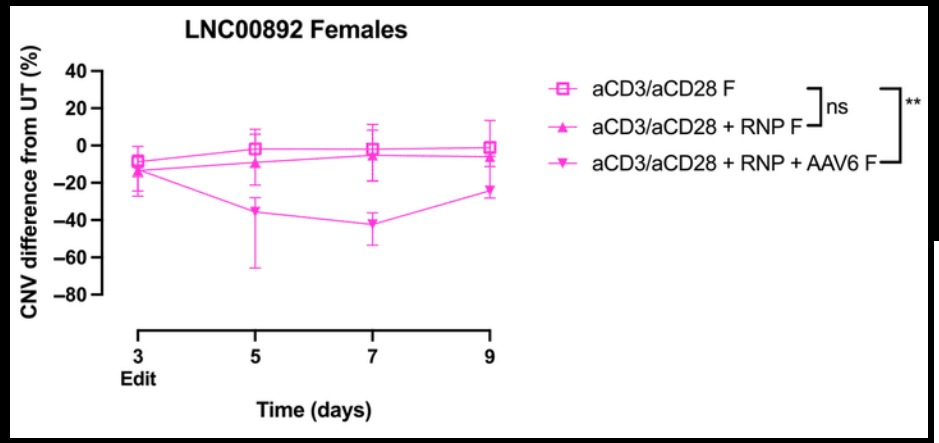


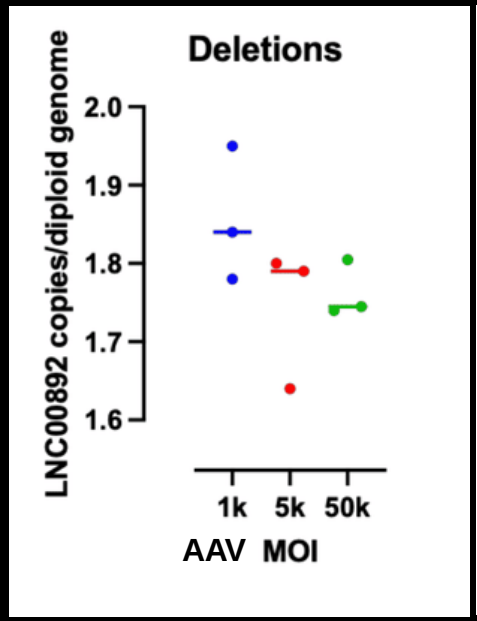


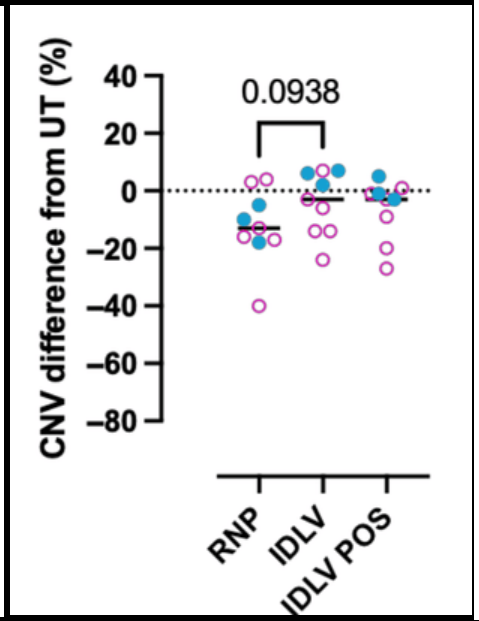
No detectable off-targets with HiFi Cas9

