Chronic Granulomatous Disease as a Model Rare Disease Target for Gene Editing

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General Gene Editing Considerations for Correction of a Monogenic Disease

- *How many patients need treatment?*
- *What should be the target cell or tissue?*
- *How much correction is required for clinical benefit or for cure?*
- *Is this a disorder in which correction is augmented by growth or survival advantage of the corrected target cell or its differentiated progeny?*
- *What are the endpoints for clinical benefit and any laboratory markers?*
- *What competing technologies are standard of care or in clinical trial?*
- *What is the cost (if known) of those alternate treatments?*
- *What advantage(s) does gene editing offer over existing technologies?*
- *For gene editing in particular, what are the alternative approaches for correction, editing/insertion tools required, and availability of those tools?*
- *Are the tools and patient cells/tissues needed for development accessible?*

Chronic Granulomatous Disease

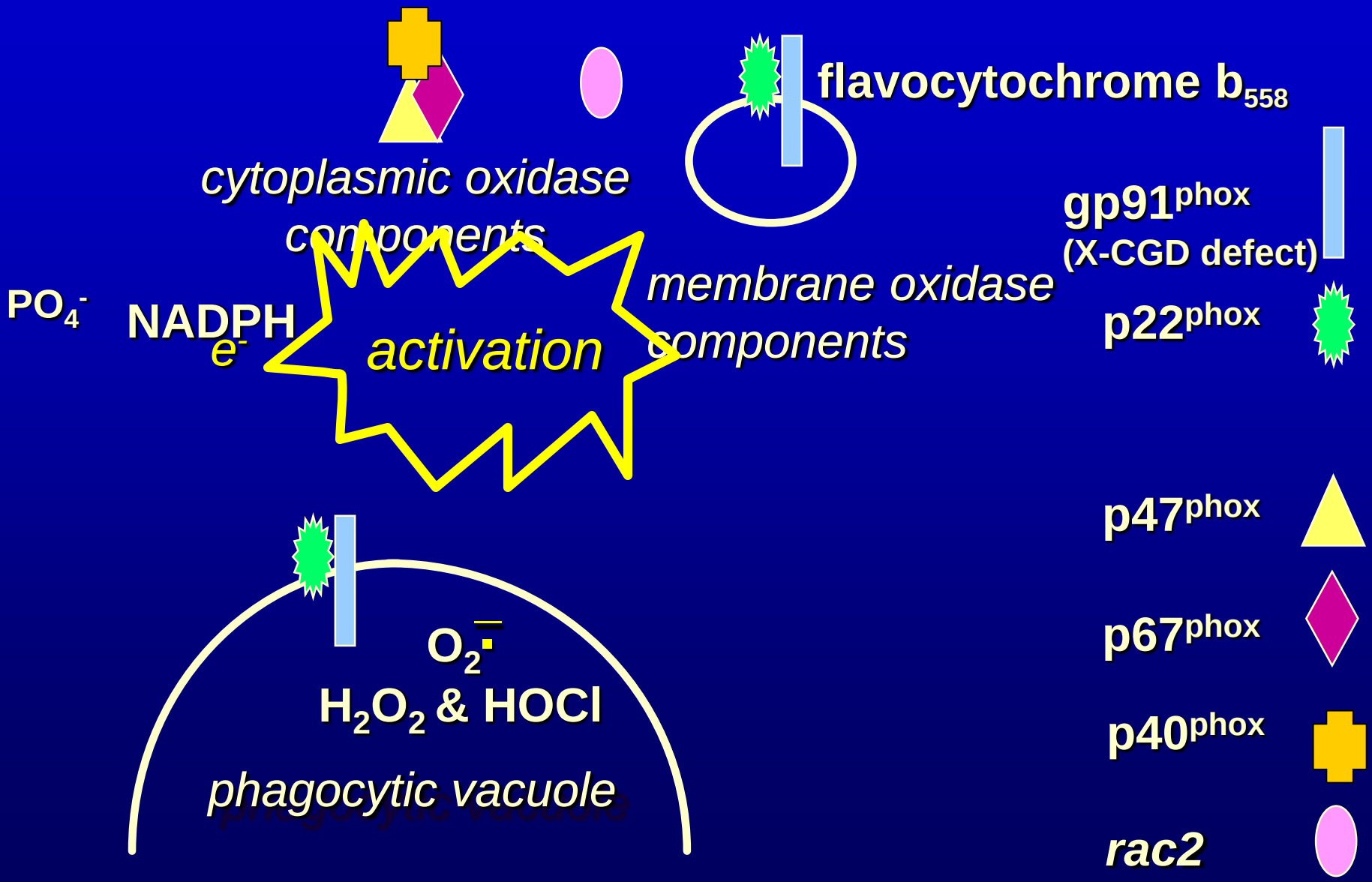
Incidence ~1:150,000 births; Most survive to adulthood, with increasing mortality after 20 years of age; there are ~1000 living patients in the US plus ~25/yr births in need of definitive therapy.

Blood neutrophils/monocytes & tissue macrophages fail to produce microbicidal oxidants. Patients experience recurrent infections & granulomatous inflammation.

Mutations in phagocyte oxidase subunits cause CGD

Type	% affected	Chromosome
X-linked:		
$gp91^{phox}$	~70%	X
Autosomal recessive:		
$p47^{phox}$	~25%	7
$p67^{phox}$	~2.5%	1
$p22^{phox}$	~2.5%	16
$p40^{phox}$	few patients	22

Phagocyte NADPH Oxidase



Chronic Granulomatous Disease – Background

- ***Bone marrow transplant achieves >80% event-free long-term cure of CGD, but donor availability, GvHD and graft failure remain unresolved issues. Demonstrates CD34+ hematopoietic stem cells are target for gene therapy:***
  Parta M, et al. 2017 J Clin Immunol. Jul 28. [Epub]; Parta M, et al. 2015 J Clin Immunol. 35:675.

- ***Female carriers of X-CGD are phenotypic mosaics for oxidase activity: >20% oxidase positive neutrophils = normal resistance to infection; <10% = increased risk of infection. Indicates how much correction is necessary:***
  Marciano BE, et al. 2017 J Allergy Clin Immunol. May 17. [Epub ahead of print]

- ***Normal vs CGD HSC/neutrophils have no selective growth or survival advantage. Gene therapy correction/engraftment must be efficient.***

- ***Increased residual oxidase activity at any level correlates with decreased mortality as a continuous function. More oxidase activity at any level provides clinical benefit and is a lab marker predictive of clinical benefit.***
  Kuhns DB, et al. 2010 N Engl J Med. 363:2600.

