Advanced Therapy Development for Erythroid Blood Disorder

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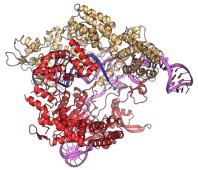
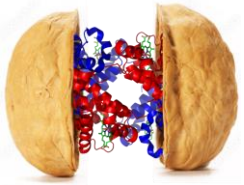
Overview

- Advanced Therapy 101



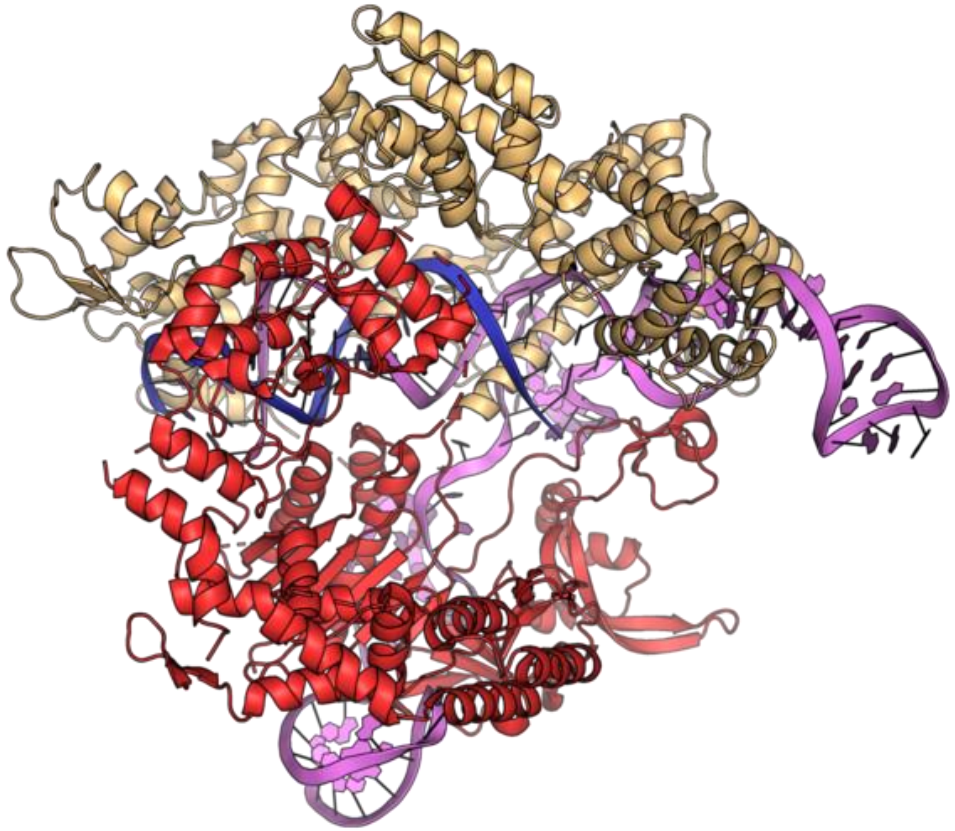
- Hemoglobinopathies in a Nutshell



- Strategies for Therapy
  (for hemoglobinopathies and beyond)

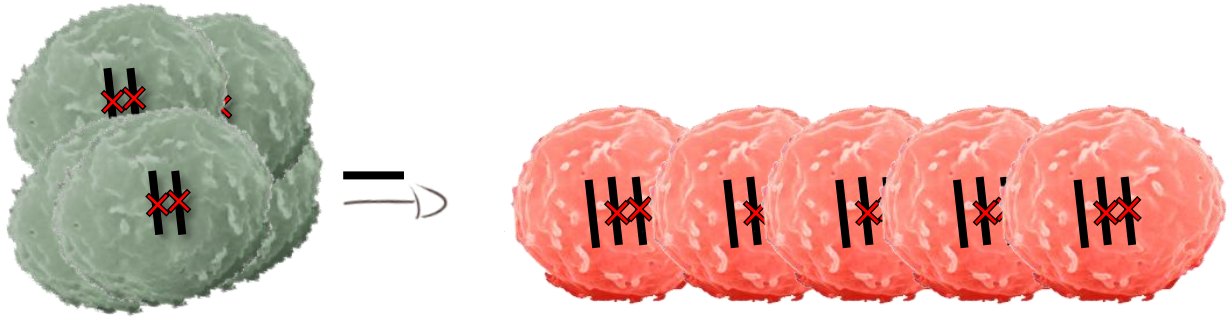
- Approaches in Cyprus

Advanced Therapy 101

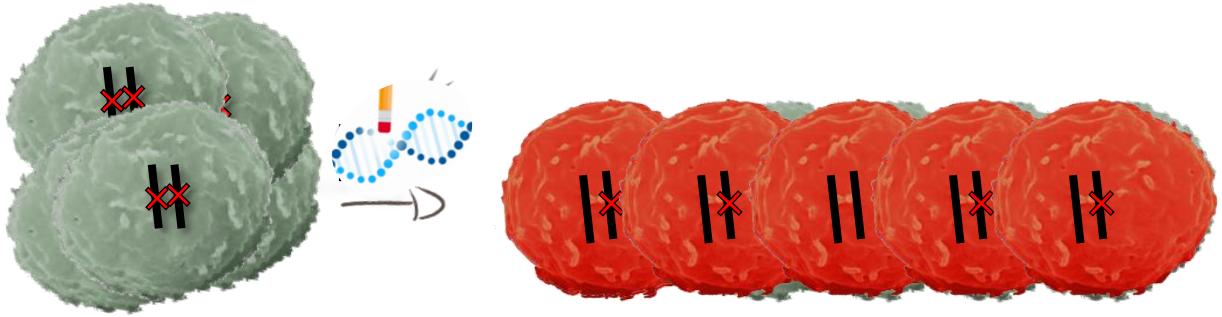


Gene Addition vs Gene Editing

- Gene addition (aka gene augmentation)
  - Addition of functional gene copy (or of a posttranscriptional silencing element)
  - For permanent cure, integration is required



- Gene editing (correction, disruption, excision)
  - DNA-level correction of defects
  - Originally based on double-strand break (DSB) repair
    - Disruption was highly efficient early-on
    - Precision repair is (still) inefficient
  - Ongoing development of DSB-independent editors (base editors, epigenetic editors, prime editors)



- Uncertainties
  - Long-term stability (true hematopoietic stem cells?)
  - Universal applicability (patient-to-patient variation in modifier genes & recognition sequences)
  - Long-term safety (emergence of clonal dominance? silencing?)

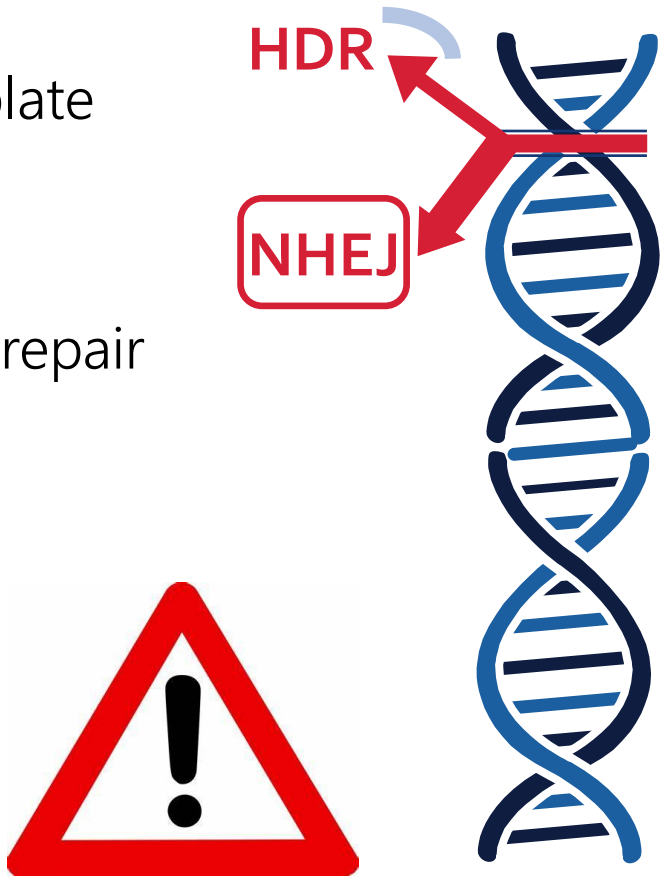
Commonly Used Classic Editors

	01 ZFN	02 TALEN	03 CRISPR
Target sequence	9–18 bp per ZFN monomer	14–20 bp per TALEN monomer	20 bp guide sequence plus PAM sequence
Recognition site	Zinc-finger protein	TALE protein RVD tandem repeat region of	Single-stranded guide RNA
Mode of recognition	Protein:DNA ZF modules; interference of neighboring recognition modules	Protein:DNA TALE RVDs; clear 2:1 amino acid:nucleotide code	RNA:DNA; 1:1 Watson-Crick basepairing
Endonuclease	FokI	FokI	Cas9
Size (kb)	~1	~3	~3.5–4.5
Ease of engineering	Complicated – Requirement of substantial protein engineering	Simplified – Requirement of complex molecular cloning procedures	Simplest – Use of 20 nucleotide sgRNA sequence per target site
Off-target effects	High	Low	Variable
Cost	High	Moderate	Low

