# Gene Therapy That Works

Inder M. Verma

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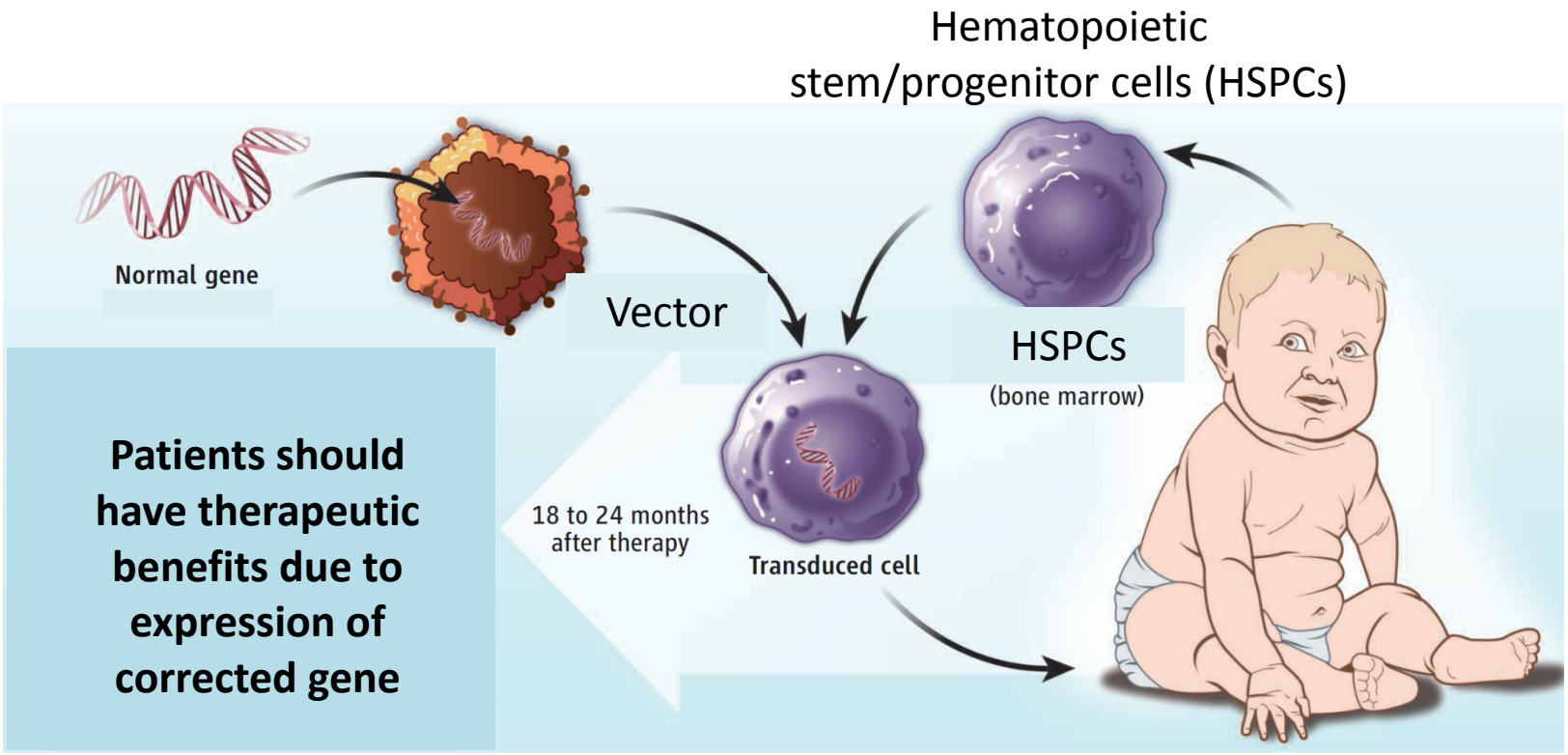

# Historical Notes: Gene therapy

- **1990**: First FDA approved gene therapy trial conducted on a four year old patient affected by ADA-SCID (Adenosine deaminase deficiency).
  - Retroviral vector
  - Effects were temporary but succesful

- **1999:** Adenovirus vector causes the death of a patient. Patient suffered from a partial deficiency of ornithine transcarbamylase (OTC).
  - Vector triggered a massive inflammatory response

- **2002–2003:** Retrovirus vector induces a lymphoproliferative disorder.
  - Gene therapy of (SCID)-XI disease.
  - The retrovirus genome had inserted near the LMO2 oncogene, activating LMO2 expression (leukemia due to insertional mutagenesis).

- In the last 12-13 years, over 50 patients affected by primary immunodeficiencies have been treated with genetically transduced autologous hematopoietic progenitors, mostly using **gamma retroviral vectors**

- **Since 1996**: lentiviral vectors have been developed and used for clinical applications.

**1.**

## Gene Therapy of Human Severe Combined Immunodeficiency (SCID)–X1 Disease

Marina Cavazzana-Calvo,*[1,2,3] Salima Hacein-Bey,*[1,2,3] Geneviève de Saint Basile,[1] Fabian Gross,[2] Eric Yvon,[3] Patrick Nusbaum,[2] Françoise Selz,[1] Christophe Hue,[1,2] Stéphanie Certain,[1] Jean-Laurent Casanova,[1,4] Philippe Bousso,[5] Françoise Le Deist,[1] Alain Fischer[1,2,4]†

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Gene therapy vector:

- Based on a **Moloney murine leukemia virus**

**2.**

## Lentiviral Hematopoietic Stem Cell Gene Therapy in Patients with Wiskott-Aldrich Syndrome WASP

Alessandro Aiuti,* Luca Biasco, Samantha Scaramuzza, Francesca Ferrua, Maria Pia Cicalese, Cristina Baricordi, Francesca Dionisio, Andrea Calabria, Stefania Giannelli, Maria Carmina Castiello, Marita Bosticardo, Costanza Evangelio, Andrea Assanelli, Miriam Casiraghi, Sara Di Nunzio, Luciano Callegaro, Claudia Benati, Paolo Rizzardi, Danilo Pellin, Clelia Di Serio, Manfred Schmidt, Christof Von Kalle, Jason Gardner, Nalini Mehta, Victor Neduva, David J. Dow, Anne Galy, Roberto Miniero, Andrea Finocchi, Ayse Metin, Pinaki P. Banerjee, Jordan S. Orange, Stefania Galimberti, Maria Grazia Valsecchi, Alessandra Biffi, Eugenio Montini, Anna Villa, Fabio Ciceri, Maria Grazia Roncarolo, Luigi Naldini

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Gene therapy vectors:

- **HIV derived** third generation self inactivating (SIN) lentivectors

**3.**

## Lentiviral Hematopoietic Stem Cell Gene Therapy Benefits Metachromatic Leukodystrophy ARSA protein

Alessandra Biffi,* Eugenio Montini, Laura Lorioli, Martina Cesani, Francesca Fumagalli, Tiziana Plati, Cristina Baldoli, Sabata Martino, Andrea Calabria, Sabrina Canale, Fabrizio Benedicenti, Giuliana Vallanti, Luca Biasco, Simone Leo, Nabil Kabbara, Gianluigi Zanetti, William B. Rizzo, Nalini A. L. Mehta, Maria Pia Cicalese, Miriam Casiraghi, Jaap J. Boelens, Ubaldo Del Carro, David J. Dow, Manfred Schmidt, Andrea Assanelli, Victor Neduva, Clelia Di Serio, Elia Stupka, Jason Gardner, Christof von Kalle, Claudio Bordignon, Fabio Ciceri, Attilio Rovelli, Maria Grazia Roncarolo, Alessandro Aiuti, Maria Sessa, Luigi Naldini*

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# Critical biosafety issue at the heart of gene therapy vector production: Prevent RCR/RCL Generation

- To date no RCR or RCL incidences have been reported for patient cell lots transduced with retroviral or lentiviral vectors in clinical studies.

- Only for early γ-retroviral vectors were RCR incidences observed in the production lot, not in the Master Cell Bank

**How to limit the possibility of RCR and RCL events:**

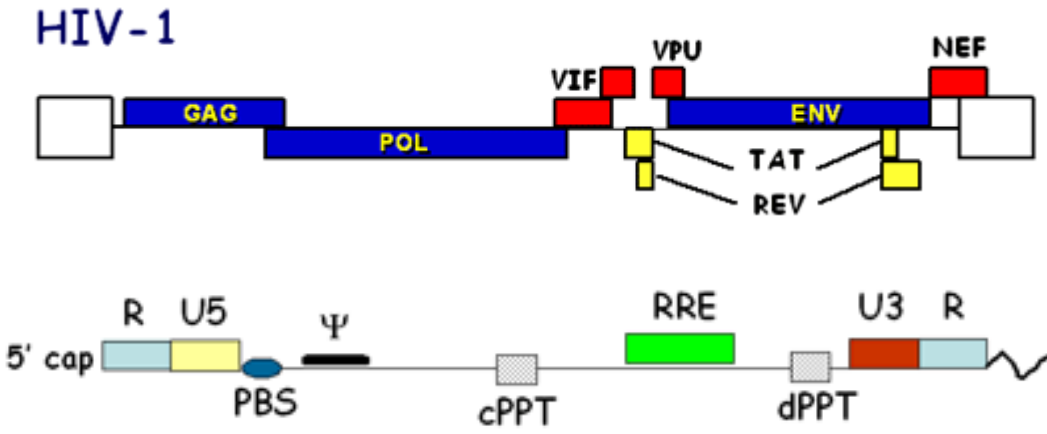
- Reduction of homology between vector and helper sequences: heterologous promoters or poly-adenylation sequences

- Reduction in homology between vector/helper sequences and cellular DNA:
Murine cells carry endogenous retroviral sequences with homology to the MLV-based gammaretroviral vectors. Using cells of a different species

# Reducing the possibility of RCR/RCL events

- distribute sequences of virus over as many independent units as possible

- maximize recombination events required to recreate a replication competent virus




**Coding sequences**

**Non- coding *cis* acting elements**

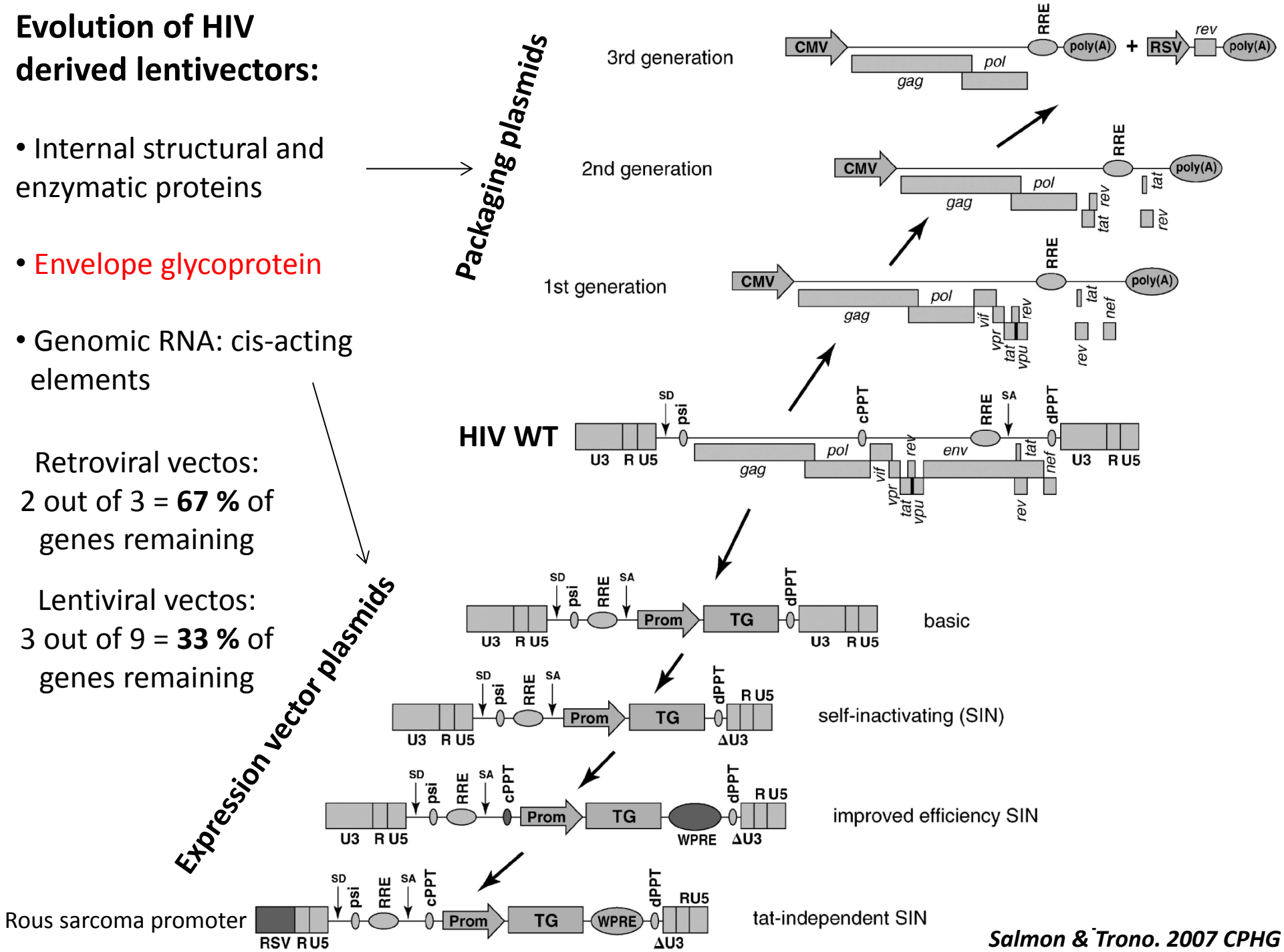
***Salmon & Trono. 2007 CPHG***

## Evolution of HIV derived lentivectors:

- Internal structural and enzymatic proteins
- Envelope glycoprotein
- Genomic RNA: cis-acting elements

Retroviral vectos:
2 out of 3 = **67 %** of genes remaining

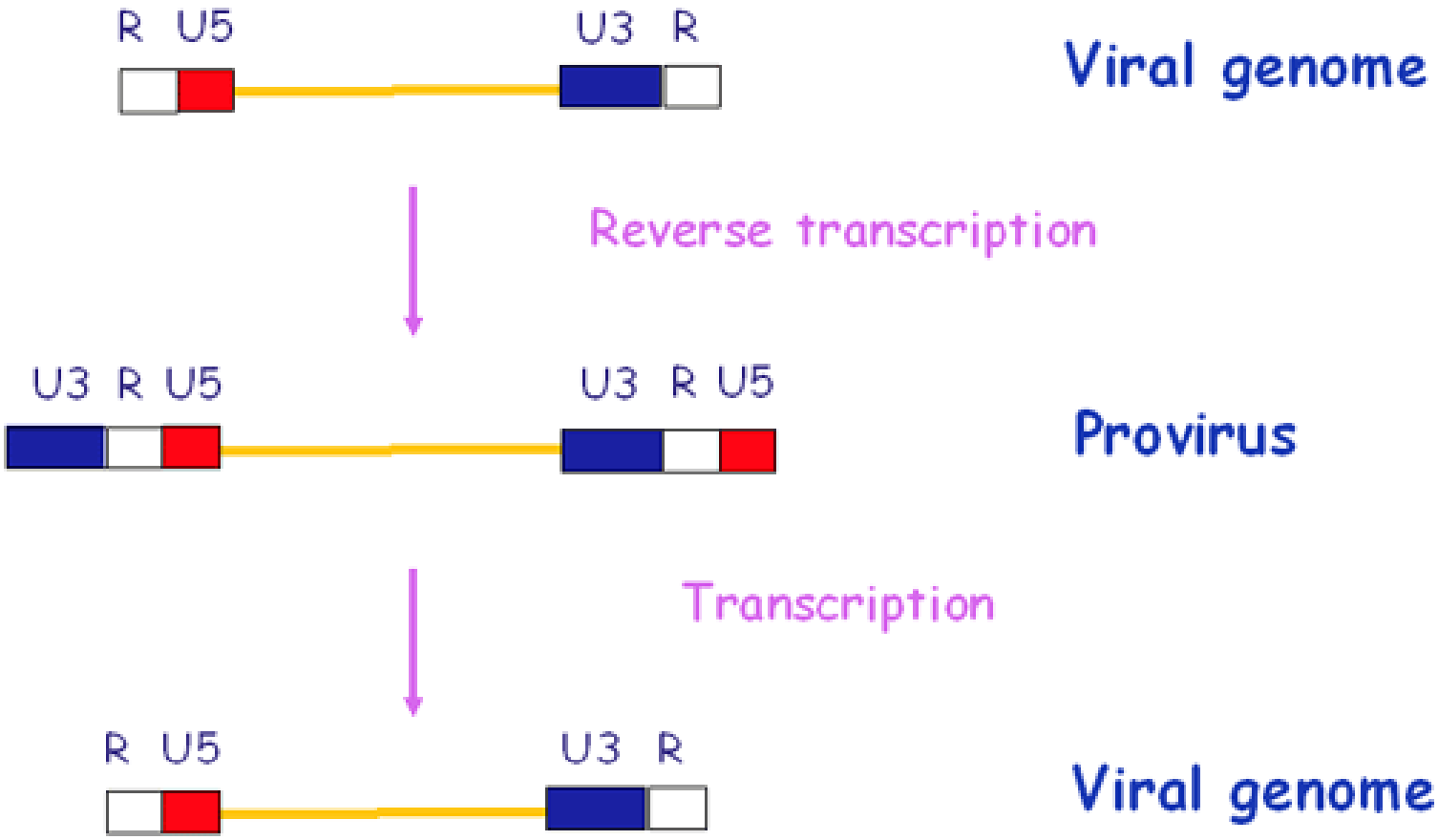
Lentiviral vectos:
3 out of 9 = **33 %** of genes remaining




*Salmon & Trono. 2007 CPHG*

## HIV replication:

- U3 for unique 3’ sequence: Duplicated during reverse transcription
- Present also at the 5’ end of the proviral DNA.
- It contains key elements for viral gene expression,  contains viral promoter.
- This is deleted to create the so called SIN lentivectors.

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# Gene Therapy of Human Severe Combined Immunodeficiency (SCID)–X1 Disease

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• **(SCID)-X1 AKA Bubble boy disease:** The body produces very few T cells and NK cells due to lack of the **interleukin-2 receptor subunit gamma** (IL2-RG)

• IL2-RG is the common cytokine receptor sub-unit protein for the following receptors: IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21.

