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Insertional Mutagenesis in X-SCID Gene Transfer: New Developments and Implications for Future Trials

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Finding cures. Saving children.

Questions for Discussion

- Is the risk for insertional mutagenesis uniquely elevated in X-SCID ?
  - Review of long term data in dogs and NHP
  - Genetics of insertional mutagenesis in XSCID
  - Results from our tumor-prone, XSCID mouse model

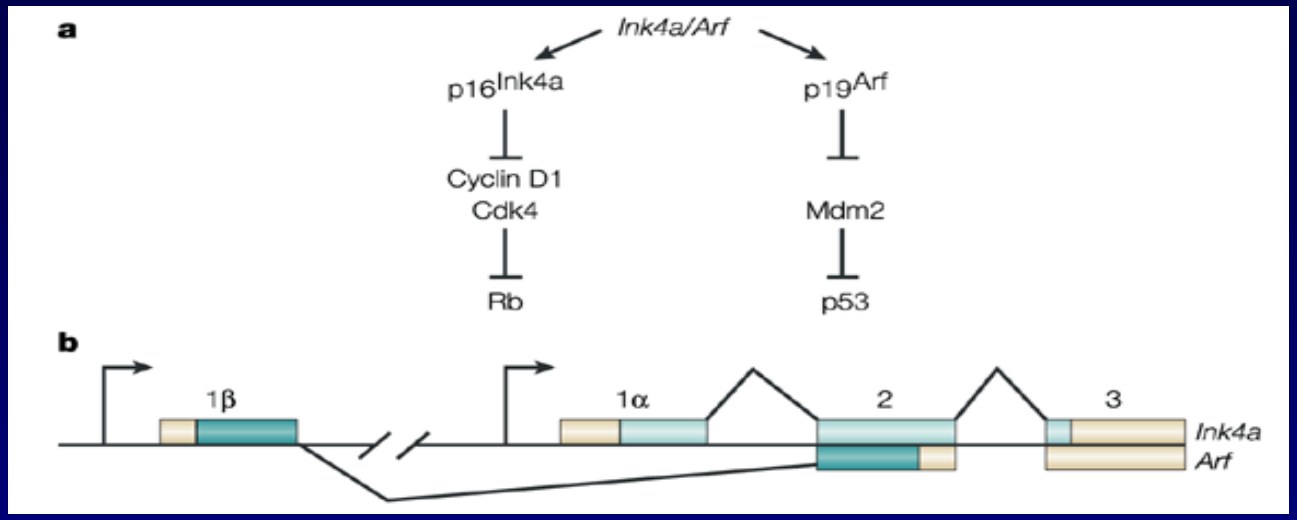
- Are lentiviral vectors a less oncogenic alternative ?
  - Differences in insertion site preferences
  - Homologous recombination studies in the LMO2 locus in human T cells
  - Results in tumor-prone mouse models

Unique risk for XSCID is suggested by long-term follow-up of large animals

- 42 rhesus macaques, 23 baboons, 17 dogs with significant levels of transduction at NHLBI and FHCRC
- CD34+ cells transduced with gamma-RV vectors expressing marker or drug resistance genes
- Median follow up 3.5 years, range 1-7 years (as of 2003)
- One animal developed a myeloid tumor at NHLBI
- All other animals had normal polyclonal hematopoiesis
- Recurrent integrations noted at the Mds1/Evi locus in NHLBI series but without clonal expansion
- No Lmo2 insertions detected

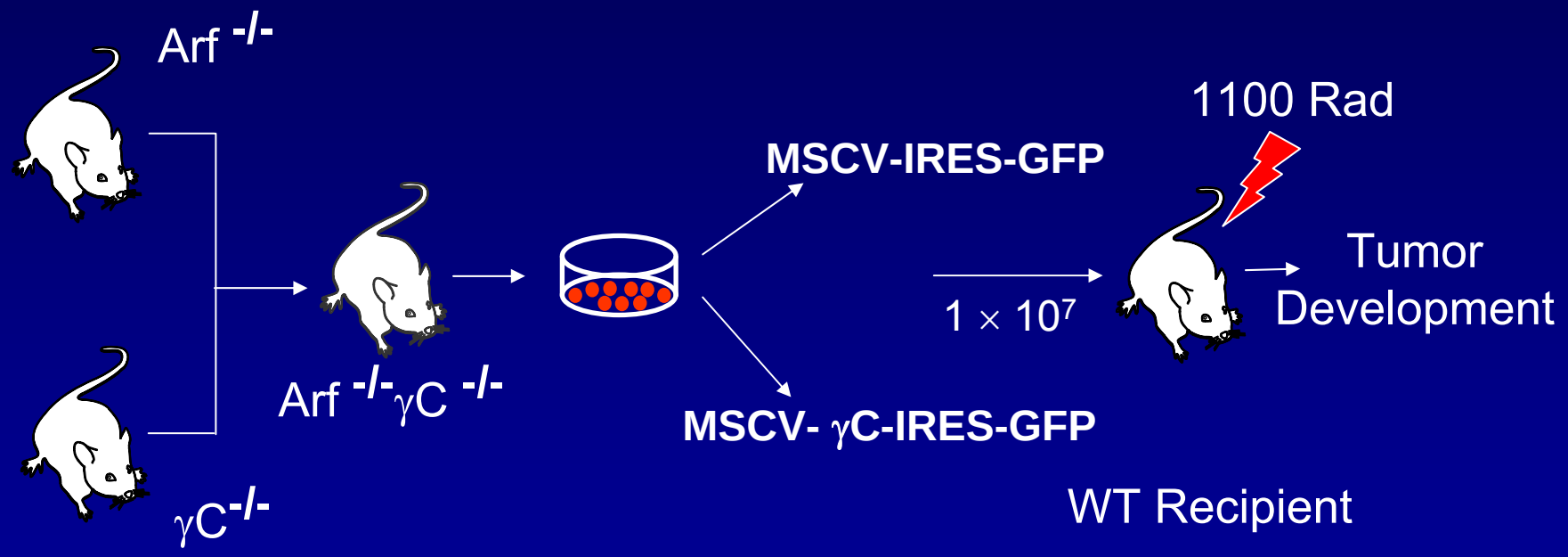
Kiem HP et al, Mol Ther, 2004
Camels B et al, Blood, 2005
Seggewiss R et al, Blood, 2006

Relevance of the Ink4a/Arf tumor suppressor locus in human T-cell leukemia and XSCID leukemia



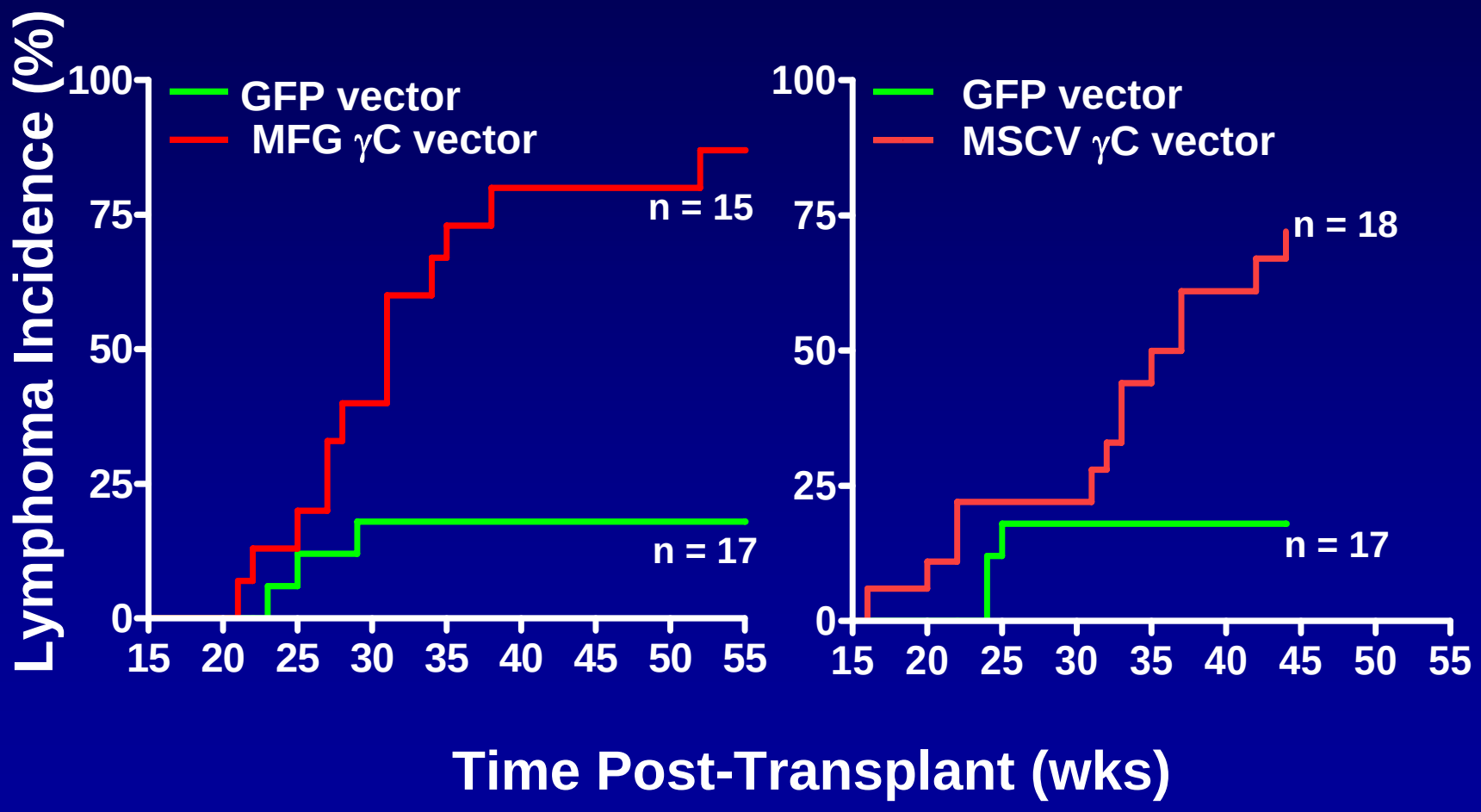
- In human T-ALL patients, the ARF locus is disrupted or deleted in all cases (149/149) (Sigaux *et al*, 1998).
- The long latency of leukemia in XSCID patients suggests the acquisition of additional collaborating mutations.
- 3 out of 4 patients have biallelic deletions of the INK4a/ARF locus. One patient has an activating insertion into the BMI-1 locus, an dominant suppressor of INK4a/ARF (M. Cavazana-Calvo, 8/2007).
- Arf-null mice are highly tumor prone and T-cell lymphoma is the second most common tumor type (Kamijo *et al,* 1999).