Patsali, Kleanthous, Lederer 2019 Mol Diagn Ther, PMID30945167; Papasavva, Kleanthous, Lederer 2019 Mol Diagn Ther, PMID30945166; Koniali et al. 2021 MDPI Cells. PMID 34198536

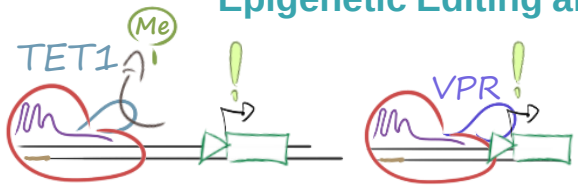
Tools in a Nutshell: Editing by Double-Strand Break

- Based on designer double-stranded-DNA nucleases → double-strand break (DSB)
- Cellular repair of DSB allows
  - Disruption (by non-homologous end joining, NHEJ)
  - Repair (by homology-directed repair, HDR) based on a repair template
- For life-long treatment, **true stem cells** need to be modified
  - Precision repair by **HDR is ±inefficient** stem cells
  - Efficient repair by **NHEJ is ±imprecise** and unsuitable for mutation repair
- Therapy by deletion or loss of function does not need precision
  - NHEJ-based clinical trials and products dominate
- Biology, not mathematics
  - HDR always has NHEJ as a side-product
  - DSBs may cause cancer by recombination with other, chance DSBs
  - Editors may inadvertently edit elsewhere, at off-target sites



Beyond the DSB – Editing v2.0

Key strategy: modular extension of the Cas protein and the gRNA sequence. Search e.g. for "David R. Liu" as a key scientist in the field.



Epigenetic Editing and Transcriptional Regulation

Addition of demethylase/methyl transferase and transcriptional regulator domains to dCas9 allows mitotically heritable changes

01

RNA Knockdown and Base Editing

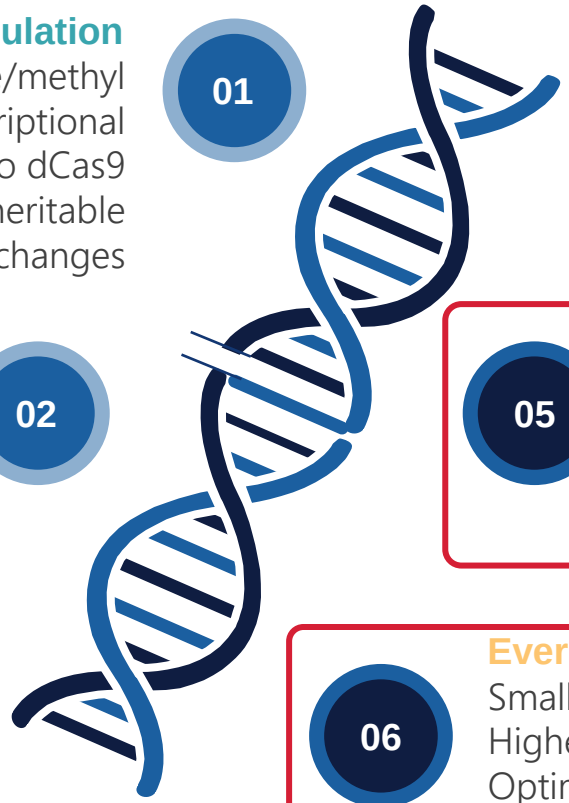
PAM-independent degradation or A>G base editing of target RNA

02

The PAM be damned

Natural and synthetic Cas variants have different or highly reduced PAM requirements

03



04

Base Editing

DSB-independent high-efficiency precision editing by nCas9 and base transitions (A→G, C→T)

05

Prime Editing

DSB-independent flexible precision editing by nCas9 and reverse transcription of a pegRNA-encoded template

06

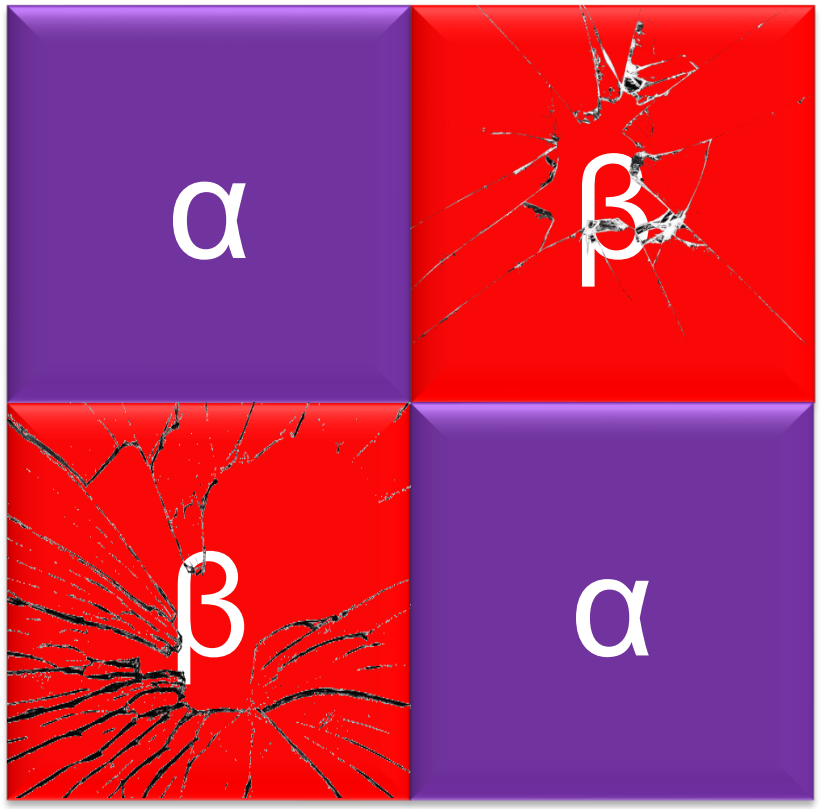
Ever-new developments

Smaller editors for *in vivo* delivery
Higher precision editors for greater safety
Optimized editors for higher efficiency
More flexible, transversion base editors

Patsali, Kleanthous, Lederer 2019 Mol Diagn Ther, PMID 30945167; Papasavva, Kleanthous, Lederer 2019 Mol Diagn Ther, PMID 30945166; Christie, Kleinstiver 2021 Trends Genet, PMID 34563399; Liu et al 2020 Nat Commun, PMID 33247095; Chen et al 2021 Cell, PMID 34653350; Chen et al 2024 Nat Biotechnol PMID 38658732

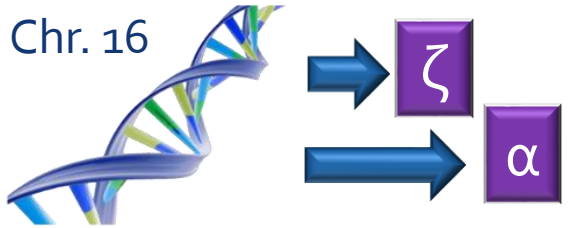
Strategies for Therapy

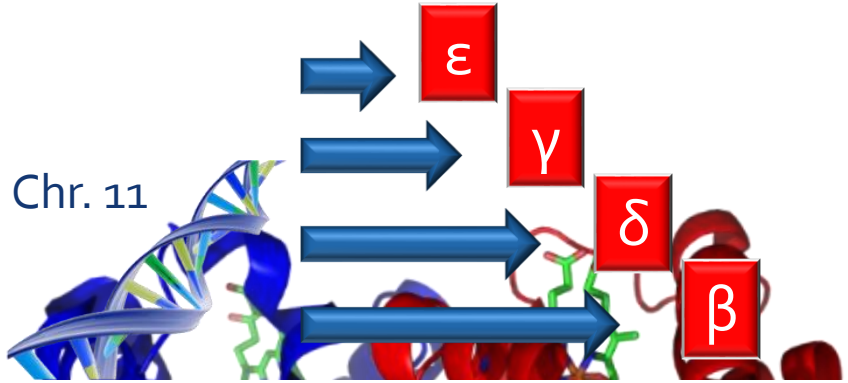
(for hemoglobinopathies and beyond)