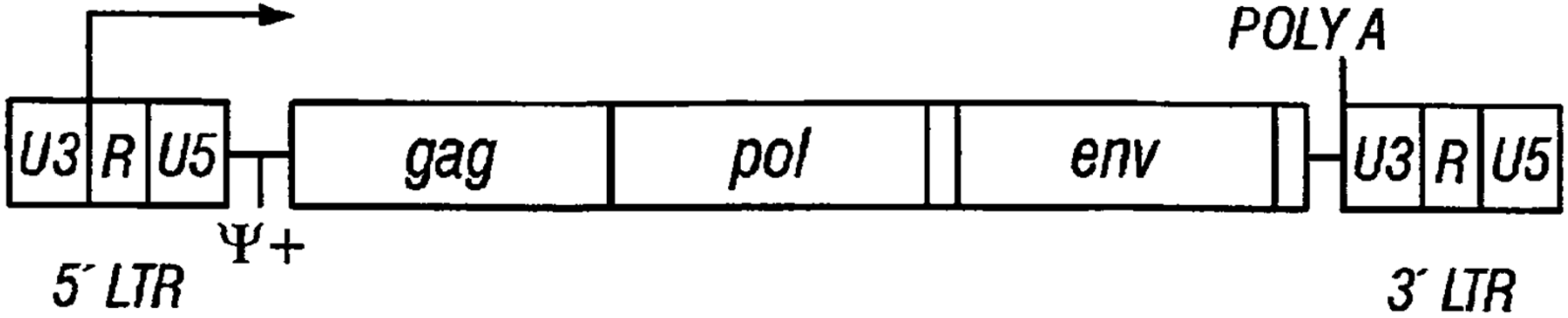
• γc gene mutations: Tailless receptor expressed at the membrane (P1)
- Protein truncated from the transmembrane domain not expressed at cell surface (P2)

• Signalling from these receptors normally promotes growth and differentiation of T cells, B cells, natural killer cells, glial cells and cells of the monocyte lineage

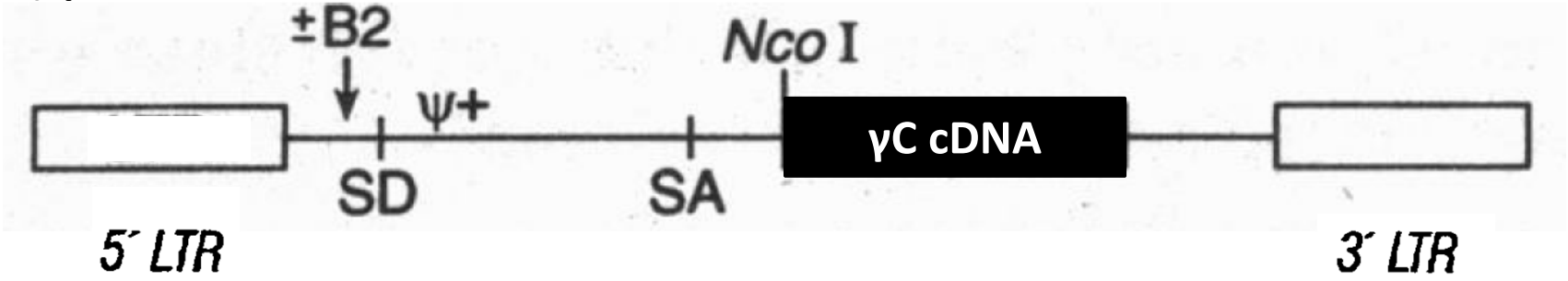
• **Gene therapy**: Reinfusion of HSPCs TRANSDUCED ex vivo with MLV vector, (MFG(B2)-γC) that carries the cDNA encoding the γc chain cytokine receptor gene

# Moloney murine leukemia vector system for (SCID) X-1 disease:

**Wild Type MLV**



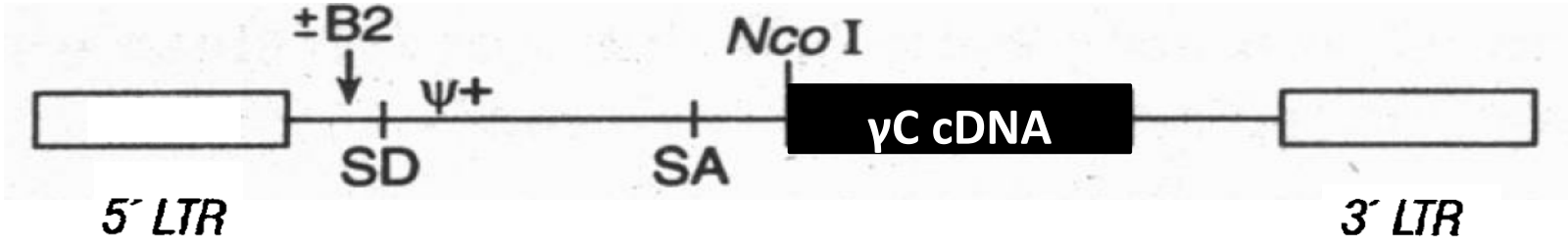

**MFG(B2)-γC vector**




- MFG(B2)-γc: Contains essential *cis*-acting elements and γc transgene, no viral genes.
- B2: Mutation in the viral tRNA primer binding site, improves vector gene expression
- SD/SA sites: Formation of sub-genomic mRNAs

***Danos et al., 1988 Proc Natl Acad Sci USA***

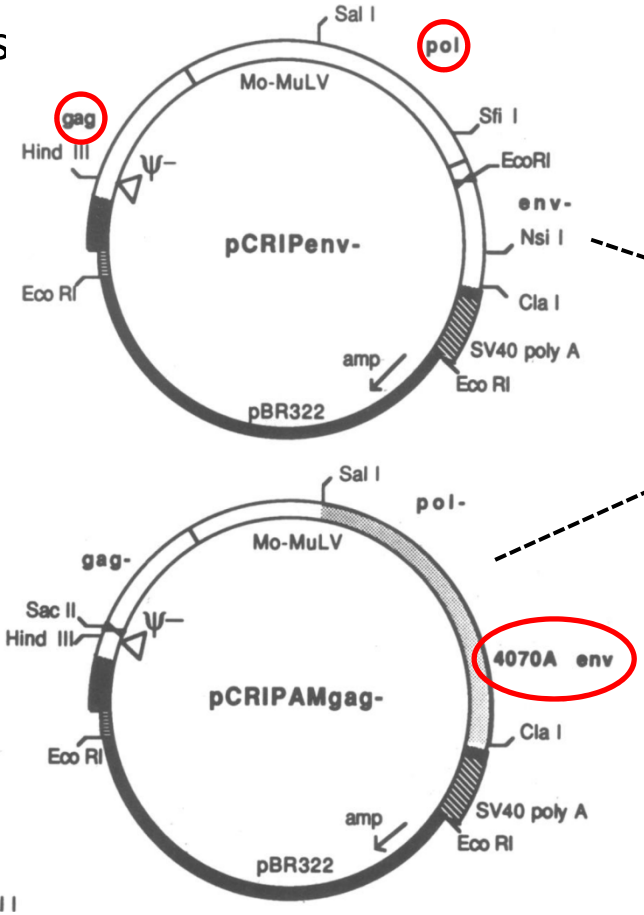
# MFG(B2)-γC vector packaged in ΨCRIP cell line (GMP):



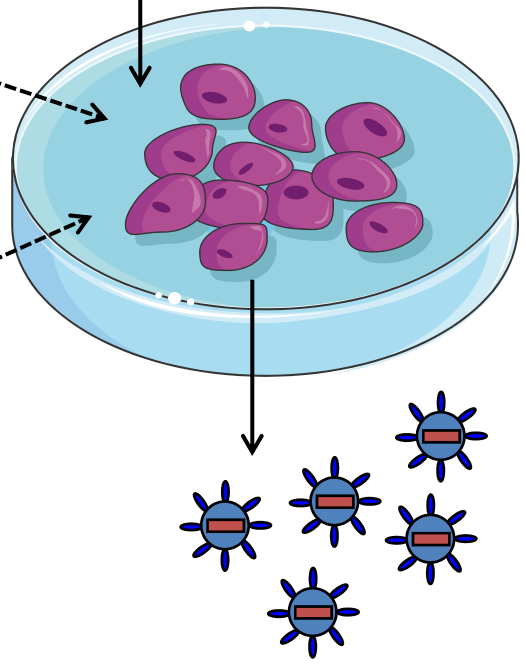

Amphotropic ΨCRIP cell lines were generated through two rounds of transfection and selection of NIH 3T3 cells:

- pCRIP env- + pSVHm (Hygromycin B)

-pCRIPAMgag- +pSV2gpt bacterial xanthine- guanine phosphoribosyltransferase gene for selection




NIH 3T3 modified mouse vector producer cell line (1988)



Harvest replication deficient MFG(B2)- γC vector

*Danos et al., 1988 Proc Natl Acad Sci USA*

## **Patient CD34+ cell isolation, culture and transduction:** (GMP)

- Bone marrow cells harvested
  - Hematopoietic progenitors positively selected by a magnetic CD34+ beads

- Magnetic selection = $9.8x10^6$ (P1) and $4.8x10^6$ (P2) CD34+ cells per kilogram

- **Preactivation:** cells cultured 24hrs at $0.5X10^6$ cells/ml

  - X-vivo 10 medium (4% fetal cell serum, stem cell factor , PEG-megakaryocyte differentiation factor, IL-3, and Flt3-L )

  - Containers were pre-coated with CH296 human fragment of fibronectin.

- **Transduction:** Retroviral containing supernatant was added every day for 3 days.

- **Cell harvest**: Cells washed twice and $19x10^6$ (P1) and $17x10^6$ (P2) CD34+ cells infused back

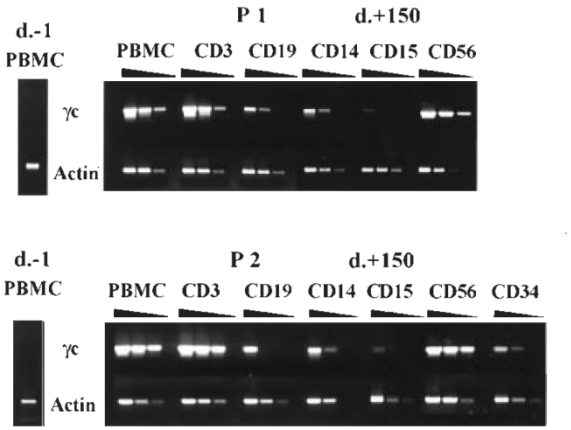
  - No prior chemo-ablation

  - Transduction rate: 20-40% (P1) and 36% (P2) of cells expressed the γc transgene

***Cavazzana-Calvo et al., 2000. Science***

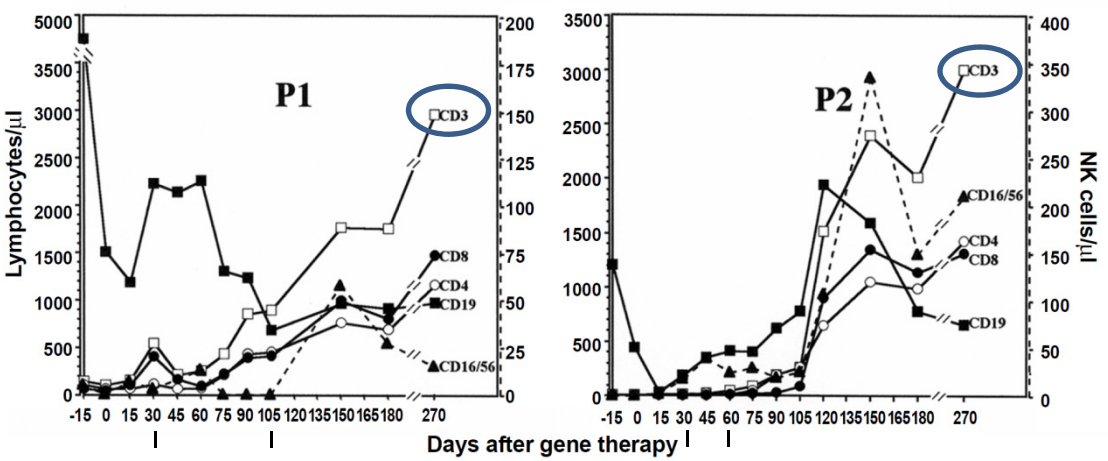
# Main Results:

## Detection of γc transgene in DNA




- Primers detect both PCR and RT PCR products. Amplify a 904bp stretch from the 3' end γc

## Recovery of T and NK cell populations




- Longitudinal study of lymphocyte subsets Absolute counts of T cells, B cells and NK cells (CD16+, CD56+)
- The scale for NK cells is on the right hand side of each panel

*Cavazzana-Calvo et al., 2000. Science*

# *LMO2*-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1

S. Hacein-Bey-Abina,[1,2*] C. Von Kalle,[6,7,8] M. Schmidt,[6,7]
M. P. McCormack,[9] N. Wulffraat,[10] P. Leboulch,[11] A. Lim,[12]
C. S. Osborne,[13] R. Pawliuk,[11] E. Morillon,[2] R. Sorensen,[19]
A. Forster,[9] P. Fraser,[13] J. I. Cohen,[15] G. de Saint Basile,[1]
I. Alexander,[16] U. Wintergerst,[17] T. Frebourg,[18] A. Aurias,[19]
D. Stoppa-Lyonnet,[20] S. Romana,[3] I. Radford-Weiss,[3] F. Gross,[2]
F. Valensi,[4] E. Delabesse,[4] E. Macintyre,[4] F. Sigaux,[20] J. Soulier,[21]
L. E. Leiva,[14] M. Wissler,[6,7] C. Prinz,[6,7] T. H. Rabbitts,[9]
F. Le Deist,[1] A. Fischer,[1,5]†‡ M. Cavazzana-Calvo[1,2]†

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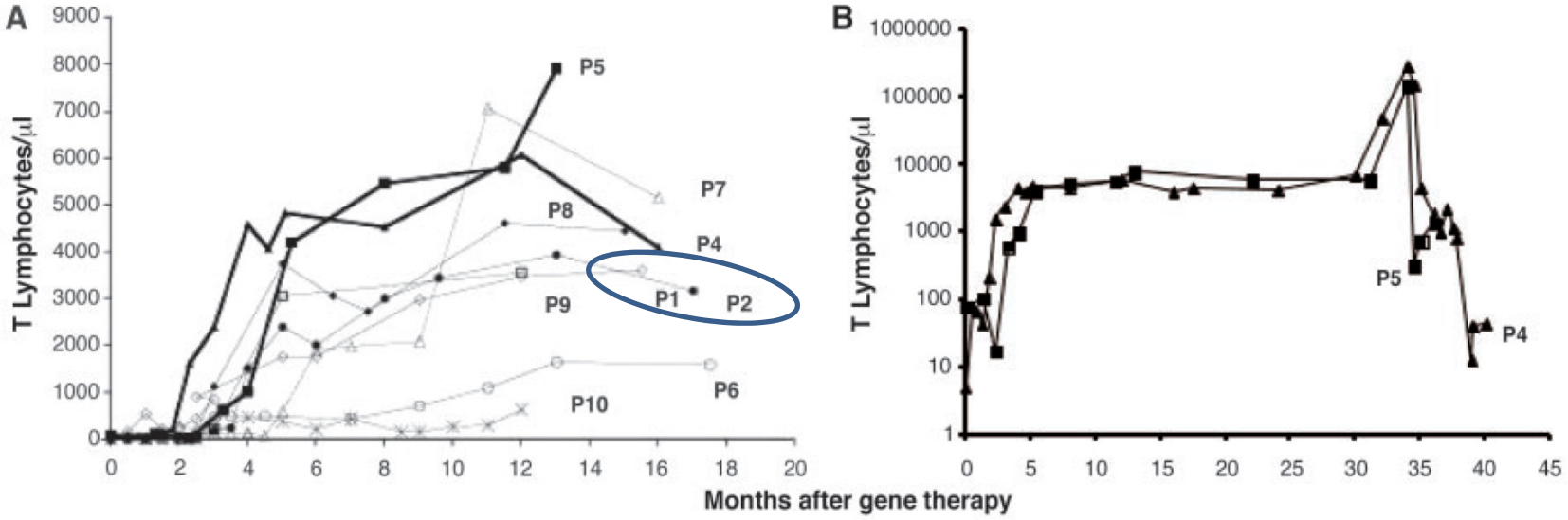
P4: M34+ T cell counts abruptly went from <10'000 to 300'000/ul




- T cell development early after gene therapy was especially rapid in P4 & P5
- P4 and P5 developed an clonal proliferation of mature T lymphocytes 30 and 34 months after gene therapy

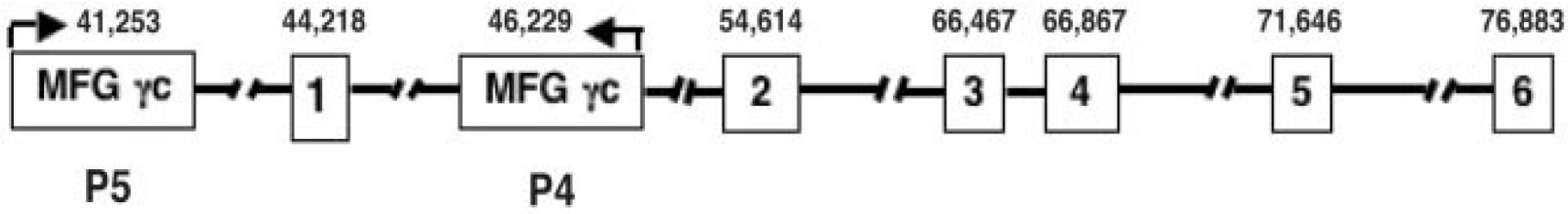
***Hacein-Bey-Abina et al., 2003. Science***

• **Activation of the LMO2 proto-oncogene**:

- LMO2 protein is a central regulator of hematopoiesis / yolk sac erythropoiesis

- LMO2 gene is downstream of a T cell translocation cluster (11p13 ttc)

-T-cell acute lymphoblastic leukemia-specific translocations occur at this site




• Retrovirus integration in close proximity to the LMO2 proto-oncogene promoter

• This resulted in aberrant transcription and expression of LMO2 and T cell leukemia

• Northern blot analysis of T cell clones from P4 and P5 revealed the 3.3-KB LMO2 transcripts

-Control T cells negative

- Levels of LMO2 protein in P4 & P5 = positive-control mouse erythroleukemia cell line

• Vector LTR exherted enhancer activity on the LMO2 promoter

• Cells with LMO2 activation due to vector integration had a growth advantage

***Cavazzana-Calvo et al., 2000. Science***

# Integration sites of viruses is not completely random

- Depending on the viruses, there is a preferential integration profile.

**Retrovirus** = MLV has a preference to integrate near transcription start sites and CpG islands.

**HIV derived LV** = Within transcriptional units, mainly introns & distant from CpG islands and transcription start sites, therefore should be safer than other types of vectors

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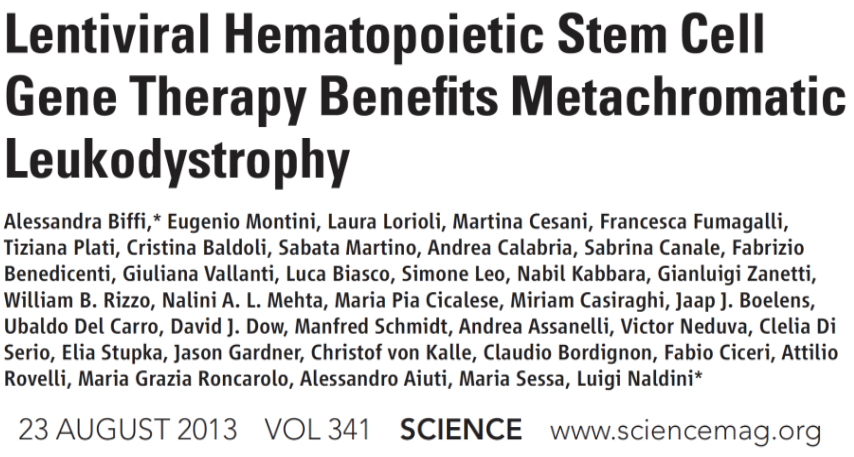

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## Outline

1. **LV-manufacturing process (GMP)**, aiming to produce replication deficient vector with high titer, infectivity, and purity

**2. Transduction protocol (GMP),** for reproducible high-rate gene transfer into BM-derived human HSPCs (infused fresh after transduction – no freeze/thawing)

**3. Assessment of the long-term safety of HSPC gene transfer IS analyses,** using deep-sequencing and bioinformatics to detect if there was polyclonal reconstitution of hematopoiesis without vector genotoxicity

## Lentiviral Gene Therapy for Wiskott-Aldrich Syndrome (WAS):

- **Wiskott-Aldrich syndrome (WAS):** X-linked primary immunodeficiency

  -Infections, microthrombocytopenia, eczema, autoimmunity, and lymphoid malignancies

  - Caused by mutations in the WAS gene - codes for WASP protein

  -WASP regulates the cytoskeleton

  -WASP-defective immune cells display alterations in proliferative responses upon activation, cell migration, immunological synapsis formation, and cytotoxicity.