Chronic Granulomatous Disease – Background

Competing Technologies:

- ***Bone marrow transplant:*** ***Accepted standard of care for patients with severe phenotype. MUD transplant cost = $300-450,000; 3-5% mortality; 3-5% graft failure; 5-10% mixed chimerism; 10-20% chronic GvHD; sub-ablative to ablative conditioning needed.***

- ***Integrating vector gene therapy:*** **Ex vivo** ***integrating SIN lentivector clinical trials are in progress, achieving 20-40% oxidase positive circulating blood neutrophils in the few patients now at 6-18 months post treatment, with per-corrected-neutrophil superoxide generation at lower end of normal range; sub-ablative busulfan conditioning needed; no adverse events.***

Gene Editing Theoretical Advantages:

- ***Single intended genomic target site.***

- ***Repair of disease gene uses natural physiologic promoter regulation.***

X-linked Chronic Granulomatous Disease – Gene Editing

Approaches taken in our lab for correction of X-linked CGD:

- ***Insertion of gp91phox cDNA minigene into a genomic safe harbor site.***
- ***Insertion of gp91phox cDNA into the* CYBB *gene.***
- ***Seamless repair of a specific mutation in the* CYBB *gene.***

Zinc finger nuclease (ZFN) mediated insertion of MND promoter – gp91phox exon 1-13 cDNA minigene donor into AAVS1 Safe Harbor to achieve correction of X-linked CGD

De Ravin SS, Reik A, Liu PQ, Li L, Wu X, Su L, Raley C, Theobald N, Choi U, Song AH, Chan A, Pearl JR, Paschon DE, Lee J, Newcombe H, Koontz S, Sweeney C, Shivak DA, Zarember KA, Peshwa MV, Gregory PD, Urnov FD, Malech HL. Targeted gene addition in human CD34(+) hematopoietic cells for correction of X-linked chronic granulomatous disease. Nat Biotechnol. 2016 Apr;34(4):424-9

AAVS1 as a genomic "Safe Harbor Site" for therapeutic gene targeting

AAVS1:

Integration site of human Adeno-Associated Virus 2.

Within intron 1 of PPP1R12C gene at 19q13.42 (myosin binding Regulatory Subunit 12C of Protein Phosphatase 1).

Heterozygous PPP1R12C knockout mice have no phenotype.

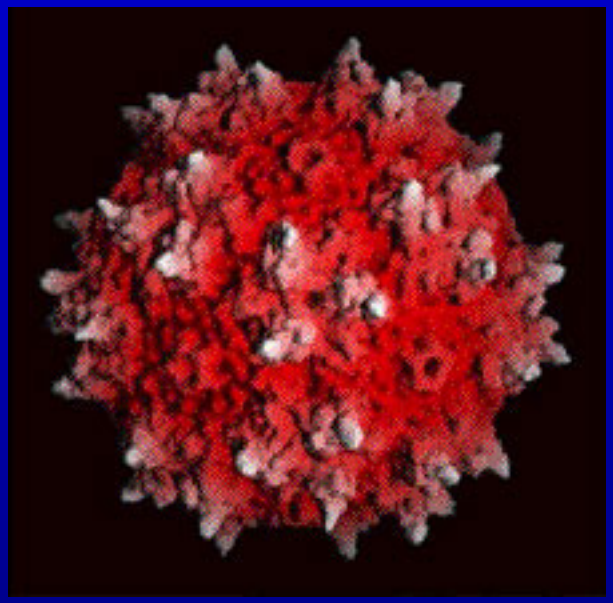
Has an open chromatin structure flanked by insulators.

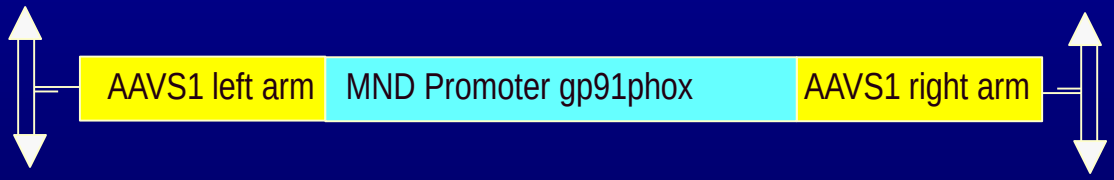
ZFN targeting of X-CGD minigene to AAVS1 safe harbor site in mobilized peripheral blood CD34^{+} HSC

- CRADA – Sangamo Therapeutics, Inc:
  - ZFN mRNAs targeting AAVS1
  - AAV6 used to deliver donor target template

- CRADA – MaxCyte, Inc: MaxCyte® GT™ high efficiency, low toxicity electroporation, capable of GMP clinical scale-up