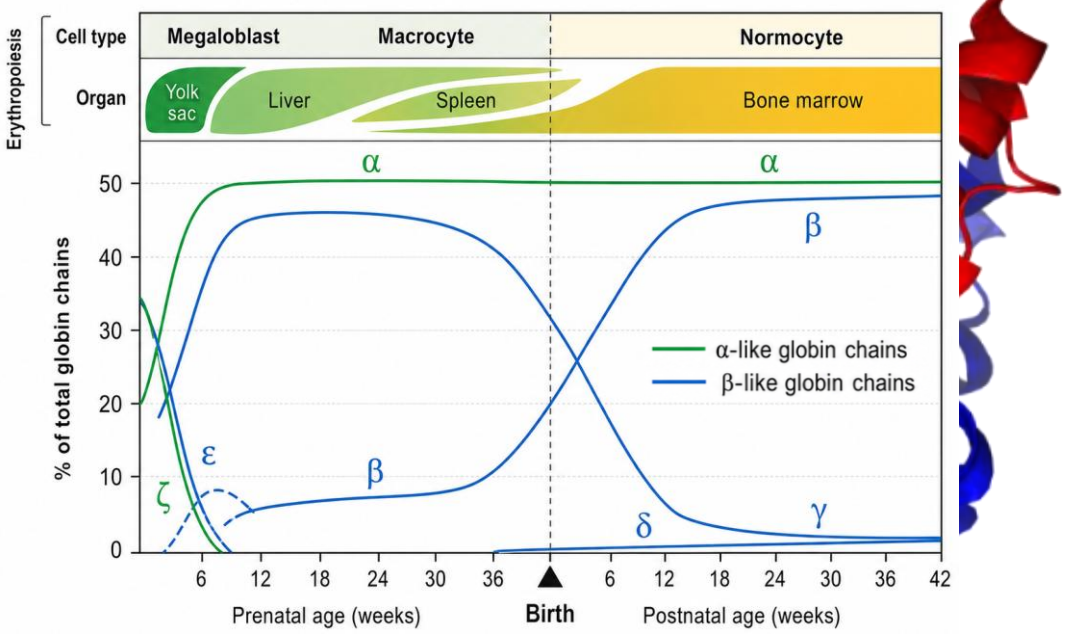


Hemoglobin
95% of Erythrocytes (in Dry Mass)

- O_2 and CO_2 transport from and to the lungs
- Two loci for H.s. globin genes
  - Embryonic: ζ & ε
  - Fetal: α & γ
  - Adult: α & δ/β
- Hemoglobin, a tetrameric metalloprotein
  - 2 α-like globin chains
  - 2 β-like globin chains
  - 4 heme molecules with central $Fe^{2+/3+}$
- Adult expression of human hemoglobins







α β / β α — HbA >95%

α δ / δ α — HbA2 <3.5%

α γ / γ α — HbF <1.0%

Hemoglobinopathies Types & Mutations

- Quantitative globin defects
  - α-thalassemia: >400 mutations (mostly deletional)
  - β-thalassemia: >400 mutations (mostly SNVs) with variably reduced β-globin levels
    - From recessive β^{++} over β^{+} and β^{0} to rare dominant-negative β^{0} mutations
- Qualitative/structural globin defects
  - α- and β-globin chain variants
  - Clinically most important: sickling variants forming sickling hemoglobins, e.g. HbS
- Hereditary persistence of fetal hemoglobin (HPFH) (globin and modifier gene mutations)
  - High-level residual HbF ($\alpha_2\gamma_2$) expression
  - Without overt pathology
  - Therapeutic for β-thalassemia and sickle cell disease (SCD)
    - γ-globin acts to replace β-globin
    - γ-globin is anti-sickling for SCD

iTHANET

www.ithanet.eu/db/ithagenes?action=stats; PMID 37451300; 26899228

Therapeutic Strategies for Hemoglobinopathies

β-thalassemia

Removal of excess α? Anemia persists.	Addition of β? Curative.
Activation of β-like γ-globin? Curative.	Repair β^{mut}? Curative.

- Hundreds of causative mutations
- Homozygous and compound heterozygous disease causation
- **Universal therapies profitable**

sickle cell disease

Removal of β^S? Anemia persists.	Addition of γ or $\beta^{anti\text{-}S}$? Curative.
Activation of β-like γ-globin? Curative.	Repair β^S? Curative.

- A single mutation
- 100,000s of patients
- **SCD & βThal universal therapies most profitable**

α-thalassemia

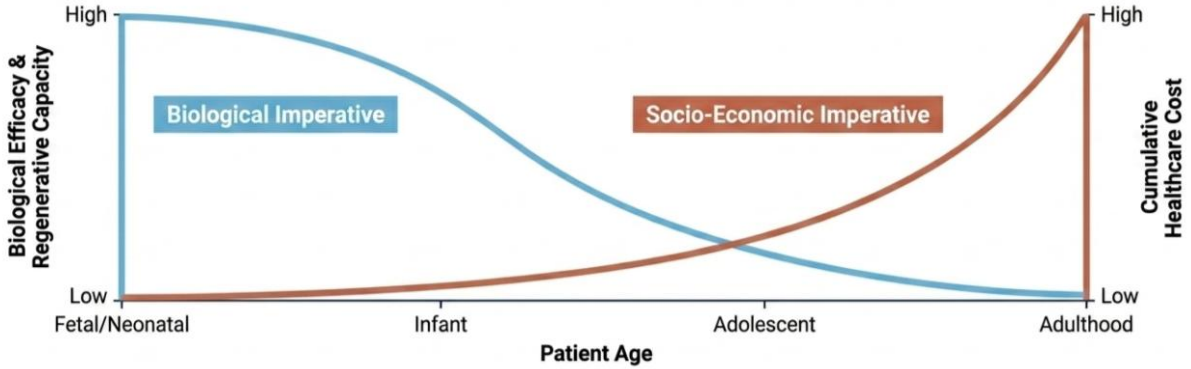
Removal of excess β? Anemia persists.	Addition of α? Curative.
Activation of α-like ζ-globin? "Tentative."	Repair α^{mut}? Curative.

- α^0 needs *in utero* therapy
- Hb H disease not lethal
- Deletional αThal tricky to fix
- **Until recently not a priority**

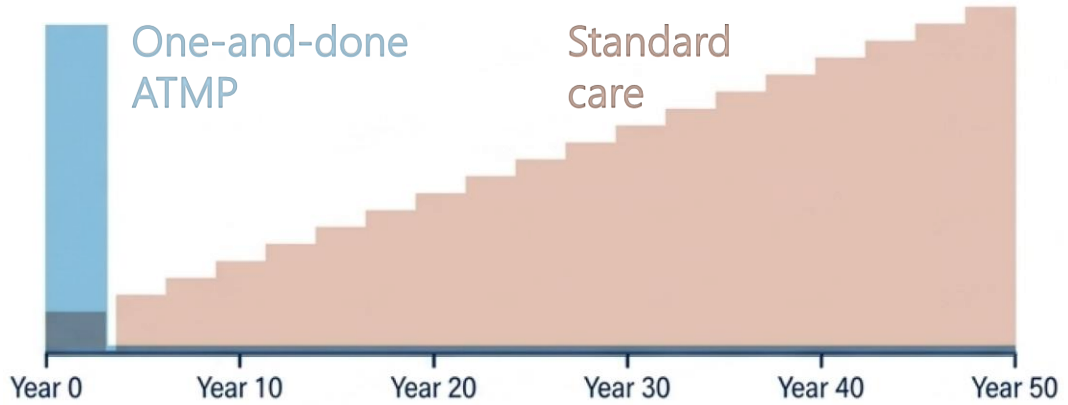
Catching Them **Early!**

- Infant gene therapy with incremental benefits
  - "Biological imperative"
    - ↑ Success rate
    - ↑ Correction of disease parameters
    - ↑ Primitive stem cell population
    - ↑ Hematopoietic reconstitution
    - ↓ Treatment-related morbidity
    - ↓ Irreversible organ damage
    - ↓ Lifetime suffering
  - "Socio-economic imperative"
    - ↓ Societal burden
    - ↓ Vector requirements
      Adult 80 kg ($100,000)
      <1 Year 8 kg ($10,000)
      ↓ Treatment cost vs ↑ profit
    - ↑ Lifetime treatment cost saving