  - Allogeneic HSPC transplantation can be curative, but with substantial morbidity/mortality

- **All three patients had <5% WASP expression**

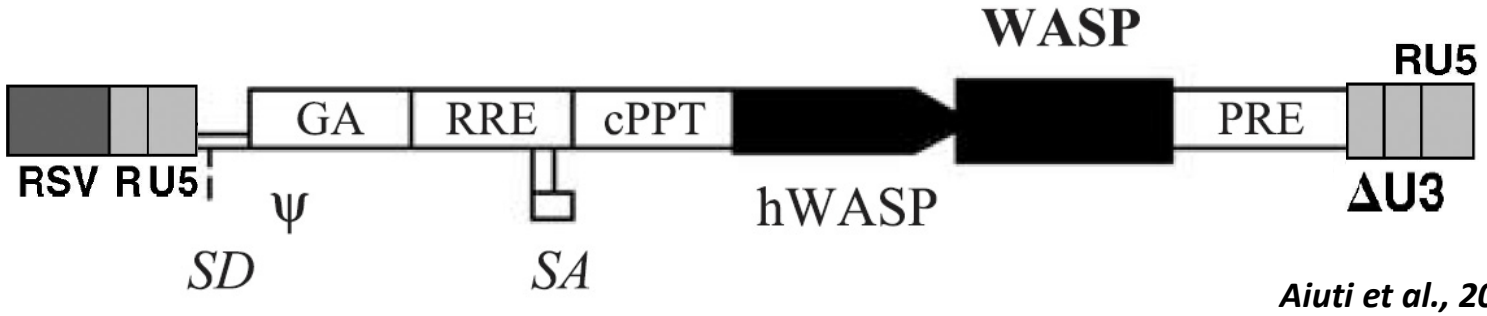
## Lentiviral Gene Therapy for WAS patients: LV-w1.6W

- Third generation SIN lentiviral vector coding for human WASP

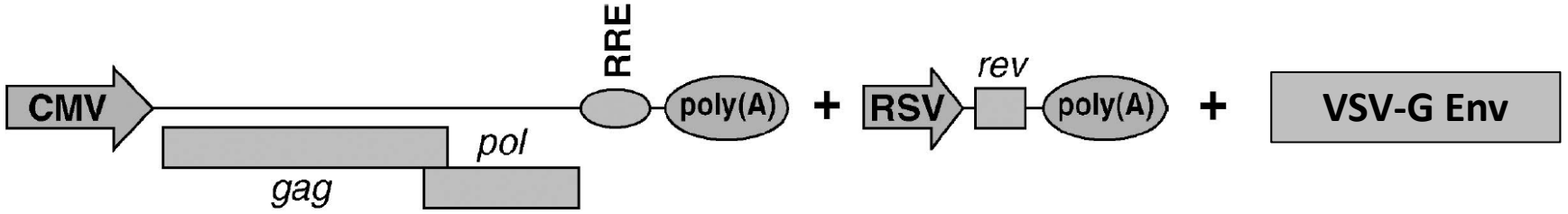
- Expression from a 1.6-kb reconstituted WAS gene promoter

- Endogenous promoter expressed WASP at physiological levels

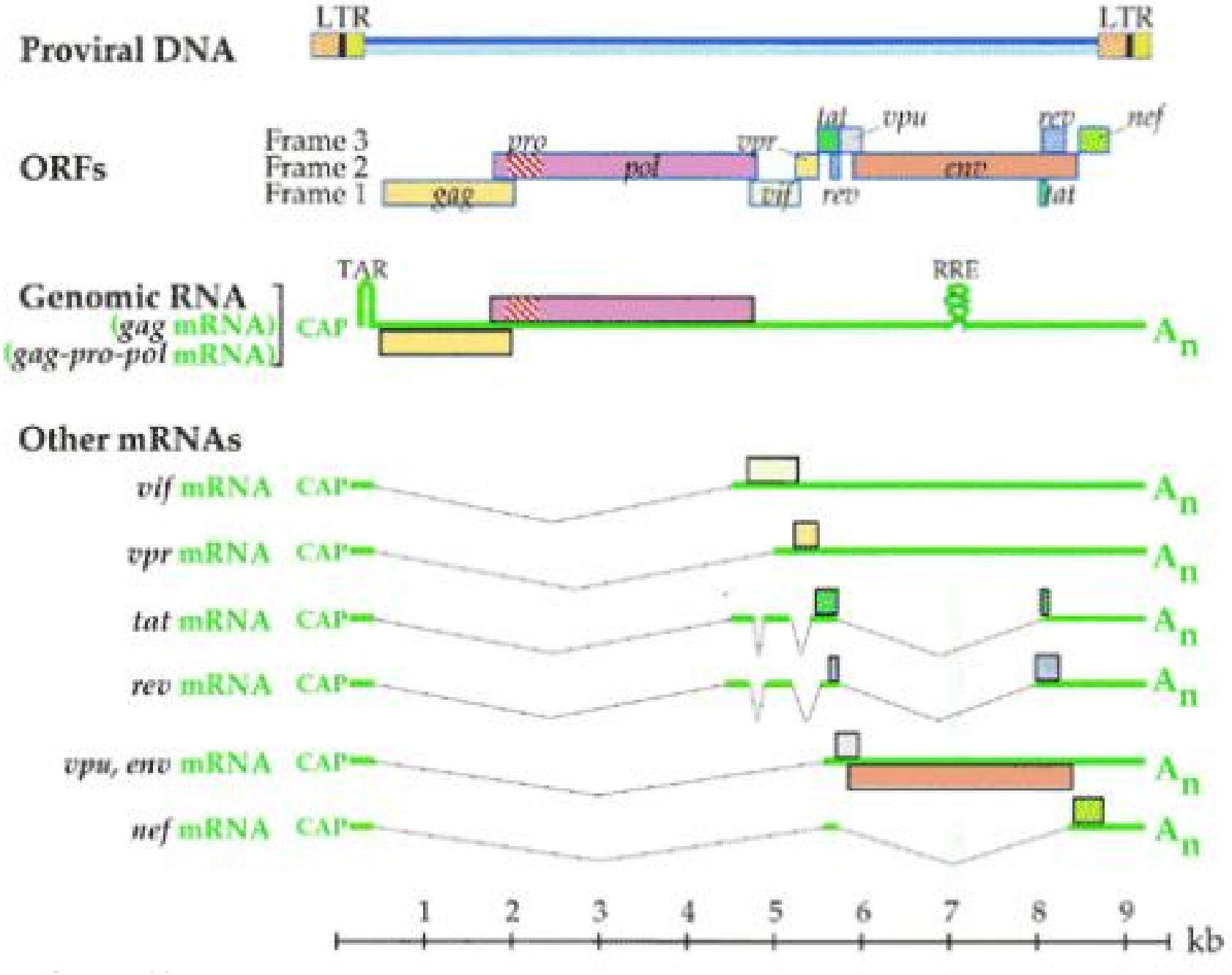
- Promoter has moderate enhancer activity/SIN LTR low risk of insertional mutagenesis




*Aiuti et al., 2013. Science*




*Salmon & Trono. 2007 CPHG*


Proviral DNA
LTR
LTR
ORFs
Frame 3
Frame 2
Frame 1
pro
pol
gag
vpr
vif
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rev
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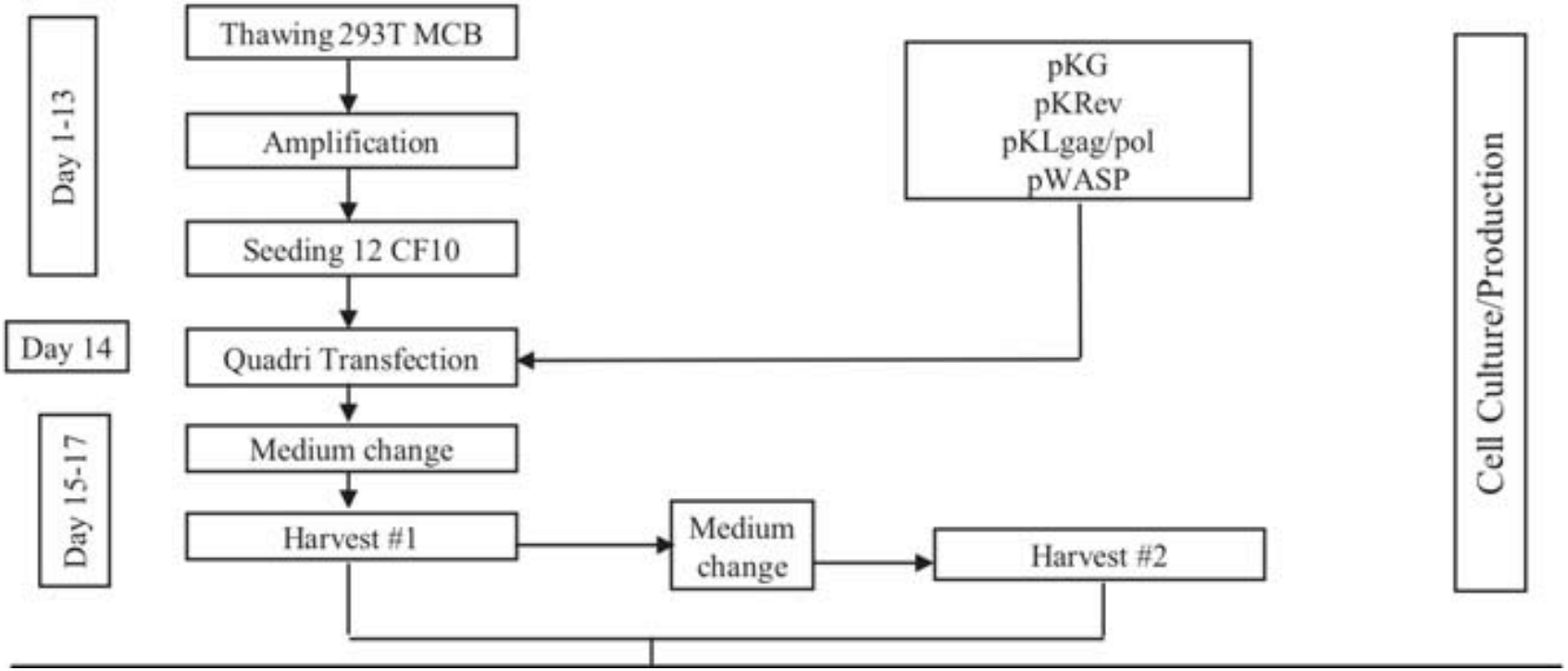
rev
nef
tat
Genomic RNA
(gag mRNA)
(gag-pro-pol mRNA)
TAR
RRE
CAP
An
Other mRNAs
vif mRNA
vpr mRNA
tat mRNA
rev mRNA
vpu, env mRNA
nef mRNA
1
2
3
4
5
6
7
8
9
kb

## 1. LV-manufacturing process

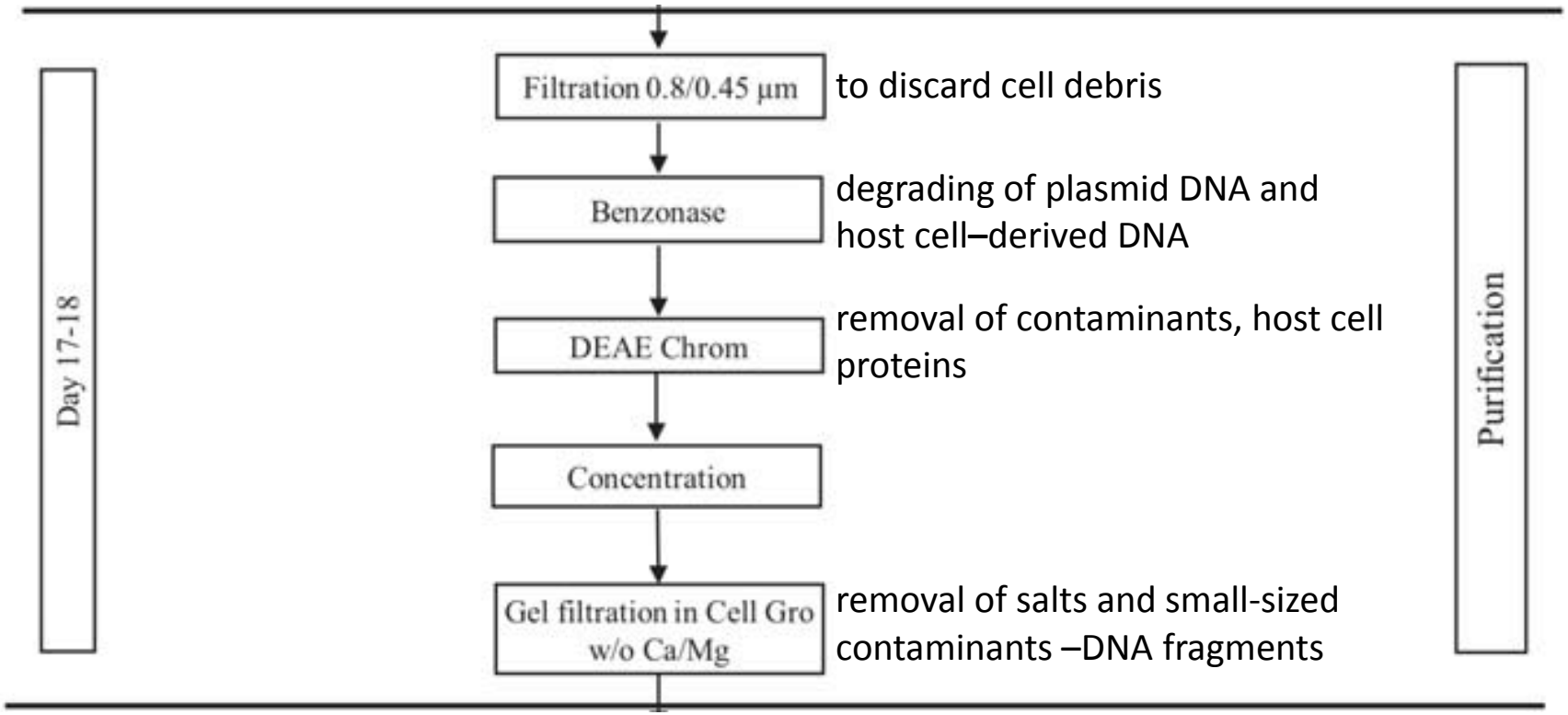
- Quadritransfection 293T cells
  - 2x harvesting of vector

SV40 origin of replication, allows episomal replication of transfected plasmids in 293T cells express the SV40 Large T-antigen, this increases protein expression levels by permitting more plasmid copies to persist in the transiently transfected cells



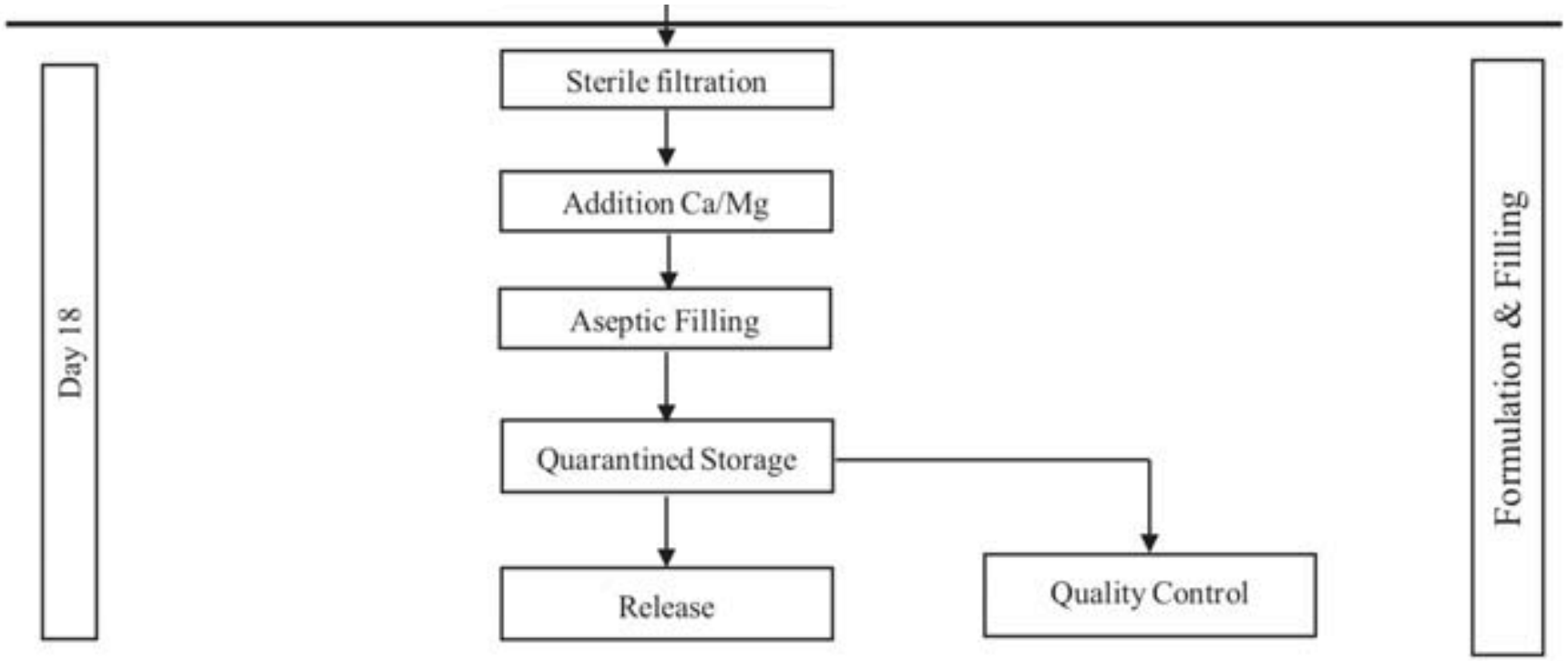

*Aiuti et al., 2013. Science*

# 1. LV-manufacturing process: Purification




***Aiuti et al., 2013. Science***

# 1. LV-manufacturing process: Sterilization




Physical titer was measured with ELISA for HIV-1 gag p24 capsid protein

Infectious titer was measured through transduction of a human T cell line with serial dilutions of vector and calculation of the copies of integrated vector per cell by quantitative PCR