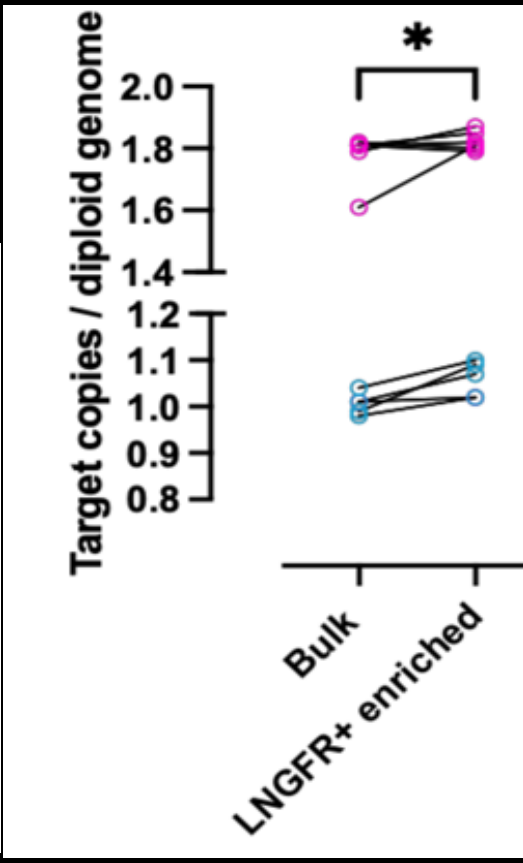
On-Target Assessment











Tiling assays on closest annotated gene(s)