Rationale for early intervention



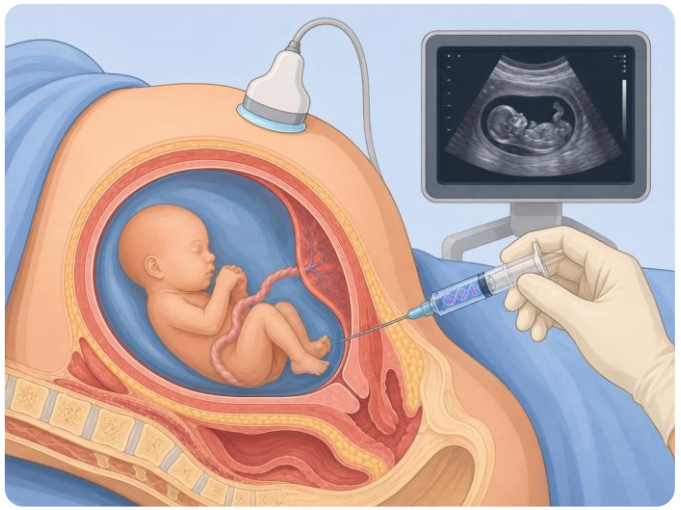
Lifetime economic trajectory

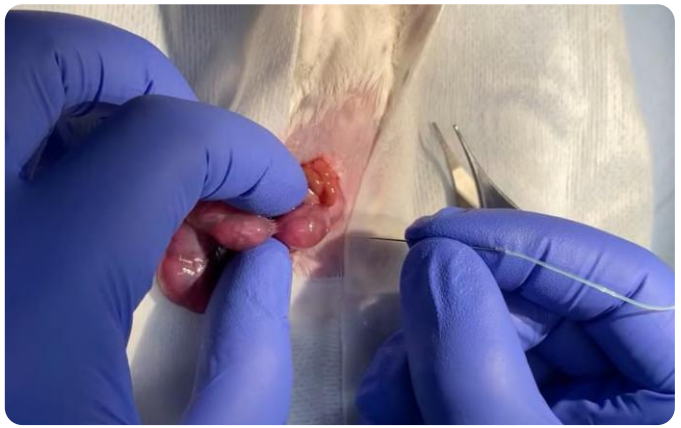


Ramachandra et al. 2014 Frontiers in Pharmacology PMID25566071
Shaw et al. 2014 Stem Cells PMID25186828; Lederer et al. 2022 Pharmaceutics PMID35456627

Catching Them **Early!**

- *In utero* gene therapy as quantum leap
  - Treatment also of prenatally fatal disorders (hydrops fetalis)
  - Resolution of accessibility bottlenecks
    - Neurological disorders (topical access challenge)
    - Muscular disorders (systemic access challenge, skeletal vs cardiac)
  - Minimal cell and vector requirements (1/1000 of that in adults)
    - *Postnatal:* *$5x10^6$ cells/kg* *$5x10^8$ TU/kg*
    - *In utero:* *$3x10^5$ cells total* *$1.5x10^7$ TU total*
  - Resolution of the GMP resource bottleneck
  - Possible (≠ ethical)
    - Direct vector injection (*in vivo* treatment)
    - Economy of scale: the vector as product
    - Application as outpatient treatment





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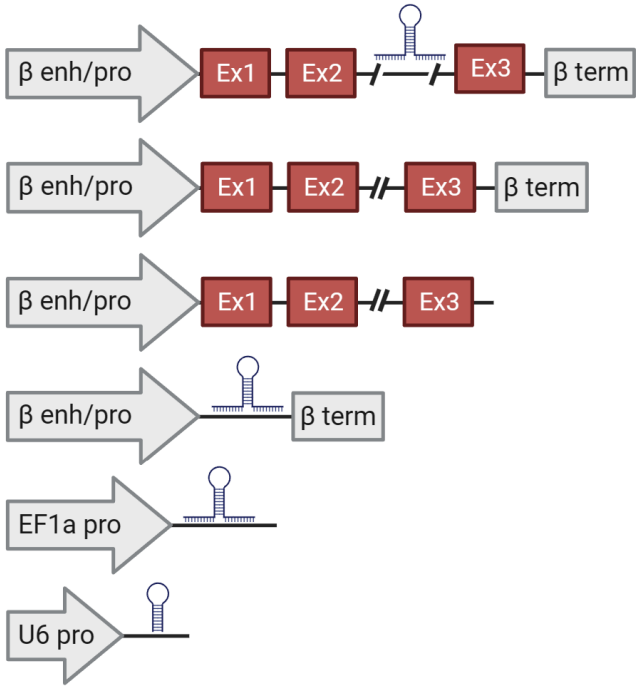
El Hoss & Shangaris Unpublished

Approaches in Cyprus

Therapy of *HBB*$^{IVSI-110(G>A)}$ by Mono- and Bifunctional LVs



○▸● – approximate relative performance indication from zero to maximum, for each evaluation stage across experiments and treatments

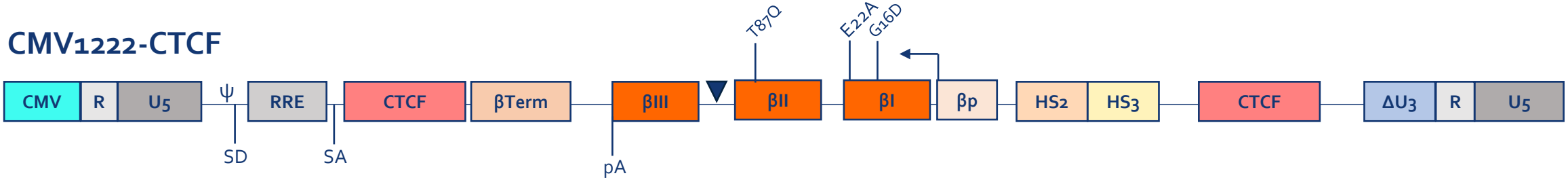
β enh/pro – β-globin enhancer and promoter elements from the GLOBE vector
β term – β-globin terminator
***EF1a* pro** – EF1a promoter
Ex1/2/3 – *HBB* exons
IVSI-110 HSPCs – *HBB*$^{IVSI-110(G>A)}$-homozygous hematopoietic stem and progenitor cells
LV – lentiviral vector
MEL-IVS – *HBB*$^{IVSI-110(G>A)}$-transgenic mouse erythroleukemia cells
VCN – vector copy number
U6 pro – U6 Promoter

GLV2-βAS3 base vector with extended β-globin terminator superior across parameters at same MOI

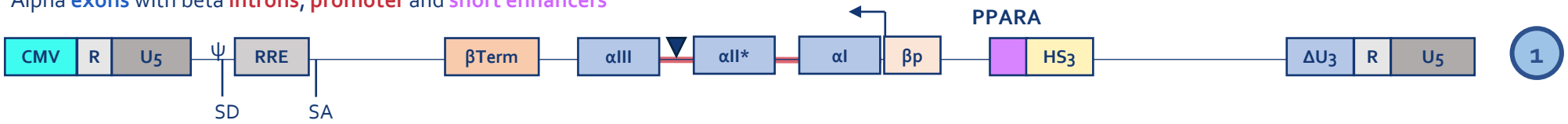
Koniali et al. 2023 Cells PMID 38132168

GLOBαL –
An α-Globin Derivative of the (Milan) GLOBE Vector

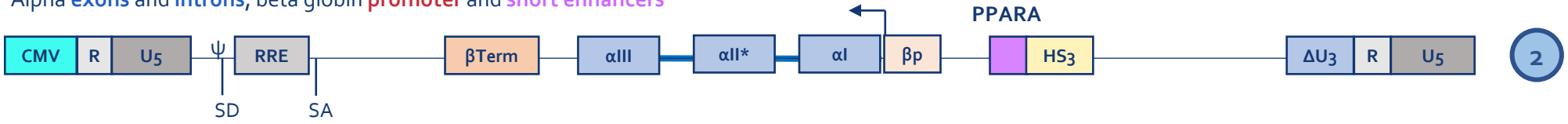
CMV1222-CTCF



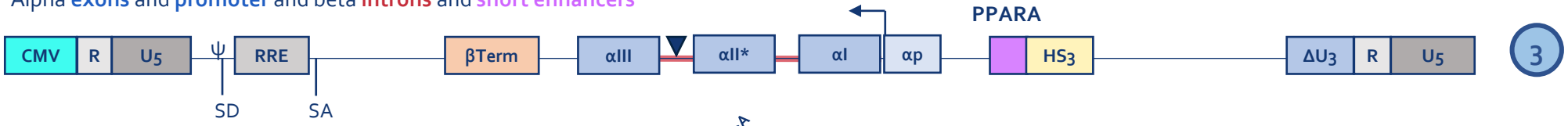
Alpha **exons** with beta **introns**, **promoter** and **short enhancers**



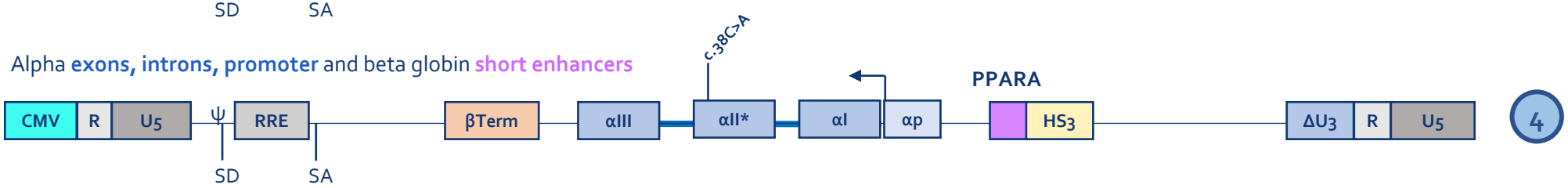
Alpha **exons** and **introns**, beta globin **promoter** and **short enhancers**