Arf $^{-/-}$ γc$^{-/-}$ mouse model

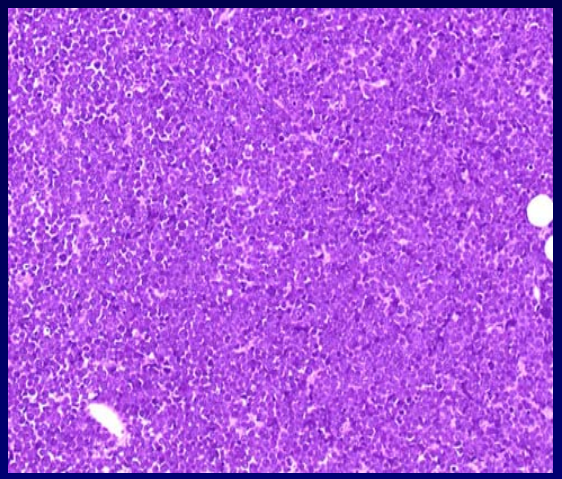


Shou Y et al, PNAS, 2006

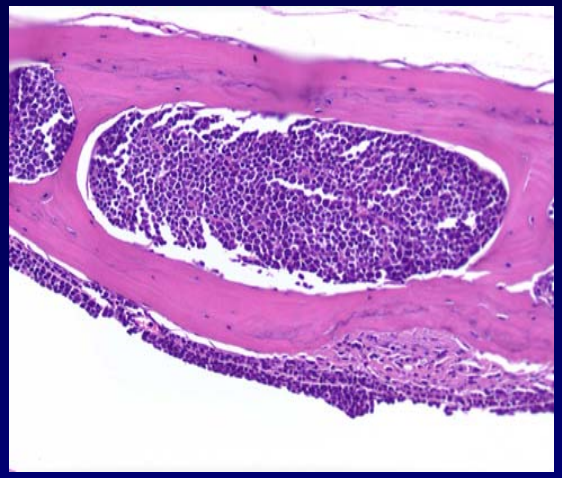
Accelerated transformation by γ_c vector integration in Arf$^{-/-}$ $\gamma_c^{-/-}$ BM cells