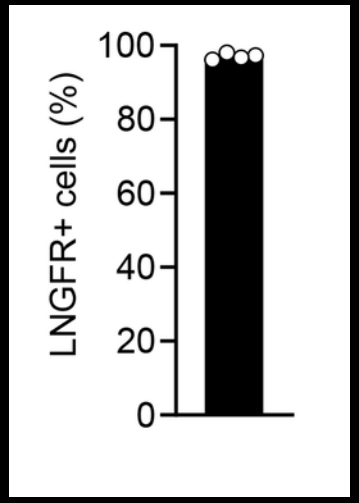
Sensitivity 5% on bulk

Large deletions purged spontaneously and by selection

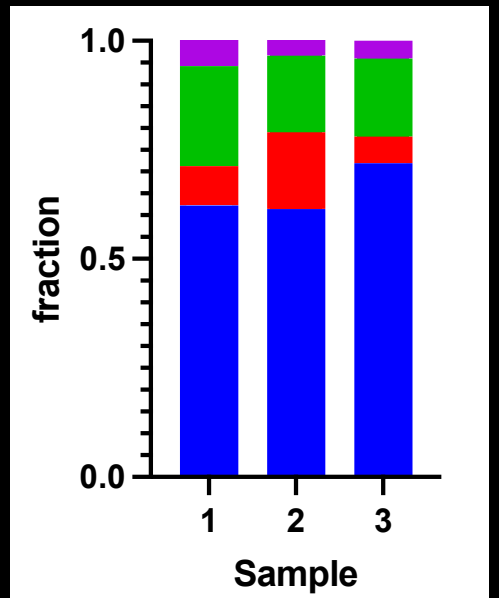
Canarutto et al., EMBO J., 2023

On-Target Assessment

Long-Read Sequencing of Target Locus by Nanopore

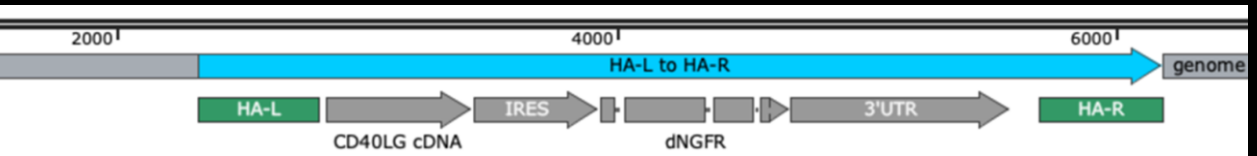


In CD40L+ pts edited cells

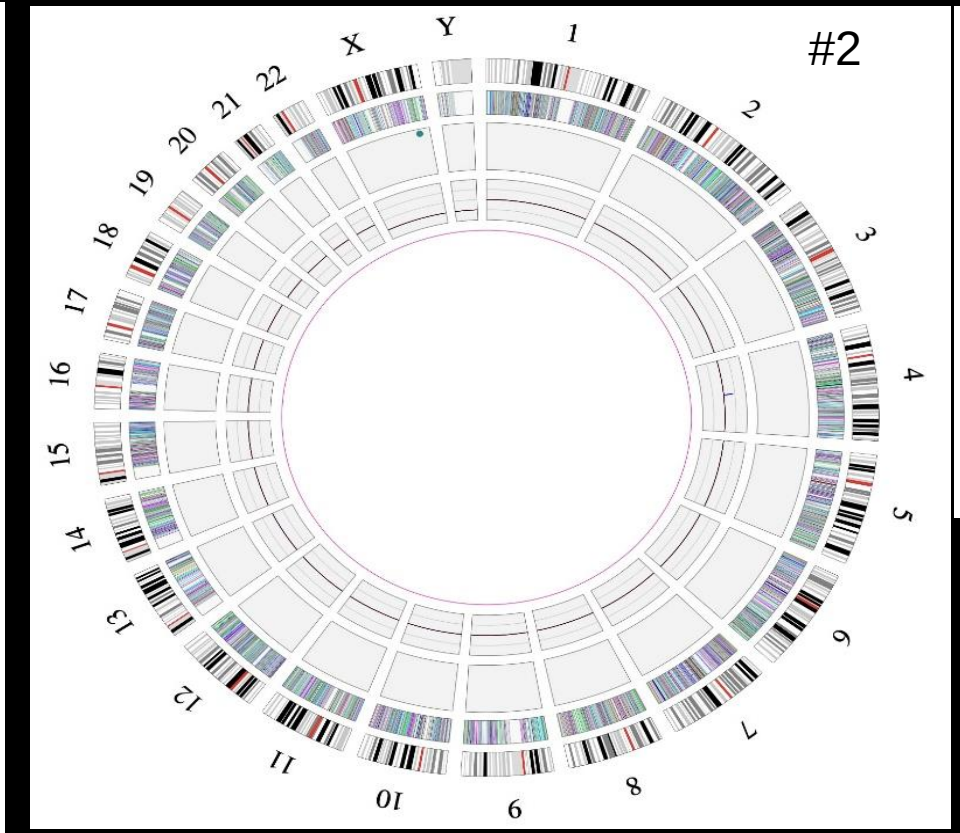


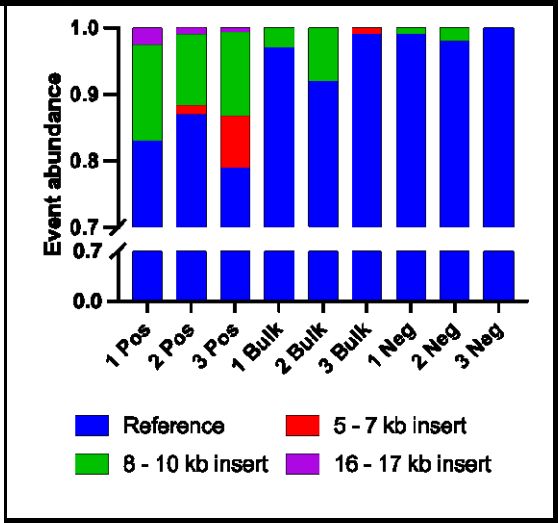
- Native, indels<50bp
- True HDR (bilateral and 0.5x monolateral HDR)
- Imprecise presumably functional HDR (concatamers + imprecise editing. Half reads counted half)
- Other

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

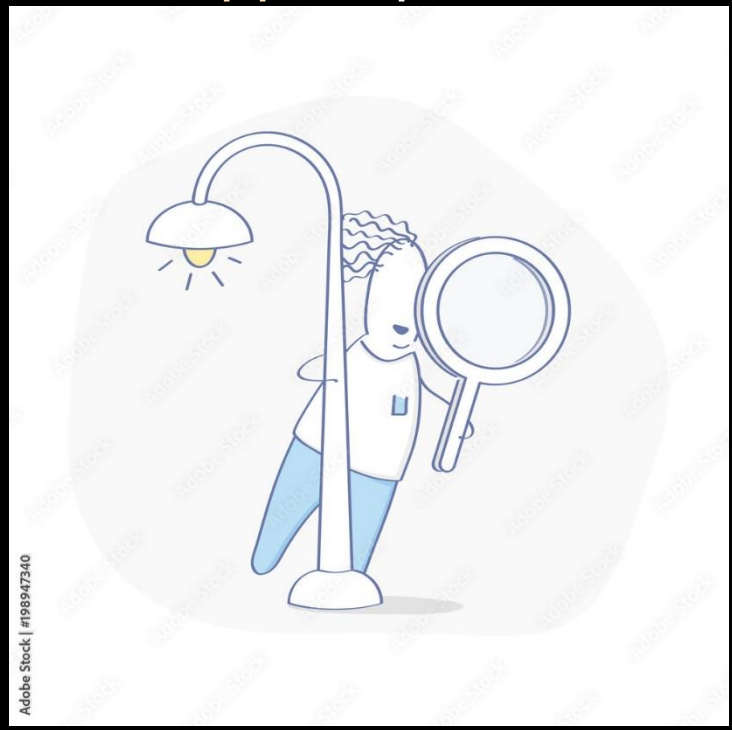
- *Development of GMP-compliant full scale process for HDR-editing of T-cells*
  - *In situ* gene correction of most *CD40L* mutations
  - Rescue of CD40L function and regulated expression
  - Selection of cells carrying intended edit coupled to purging adverse on target outcomes
  - Some heterogenous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