***Aiuti et al., 2013. Science***

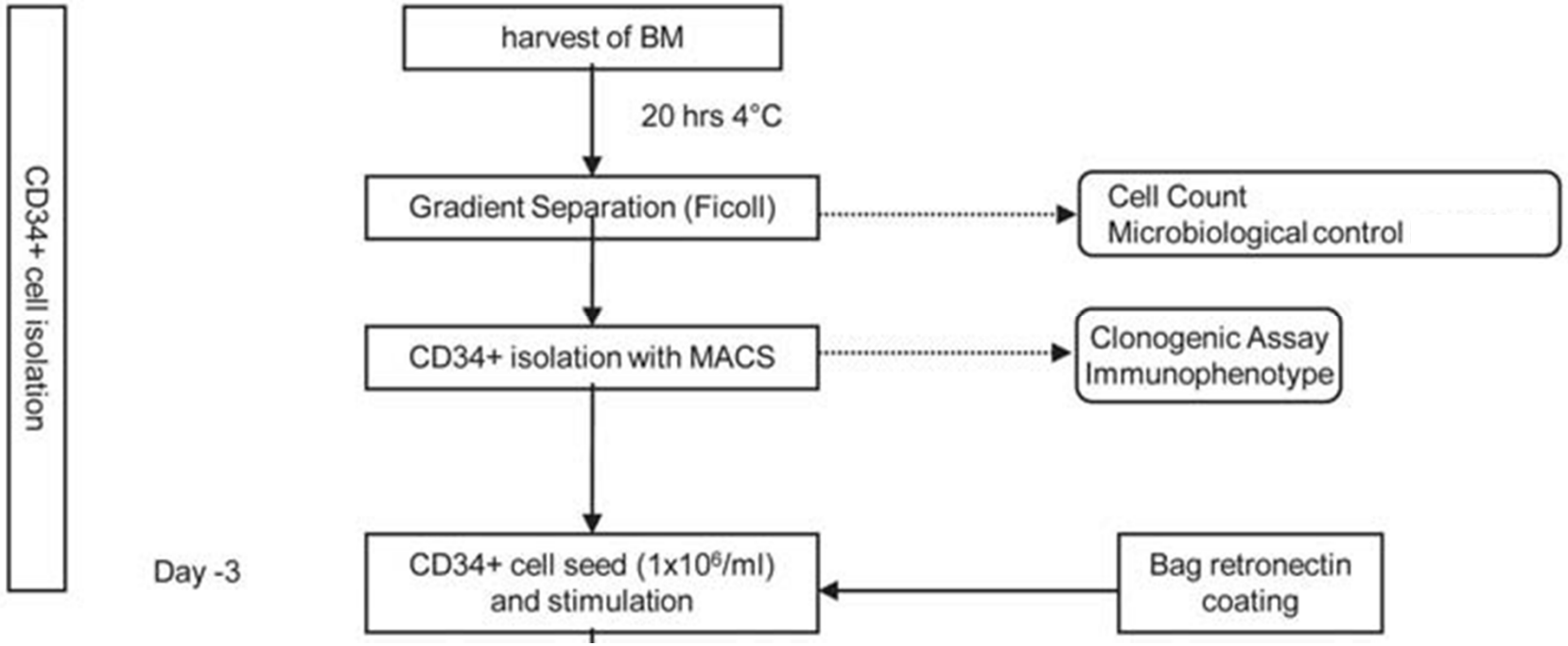
# Vector quality control: Titer, potency, purity and safety

| PARAMETER | GMP BATCHES | | |
|---|---|---|---|
| | *08073* | *08078* | *09010* |
| Initial titer (TU/ml) | $1.2x10^7$ | $1.7x10^7$ | $1.0x10^7$ |
| Initial Physical particles (ng p24/ml) | $3.0x10^2$ | $3.5x10^2$ | $3.6x10^2$ |
| Initial Infectivity (TU/ng p24) | $4.0x10^4$ | $5.0x10^4$ | $3.0x10^4$ |
| Initial Volume (Litres) | 25 | 25 | 25 |
| Final titer (TU/ml) | $7.6x10^8$ | $5.0x10^8$ | $2.1 x10^8$ |
| Final Physical particles (ng p24/ml) | $1.2x10^4$ | $1.3x10^4$ | $8.6x10^3$ |
| Final Infectivity (TU/ng p24) | $6.1x10^4$ | $3.8x10^4$ | $2.4 x10^4$ |
| Final Volume (ml) | 180 | 180 | 180 |
| Overall Yield (TU) | 45% | 21% | 14% |
| Overall Yield (p24) | 29% | 27% | 17% |
| Total TU | $1.4x10^{11}$ | $9.0x10^{10}$ | $3.8x10^{10}$ |

| TEST | SPECIFICATION | BATCH NO. | | |
|---|---|---|---|---|
| | | *08073* | *08078* | *09010* |
| *Physicochemical and Identity* | | | | |
| Osmolality (mOsm/Kg) | 260-350 | 303 | 304 | 305 |
| pH EP 2.2.3 | 7.0-8.0 | 7.6 | 7.7 | 7.7 |
| WASP transgene sequence | Corresponding | Corresponding | Corresponding | Corresponding |
| Vector integrity | Corresponding to reference | Corresponding | Corresponding | Corresponding |
| Lentiviral proteins | Corresponding to reference | Corresponding | Corresponding | Corresponding |
| *Potency and Bioactivity* | | | | |
| InfectiousTiter (TU/ml) | $\geq 2.0 \times 10^8$ | $7.6 \times 10^8$ | $5.0 \times 10^8$ | $2.1 \times 10^8$ |
| Physical titer (HIV Gag p24 Antigen) (ng/ml) | FIO | $1.2 \times 10^4$ | $1.3 \times 10^4$ | $8.6 \times 10^3$ |
| Infectivity (Transducing unit/ng p24) | $\geq 2.0 \times 10^4$ | $6.1 \times 10^4$ | $3.8 \times 10^4$ | $2.4 \times 10^4$ |
| Transgene expression | WAS protein presence | Present | Present | Present |
| *Microbial purity and safety* | | | | |
| Sterility EP 2.6.1 | Negative | Negative | Negative | Negative |
| Mycoplasma EP 2.6.7 (cultural assay) | Negative | Negative | Negative | Negative |
| Endotoxin EP 2.6.14 (quantitative assay) (EU/ $2x10^8$ TU) | $\leq 25$ | 14 | 21 | 9 |
| In vitro Adventitious viruses | Negative | Negative | Negative | Negative |
| In vivo Adventitious viruses | Negative | Negative | Negative | Negative |
| RCL | Negative | Negative | Negative | Negative |
| *Process and product impurities* | | | | |
| Host cell proteins (ng/ $2x10^8$ TU) | FIO | 21 | 33 | 79 |
| Plasmid residual DNA (VSV-G) (copies/ $2x10^8$ TU) | $\leq 4x10^8$ | $0.4 \times 10^8$ | $0.7 \times 10^8$ | $2.3 \times 10^8$ |
| Large T antigen protein contamination (ng/ml) | $\leq$ LLOQ | $\leq$ LLOQ | $\leq$ LLOQ | $\leq$ LLOQ |
| Large T antigen Residual DNA (copies / $2x10^8$ TU) | $\leq 2x10^5$ | 1.1 x 104 | 1.9 x 104 | 9.2 x 104 |
| Benzonase contamination (ng/ml) | $\leq 0.2$ | $< 0.1$ | $< 0.1$ | $< 0.1$ |
| E1A residual DNA (copies/ $2x10^8$ TU) | $\leq 2x10^5$ | 2.0x 104 | 4 .0x 104 | 9.1 x 104 |

***Aiuti et al., 2013. Science***

# 2. Transduction protocol: preparation of transduced CD34$^{+}$ cells (I)



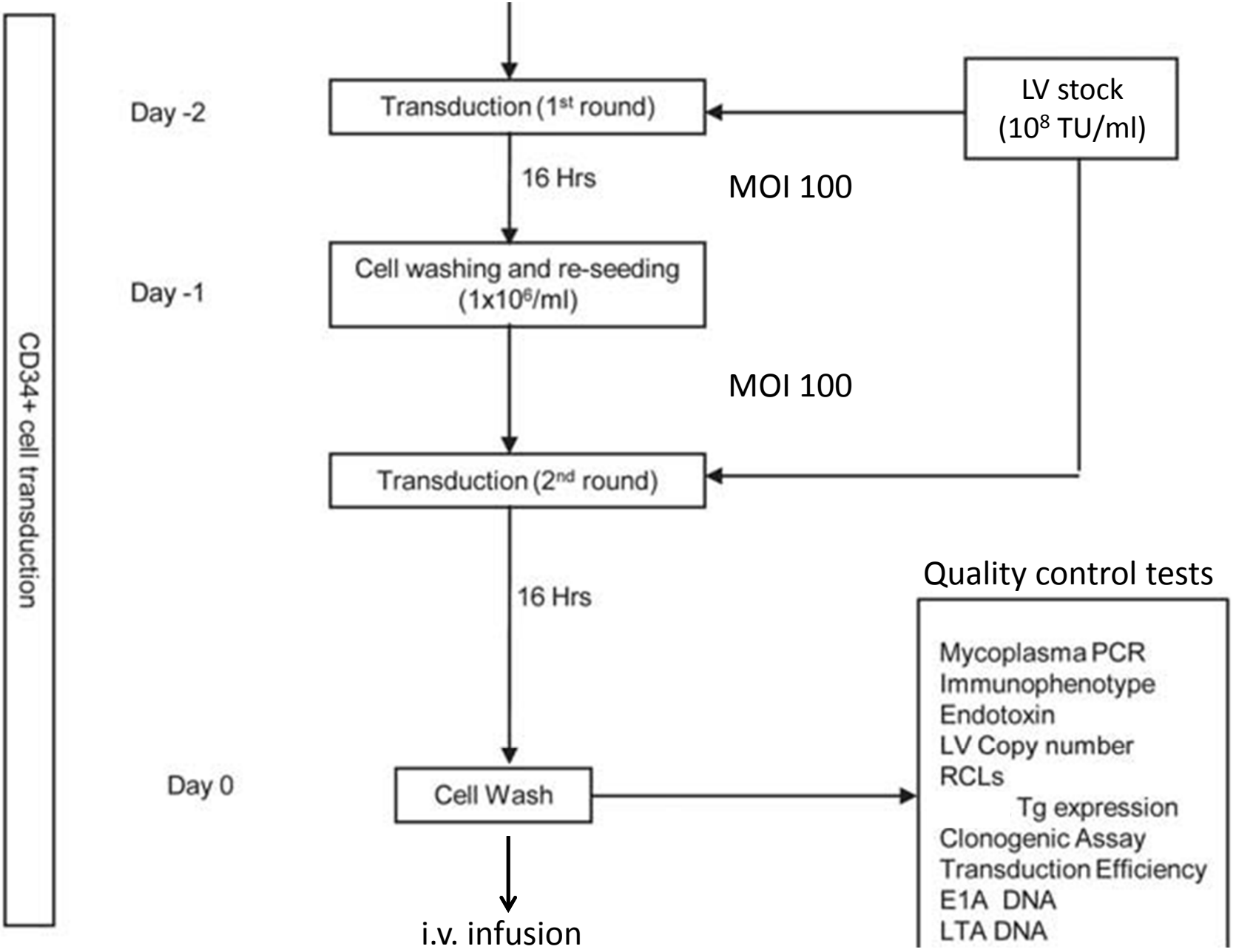

- CellGro SCGM medium (serum free, SCF, TPO, IL-3, and Flt3-L)

VueLife bags

WAS patient treatment:
Busulfan & Fludarabine for ablation and monoclonal anitbody CD20 for immunosupression

***Aiuti et al., 2013 & Biffi et al., Science 2013***

# 2. Transduction protocol: preparation of transduced CD34$^{+}$ cell (II)




Aiuti et al., 2013 & Biffi et al., Science 2013

**Integration profile (LAM-PCRs & NGS)**

## Quality control tests

| | Specification | Available at infusion |
|---|---|---|
| ***Drug Substance*** | | |
| Mycoplasma PCR (EofT) | Negative | yes |
| LV Copy number (LC) | FIO | no |
| ARSA Transgene product expression (LC) | FIO | no |
| RCL (EofT) | Negative | no |
| Large T antigen DNA (EofT) and (LC) | *EofT*: FIO<br>*LC*: ≤ LLOQ | yes (LC) |
| Endotoxin (EofT) | ≤ 2.5 EU /ml | yes |
| Clonogenic test (EofT) | FIO | no |
| Transduction efficiency (EofT) | FIO | no |
| E1A DNA (EofT) and (LC) | *EofT* : FIO<br>*LC*: ≤ LLOQ | no |
| IF (CD34, CD45, CD19, CD3, CD15) | FIO | yes |
| ***Drug Product*** | | |
| Sterility – microbiological control of cellular products | Negative | yes |
| Cell viability | >80% | yes |

If no, Available 2 to 5 weeks after infusion

**Table S4**. Tests and specifications for Drug Substance and Drug Product quality control. EofT: end of transduction; LC: 14 days liquid culture; FIO: for information only; RCL: replication competent lentivirus; LLOQ: lower limit of quantification; IF: Immunophenotyping.

Biffi et al., Science 2013

**Characteristics and treatment of WAS patients :**

| | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|
| Infectious manifestations | Recurrent ENT | Pneumonias, colitis arthritis/cellulitis, URTI, UTI | Pneumonia with respiratory distress, URTI, otitis |
| Pathogens | VZV, CMV, HSV, EBV | CMV, HHV-6, candida | *Pneumocystis jirovecii*, CMV |
| Thrombocytopenia manifestations | Skin petechiae | Skin petechiae, GI bleeding | Skin petechiae, GI bleeding, epistaxis |
| Eczema | Moderate-severe | Moderate-severe | Severe |
| Other | Developmental disorder, allergy | Failure to thrive, elevated inflammatory indexes/vasculitis, hepatosplenomegaly | GE reflux/food aversion (fed by nasogastric tube), allergy |
| WAS mutation | Exon 10: C>T 995 (R321X) | IVS10del11nt | 37C>T (R13X) |
| WASP expression | <5% | <5% | <5% |
| Zhu score | 3 | 4 | 4 |
| Age at treatment (years) | 5.9 | 1.6 | 1.1 |
| Infused $CD34^+$ cells ($\times 10^6$/kg) | 3.66 (BM) + 5.25 (MPB) | 14.1 | 10.2 |
| Vector copies/genome | 1.9 (BM) – 1.4 (MPB) | 2.4 | 2.8 |
| Transduction efficiency (CFC) | 92% (BM) – 88% (MPB) | 97% | 100% |
| Follow-up (months) | 32 | 23 | 20 |
| Current clinical conditions | A&W, no eczema, no major bleeding or petechiae, off IVIG | A&W, no eczema, no major bleeding or petechiae | A&W, no eczema, no major bleeding or petechiae |