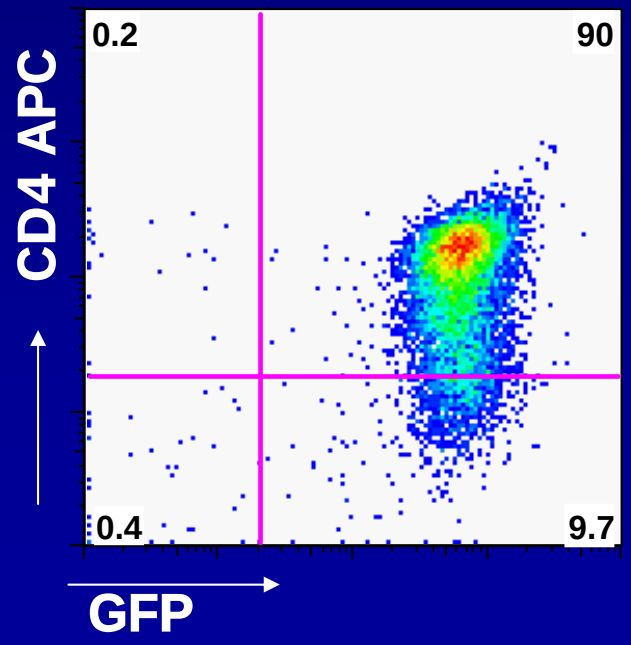
T cell lymphomas with vector insertions

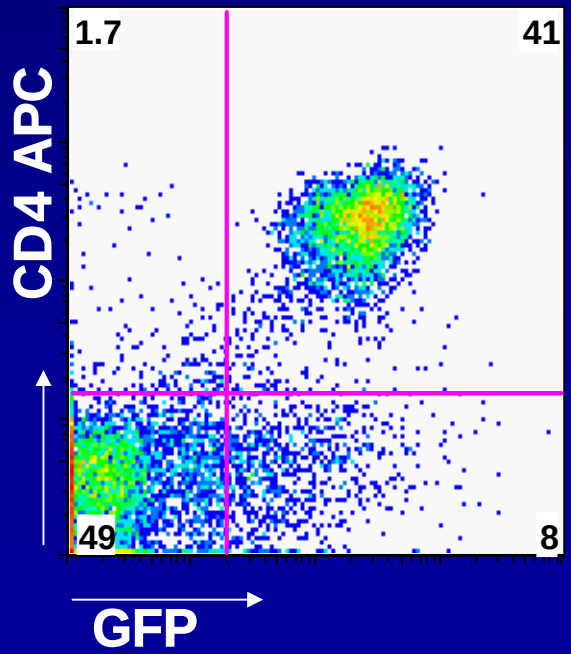


Thymus 20x

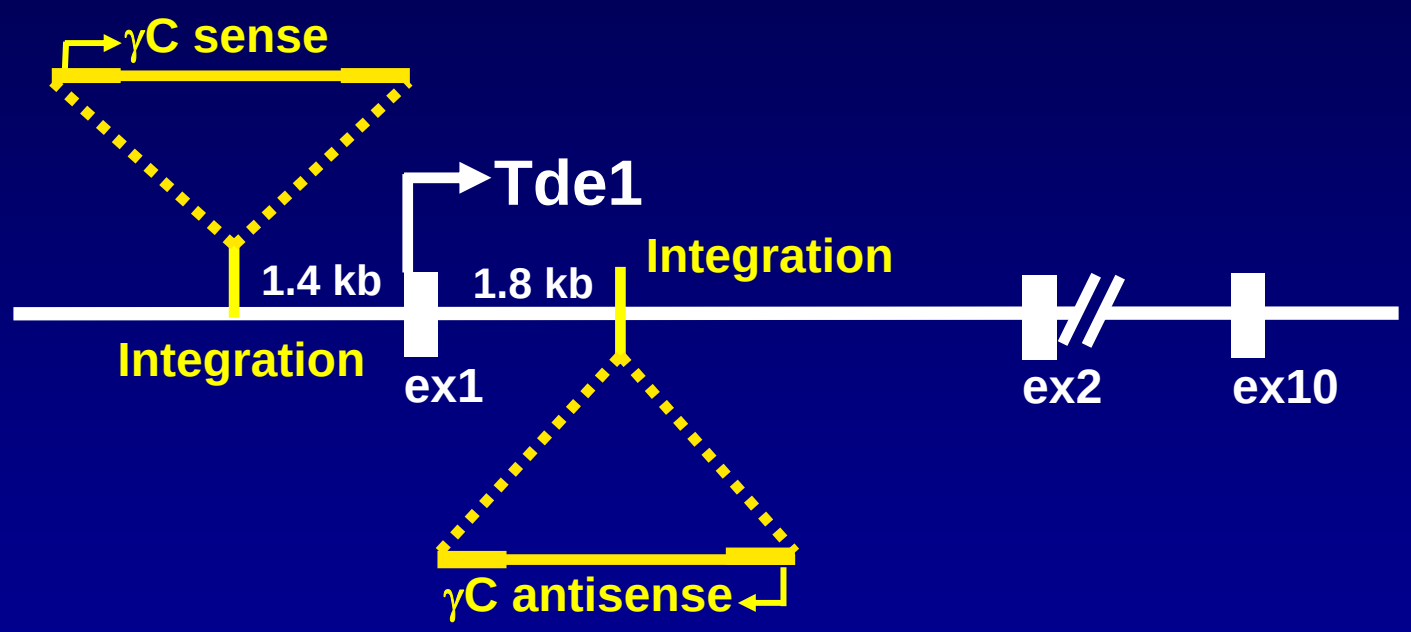


Bone marrow 20x





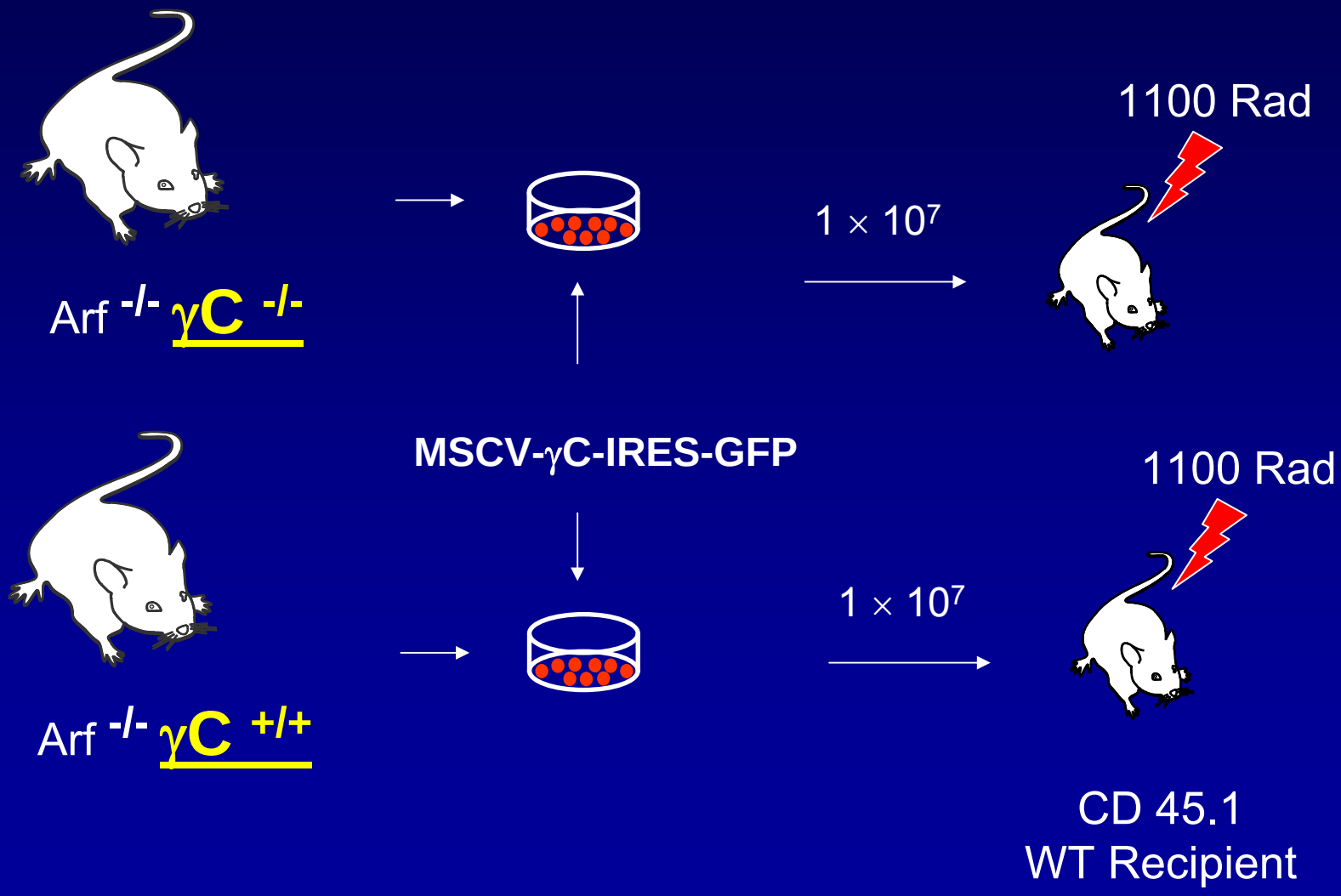
Multiple independent insertions into a single allele



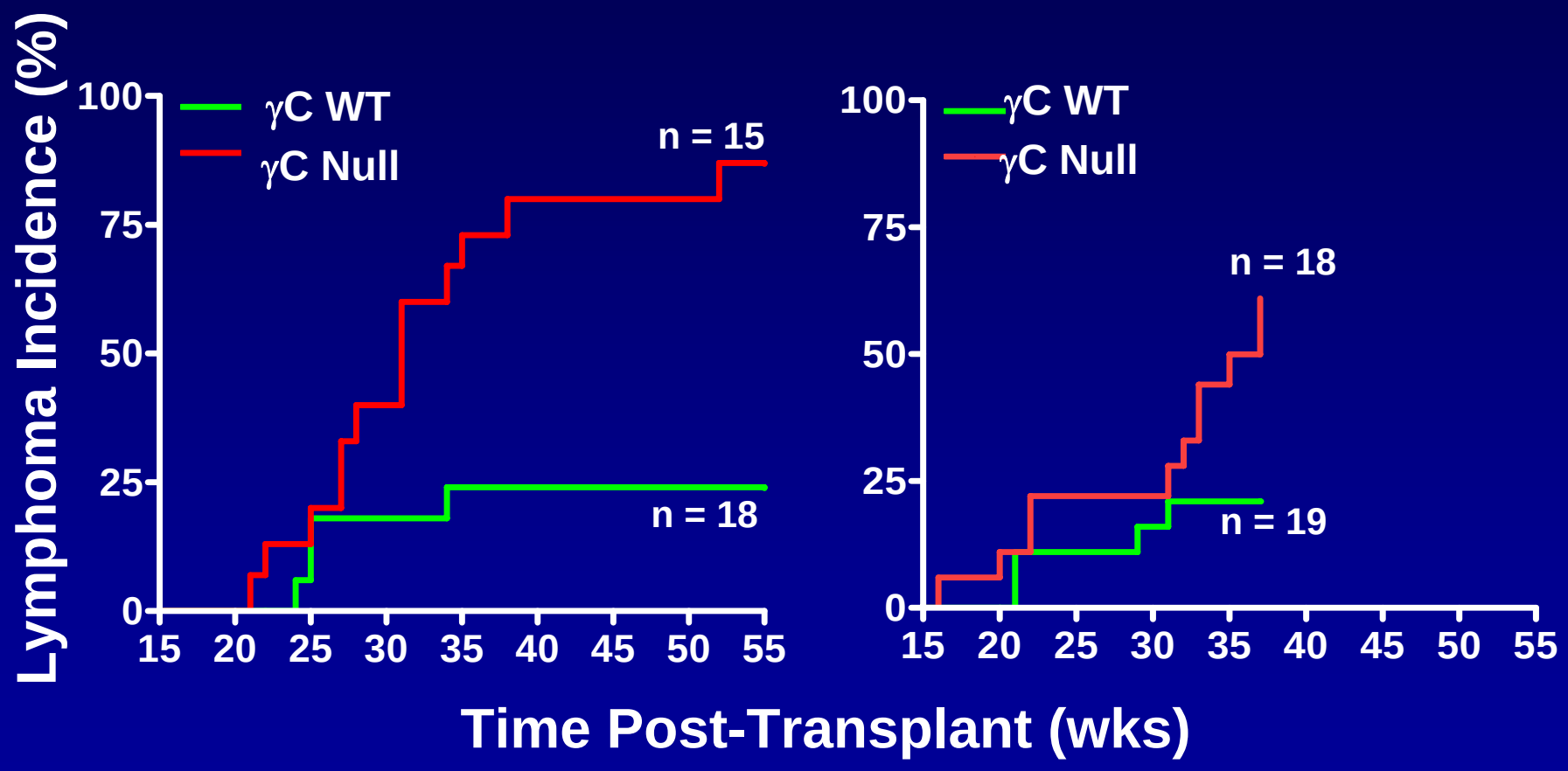
Clonal selection for activation of specific genes

Bossolasco M et al, Human TDE1, a TDE1/TMS family member, inhibits apoptosis in vitro and stimulates in vivo tumorigenesis. Oncogene, 2006

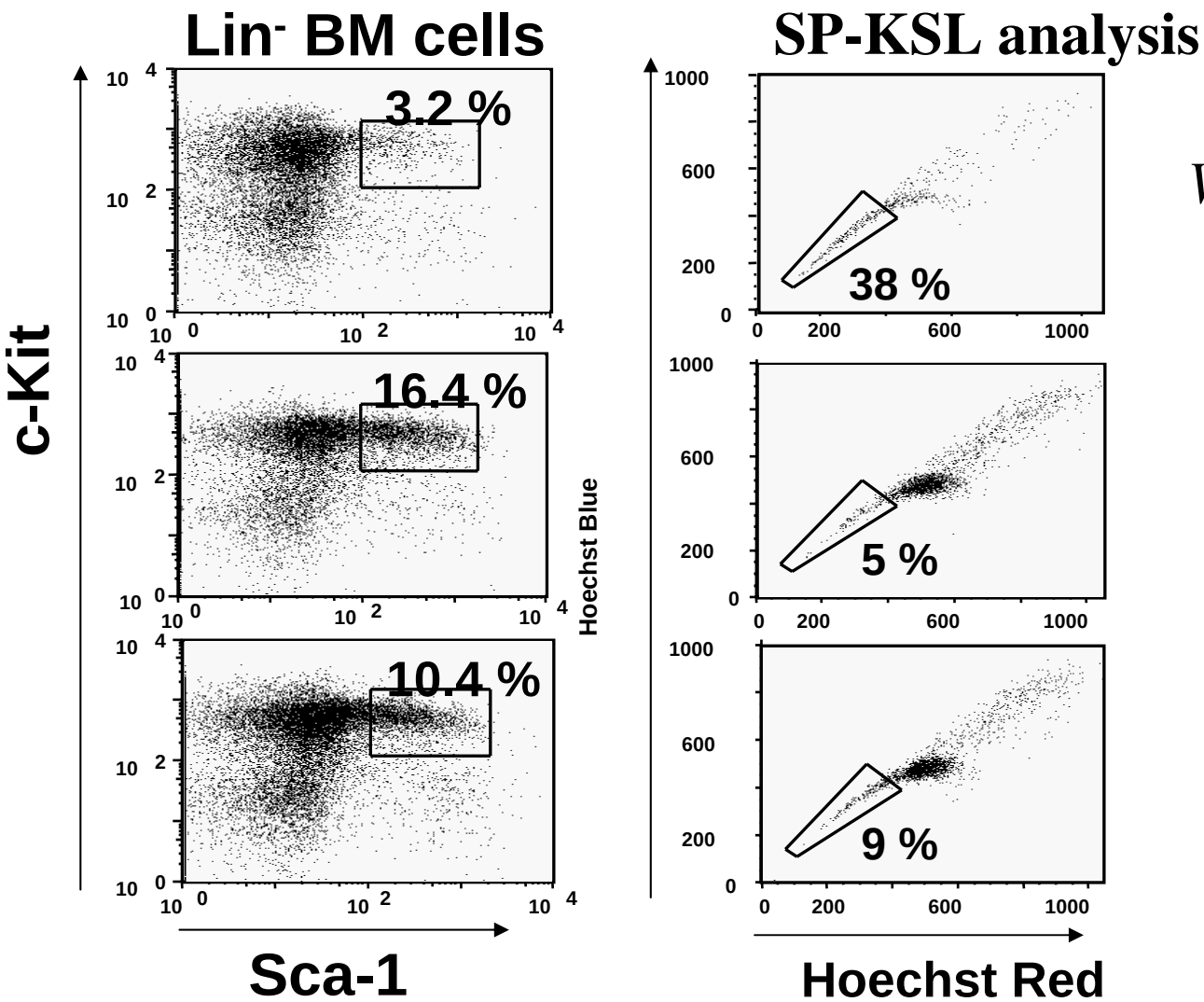
Does the XSCID background confer a specific risk factor ?