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

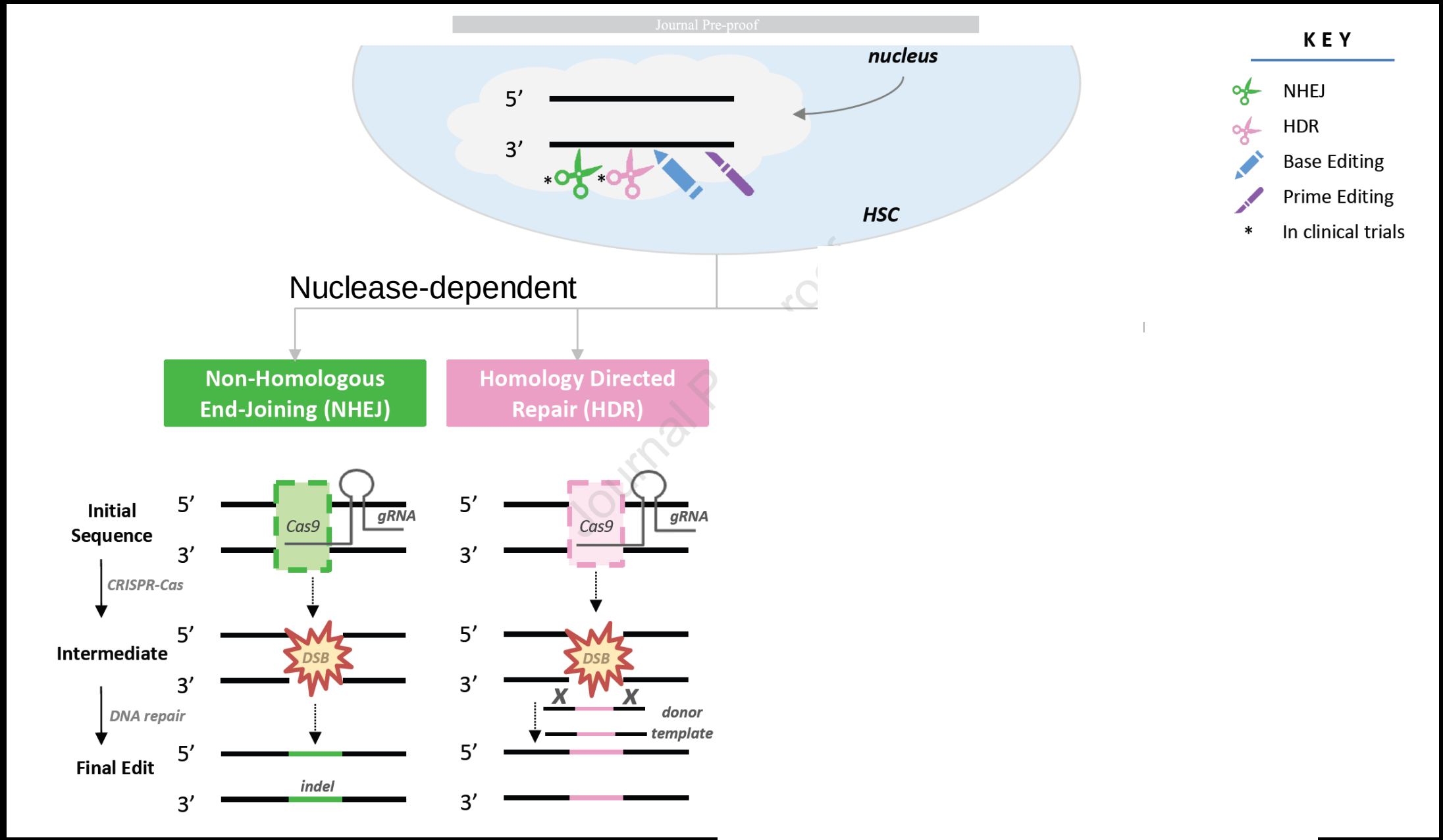
Nuclease-based Gene Editing: Where We Stand Today