CD34+ cells

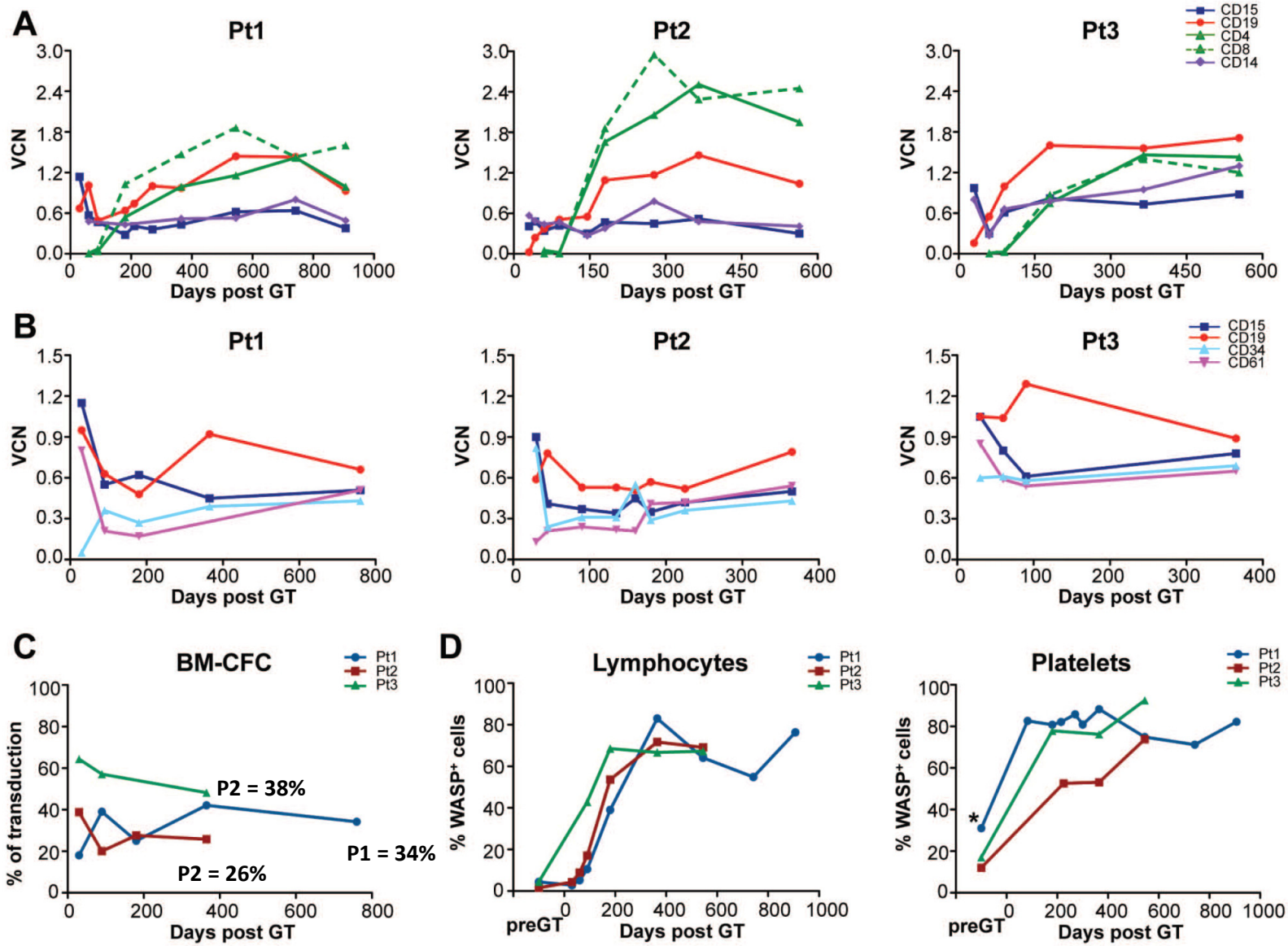
CD34+ cells

***Aiuti et al., 2013. Science***

# Vector copy number (gene-marking) and WASp expression after engraftment

PB cells

BM cells




***in vivo*** engraftment of transduced HSPCs

Selective advantage of WAS+ lymphocytes & platelets

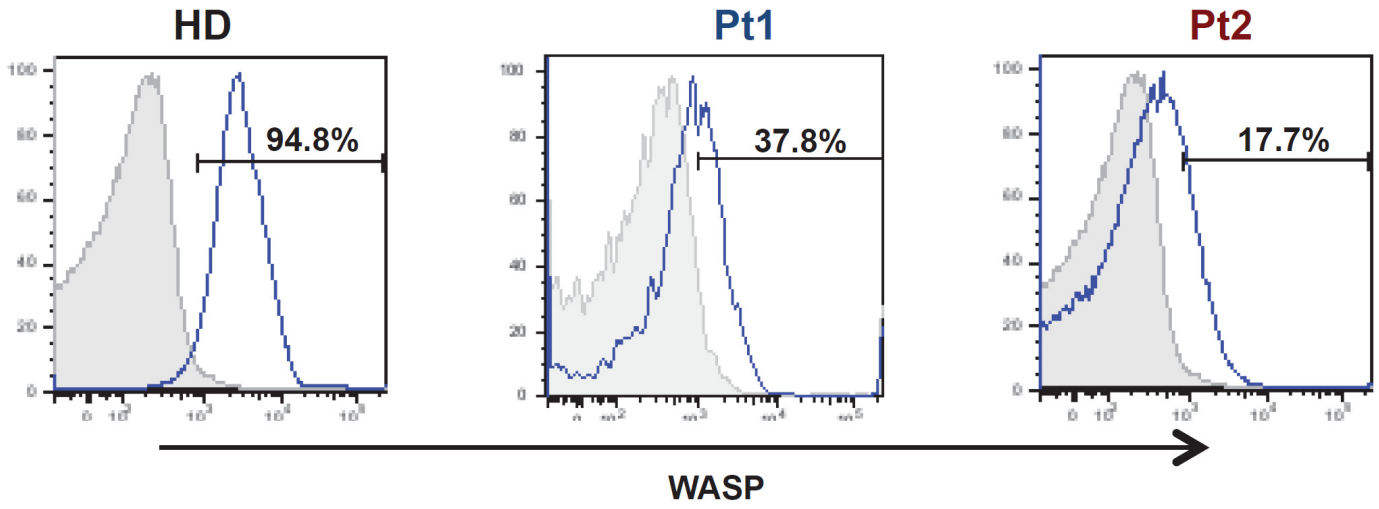
VCN stabilized at a range of 0.4 to 0.9

Colonies that harbored the LV genome 1yr post GT ranged 26 and 38%

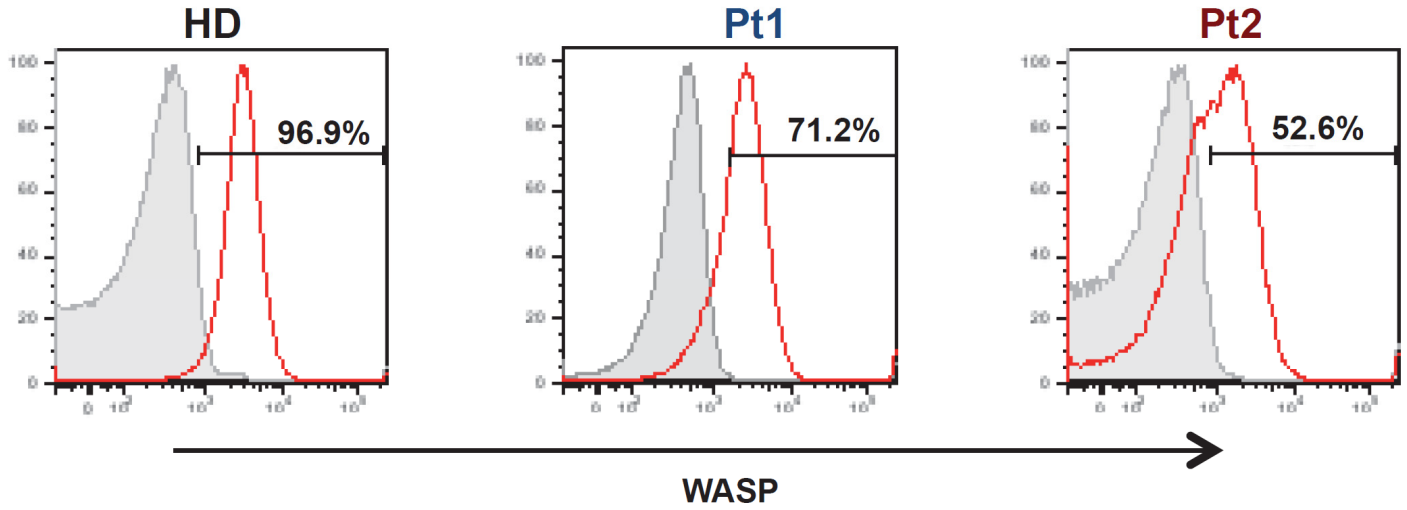
***Aiuti et al., 2013. Science***

**WASP expression on platelets 1yr after gene therapy:**

## A Bone Marrow




## B Peripheral blood: Higher frequency in periphery compared to BM. ⟶ Migration or selection survival advantage of corrected platelets in periphery




*Supplementary Fig. Aiuti et al., 2013. Science*

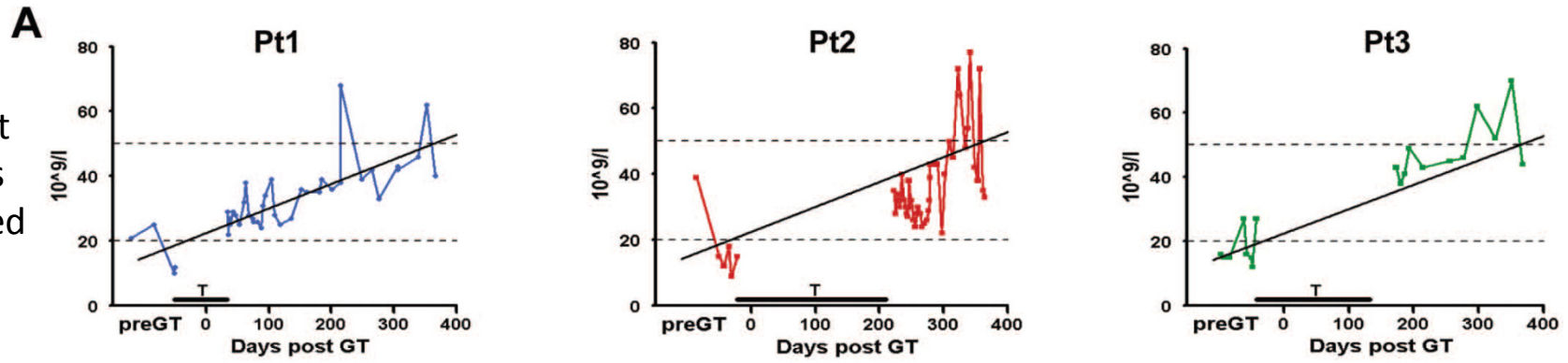
**Results described:**

• No abnormal cellular expansion detected in BM or periphery, immunophenotypic, morphologic and karyotyping analyses.

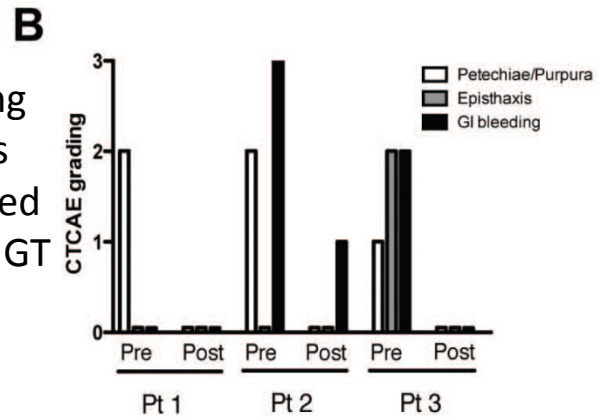
• Analyses to detect HIV gag p24 proteins and Replication competent lentivurs (RCLs) were all negative

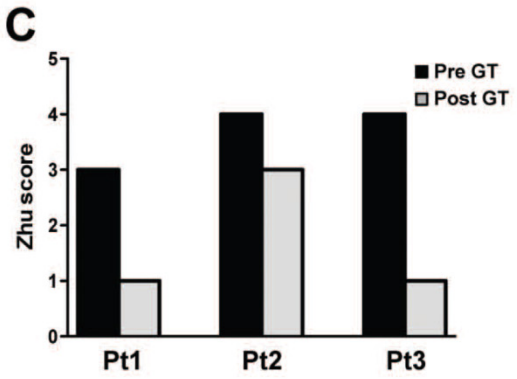
# Clinical features and immune function of WAS patients after gene therapy

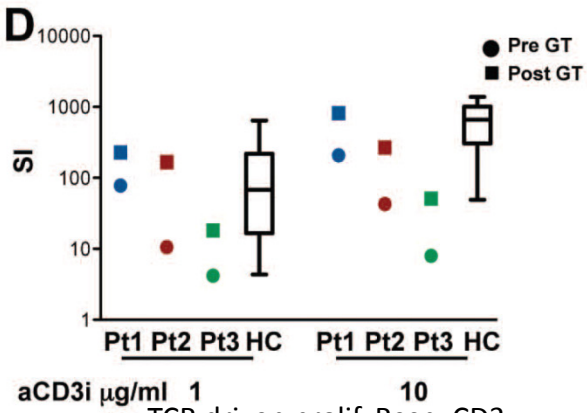
Platelet Counts improved



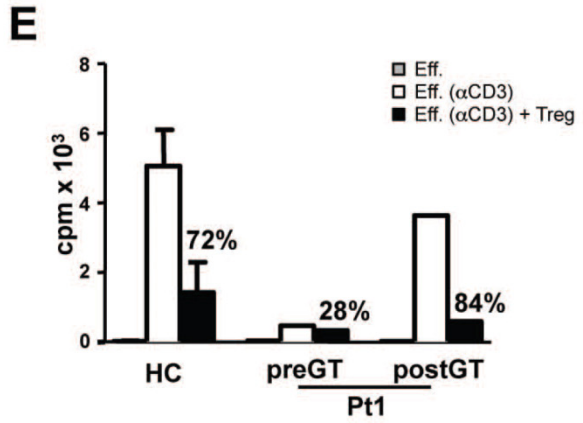

Bleeding events decreased 1yr post GT

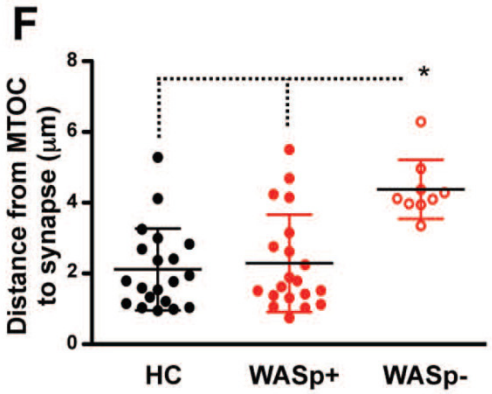


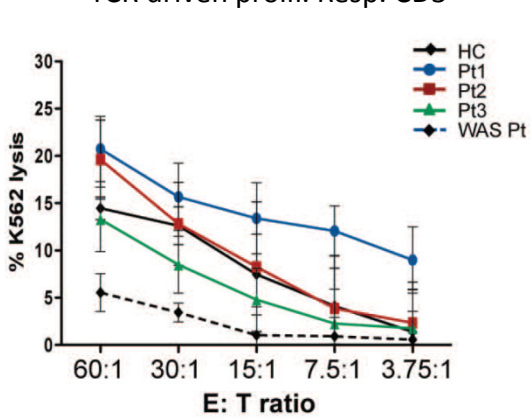




TCR driven prolif. Resp. CD3



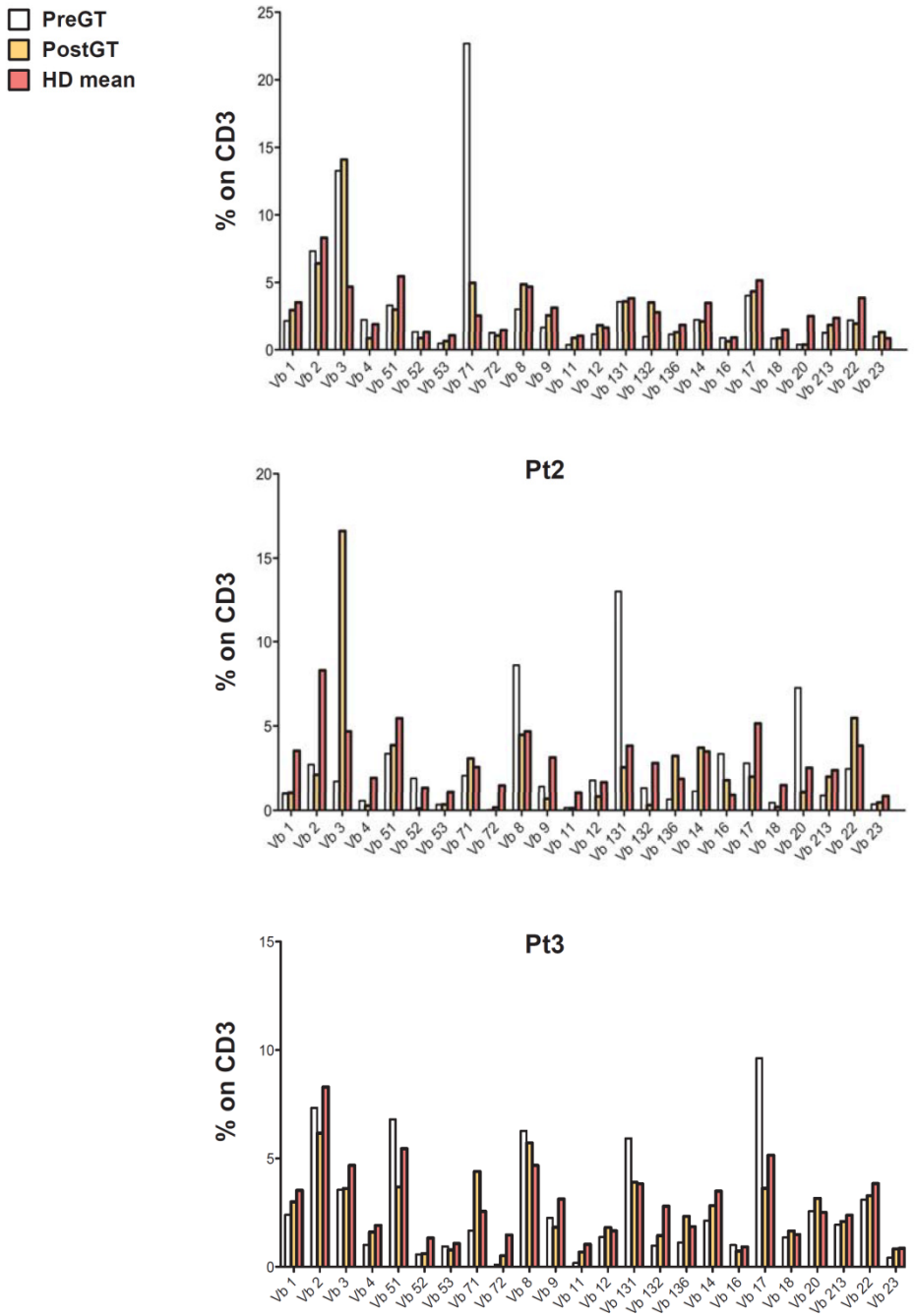

***Aiuti et al., 2013. Science***

$T_{reg}$ cell function, suppress effector cells

NK cell synapse formation (microtubule orginizational centre)

NK cytotoxicity is restored Human erythroleukemia cells

## Pt1 Determine if TCR Vβ repertoire is polyclonal

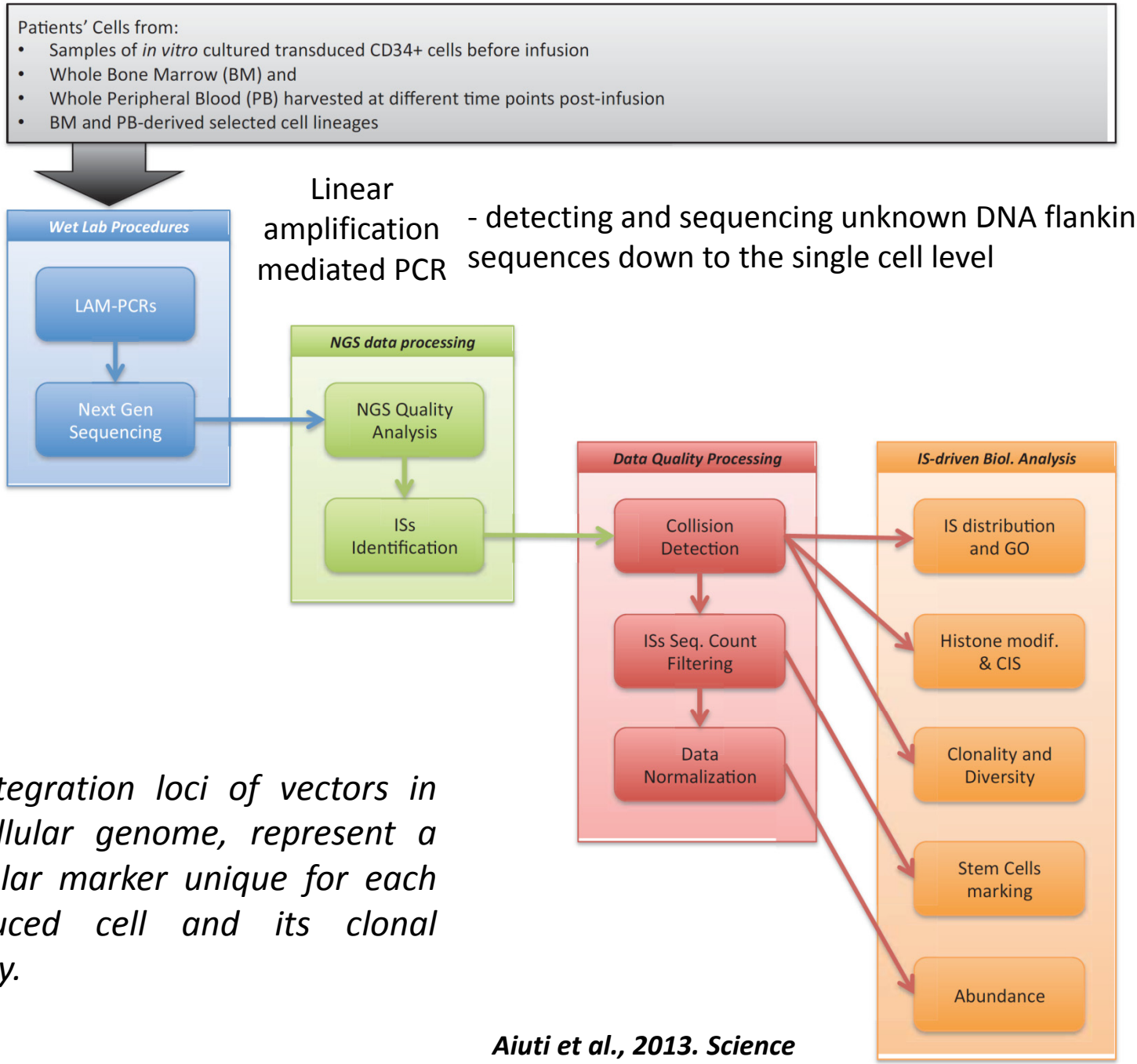



## T cell receptor (TCR) Vβ profiling:

- All three WAS patients displayed broader polyclonal repertoire after gene therapy
- Correlates with polyclonal reconstitution
- 24 different Vβ specificities were analysed by flow cytometry.
- The average % of each TCR Vβ on CD3+ cells is shown
- 8 vials with mixtures of conjugated TCR Vβ antibodies
- corresponding to 24 different specificities (about 70% coverage of normal human TCR Vβ repertoire).