Decreased incidence of lymphoma associated with Arf$^{-/-}$ γc$^{+/+}$ cells



An abnormal accumulation of progenitor cells is detected in the bone marrow of XSCID mice



WT mouse BM cells

$Arf^{+/+}$ $\gamma c^{-/-}$

$Arf^{-/-}$ $\gamma c^{-/-}$

Is the risk uniquely elevated in XSCID ?

- YES, based on data from several animal studies.
- Specific genetic alterations are associated with XSCID T-cell leukemia including LMO2 activation and the loss of function of the ARF tumor suppressor.
- Lymphoid specific transformation systems are most relevant for testing vector safety modifications.
- The target cell for transformation has not been defined but may be a lymphoid progenitor cell with specific characteristics defining vector insertion preferences.
- Unknown to what degree ADA-deficient SCID will be similar or different from XSCID with regard to risk. LMO2 insertions have been detected in ADA SCID clinical trials.

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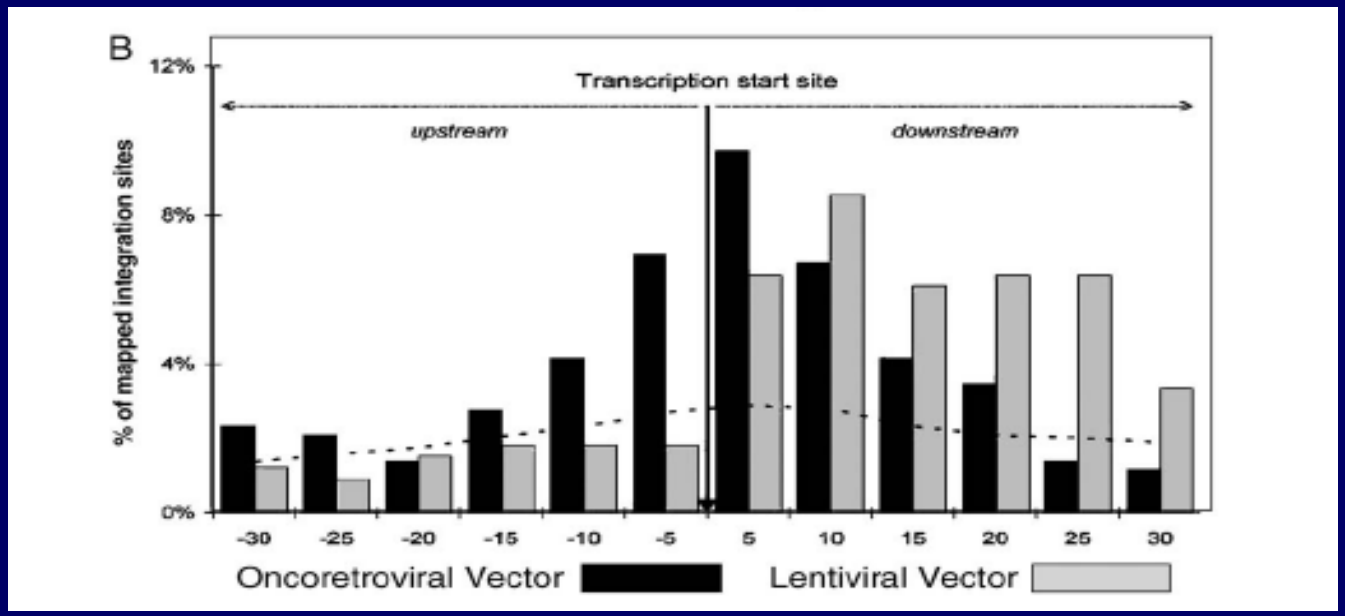
  - Review of long term data in dogs and NHP
  - Genetics of insertional mutagenesis in XSCID
  - Results from our tumor-prone, XSCID mouse model

- **Are lentiviral vectors a less oncogenic alternative ?**
  - **Differences in insertion site preference**
  - Homologous recombination studies in the LMO2 locus in human T cells
  - Results in tumor-prone mouse models

Potential advantages for lentiviral vectors

- HIV virus has not been observed to cause tumors with wildtype infections, as opposed to MLV virus
- Lentiviruses have a fundamentally different pattern of genomic integration that has not been associated with integrations in know "problem" genes. (LMO2, MDS1/Evi1, Prdm1)
- Can easily be created that lack all viral transcriptional regulatory elements and enhancers. (so called SIN or self-inactivating design)
- Superior transduction efficiency in non-human primate hematopoietic stem cells
- Can accommodate larger, more complex gene-expression units

Integration sites of lentiviral vectors in blood cells



Camels B, PLOS, 2006

- Cattoglio C et al, Blood, 2008
  - Map insertions into human CD34+ cells
  - Hot spots account for 21% of gamma-RV sites
  - Significantly less hot spots for lentivirus
  - Lentiviral hotspots were less likely to be enriched in oncogenes or growth-controlling genes

- Persons DA et al, Molec Ther, 2008
  - Globin lentiviral vector insertions mapped in CFU-S
  - Several examples of integrations near or within potential oncogenes
  - Alteration of expression noted in some cases, however the functional significance could not be determined in this assay system.

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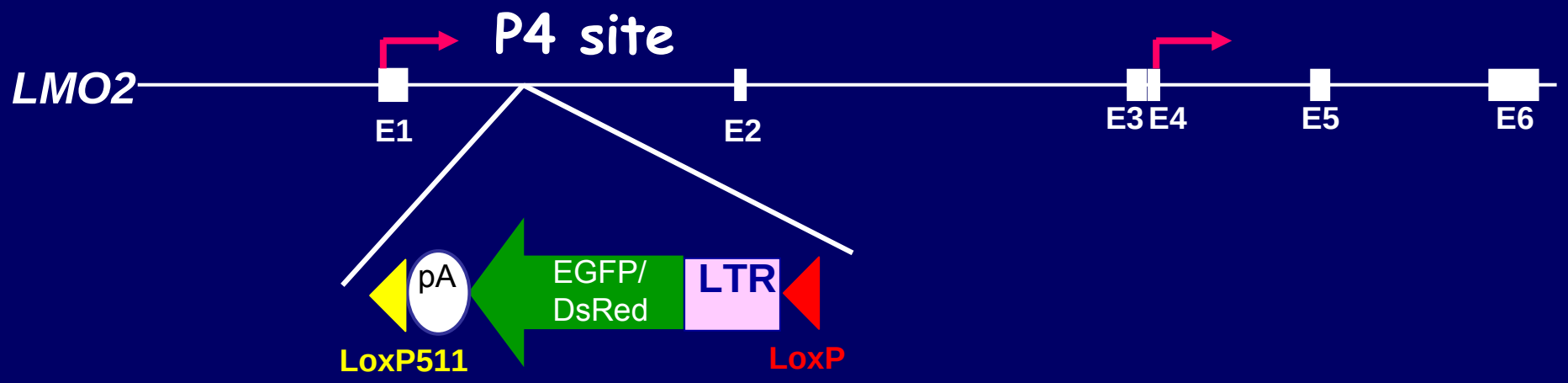
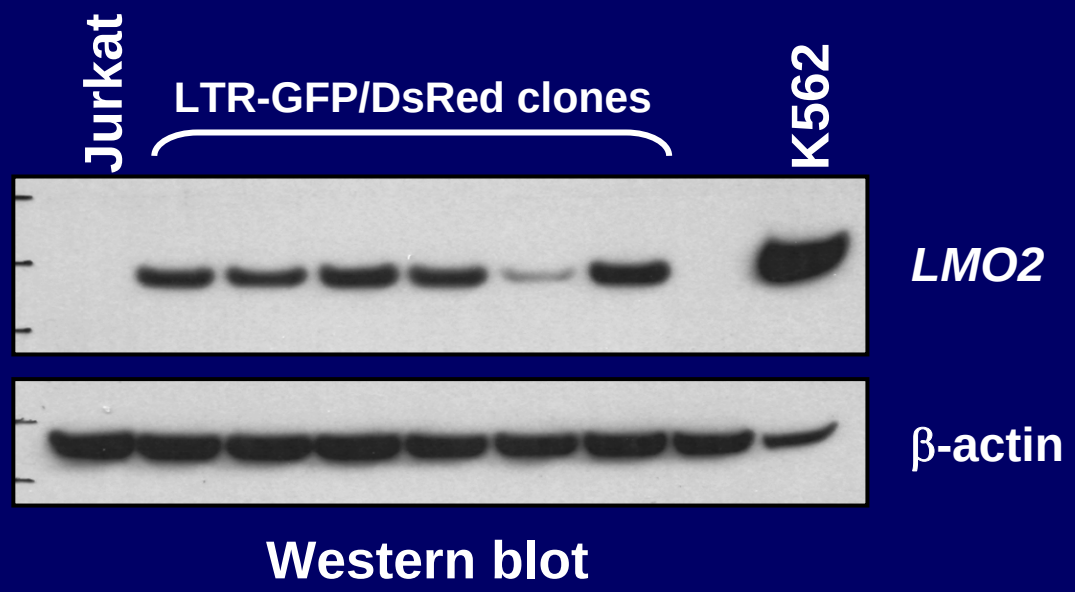
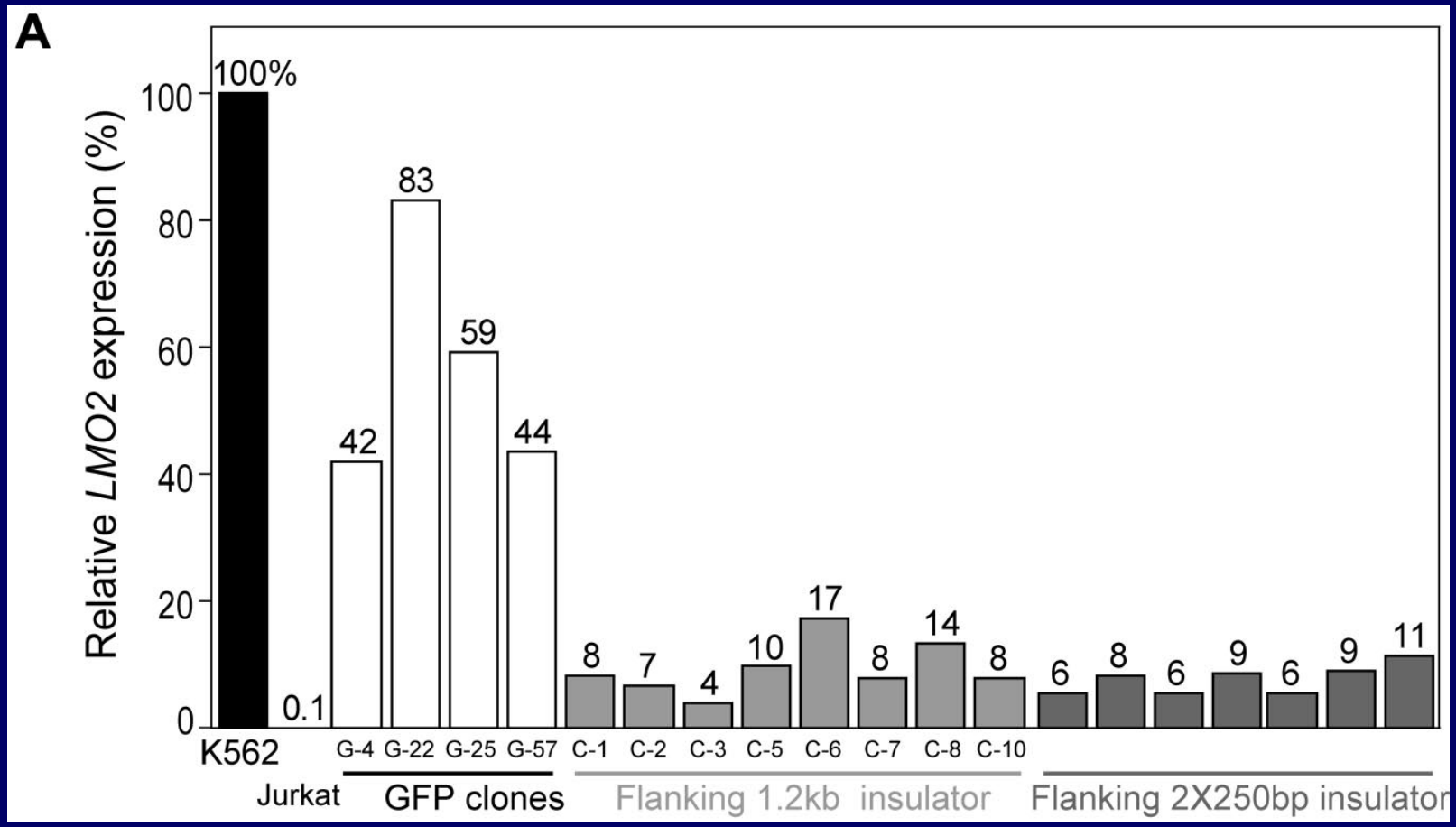
Knock-in of LTR-GFP cassette activates LMO-2 expression in human Jurkat T cells