Alpha **exons** and **promoter** and beta **introns** and **short enhancers**



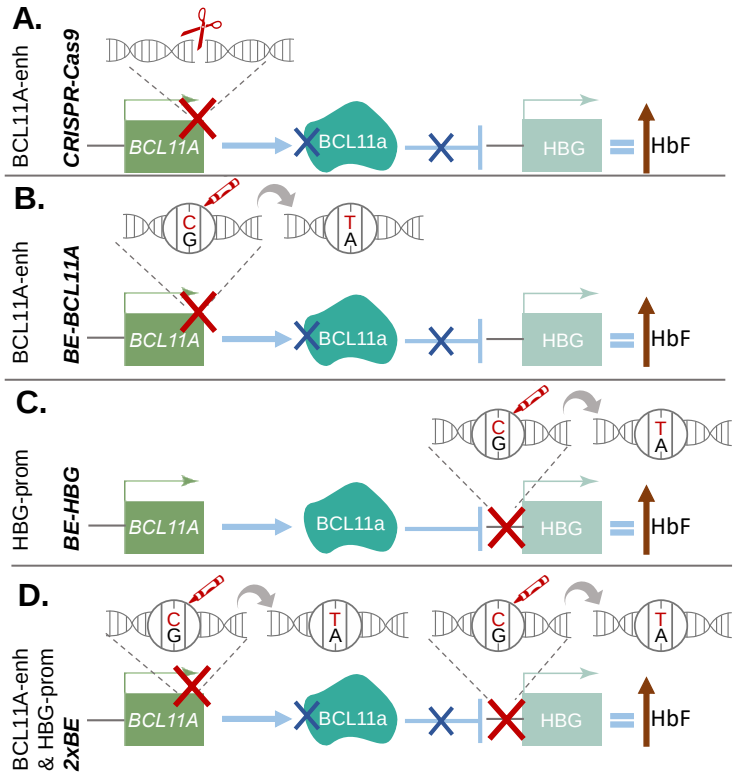
Alpha **exons, introns, promoter** and beta globin **short enhancers**



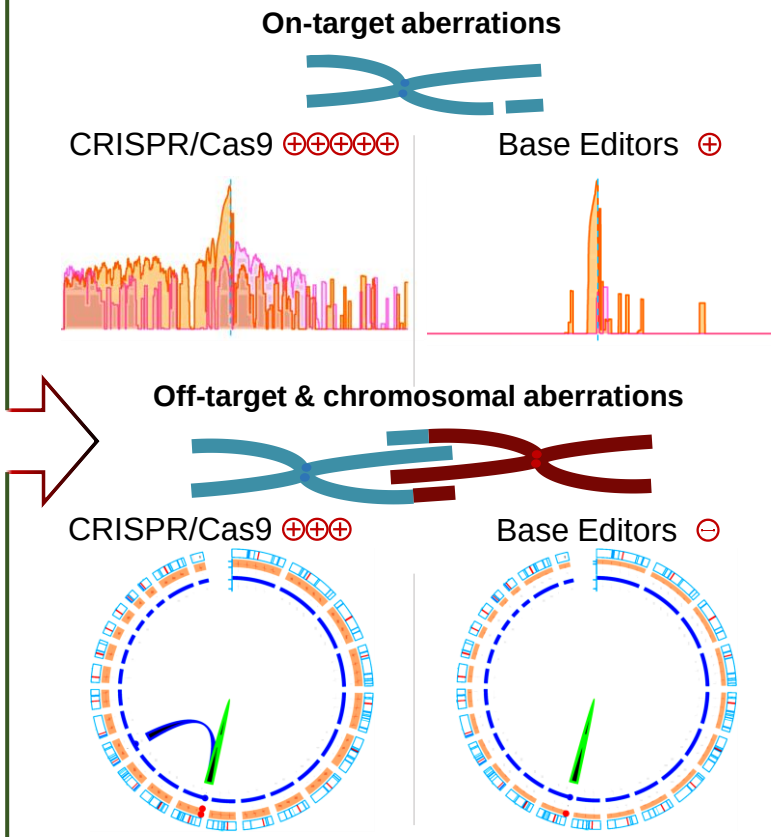
Unpublished data.

Universal Therapy for β-Hemoglobinopathies

Simplex and duplex DNA base editing of *cis* (HBG-prom) and *trans* (BCL11A-enh) regulatory elements for therapeutic fetal hemoglobin (HbF) induction by CRISPR/Cas9 & base editors (BEs)