***Aiuti et al., 2013. Science***

## 3. Assessment of the long-term safety of HSPC gene transfer: Vector integration site (IS) analysis




*The integration loci of vectors in the cellular genome, represent a molecular marker unique for each transduced cell and its clonal progeny.*

***Aiuti et al., 2013. Science***

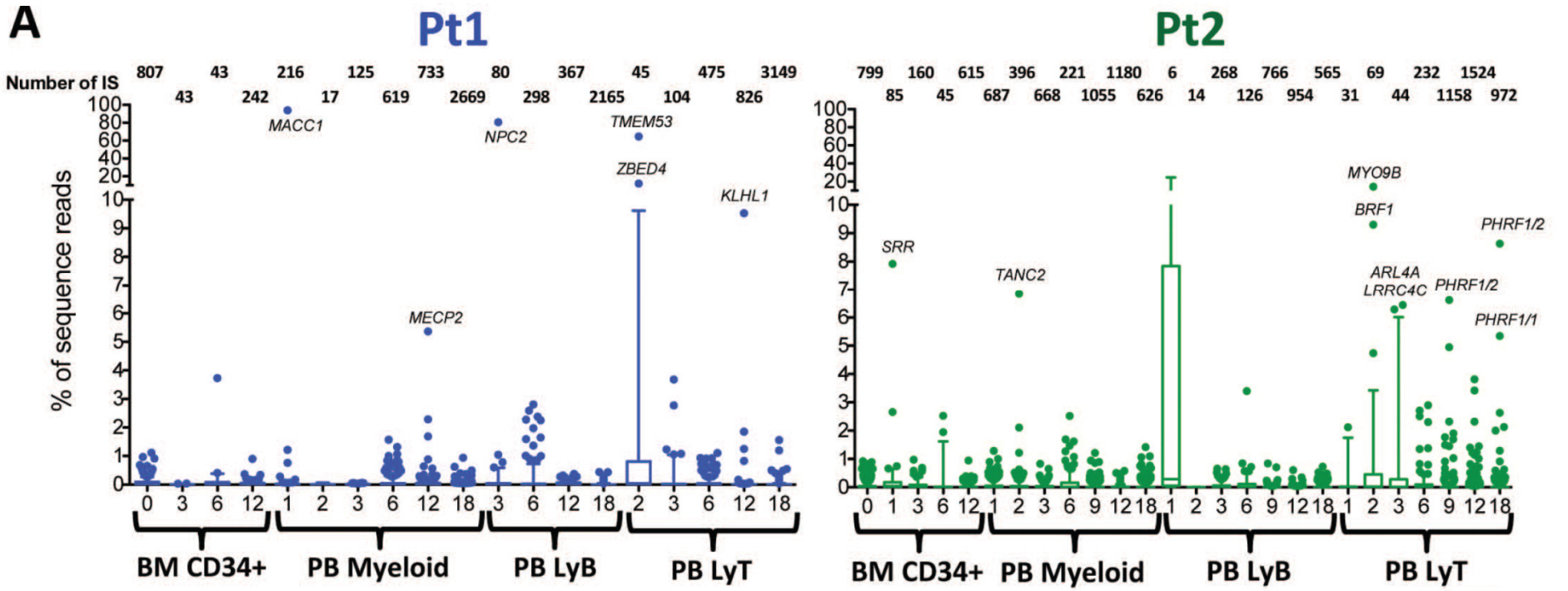
**Measurement of the clonal contribution of gene corrected cells to hematopoiesis,**
**High-throughput IS analysis:** BM CD34+ cells, PB myeloid, T and B cells

Detected >2'400'000 IS sequences, mapped to 33'363 unique chromosomal positions (Total)
800'000 ISs per patient on average
P1 = 11'137 P2 = 10'889 P3 = 10'333 unique ISs.

Y axis: % sequence reads: ISs of each lineage expressed as a % <u>of the total reads</u>

Show only the top 5% most abundant reads, because the other 95% are very low, can conclude that there is no clonal dominance/expansion

Vast majority of ISs from all lineages was below 5% of the total reads of particular lineage



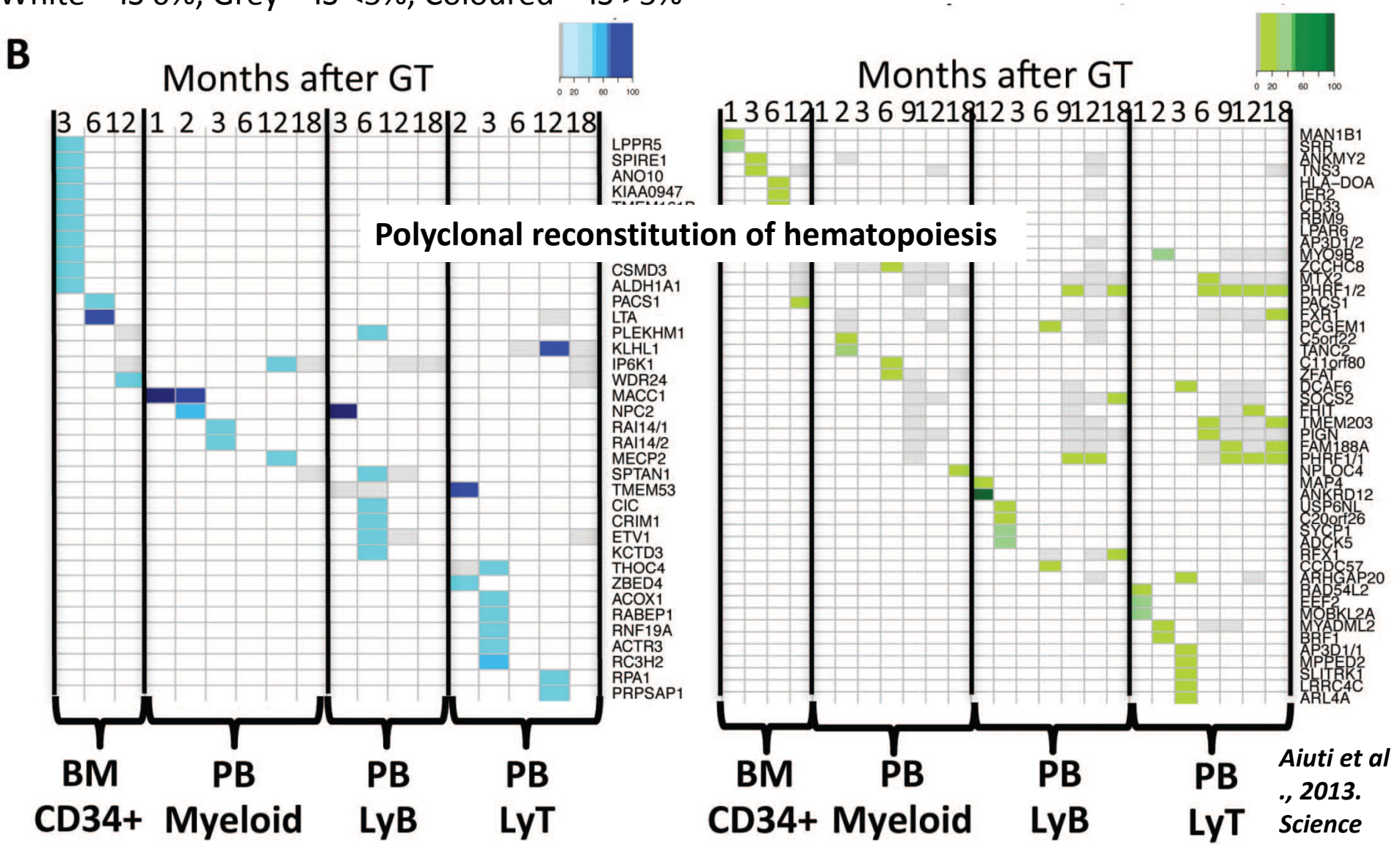

*Aiuti et al., 2013. Science*

**Monitoring of the most frequent ISs in each lineage over time:**

The Highly representative ISs were different at each time point and fluctuated over 12 months

Persistent multilineage contribution of several ISs, remaining below 10% of total reads

White = IS 0%, Grey = IS <5%, Coloured = IS >5%




*Aiuti et al ., 2013. Science*

**Wanted to further compared the ISs retrieved from BM CD34+ and CFC with that of myeloid and lymphoid lineages in each patient in order to determine if there are a significant number of shared ISs across the lineages**

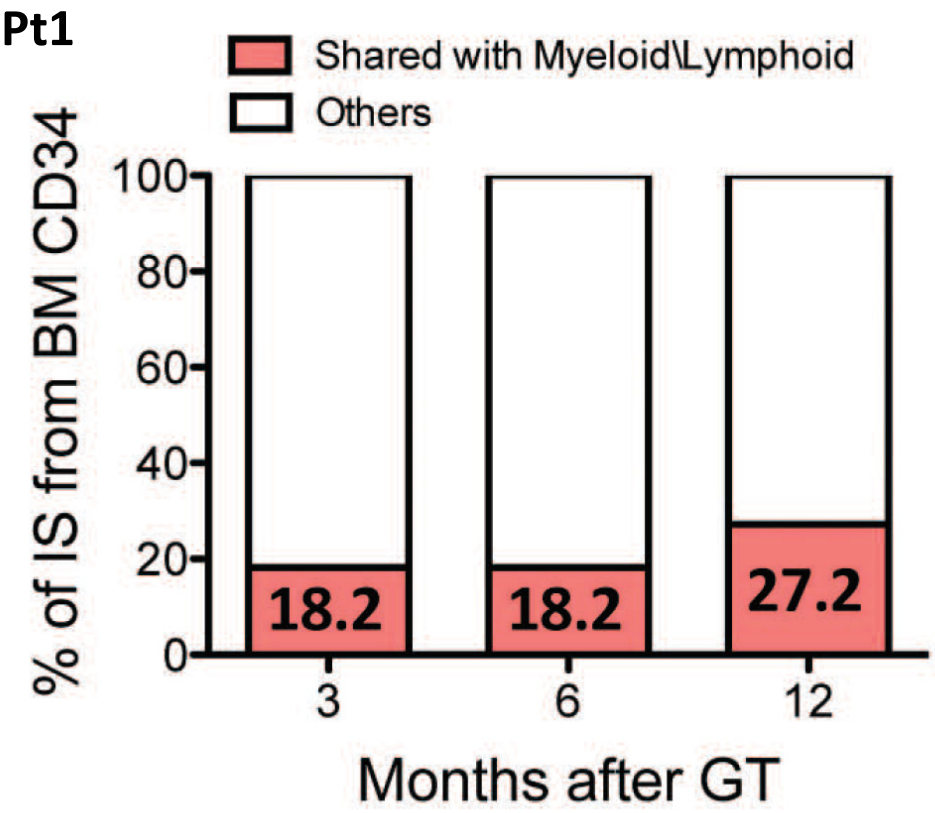
**Applied stringent analytical filters to take into account sample impurity and cross-contaminations**

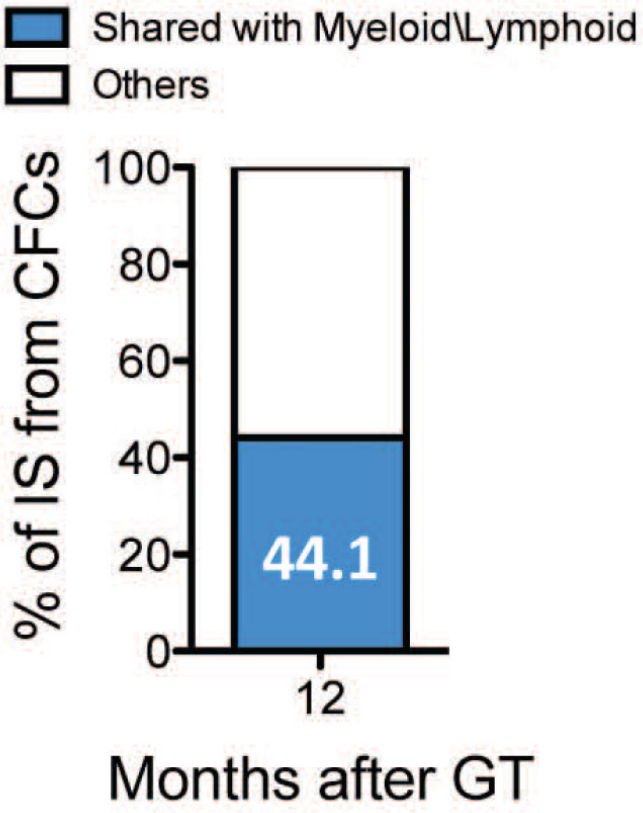
**Evidence of efficient engraftment of self-renewing transduced HSPCs:**

Indeed the analysis showed a group of ISs shared among the different lineages

Shared ISs increased from 18.2 – 27.2% in a year for Pt1.

**Pt1**

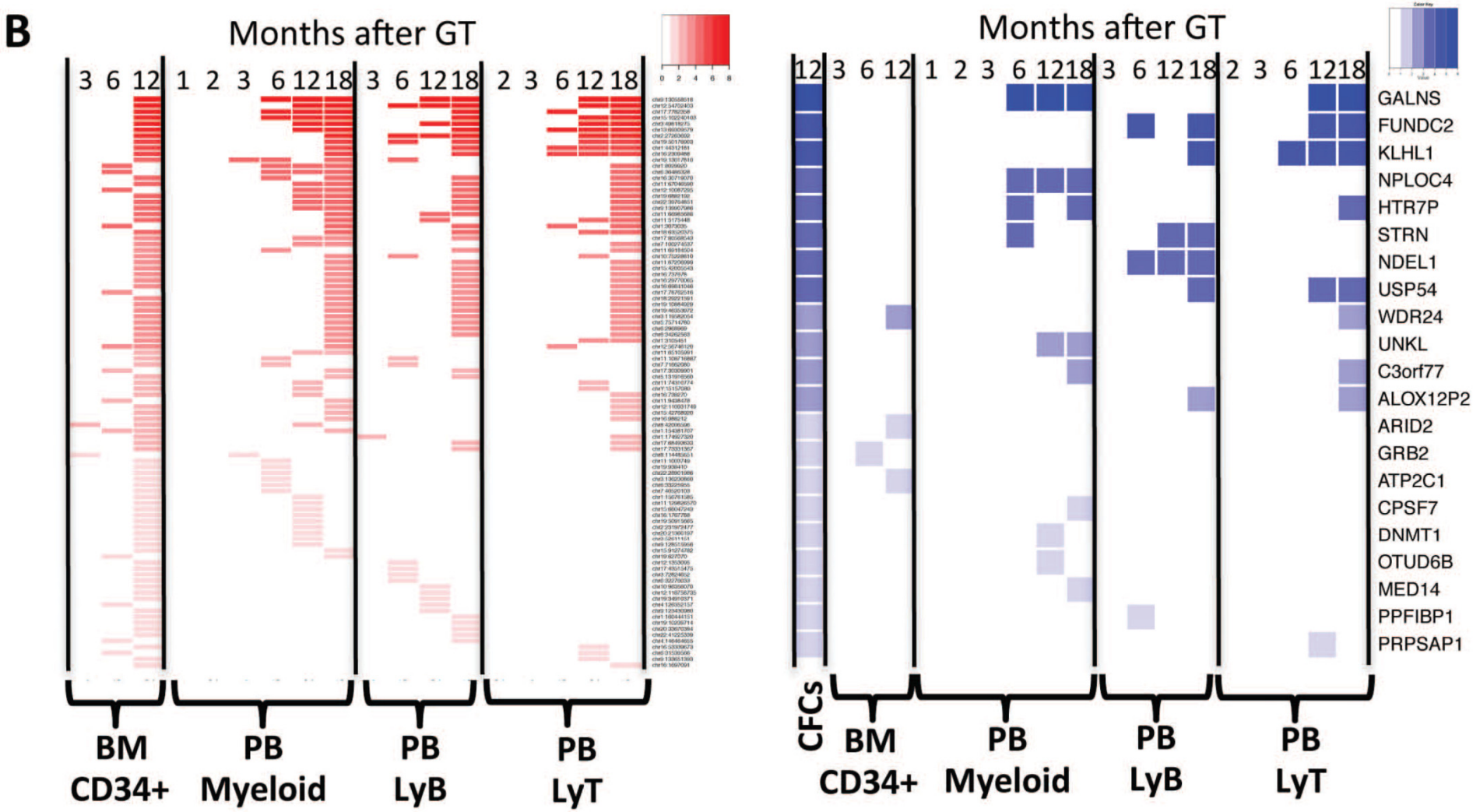



*Aiuti et al., 2013. Science*

Many ISs from BM CD34+ cells or CFC 1 year after gene therapy could be traced back to ISs in mature cells at earlier time points

Again pointing to efficient engraftment of self-renewing transduced HSPCs




*Aiuti et al., 2013. Science*

## Genomic distribution of lentiviral vector and γ-retroviral vector ISs:

- Chromosome number indicated

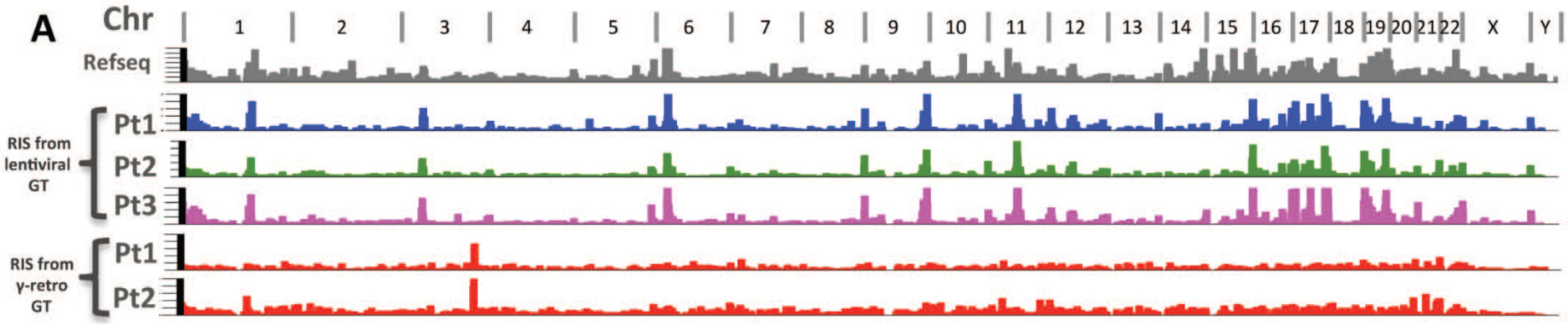
-Refseq: Classical distribution of lentivirus ISs:
  - Each bar represents a bin of 1mio bp
  - Hight of individual bars indicates frequency of ISs.