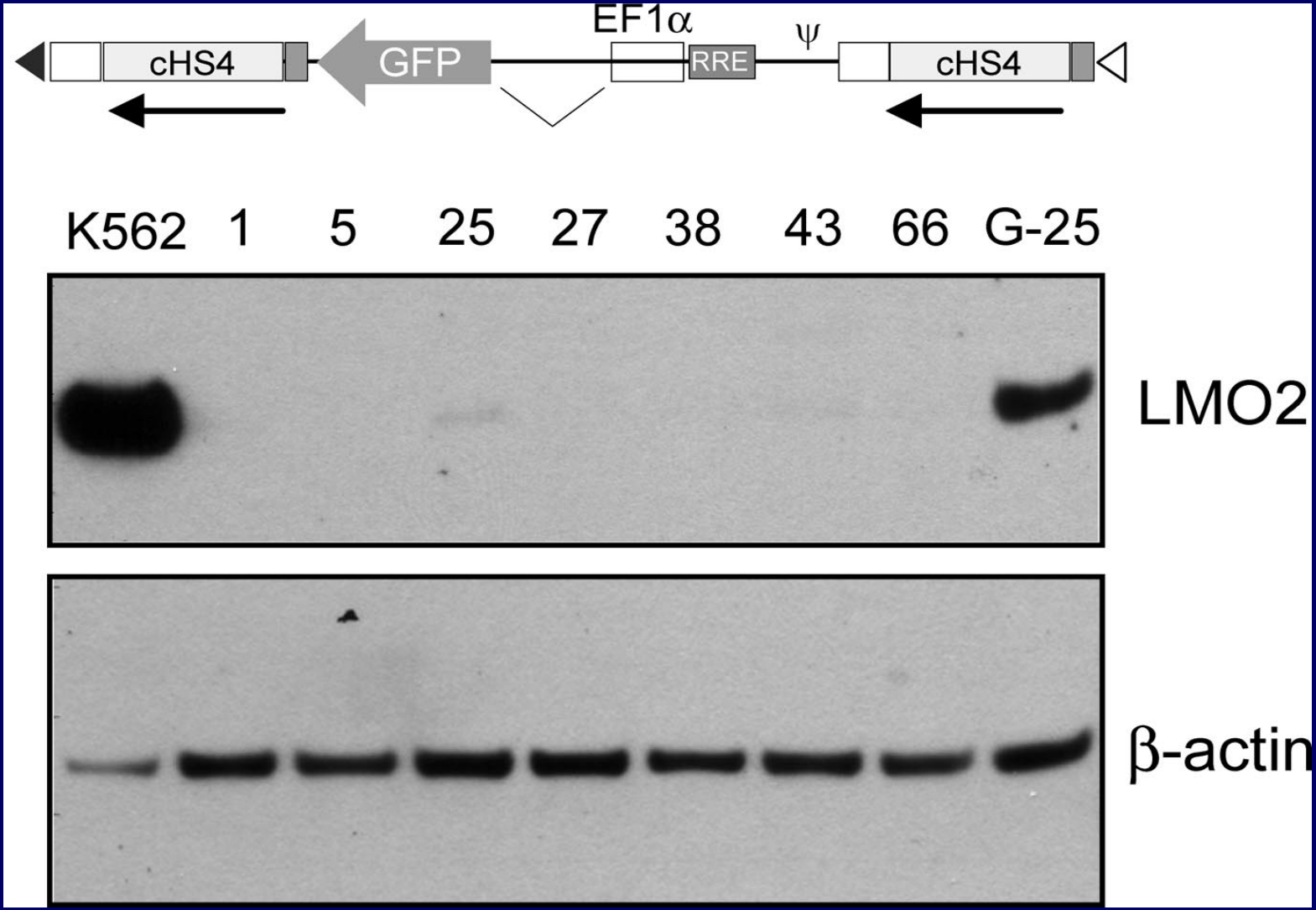
(B Ryu et al Blood. 2008)

The cHS4 insulator only partially blocks activation of LMO2 expression



(B Ryu et al Blood. 2008)

Lack of LMO2 activation with an insulated lentiviral vector



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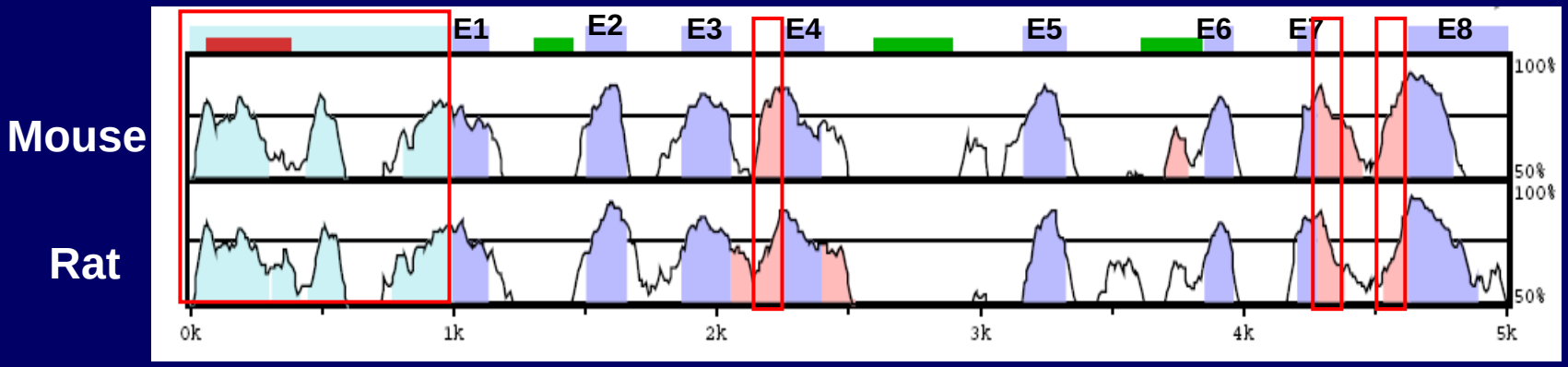
Tumor prone mouse models

- Montini E et al, Nat Biotech, 2006
  - Ink4a KO mouse model
  - Gamma-RV vectors triggered tumor acceleration
  - Tumor formation rate unaffected by lentiviral vectors
  - Both lymphoid and myeloid tumors were noted

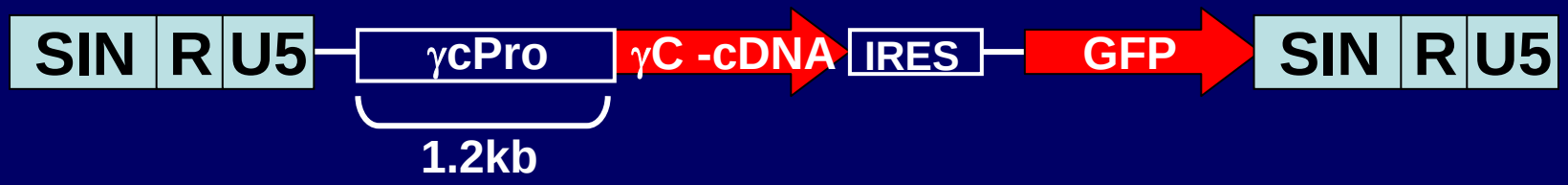
Zhou and Sorrentino, unpublished data

	Number of animals treated	GFP+ donor lymphoma
MSCV- γC -IGFP	36	4
CL20-MSCV- γC-IGFP	40	1
CL20Ins-MSCV-γC-IGFP	40	0