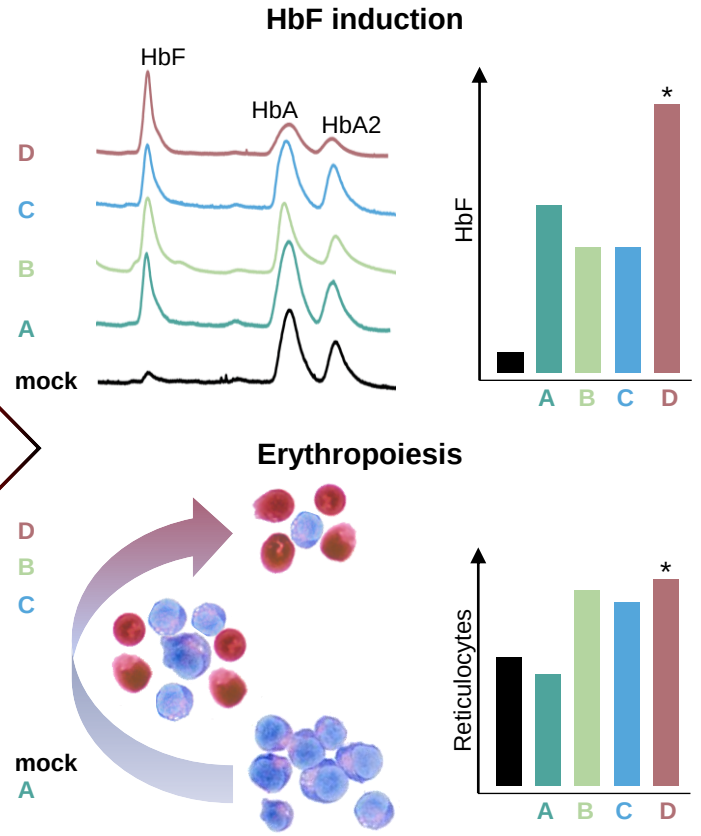


Genome integrity



Superiority of BEs over CRISPR/Cas9

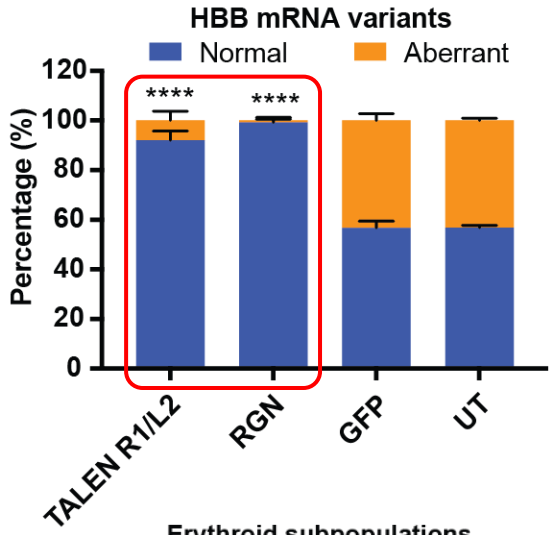
Functional correction

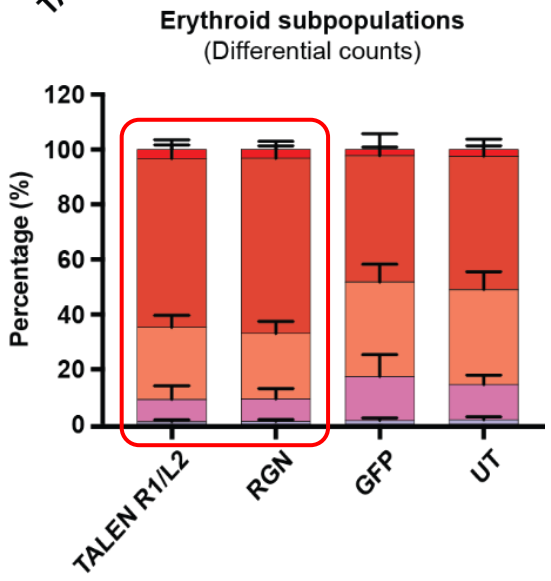


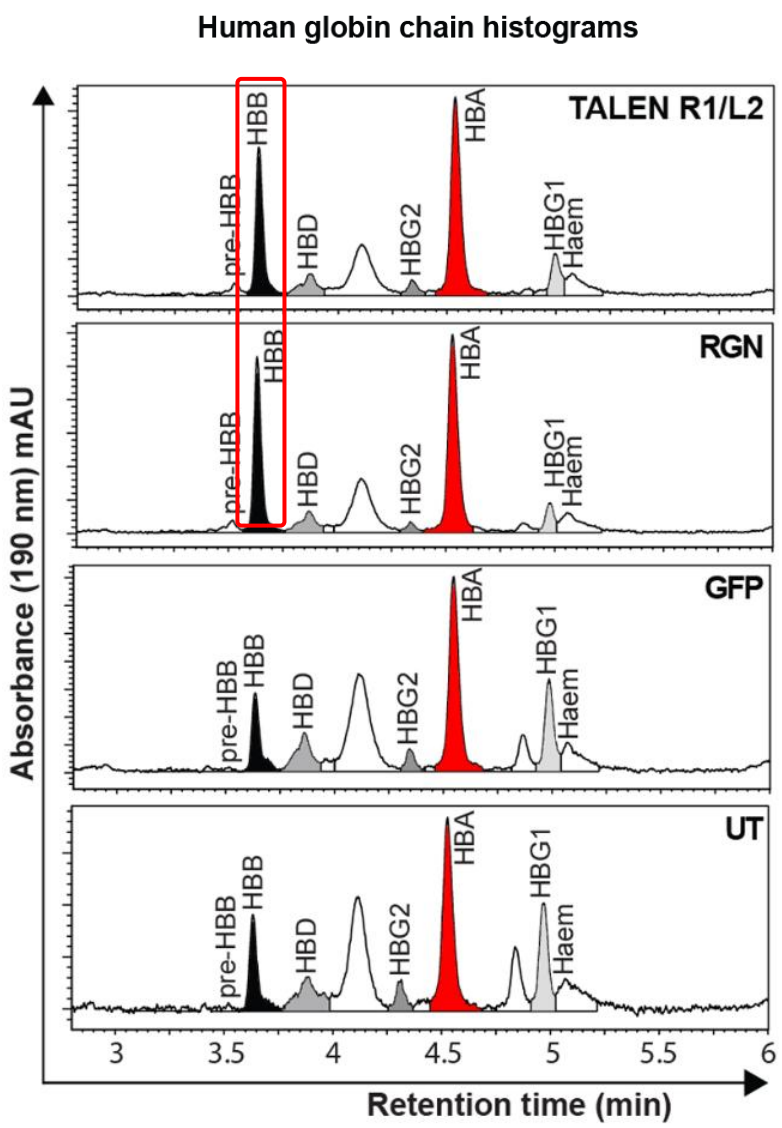
Superiority of BE and especially duplex BE correction

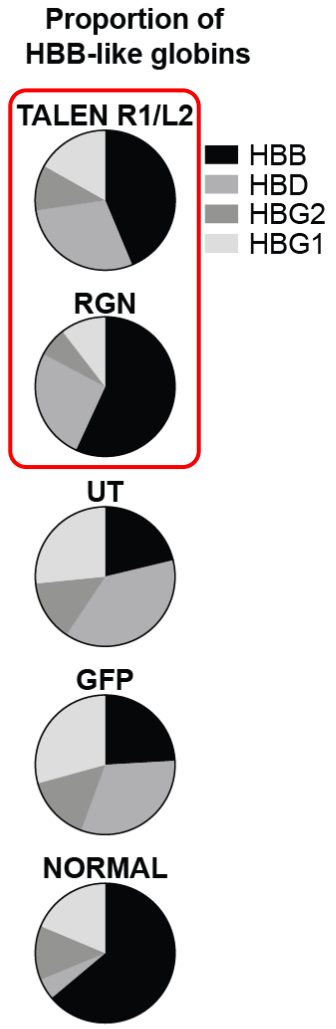
Papaioannou et al. 2026 Genome Biology PMID: 41629994

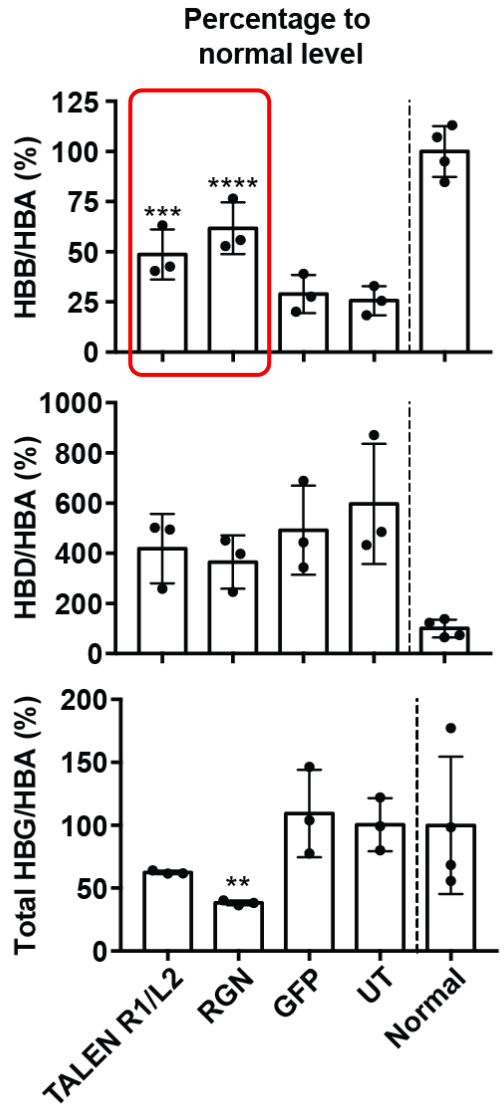
Disruption-based Therapy of *HBB*$^{IVSI-110(G>A)}$ in Patient-derived CD34^{+} Cells