- ISs profile of 3 WAS patients is very similar to the refseq

- ISs profile γ-retroviral gene therapy different:

- LVV: Within transcriptional units / gene rich regions

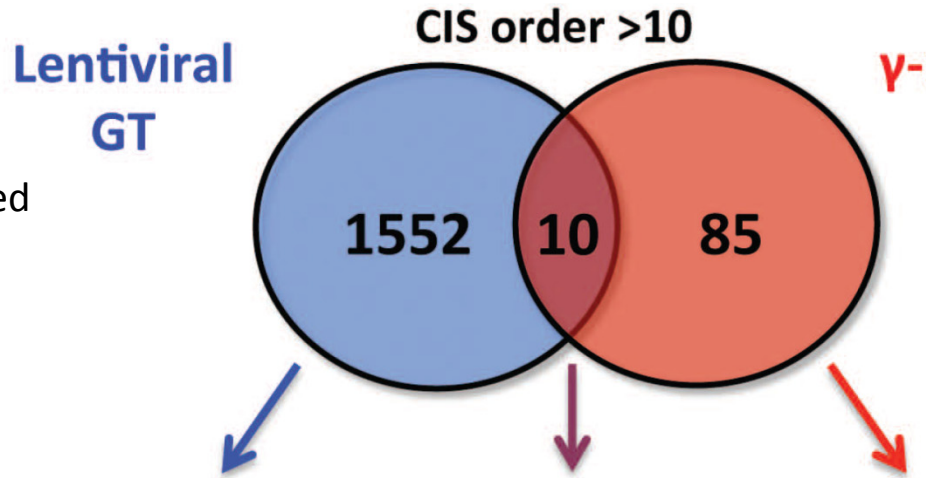
- RVV: Skewed towards transcription start sites




*Aiuti et al., 2013. Science*

**Analysis of common insertion sites (CIS):** Identify insertional hotspots during transduction allowing for definition of surrogate markers of *in vivo* selection of clones

CIS are clusters of integrations within a pre-deteremined window size: defines a CIS gene (This defines the maximum distance between ISs forming a cluster)




LVV CIS genes are well known and not associated with clonal expansion
Total ISs = 33.363
KDM2A (125 ISs)
PACS1 (117 ISs)
TNRC6C (94 Iss)

RVV CIS genes associated with clonal expansion & known proto-oncogenes
Total ISs = 11'294
MECOM (MDS-EVI1) (67 ISs)
LMO2 (15 Iss)

LVV: MECOM and LMO2 CIS genes were hit 10 and 5 times out of 33'393

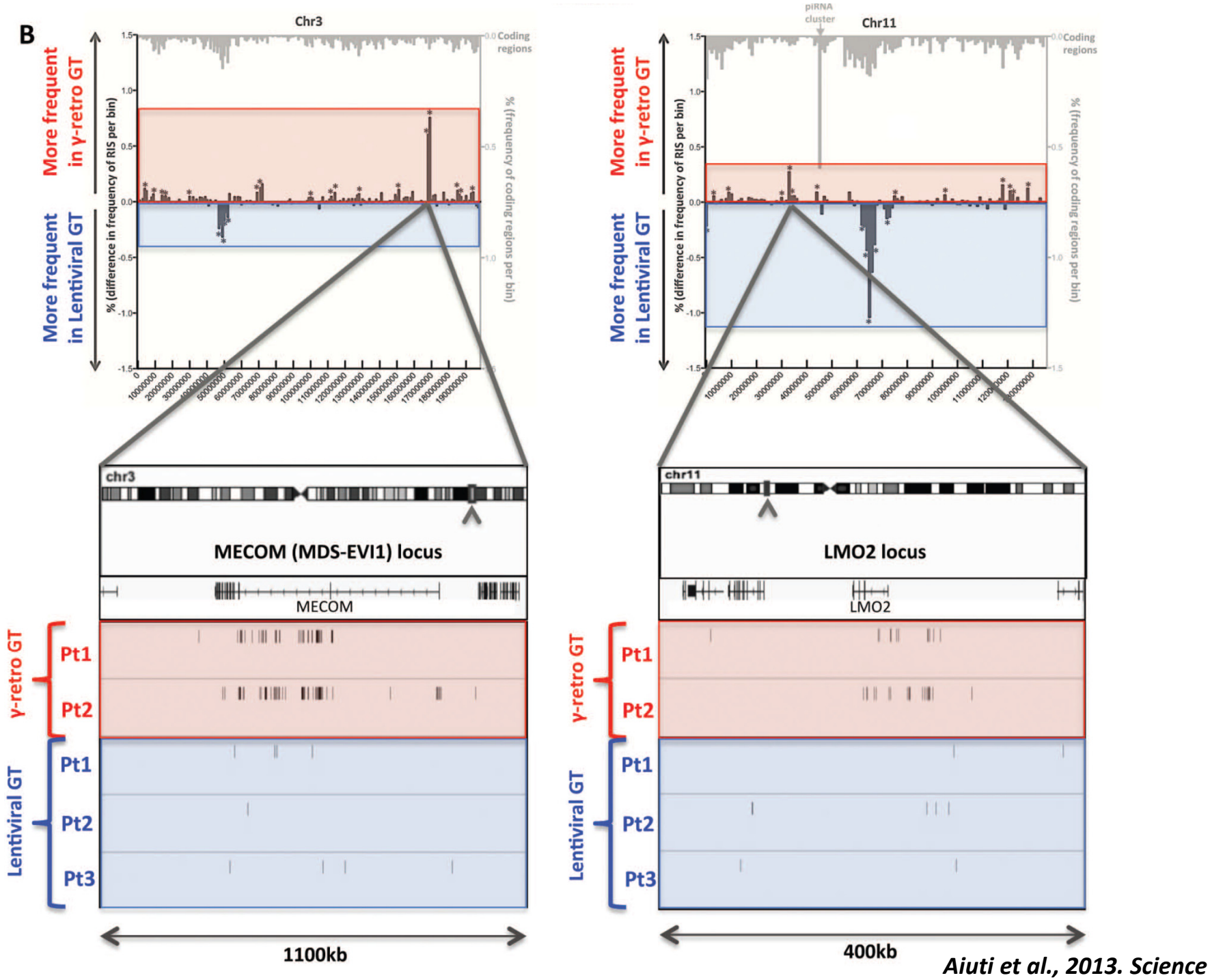
Frequency of sequence reads of these ISs compared to total reads: 0.44% and 0.05%

MECOM and LMO2 integrants were not consistently detected

ENSA
EGFL7
FAM69B
AMICA1

MECOM

X-linked chronic granulomatous disease (X-CGD) treated by gene therapy in 2004. Silencing of transgene - methylation of viral promoter, and myelodysplasia with monosomy 7 resulting from insertional activation of ecotropic viral integration site 1 (EVI1).




*Aiuti et al., 2013. Science*

# Main Conclusions of WAS Gene therapy:

- Aiuti *et al. Showed post GT that pretreatment* eczema resolved between 6 and 12 months

- Decreased, progressively, the frequency of infections; and improved platelet counts

- No clonal domination was observed

- Analysis of the vector insertion site in hematopoietic cells showed no preferential integrations in a particular gene locus, thus decreasing the likelihood of generating an abundance of abnormally proliferating cells.

- Increasing presence over time of CD34+ progenitors and mature cells of myeloid and lymphoid lineages marked by identical integration sites of the delivered gene is strong evidence of self-renewal and multilineage potential of vector-transduced hematopoietic stem cells after engraftment

# Gene therapy for Metachromatic LeukoDystrophy (MLD)

MLD is a lysosomal storage disease, which affects the metabolism of sphingolipids.

Autosomal recessive disease caused by mutations in the *ARSA* gene encoding for the arylsulfatase A enzyme

This enzyme is located in lysosomes, where mediates the breakdown of cerebroside 3-sulfate into cerebroside and sulfate

Deficiency in arylsulfatase A activity →accumulation of toxic levels of sulfatide in oligodendrocytes, microglia and certain neurons (CNS) + Schwann cells and macrophages (PNS) → demyelination and neurodegeneration

Severe progressive motor and cognitive impairment

No pharmacological treatment available

Biffi et al., Science 2013

# Background: gene therapy for MLD

In a mouse model of MLD, disease was prevented with LV hematopoietic stem cell gene therapy, BUT NOT with hematopoietic stem cell transplantation. (Biffi et al., JCI, 2004; Biffi et al., JCI, 2006)

Similarly, hematopoietic stem cells transplantation failed to provide consistent benefit in MLD patients (Biffi et al., Hum Mol Genet, 2011)

→ **Supraphysiological expression of the functional ARSA gene is necessary**

Objective: as shown in mice → LV-mediated gene therapy of human HSPCs, which in turn can differentiate and migrate to CNS and PNS leading to widespread enzyme transfer and robust cross-correction of neural cell targets

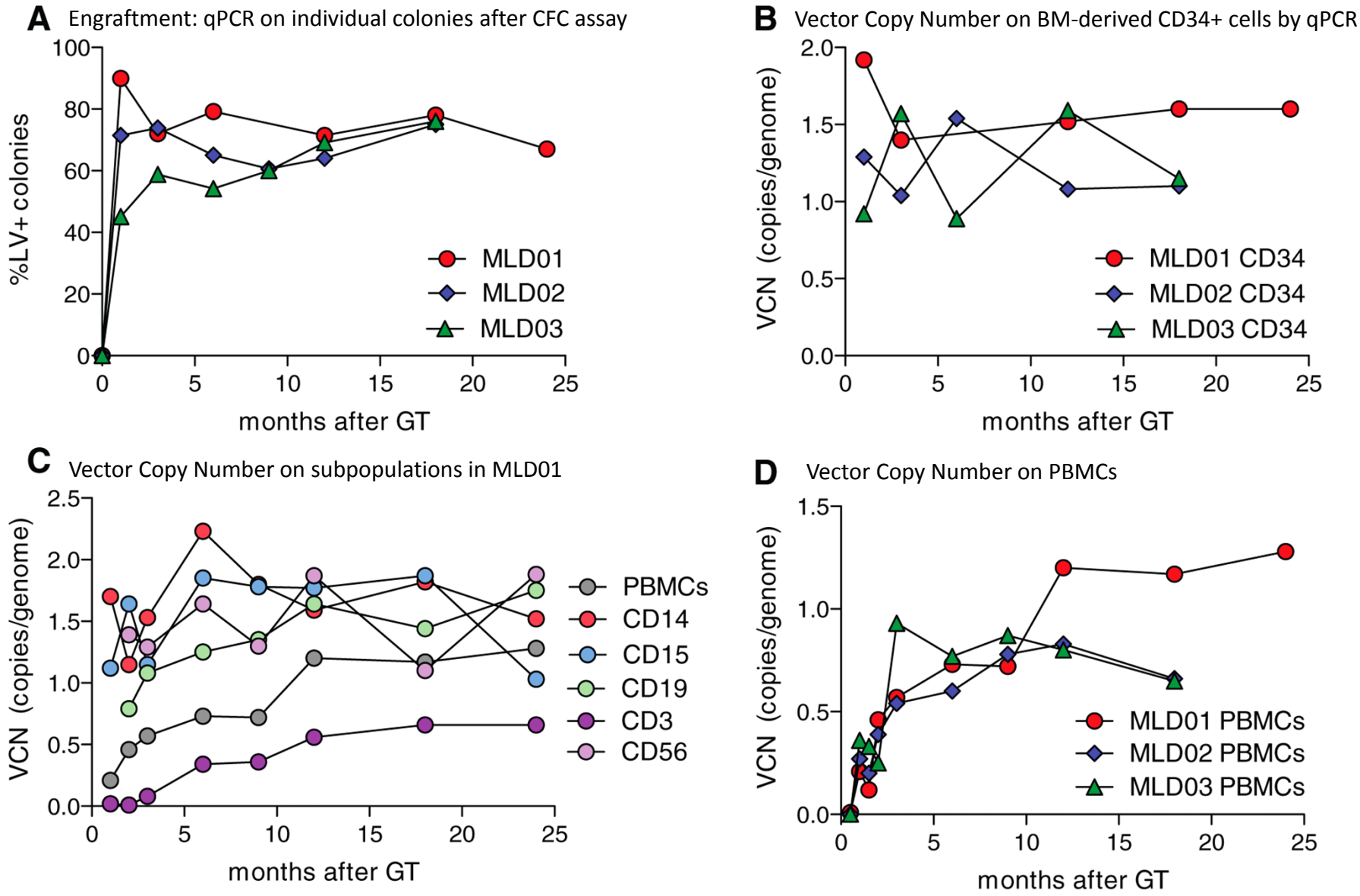
Study design: autologous HSPCs transduced ex vivo with ARSA-encoding LVs and reinfused after myeloablative regimen with alkylating agent busulfan

9 patients with early onset MLD in a phase I/II trial

Reported: outcome of first 3 patients (MLD01 at 24 months follow-up, MLD02, MLD03 at 18 months follow-up)

Biffi et al., Science 2013

## Fig. 1. Engraftment and gene marking in patients after HSPC-GT.



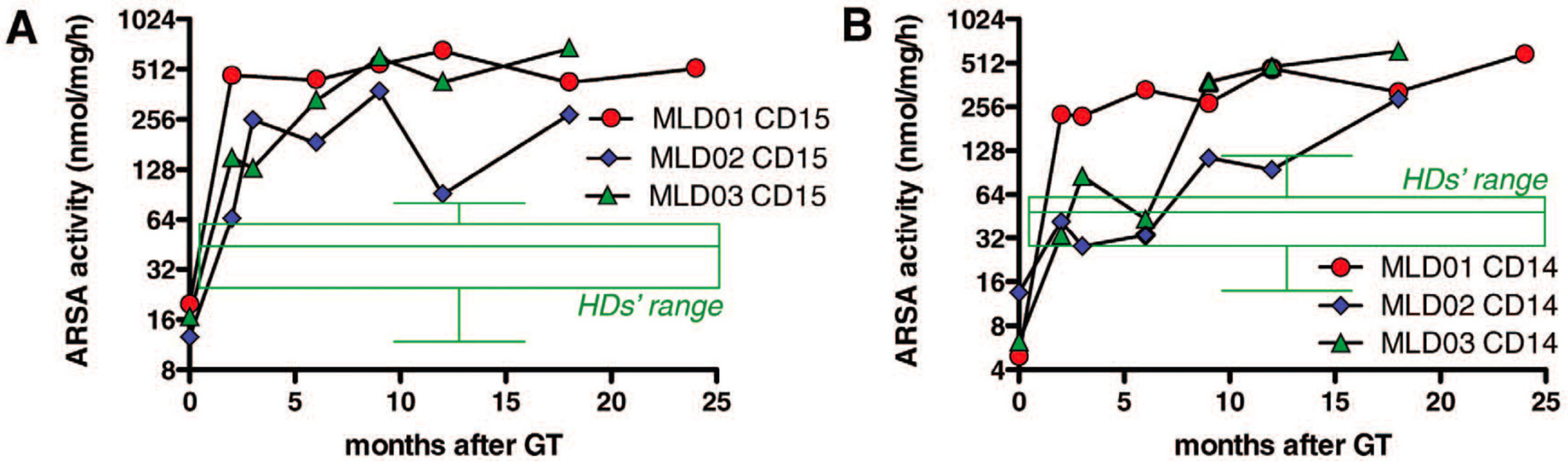

Between 45 and 80% of the colonies harbored the LV genome

VCN ranging from 0.9 to 1.9 up to the latest followup time

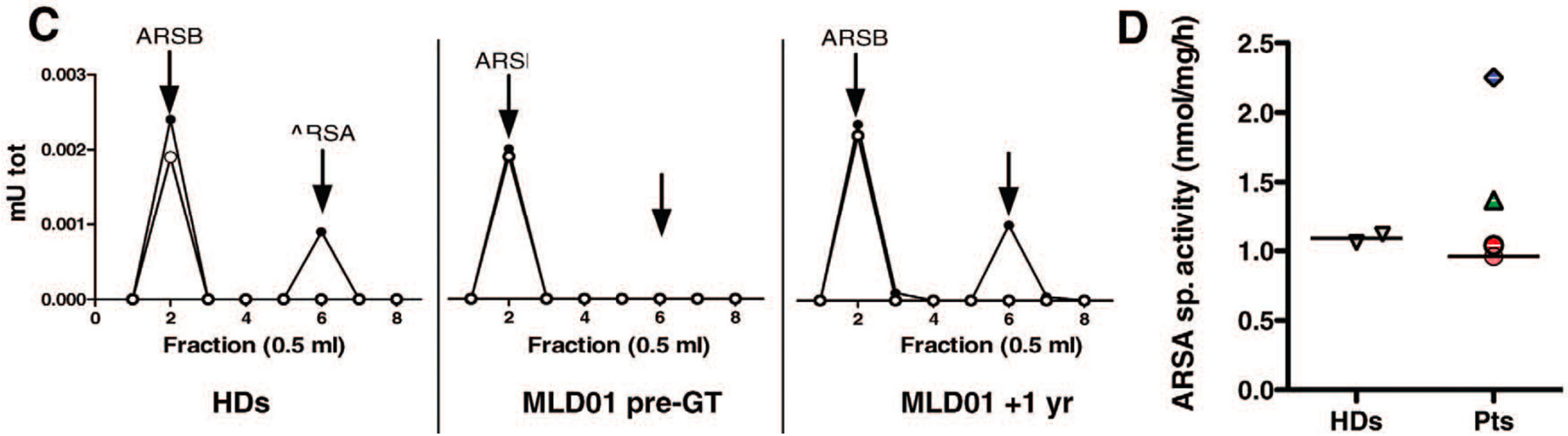
Biffi et al., Science 2013

**Fig. 2. ARSA expression and activity in patients after HSPC-GT.** Biffi et al., Science 2013

**(A-B) p**-nitrocatechol sulfate (PNCS) assay: ARSA activity up to above-normal values in the most therapeutically relevant myeloid populations as well as in other circulating cells



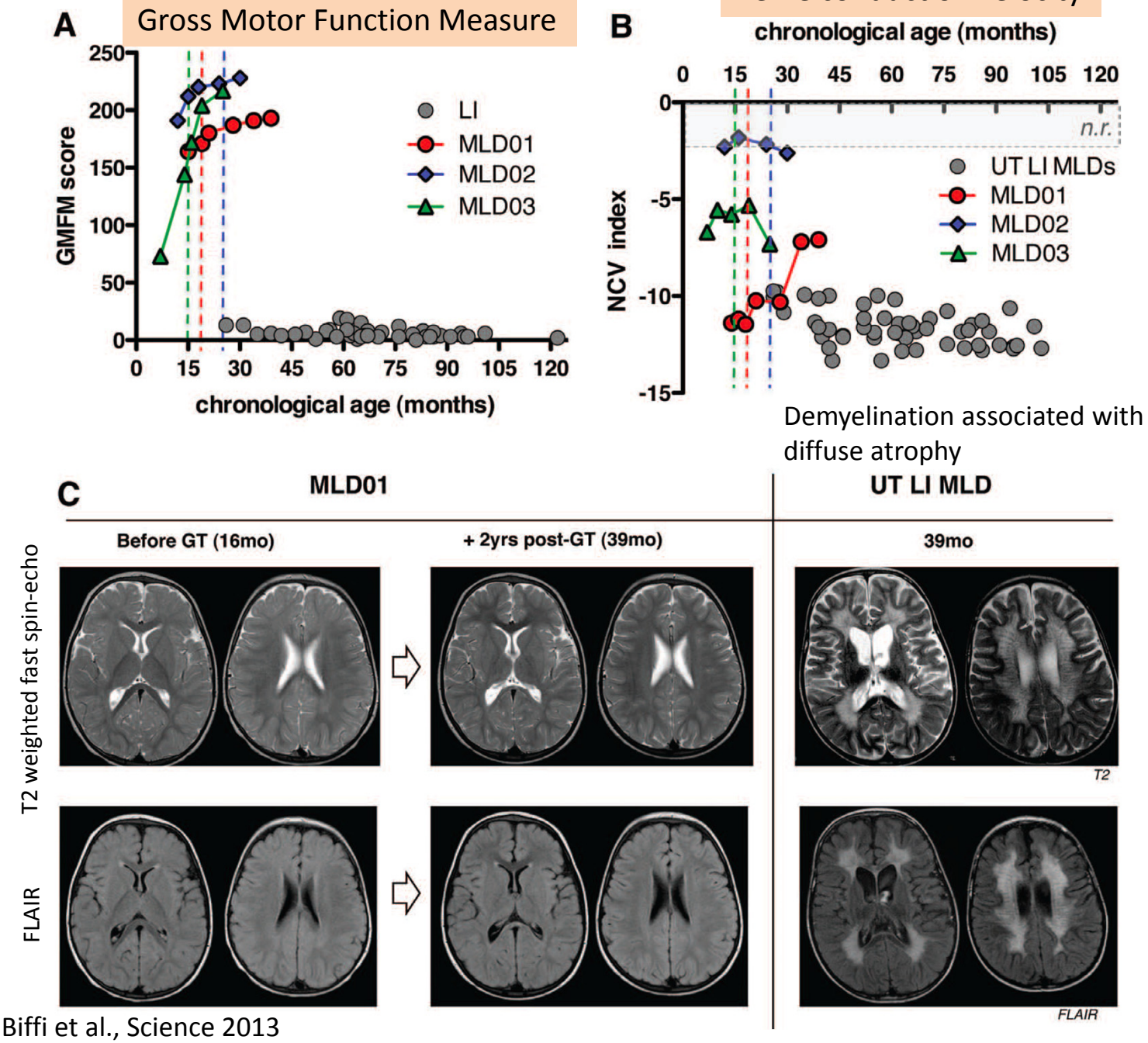

**(C)** ARSA expression (DEAE cellulose-chromatography analysis on 500**u**l of cerebrospinal Fluid) **(D)** ARSA activity in CSF samples (circles, MLD01 12 and 24 months; blue diamond, MLD 02 12 months; and green triangle, MLD03; 12 months)