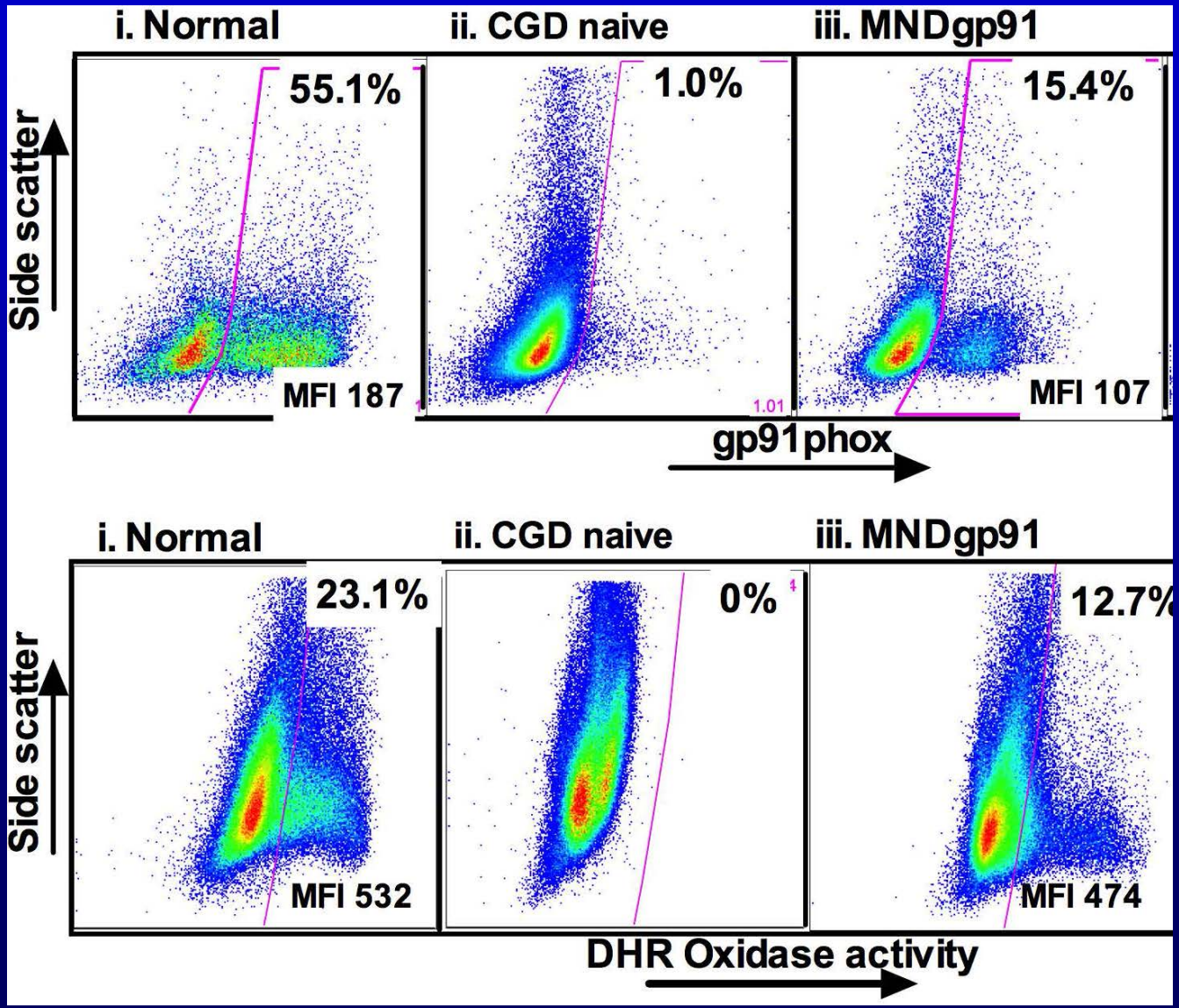
AAV-6 for donor delivery to CD34+ HSC





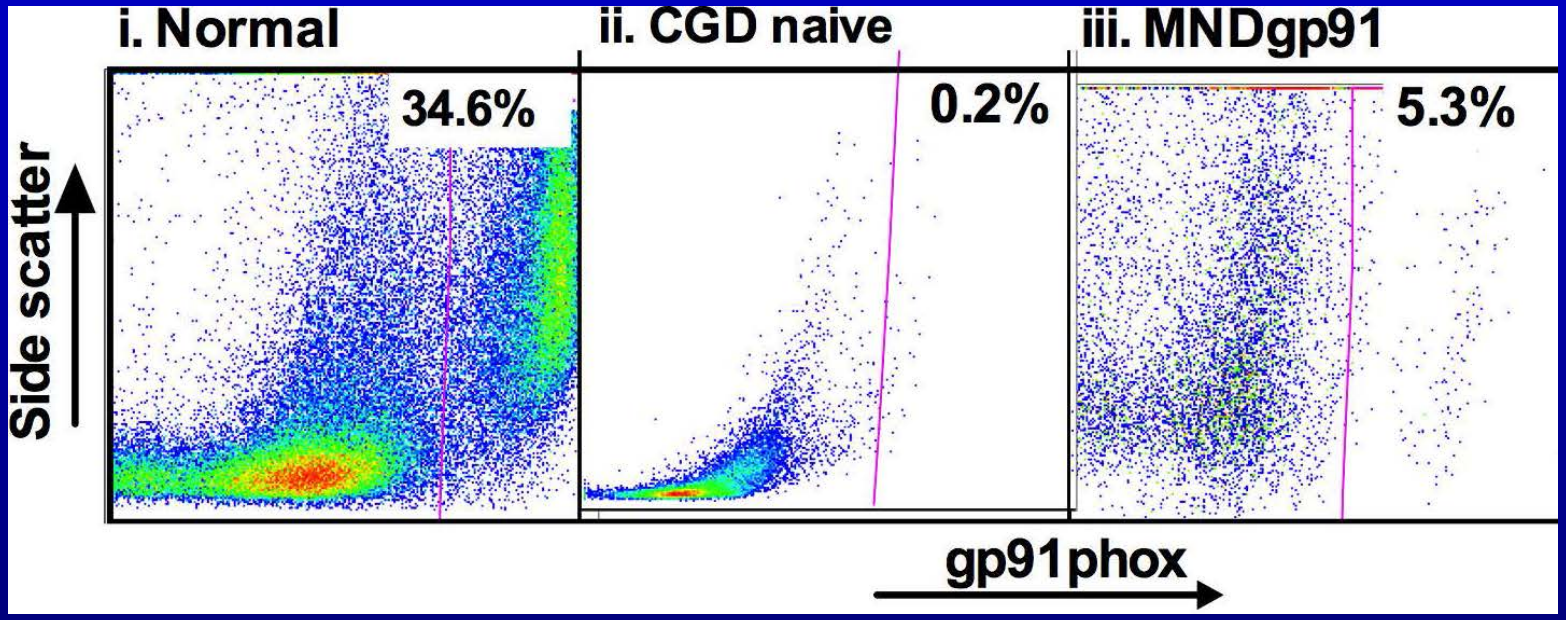
ZFN AAVS1 targeting of MNDgp91 to X-CGD patient peripheral blood mobilized CD34^{+} HSCs

Ex vivo correction 2 wks culture post treatment



ZFN AAVS1 targeting of MNDgp91 to X-CGD patient PB CD34^{+} HSCs

In vivo **NSG mouse marrow xenograft at 20 weeks after transplant (gated on human CD45+ cells)**



Sequence analysis indicates minigene insertion in ~15% of human cells in these 20 week xenografts.