- *(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



A Versatile & Constantly Evolving Platform for Gene Engineering



D. Bauer, Mol Ther, 2021

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

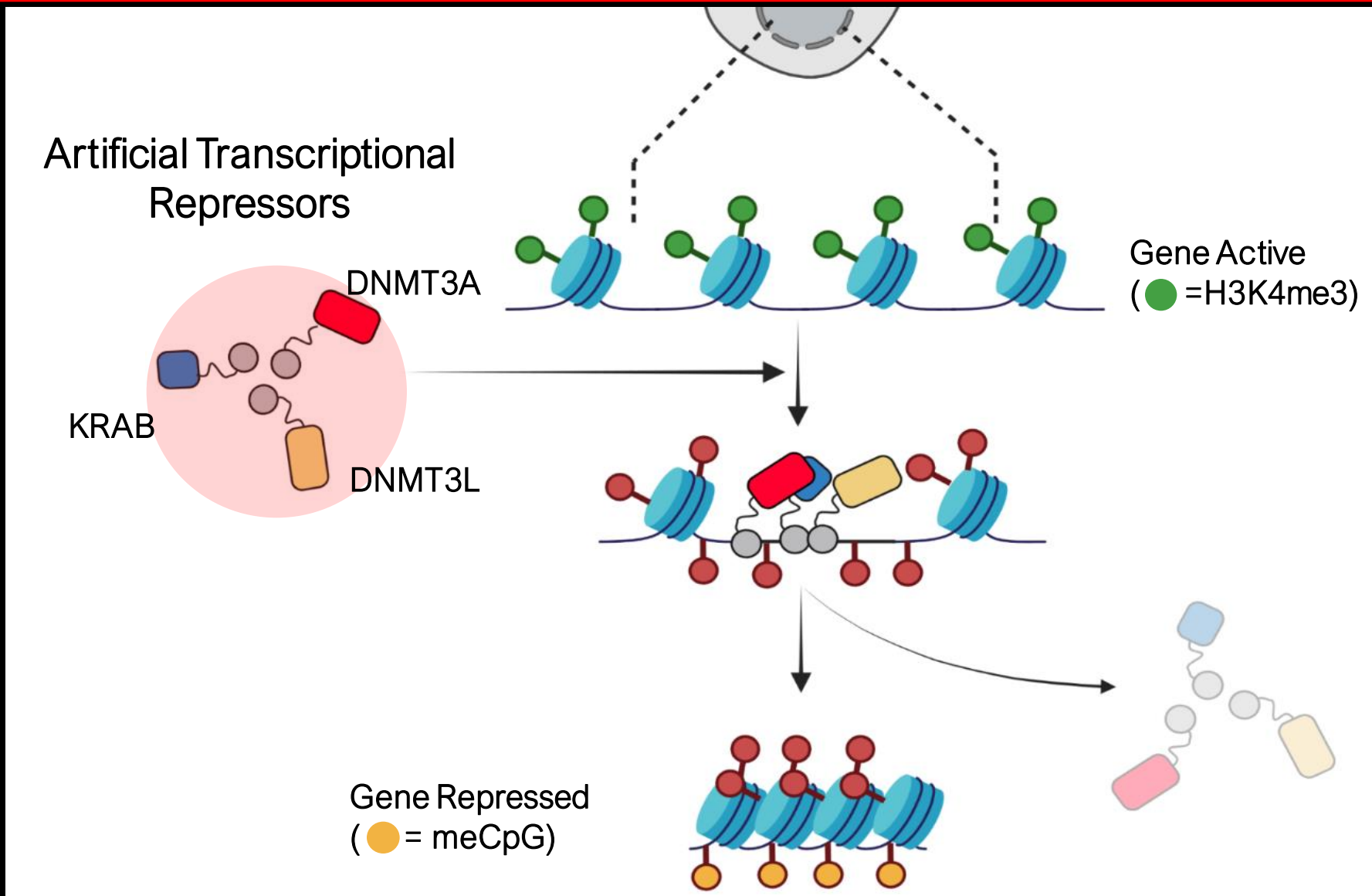
Addressing the Genotoxicity Risk of Gene Editing