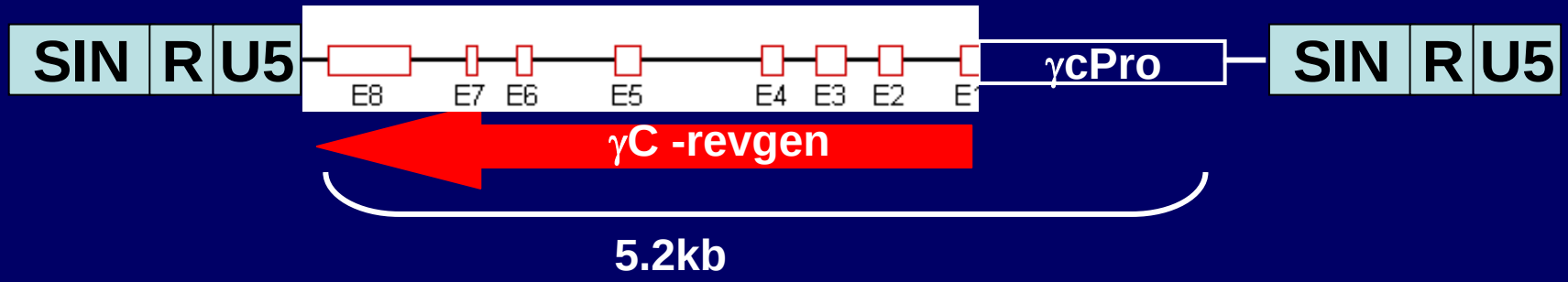
Use of γc elements for an XSCID lentiviral vector



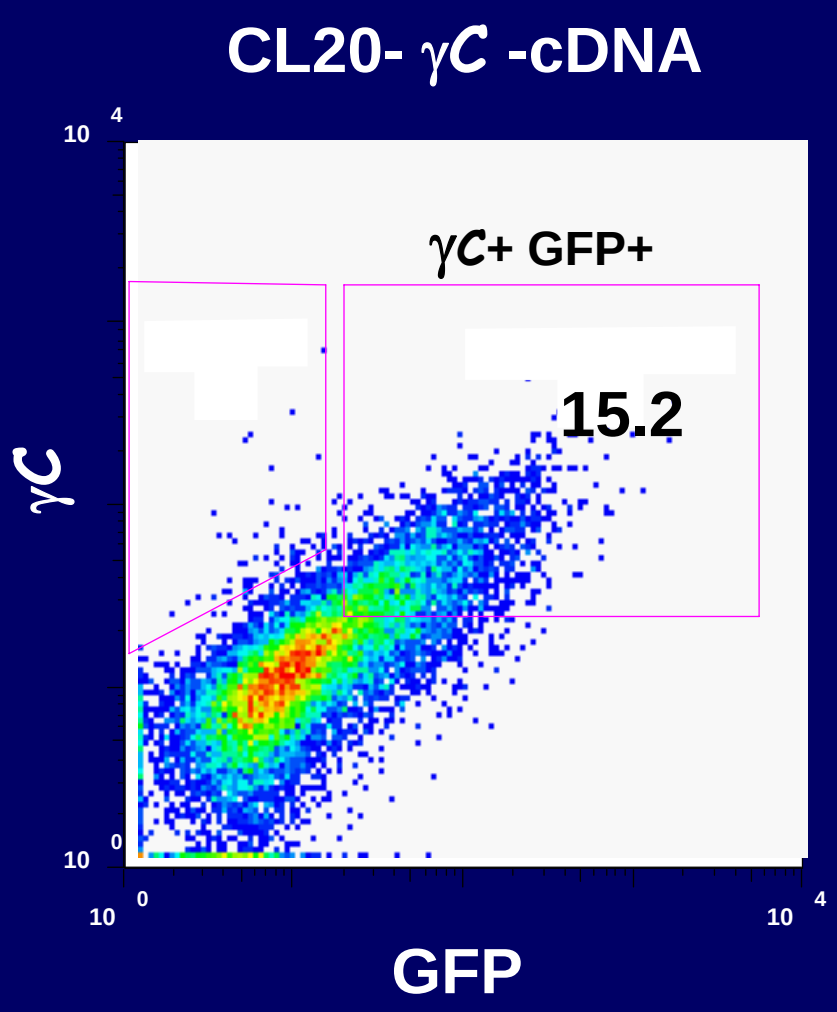
1. CL20- γc-cDNA

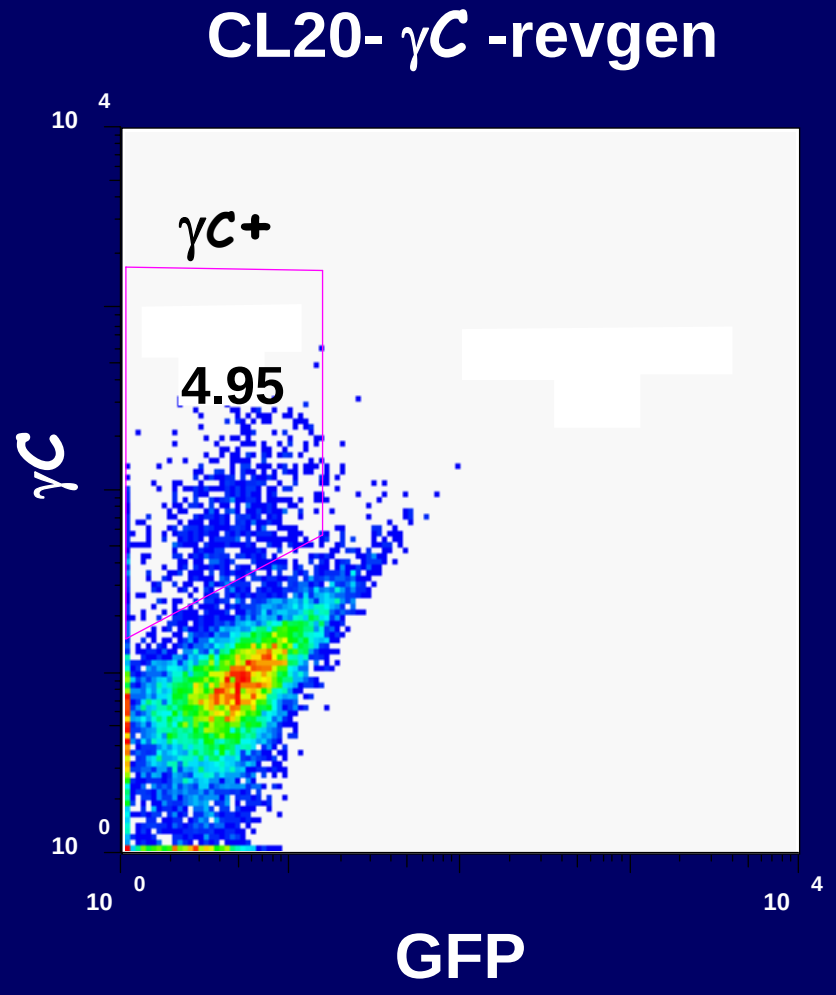


2. CL20- γc-revgen



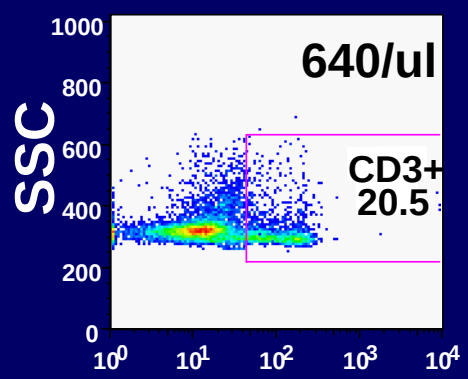
γc promoter is sufficient for expressing γc in EBV- γC ^{null} B lymphocytes

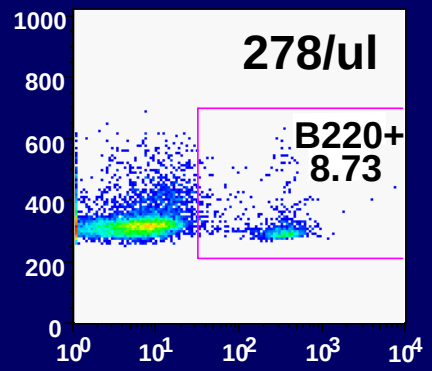




Only the CL20-γC -revgen vector leads to immune reconstitution (15 weeks)