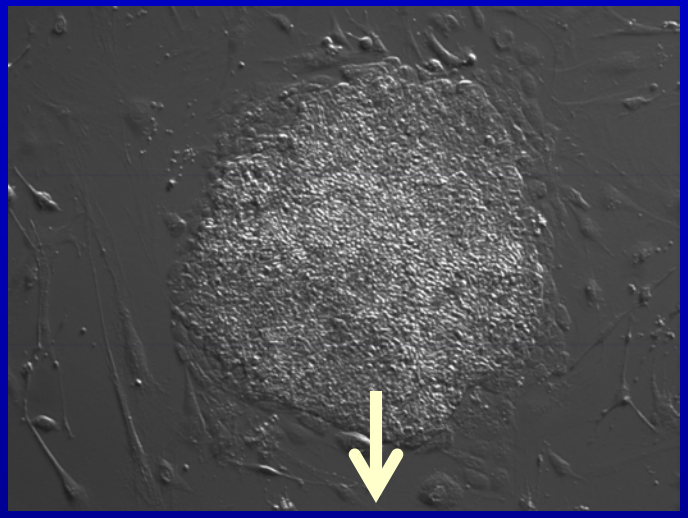
CRISPR/Cas9 mediated insertion of gp91phox exon 2-13 cDNA donor into CYBB gene at exon 2 to achieve correction of X-linked CGD

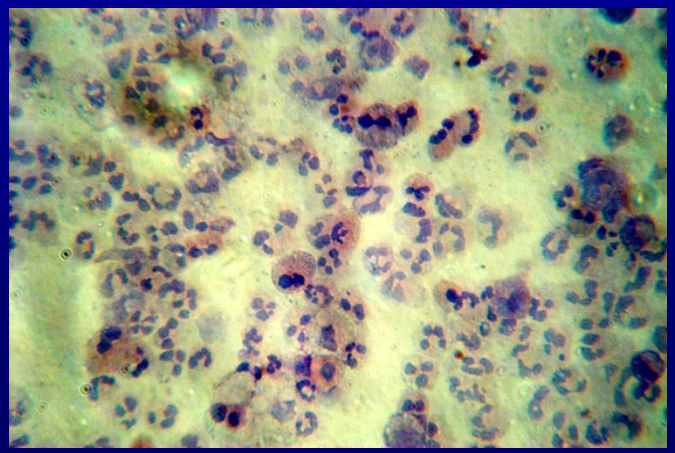
Studies preliminary to correction of CD34+ HSC were performed in induced pluripotent stem cells derived from X-CGD patients, showing that retention of intron 1 sequence is required.

Sweeney CL, Zou J, Choi U, Merling RK, Liu A, Bodansky A, Burkett S, Kim JW, De Ravin SS, Malech HL. Targeted Repair of CYBB in X-CGD iPSCs Requires Retention of Intronic Sequences for Expression and Functional Correction. Mol Ther. 2017 Feb 1;25(2):321-330.

Mature functional neutrophils from iPSC (left); DHR oxidase assay (right) shows absence of oxidase positive neutrophils differentiated from from X-CGD iPSCs