- *Human genome sequence variation and unbalanced representation of regional differences in available database*
  - Limits off-target prediction by testing a generic / individual donor human genome
  - Animal models useless except for target sequence-independent effects
  - Patient-specific emerging toxicity to be addressed in the clinic
- *Complexity of interaction with cellular DNA and repair machinery*
  - Need better understanding of biology
  - Emerging risk of interfering/inhibiting alternative repair pathways
  - Requires novel *ad-hoc* testing
  - Long-term clinical follow-up needed coupled to monitoring clonality
- *Hit-and-run process makes difficult to trace eventual adverse events*

Gene Silencing by Epigenetic Editing

Transient Administration (Hit and Run)

Stable Silencing Across cell progeny

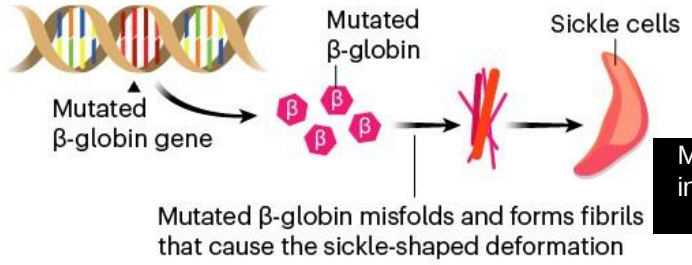


Amabile… Naldini and Lombardo; Cell 2016

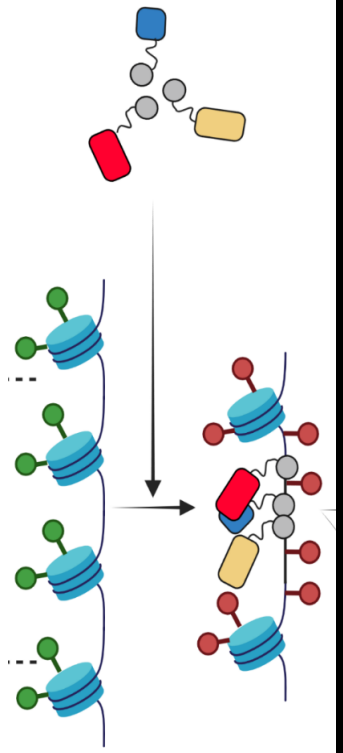
HSC Gene Therapy for SCD / β-Thalassemia

A TRIO OF TACTICS

Some gene therapies for sickle-cell disease restore healthy red-blood-cell function even if expression of the mutant protein continues uninterrupted. By contrast, CRISPR-based efforts aim to fully repair the root cause of the disease.



Modify BCL11A binding sites in y-globin promoter by Base Editing



Silence BCL11A by epigeneitic editing

Correct mutation by Prime Editing

Modified from
Nature **596**, S2-S4 (2021)
https://doi.org/10.1038/d41586-021-02138-w

Summary: Choosing the Right Tool

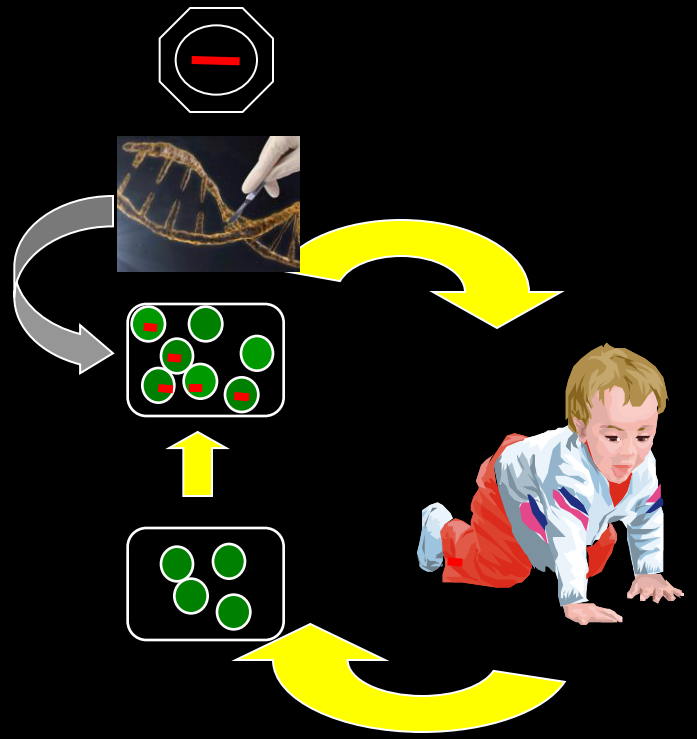
Lentiviral Vectors
Genome-wide insertion
Highly efficient
ex vivo in proliferating cells
Solid clinical track record
in HSC and T cells
Insertional genotoxicity minimized by vector design