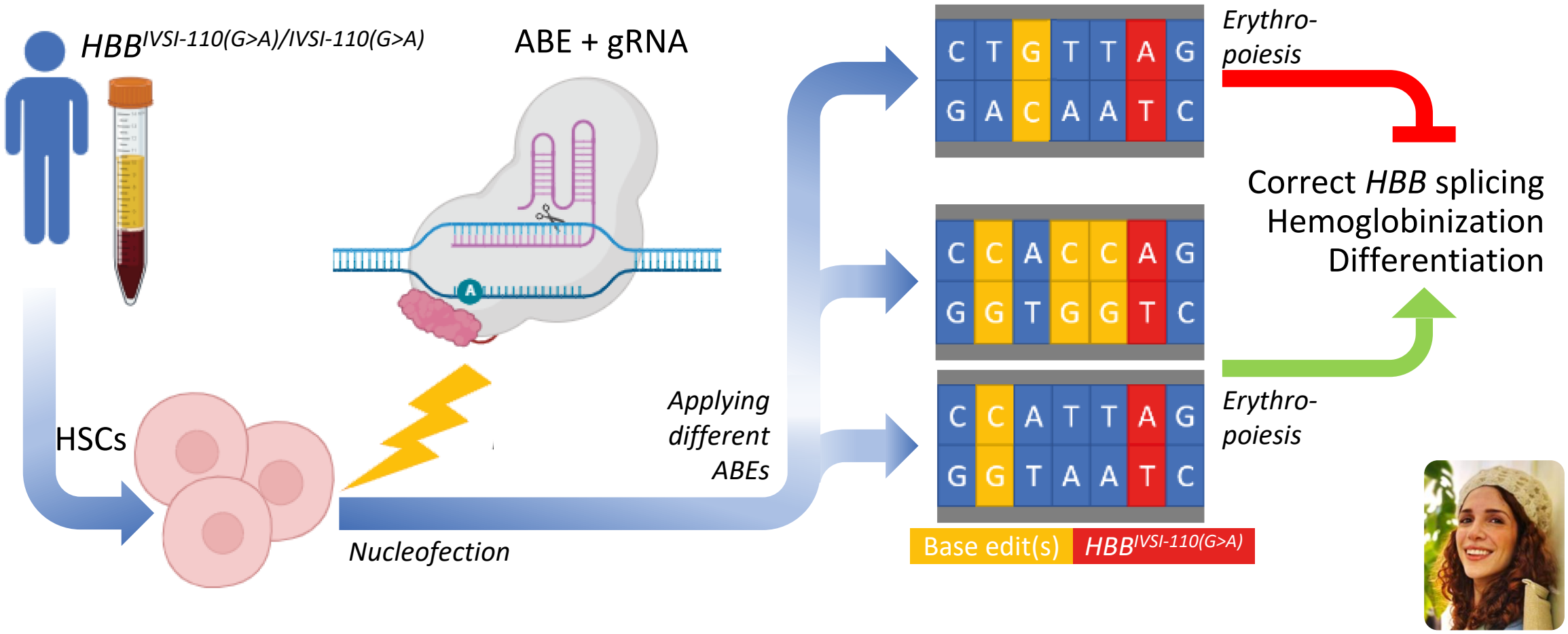






Patsali et al. 2019 Haematologica PMID 31004018

Disruption-based therapy of *HBB*$^{IVSI-110(G>A)}$ Therapy by Base Editing



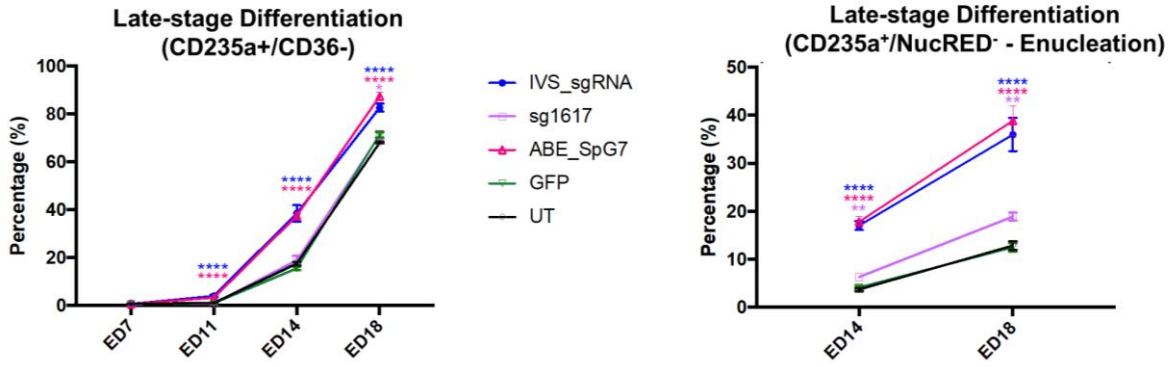
Naiisseh et al. 2024 Molecular Therapy Nucleic Acids PMID: 38706633

Comparative Therapy of *HBB*$^{IVSI-110(G>A)}$ in Primary Erythroid Cultures

- Testing of TrueCut vs SpG7 BE vs clinically applied (CASGEVY-type) Cas9 therapy
  - Evaluation in primary erythroid cultures

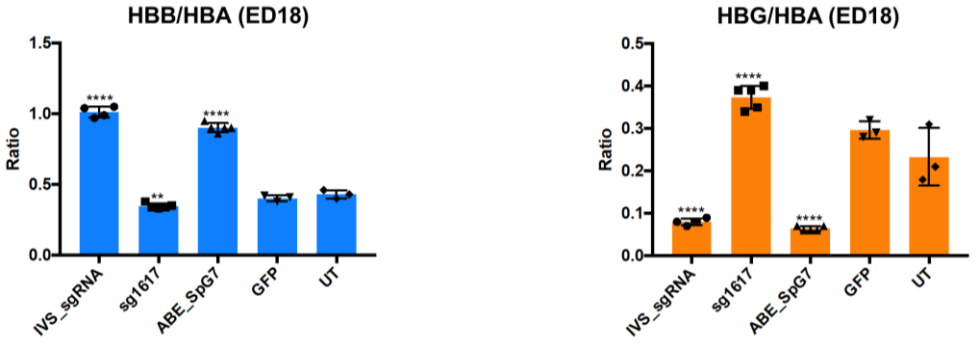
Restoration of Late-stage Erythropoiesis

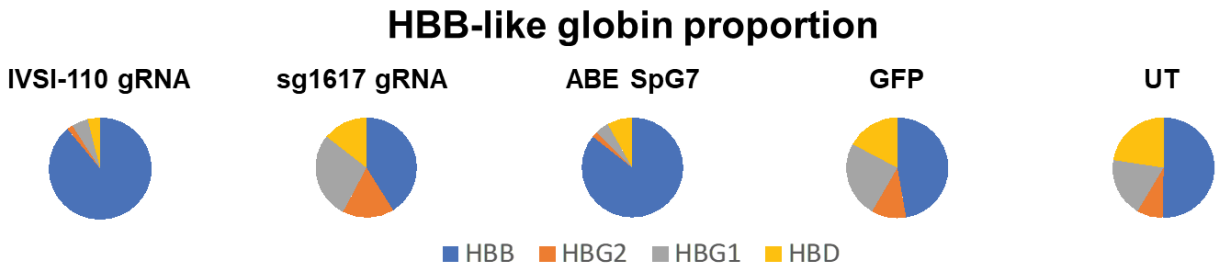
(via flow cytometry)



Restoration of Protein Levels

(via RP-HPLC)

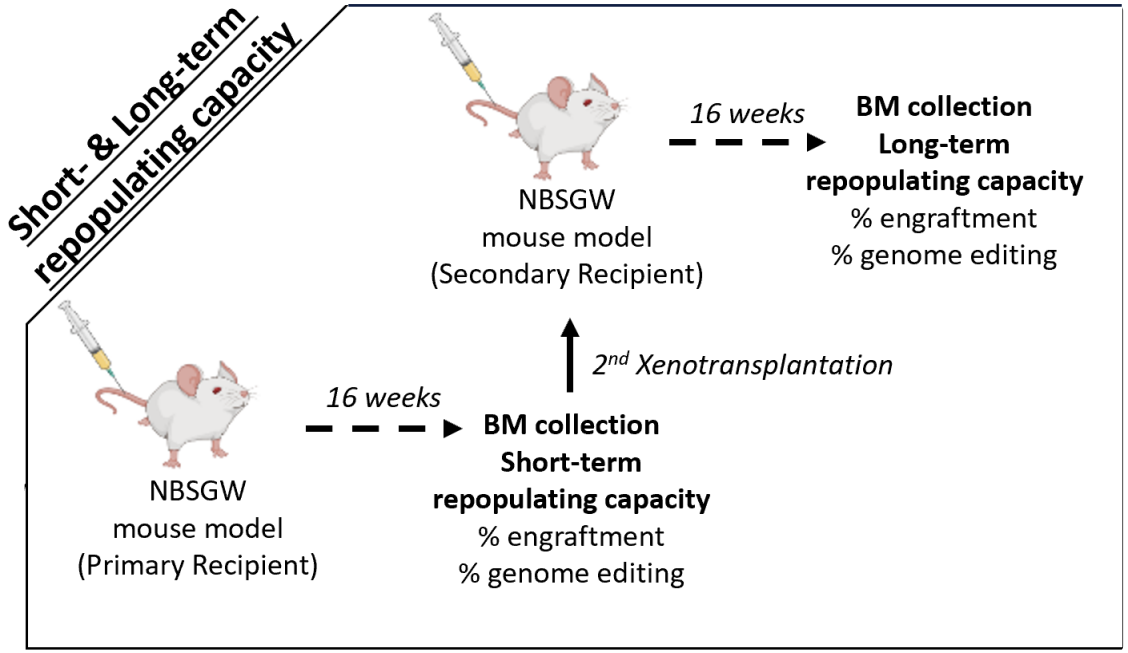




Unpublished data.

Comparative Therapy of *HBB*$^{IVSI-110(G>A)}$ Preclinical Evaluation in NBSGW Mice

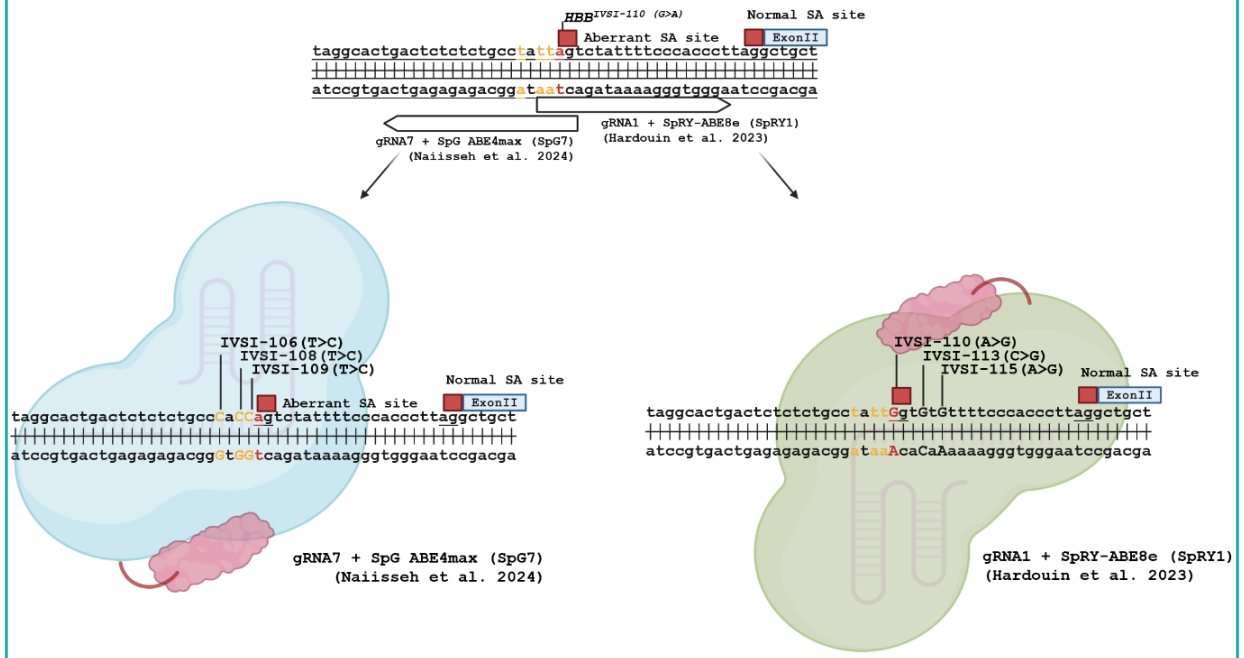
- Immunodeficient mice transplanted with edited LT-HSCs from homozygous *HBB*$^{IVSI-110(G>A)}$ thalassemia patients
  - On- and off-target efficiency
- Mutation-specific tools (CRISPR/Cas9 IVSI-110 gRNA and Base Editor ABE SpG7) compared to CASGEVY-type therapy
  - Higher editing efficiency
  - Improved erythropoiesis & RNA/protein correction
- The SpG7 ABE demonstrated superior long-term repopulation and precision
  - Choice for downstream analysis in HBB$^{IVSI-110}$ heterozygotes: **ABE SpG7 mRNA**
  - Comparison with Annarita Miccio BEs (Institut Imagine)