X-CGD iPSC clone from patient

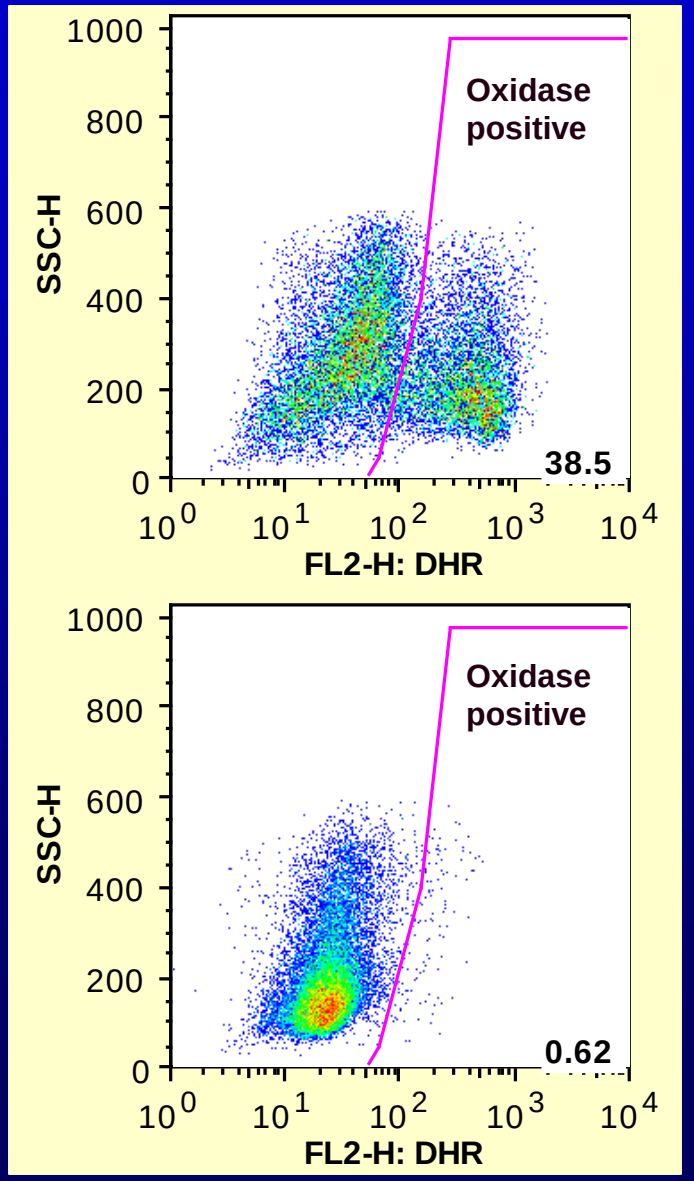




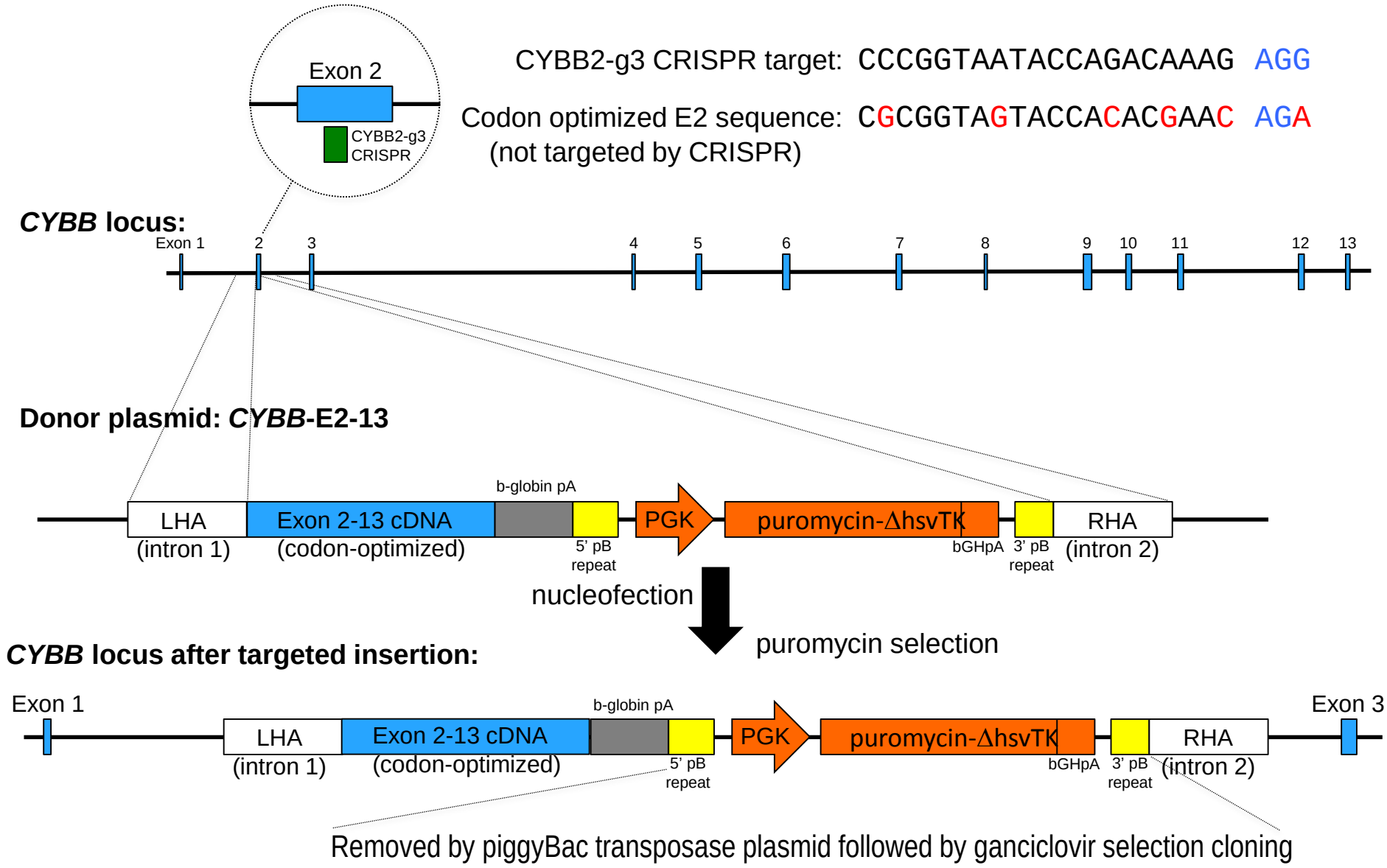
Neutrophils differentiated from X-CGD iPSC

Normal iPSC-derived neutrophils

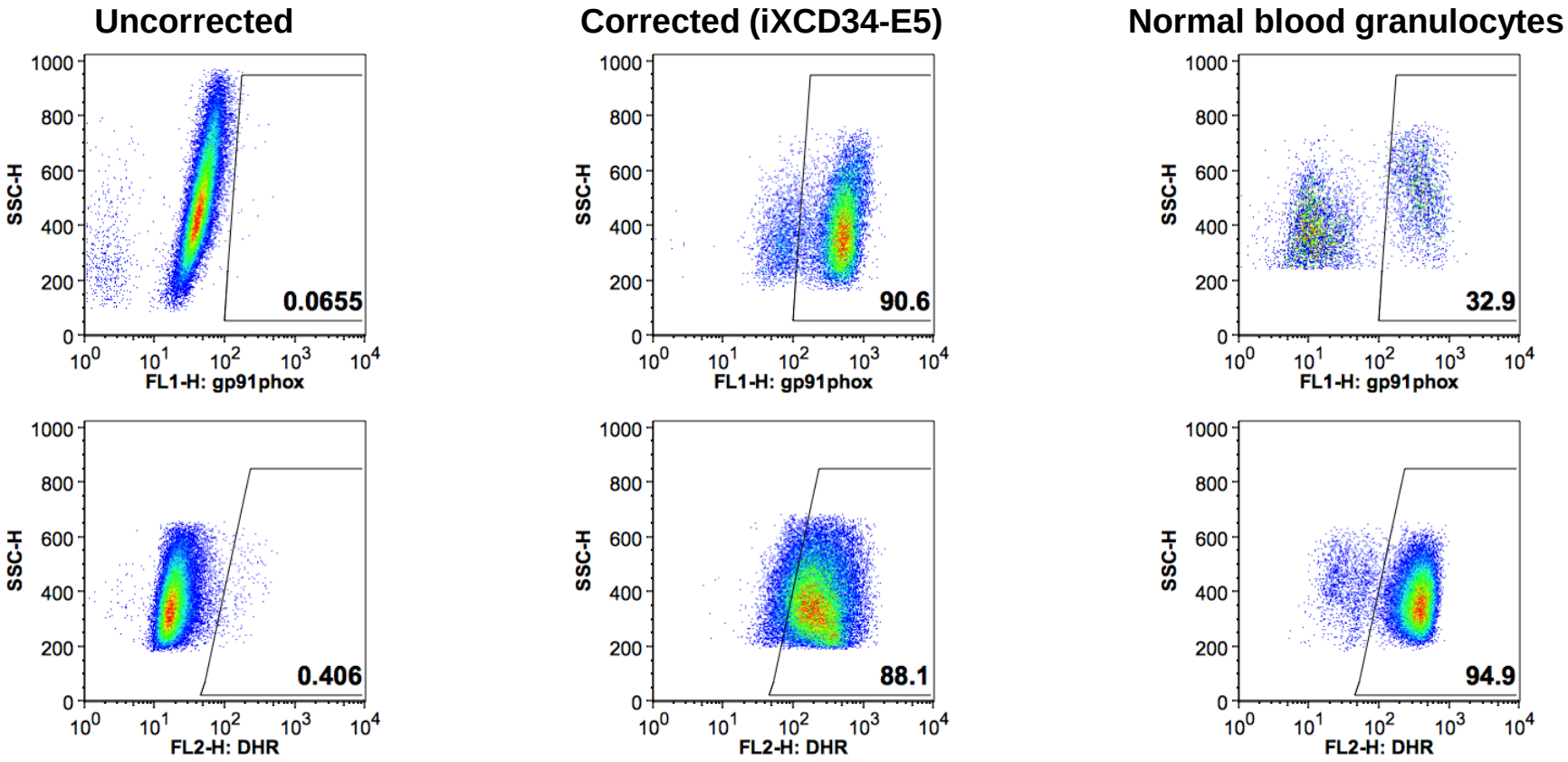
X-CGD iPSC derived neutrophils



CRISPR-mediated targeted insertion of codon-optimized *CYBB* cDNA encoding exons 2-13 into iPSC