Nuclease-based
Efficient for disruption
Suitable - with constrains -
for gene-size edits
Early clinical stage
in HSC and T cells
Off-target activity, DNA breaks, deletions, genomic rearrangements

Nickase-based (B & P)
Highly efficient for
correcting mutations
Just in the clinic
Bystander edit
Sequence-independent off-target activity

Epigenetic Editing
Stable gene silencing
w/out modifying DNA
sequence
R&D stage

In vivo Genetic Engineering of HSC and T Cells



Bypass the challenges of ex vivo engineering and conditioning

In vivo Gene Transfer into HSC and T Cells

- *Direct intra-venous administration of LV*
  - Surface engineered for
    - Specific targeting
      - T cells for CAR-T: need for cell activation (Anti-CD3/CD28…)
      - HSC: need for mobilization in the circulation
    - De-targeting liver & immune-shielding
      - Mutant VSV.G or alternate fusogen
      - CD47 overexpression
      - MHC-I knockout
  - No conditioning required
  - Limited efficiency but effective if transduced cells expand
  - *Poor control on target cells, product profile & biodistribution, durability TBD*
  - *Higher risk of immunogenicity preventing serial dosing*

In vivo Gene Editing of HSC and T Cells

- *Editor choice*
  - Cas nuclease (difficult to achieve HDR because of template co-delivery)
  - Base editors
  - Retrotransposons, Prime editor, target-primed retrotransposition
- *Delivery*
  - mRNA packaged by Lipid NanoParticles (LNP)
    - specific targeting by surface engineering
    - short-lived editor expression
    - easier to manufacture & scale-up
    - *low efficiency (high dose required)*
    - *challenging to de-target from liver to intended tissue*
  - Viral Like Particles (VLP)
    - combine efficient viral machineries for transient & targeted delivery
    - *challenging to manufacture*

Advanced Therapy Medicinal Products (ATMPs)

- *Transformative Medicines*
  - Complex “live” medicines made with genes, modified viruses, cells and/or designer DNA-modifying enzymes…
  - *“Once and done”* treatments with potential for “cure” of otherwise severe-lethal diseases
  - Life-long benefits but also possible delayed adverse effects
  - Fully personalized if made with own patient’s cells
- *Disruptive procedures*
  - Complexity & high costs of manufacturing and supply chain
  - High market values can be claimed, but spread over decades ahead
  - Staff and structure at point-of-care actively involved in the process
  - Challenging to provide fair, equitable and broad access

A Perfect Storm (for Rare Disease Gene Therapies)

- *They paved the way*
  - Validated rationale of cure at the genetic bases of disease
  - Provided proof of safety and efficacy of all major platforms
- *First to reach the market*
  - Claiming highest price of any other medicine
  - Yet failed to achieve sustainable deployment by pharma industry
- *Pharma disinvesting from rare to privilege common diseases*
  - CMC / supply chains not developed to enable economy of scale
- *Many orphan diseases potentially amenable to treatment*
  - sometimes undertaken by startups with limited resources

Towards more Sustainable ATMP Deployment

- *Must alleviate burden and cost of development/product release*
  - Working together with regulatory agencies leveraging on
  - Platform approach, where same vector and process are used for different products
  - Increasing confidence gained on clinical experience with major vector types
- *Manufacturing designed to contain costs*
  - Meeting safety but not always commercial manufacturing standards
    - platform approach, master data files, shared tools, universal safe harbors
- *Non-Profit ATMP supply Centers supported by public sponsored schemes*
  - Using harmonized & centralized procedures & point-of-cares
  - Provide education and training of specialized personnel
  - Foster innovation
  - May ease introducing “platform” improvements in vector design & process

Ensuring a Future for Gene Therapy for Rare Diseases

Nature Medicine volume 28, pages1985–1988 (2022)

comment

Ensuring a future for gene therapy for rare diseases

Alessandro Aiuti, Francesca Pasinelli and Luigi Naldini