## Fig. 3. Clinical follow up of MLD patients

Gross Motor Function Measure

nerve conduction velocity




Biffi et al., Science 2013

MLDO1 treated at 15 months

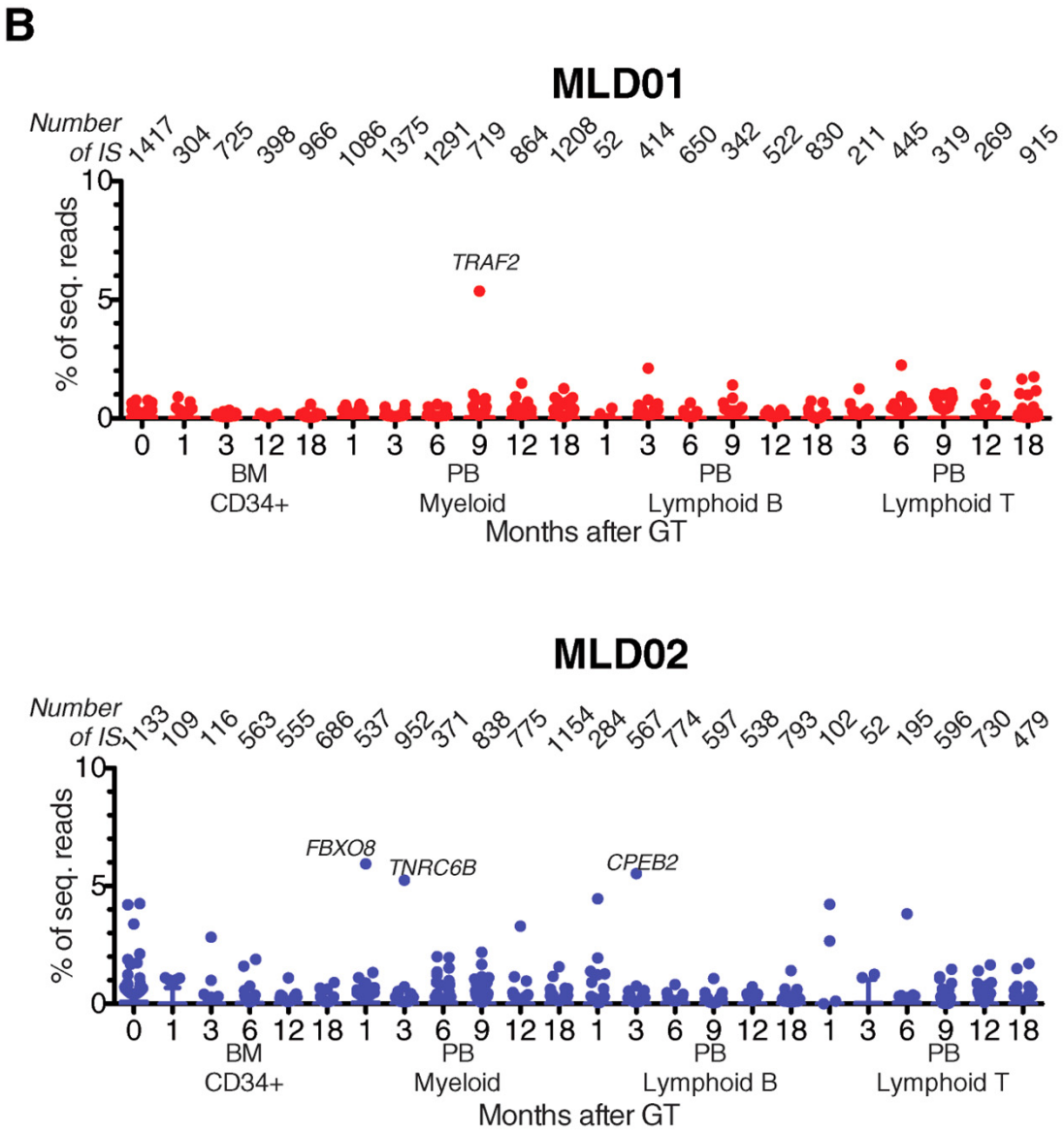
Demyelination associated with diffuse atrophy

MLD01 siblings had rapid disease progression after onset and reached level 6 of GMFM by 30 months of age, when they were wheelchairbound and unable to support their head and trunk + Totally impaired speech

**In contrast, at 39 months of age MLD01 was able to stand independently and to walk and run with single aid + Normal composite IQ score for age, with normal language and cognitive abilities**

**MLD02 and MLD03: Remain asymptomatic**

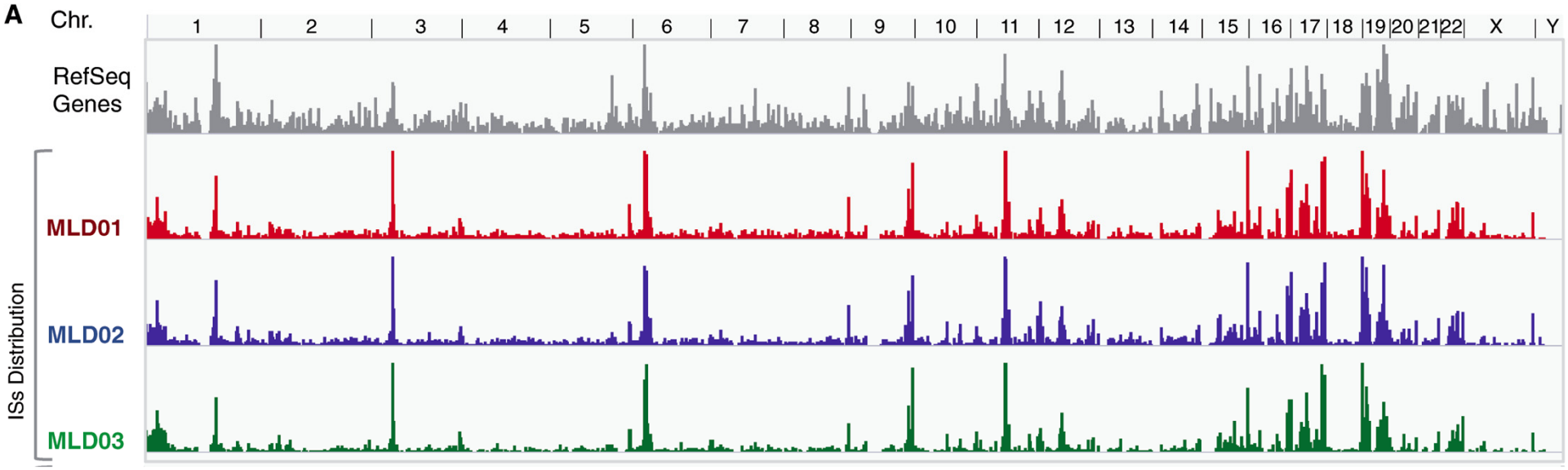
**Fig. 4. LV genomic integration profile.**




Box plot of the percentage of sequence reads (y axis) for unequivocally mapped ISs from patients MLD01 and MLD02 in different cell types

Biffi et al., Science 2013

**Fig. 5. Common insertion site analysis.**




Frequency distribution along chromosomes of RefSeq genes

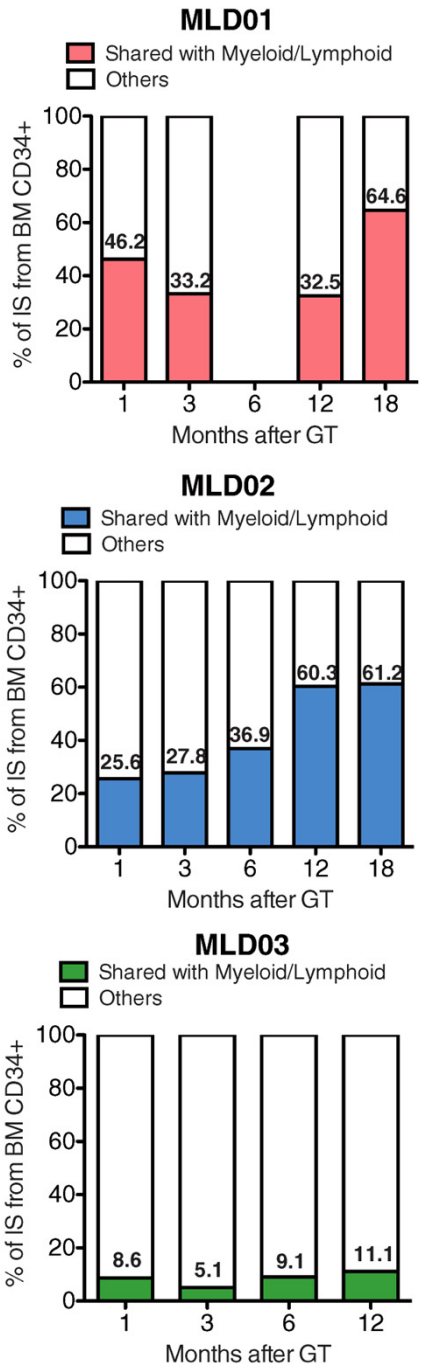
LV integrations for each patient highly similar to that of the RefSeq genes

Biffi et al., Science 2013

## Fig. 6. Clonal dynamics.

Percentage of shared ISs (y axis) between BM-derived CD34+ and myeloid or lymphoid lineages during time (months after GT, indicated below).

**Polyclonal engraftment of transduced HSPCs**




Biffi et al., Science 2013

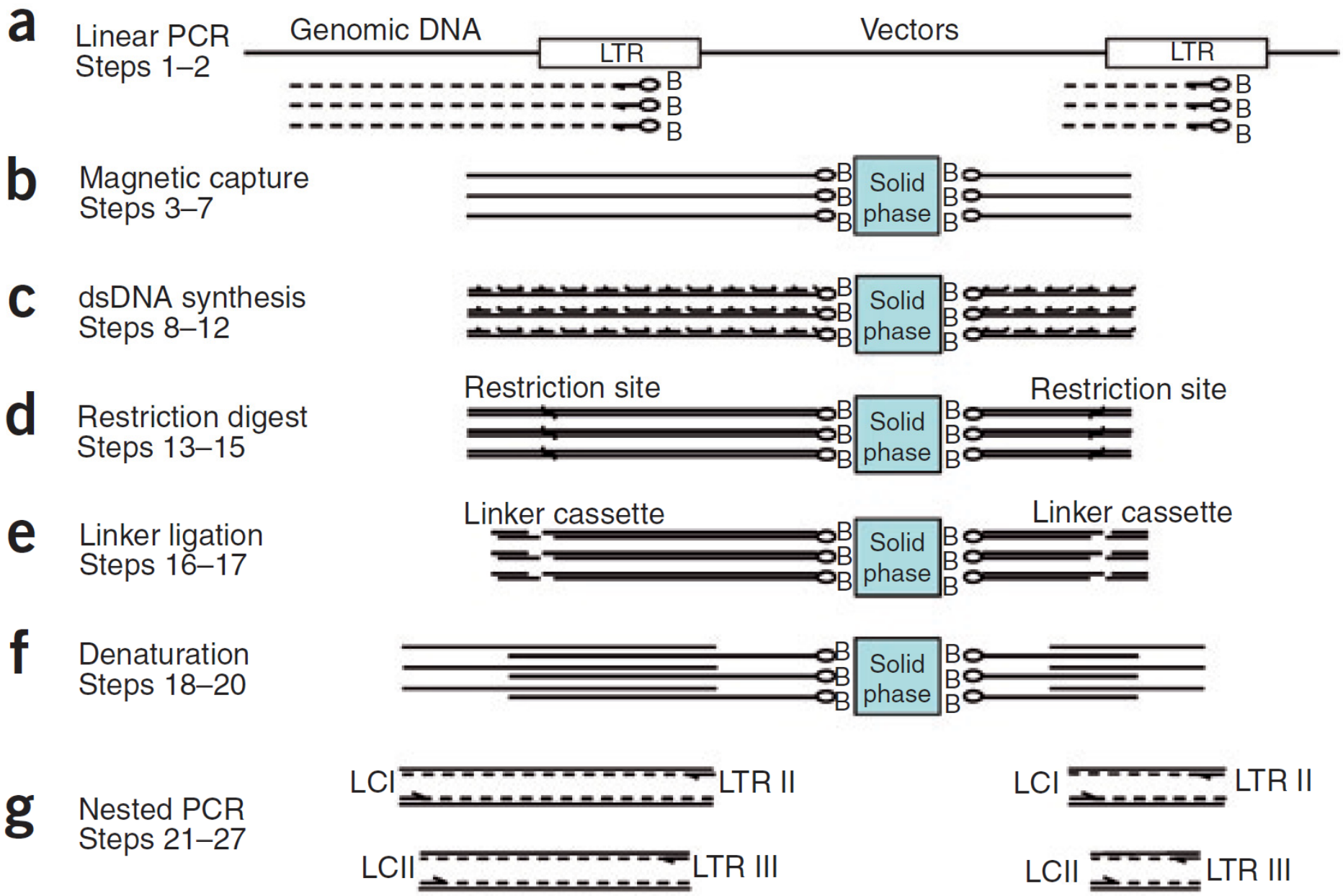
## Conclusion

Overall, these data provide evidence that **efficient ex vivo gene transfer** was followed by **substantial engraftment** and **sustained clonogenic activity** of the transduced HSPCs in the patients, resulting in **extensive polyclonal reconstitution of hematopoiesis with gene-corrected cells**.

Biffi et al., Science 2013

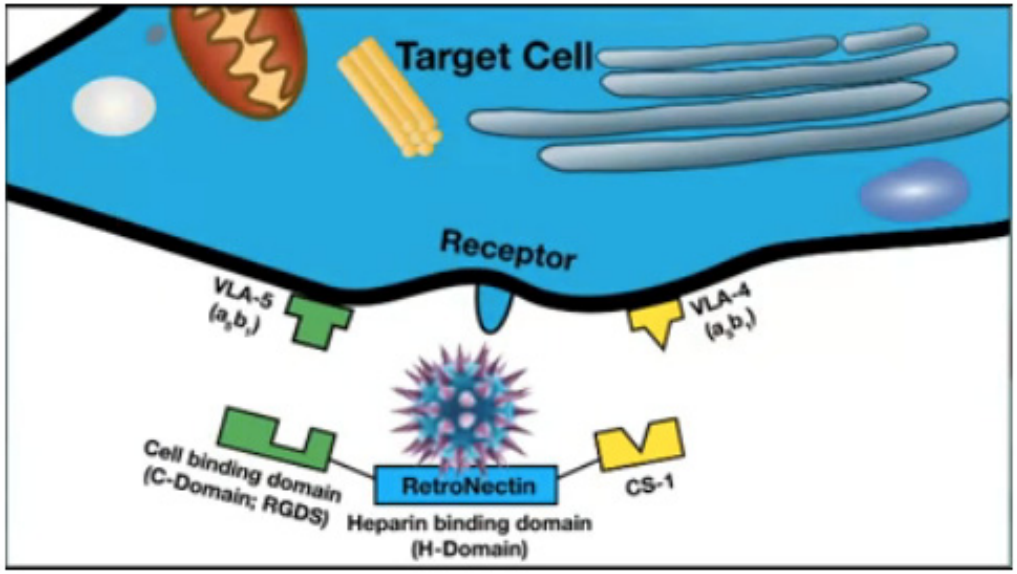
So far, no evidence that the LVs trigger leukemia through insertional mutagenesis

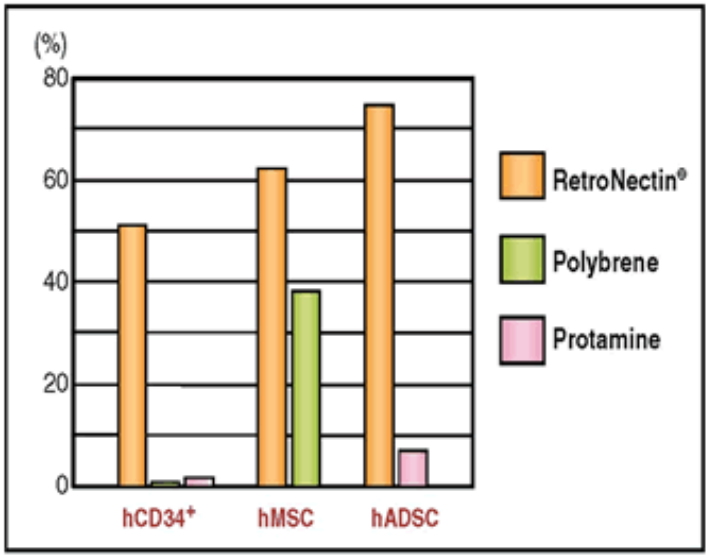
Dr. Biffi


a
Linear PCR
Steps 1–2
Genomic DNA
LTR
Vectors
LTR
B
b
Magnetic capture
Steps 3–7
Solid phase
c
dsDNA synthesis
Steps 8–12
d
Restriction digest
Steps 13–15
Restriction site
e
Linker ligation
Steps 16–17
Linker cassette
f
Denaturation
Steps 18–20
g
Nested PCR
Steps 21–27
LCI
LTR II
LCII
LTR III

# Retronectin

- Chimeric peptide composed of E. coli-derived recombinant human fibronectin fragment that contains three functional domains: a central cell-binding domain (type III repeat, 8-10), a heparin-binding domain II (type III repeat, 12-14), and a CS1 site within the alternatively-spliced IIICS region.
- Allow for enhanced gene transfer efficiency
- Using closed bags for transduction and cell culture: Reduced risk of culture contamination




Comparison of Retrovirus-Mediated Gene Transduction Efficiency in Various Methods into Human Stem Cells.

http://www.clontech.com/takara/US/Support/Videos/Gene_Transfer/RetroNectin

# RCL issue

- Since 1996, lentiviral vectors have been developed and used for clinical applications.

- So far replication-competent lentivirus (RCL) has not been reported with the commonly used lentiviral production systems that lack accessory protein functions (Sastry and Cornetta 2009).

- FDA collaborated with industry and academia and addressed the specific issues related to RCL testing during the 6th annual American Society for Gene Therapy (ASGT) meeting in 2003.

- This effort resulted in a publication outlining the recommendations for developing assays and reference materials for detecting replication-competent lentivirus in production lots of lentiviral vectors:

KIERMER V, BORELLINI F, LU X, SLEPUSHKIN V, BINDER G, DROPULIC B, AUDIT M, ENGEL B, CORNETTA, WILSON, TAKEFMAN D, ZHAO Y, and CARSON K. (2005). Report from the Lentivirus Vector Working Group: Issues for Developing Assays and Reference Materials for Detecting Replication-Competent Lentivirus in Production Lots of Lentivirus Vectors. www.**bioprocessingjournal**.com. March/April pp 39-42.

# RCL assay methods

Cell culture based RCL assay with an appropriate permissive cell line (C8166) is used to allow viral amplification and end point detection.

→ Vector aliquots are used to inoculate a human T-cell line (C8166)

→ The cells are cultured for 21 days to amplify any potential RCL.

→ The medium from amplified cells is then used to infect naïve C8166 indicator cells

→ culture for an additional seven days and analysis for the presence of viral proteins or nucleic acids

Sensitive end-point assays:

- ELISA-based p24 Gag antigen assay (Mochizuki *et al*. 1998)
- Product enhanced reverse transcriptase (PERT) assay that involves the vector's reverse transcriptase (Miskin *et al*. 2006; Sastry *et al*. 2005)
- PCR-based assay that detects Psi-Gag sequences from a recombination event between vector and packaging constructs
- PCR-based assays to detect the VSV-G Env used for pseudotyping (Sastry *et al*. 2003)