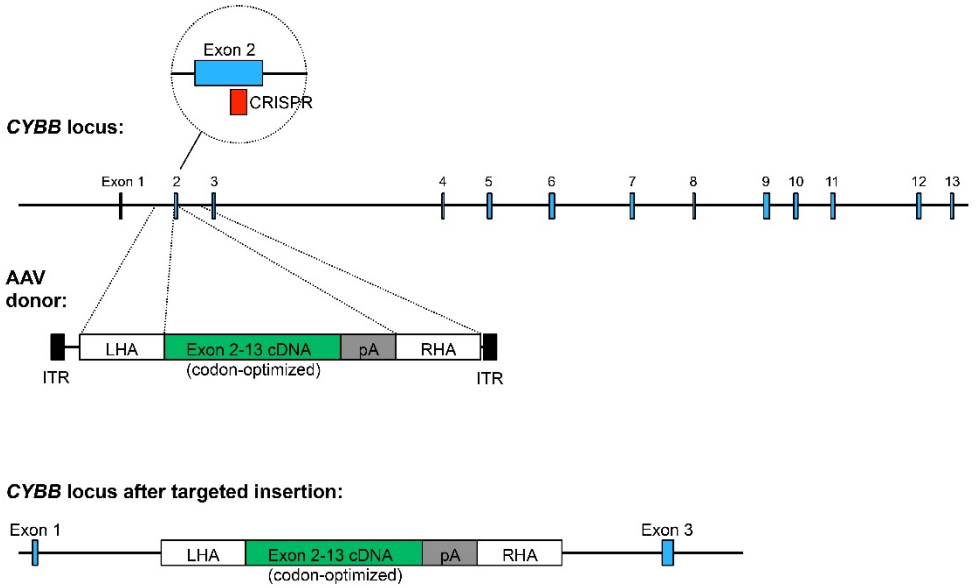
CRISPR-mediated insertion of *CYBB* exon 2-13 cDNA into exon 2 of the CYBB gene restores gp91^{phox} expression and oxidase activity (DHR assay) in iPSC-derived granulocytes



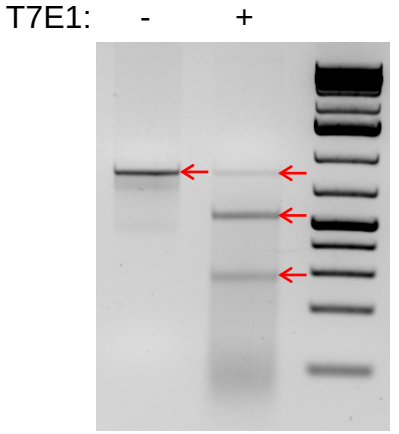
Targeted gene correction of X-CGD patient CD34^{+} cells with CRISPR RNP and *CYBB* exon 2-13 cDNA donor (AAV-6)

(unpublished data Sweeney & Malech)

CYBB targeted gene correction scheme:

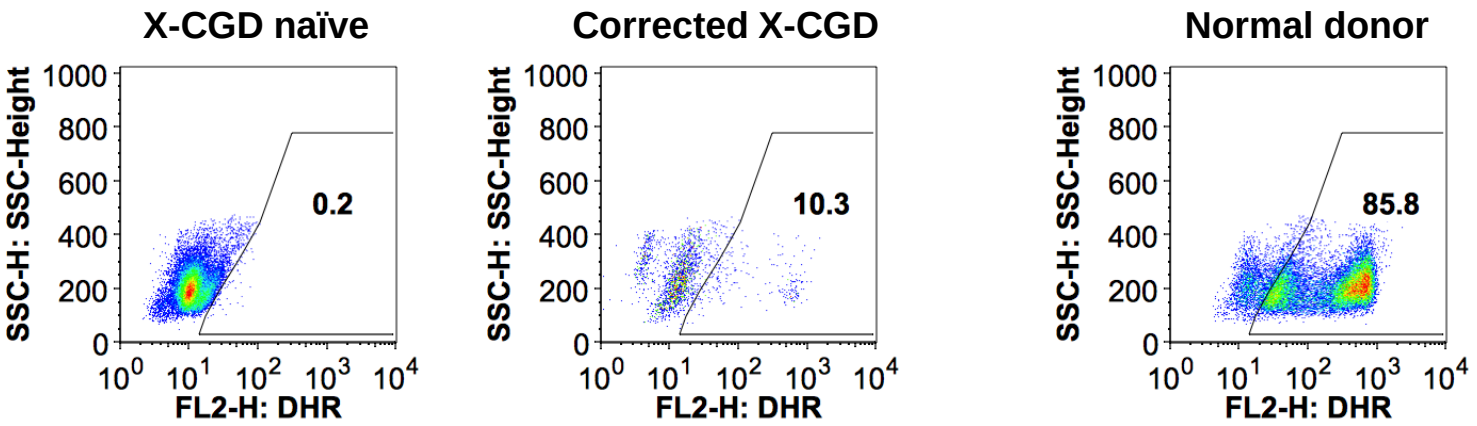


CRISPR activity (T7E1 assay) in nucleofected X-CGD CD34^{+} cells:



Indel activity: 87%

Oxidase activity (DHR assay) in neutrophils and monocytes differentiated from CD34^{+} cells *in vitro*:



CRISPR/Cas9 mediated seamless repair of CYBB *exon 7 point mutation using ssODN donor to mediate correction of X-linked CGD*

De Ravin SS, Li L, Wu X, Choi U, Allen C, Koontz S, Lee J, Theobald-Whiting N, Chu J, Garofalo M, Sweeney C, Kardava L, Moir S, Viley A, Natarajan P, Su L, Kuhns D, Zarember KA, Peshwa MV, Malech HL. CRISPR-Cas9 gene repair of hematopoietic stem cells from patients with X-linked chronic granulomatous disease. Sci Transl Med. 2017 Jan 11;9(372). pii: eaah3480.