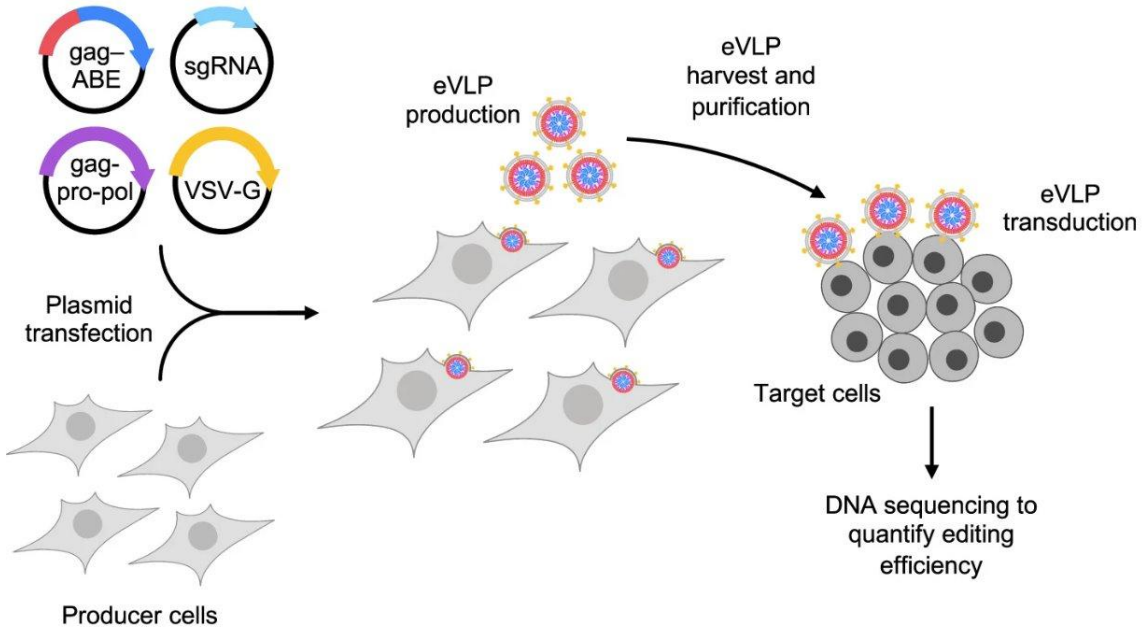
Unpublished data.

BE-based *HBB*$^{IVSI-110(G>A)}$ In Compound Heterozygotes

Evaluation of therapeutic efficiency of Adenine Base Editors (ABEs) in compound heterozygote-derived HSPCs



- Development of an eVLP-based in vivo delivery platform

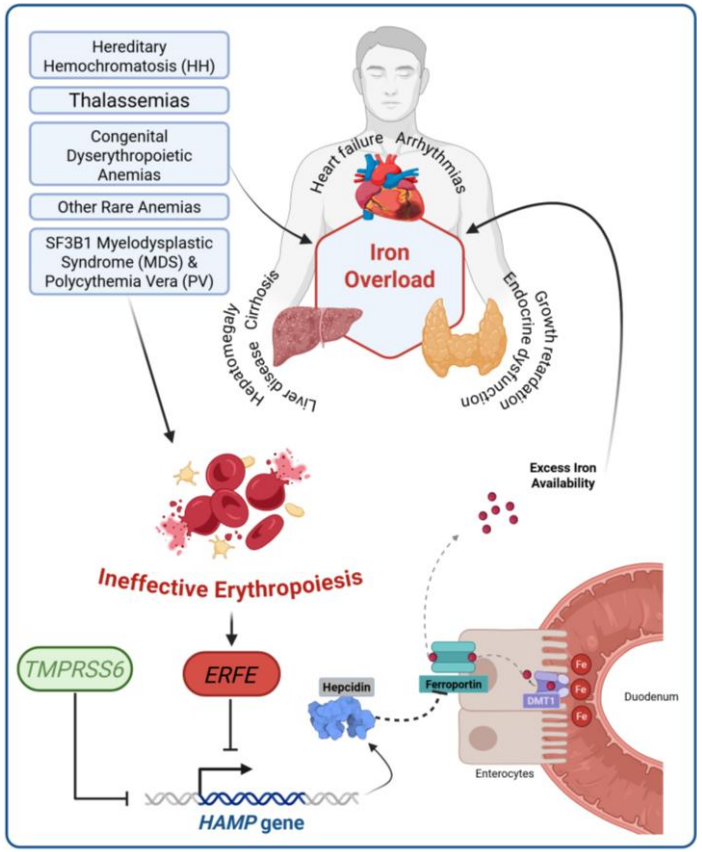


The project BETA-BET (EXCELLENCE/0524/0521) is co-financed by the European Regional Development Fund and the Republic of Cyprus through the Research and Innovation Foundation.

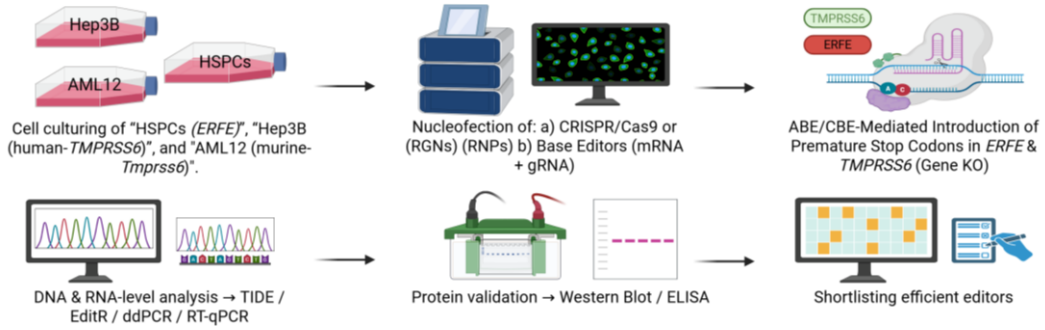
Unpublished data.

BETA-BET
Targeted Base Editing for Beta Thalassemia

Genome Editing for Iron-Restrictive Therapy of Hereditary and Acquired Hematological Disorders



In Vitro Editing & Validation



Lentiviral Vector Production & *In Vivo* Delivery

Cloning shortlisted *TMPRSS6* editors into LVs

Tail-vein injection for liver-specific delivery

β-Thalassemia ($Hbb^{th3/+}$)
HH (*HFE* C282Y)
PV (*JAK2* V617F)

Functional Evaluation (*Ex-Vivo* & *In Vivo*)

Ex-vivo *ERFE* KO HSPCs

- Erythroid differentiation → Flow cytometry
- Clonogenic assays / HPLC for Hb profile / ELISA for *ERFE*

Liver Tissue Analysis
Peripheral Blood Analysis
post-LV injection

- Hepcidin mRNA ↑ – Serum iron ↓ – Ferritin ↓
- Organ iron quantification (liver, spleen, kidney, heart)
- CBC and reticulocyte counts

In Silico Design & Selection

- Design of gRNA for *ERFE* and *TMPRSS6*
- **RGNs (Cas9)** and **Base Editors (ABE/CBE)**
- Human + Murine-compatible target sites

Specificity & Safety Evaluation

- GUIDE-seq / CIRCLE-seq → off-target mapping
- EndoV-seq for BEs
- Whole Exome + RNA-seq → genomic & transcriptomic safety
- CRISPECTOR analysis pipeline

Edit-4-IRON

BILATERAL/ISRAEL (MOST)/0224/0024

Unpublished data.

The Cyprus Institute of Neurology & Genetics – Blood Disorder Genetics & Thalassemia

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Thank you…!

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