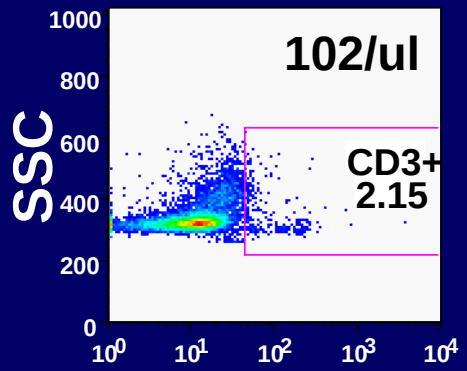
CL20- γC -revgen

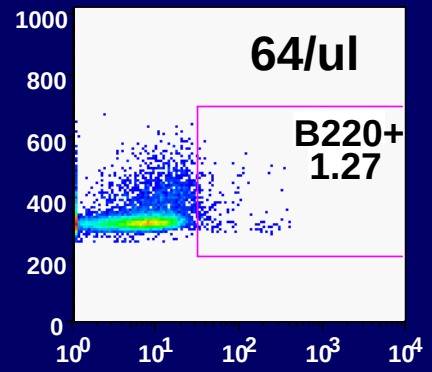




7/11 mice

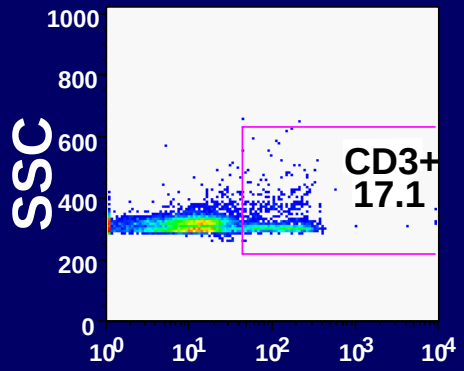
CL20- γC -cDNA

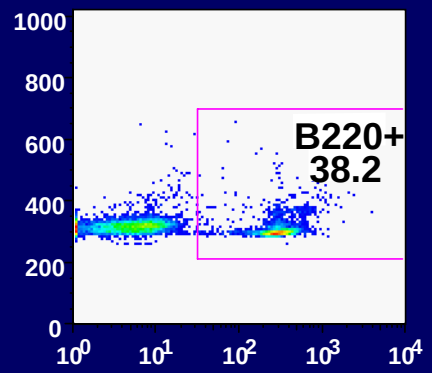




0/6 mice

WT control



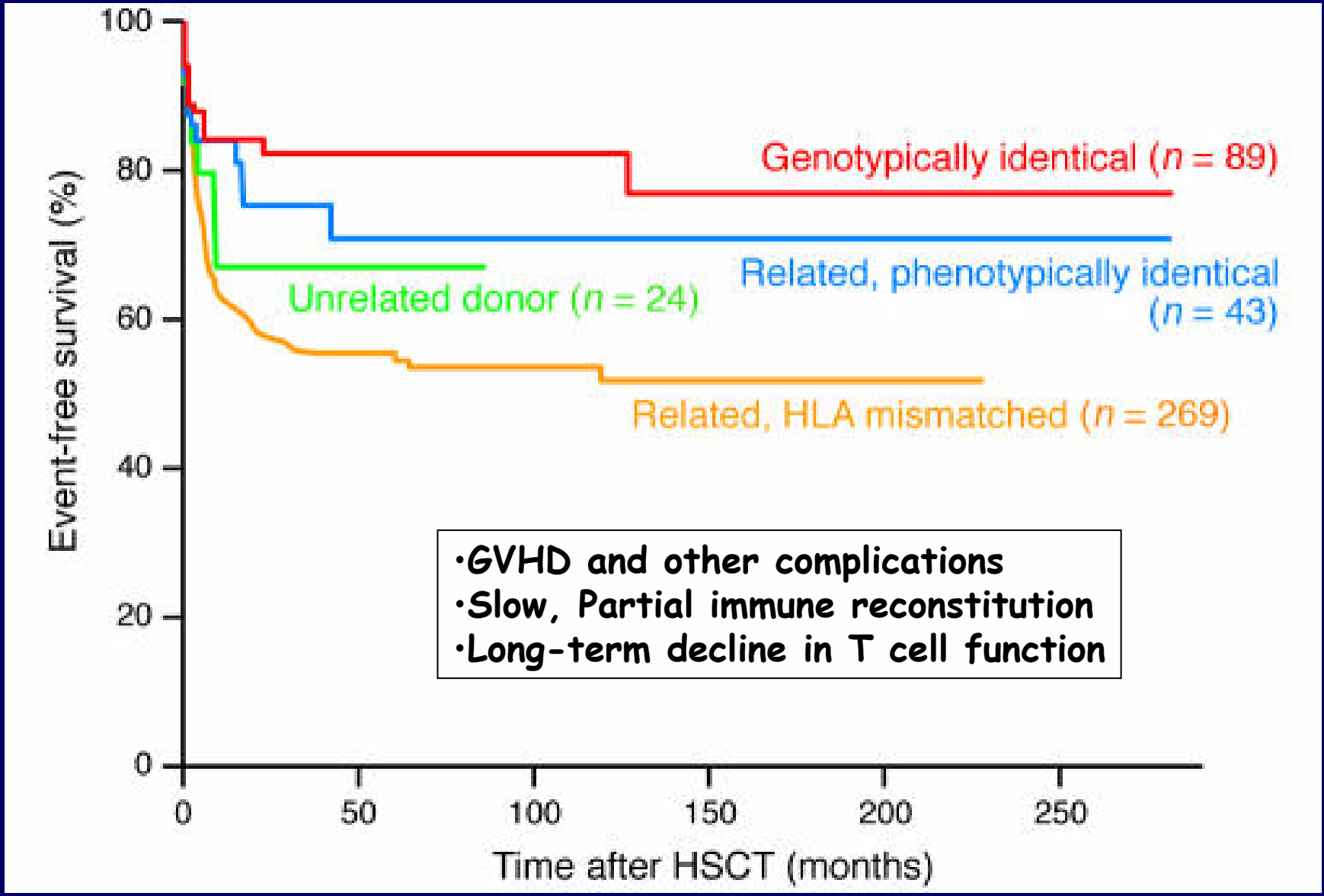


CD3 B220

Conclusions

- Safety can likely be improved by eliminating viral enhancers and using appropriate cellular promoters.
- Insulators may also decrease the risk of insertional mutagenesis although their effect is not absolute in lymphoid cells.
- It is not clear how the safety profiles of SIN lentiviral vectors will compare with that of SIN gamma-retroviral vectors.
- An important new focus is developing and testing new vector designs for efficacy and safety in appropriate animal models.

Allogeneic HSCT for SCID (45% are X-SCID)



- GVHD and other complications
- Slow, Partial immune reconstitution
- Long-term decline in T cell function

Marina Cavazzana-Calvo, et al. *J. Clin. Invest,* 117:1456

Acknowledgements

Brian Sorrentino

Taihe Lu

Zhijun Ma

Mike Greene

Soghra Fatima

Maggie Evans-Galea

Andy Wilbur

Phillip Hargrove

Matt Wielgosz

John Gray

Derek Persons

Arthur Nienhuis

Mary Ellen Conley

Charles Sherr

Hartwell Center

Jin Ma

Flow Cytometry Core

Anima Research Center

ARC diagnostic lab

Hartwell center

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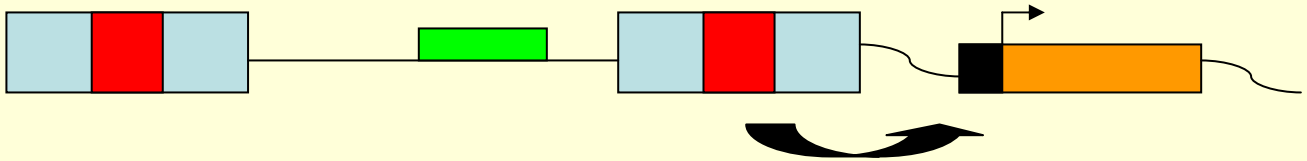
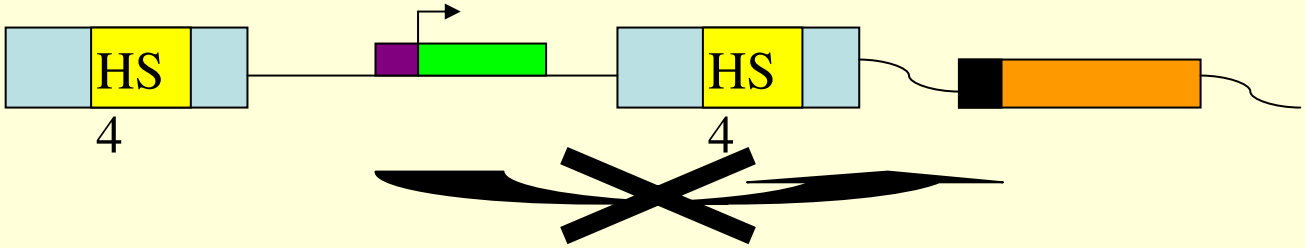
Safety strategies for vectors



Internal promoter and chromatin insulators



X-SCID gene therapy clinical studies

- Started in 1999 in France and later in London
- Patient's CD34+ cells from bone marrow.
- Used retroviral vector (Based on Moloney murine leukemia virus –MLV) carrying therapeutic wild type γC cDNA (MFG- γC)
- To date, 20 patients treated, 17 successful in immune function restoration, most off of all medications

"Thus, the efficacy of gene therapy in conferring immune function in those infants with SCID-X1 seemed to be far superior to that of allogeneic marrow stem-cell transplantation."
-R Buckley, Annu. Rev. Immunol. 2004. 22:625–55

What we have learned:

- Gene therapy could be very efficacious
- MLV based vector cause leukemia due to insertional mutagenesis

What we need to do next:

- Develop safer vectors
- Develop sensitive, disease relevant models to evaluate vector safety

The newly developed vectors and the principles of vector safety evaluation systems could be used for developing gene therapy for other hematological diseases

Data regarding integration sites of lentiviral vectors

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  - Ink4a KO mouse model
  - Gamma-RV vectors triggered tumor acceleration
  - Tumorigenesis unaffected by lentiviral vectors
  - Not a lymphoid specific system
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  - Several examples of integrations near or within potential oncogenes
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Overall hypotheses