We identified 17 patients (12 kindreds) with X-CGD followed at NIH with X-CGD caused by a single bp mutation in Exon 7 CYBB (a relative "hot-spot" affecting 6% of X-linked CGD patients).

1. **Mutation:** GAA ***C*****CGA** A **to** GAA ***T*****TGA** A C>T 676 leading to Arg(226)stop.

2. **Oxidase activity:** All patients in the group are gp91phox protein null and have no residual oxidase activity.

3. **Clinical significance:** 3 have died; 3 have received successful allogeneic bone marrow transplant at NIH; one received successful lentivector gene therapy at the NIH; remaining 10 living patients including both children and young adults have had recurrent life-threatening infections.

Gene editing using MaxCyte ® GT electroporation instrument for C>T 676 Exon 7 mutation repair

1. **CRISPR-Cas9 mRNA.**

2. **20 bp gRNA designed to be efficiently mutation specific and which poorly targets normal sequence.**

3. **100 bp single-stranded oligonucleotide donor DNA (2p phosphorothioate-modified) with normal *CYBB* sequence centered on mutation site.**

gRNA 2 homology relationship to mutation site

Splice acceptor

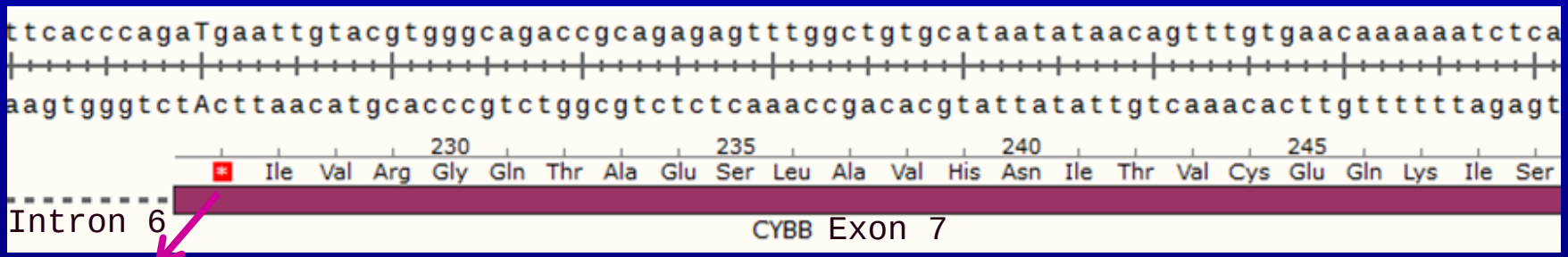
```
TTCACCCAGACGAATTGTACGTGGGCAGACC   Normal
TTCACCCAGATGAATTGTACGTGGGCAGACC   Mutant
  CACCCAGATGAATTGTACGT            gRNA
```

Mutation

Mutation C>T 676

Cut site

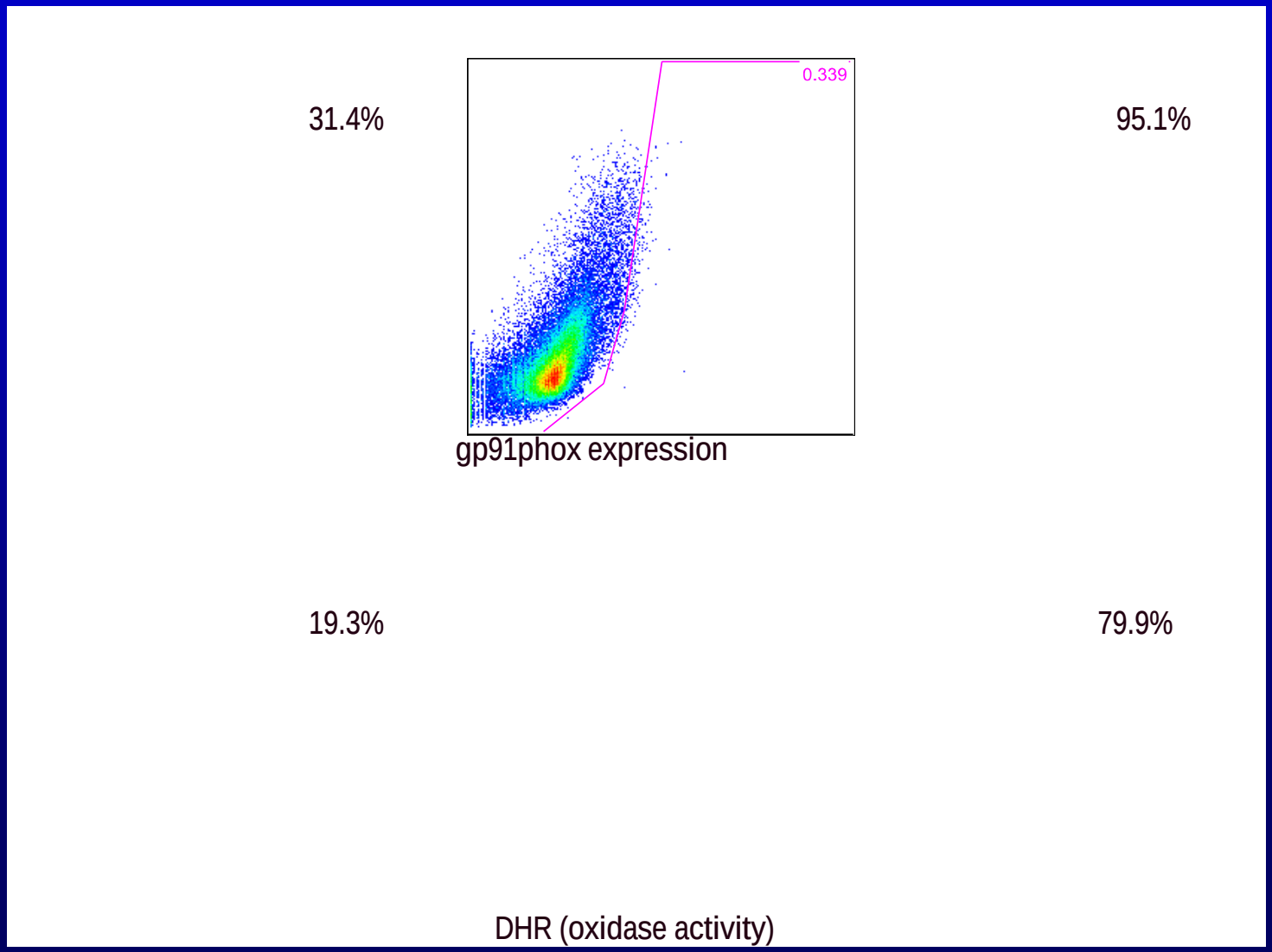
PAM



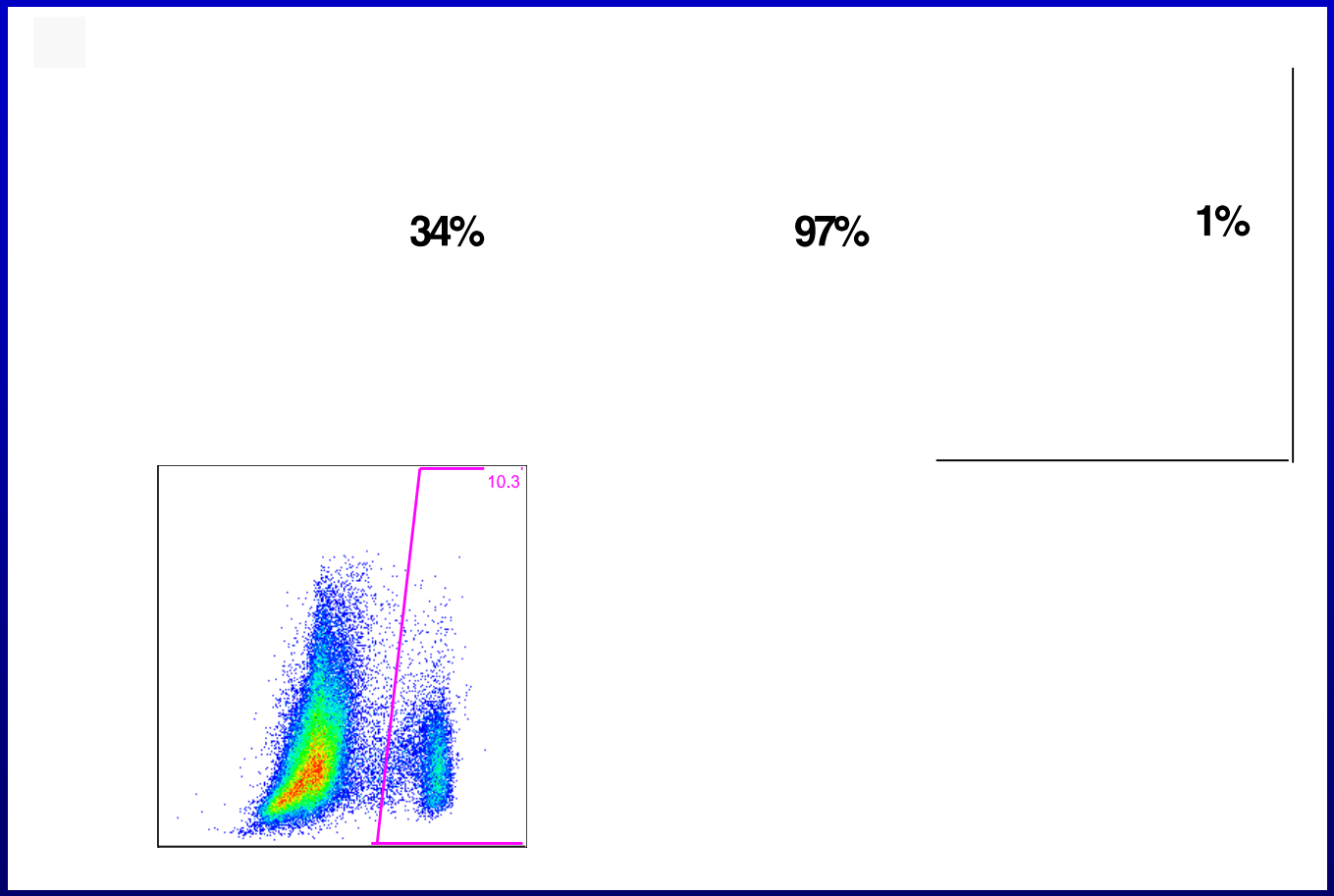
TGA Stop Codon → CGA Arg → g91 expression

Electroporate culture day 2 with CRISPR/Cas9 mRNA100 µg/ml, sgRNA 200 µg/ml, phosphorothioate-modified ssODN donor 200 µg/ml.

gp91phox expression and oxidase activity in neutrophils and monocytes differentiated in vitro at 3 weeks after CYBB *exon 7 mutation repair of patient (P1) CD34⁺ HSC*



gp91phox expression and oxidase activity in mouse bone marrow at 20 weeks after transplant of CYBB exon 7 mutation repaired patient (P1) CD34+ HSC



Gated on human CD45+ myeloid cells in the transplanted nsg mouse marrow

Gated on all human CD45+ cells in the transplanted nsg mouse marrow

High throughput sequencing of human CYBB exon 7 from all corrected P1 CD34+ HSC transplanted mice averaged ~20% corrected, ~40% indels, ~40% uncorrected mutant sequence. No off-target indels detected in top 10 predicted off-target sites.

Summary of Gene Editing for Correction of X-linked Chronic Granulomatous Disease

- *ZFN + AAV6 donor insertion of gp91phox cDNA minigene into the AAVS1 genomic safe harbor site in patient CD34+ HSC:*
  ~28% average insertion rate in vitro
  ~15% average insertion rate in vivo *in long term engrafting HSC*

- *CRISPR/Cas9 RNP + AAV6 donor insertion of gp91phox exon 2-13 cDNA into the* CYBB *gene in patient CD34+ HSC:*
  ~12% average insertion rate in vitro

- *Seamless repair of a specific mutation in the* CYBB *gene.*
  ~30% average mutation repair rate in vitro
  ~20% average mutation repair rate in vivo *in long term engrafting HSC*

Thank you.

LHD Lab / Clinical Team and Collaborators (Gene editing)

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