1. Lentiviral vectors are safer than MLV retrovirus vectors

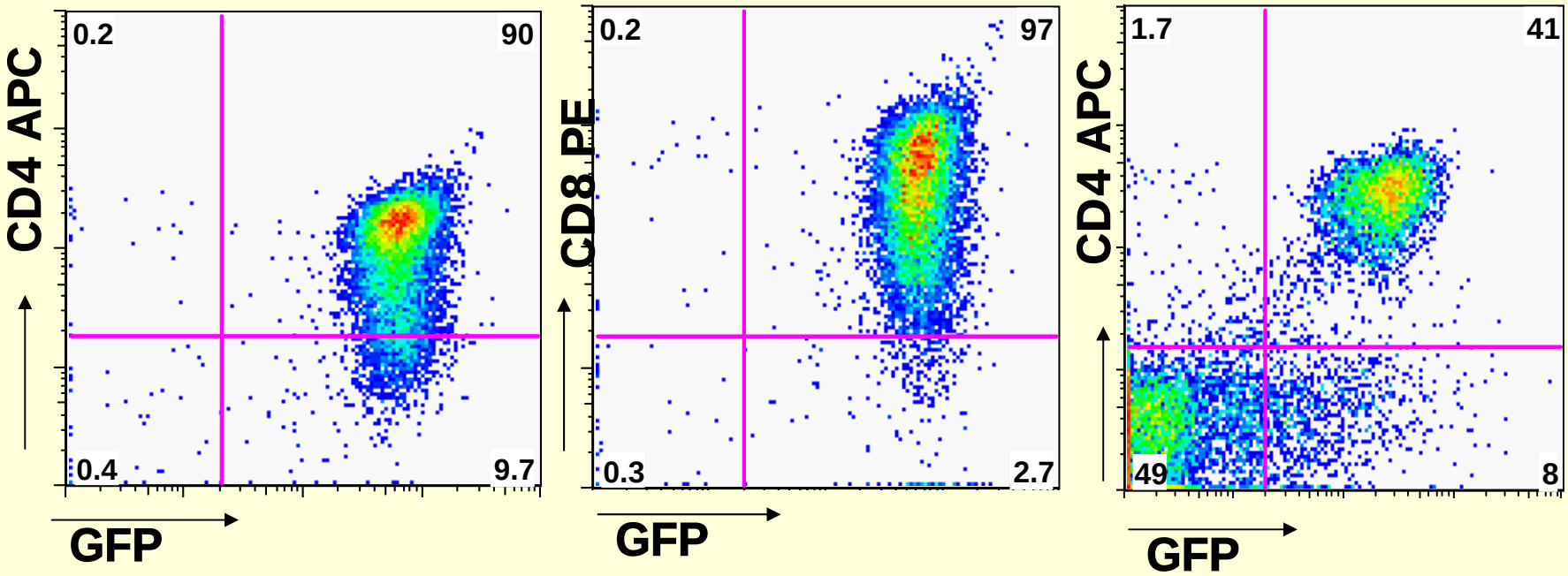
2. Insulator elements further reduces insertional mutagenesis

3. Appropriate internal cellular promoters can be identified to safely drive expression of the gamma-chain transgene

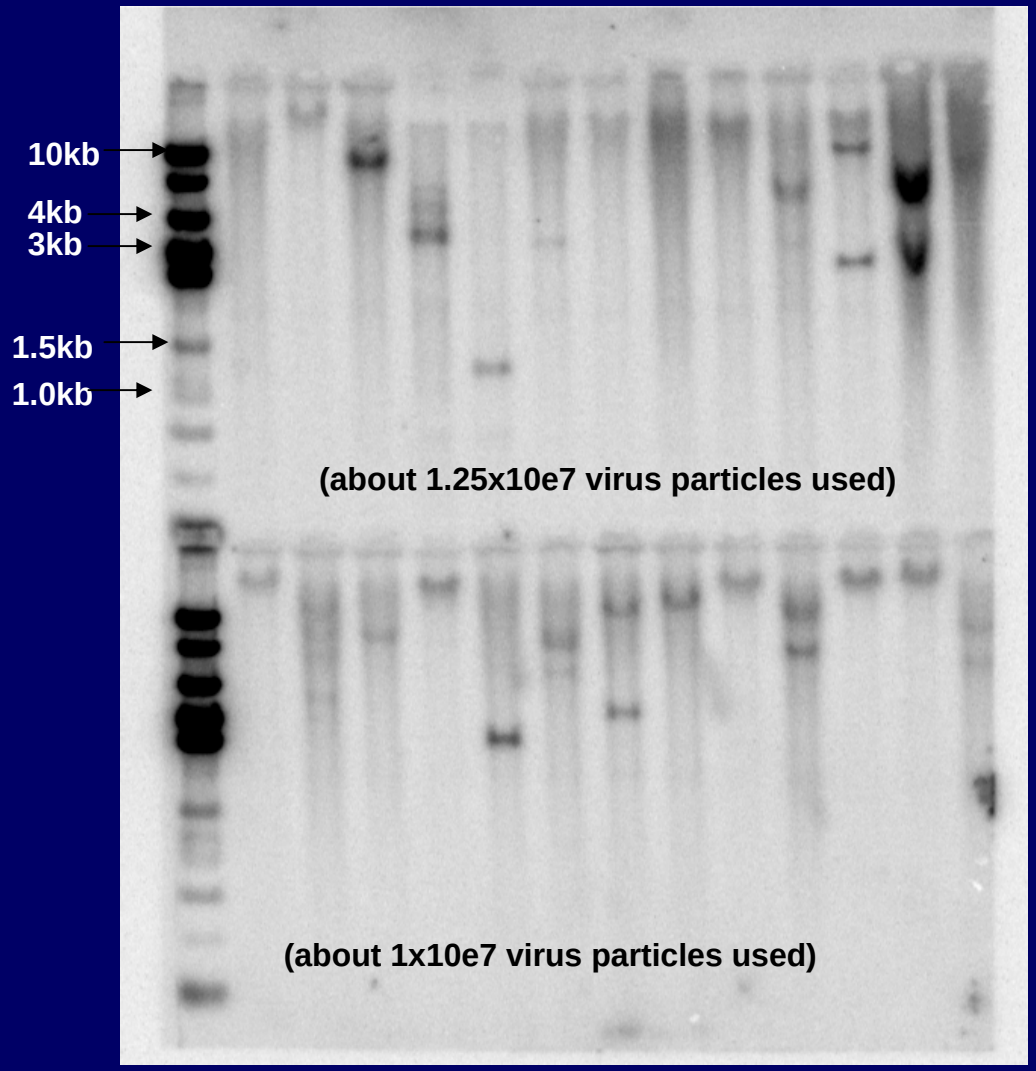
T-cell tumors express the γ_c GFP vector

Thymus

Bone marrow



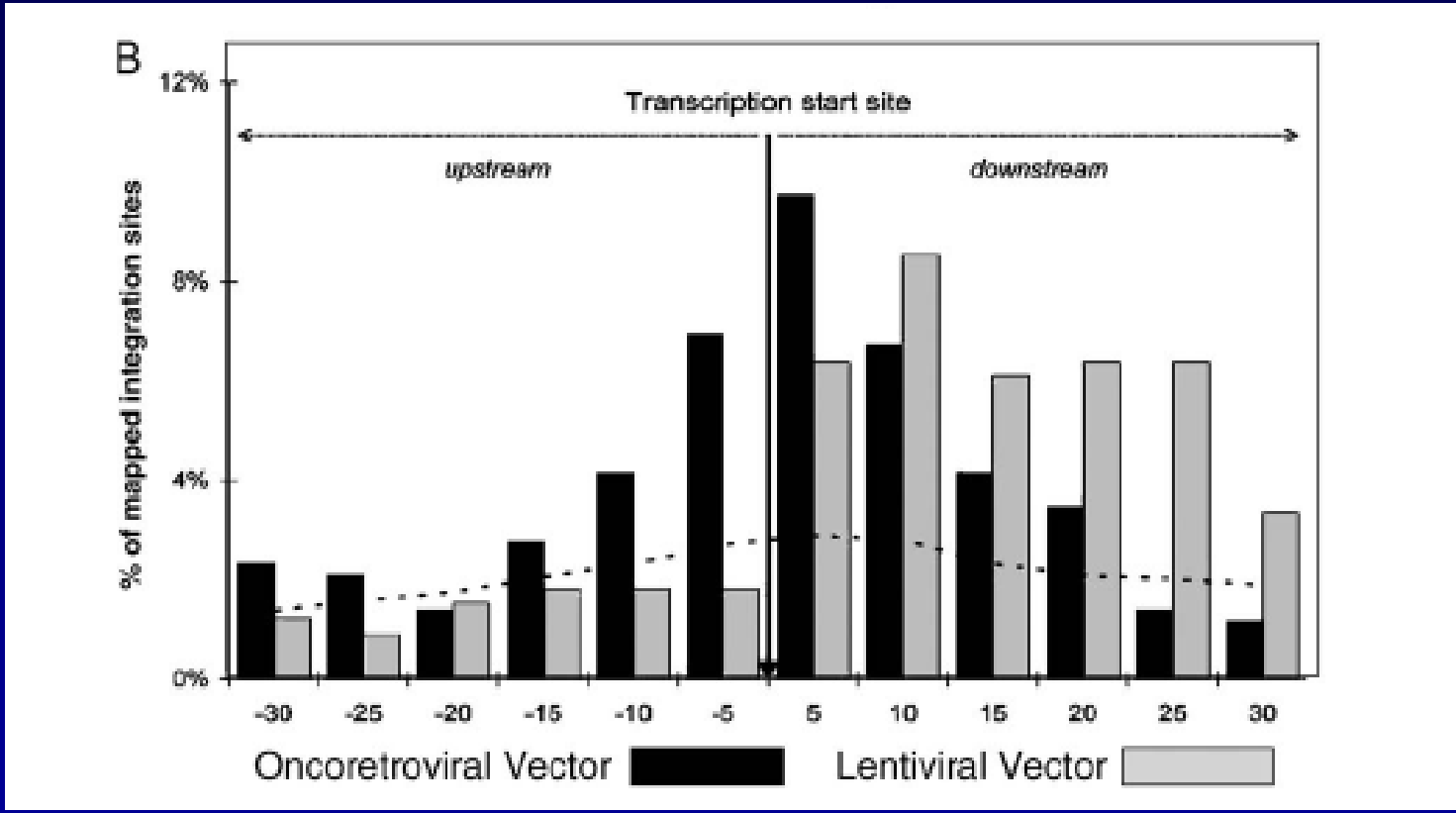
CFU-S analysis shows 1-2 proviral copies per clone with lentiviral vectors



CL20Ins-MSCV- γC -IGFP

CL20-MSCV- γC -IGFP

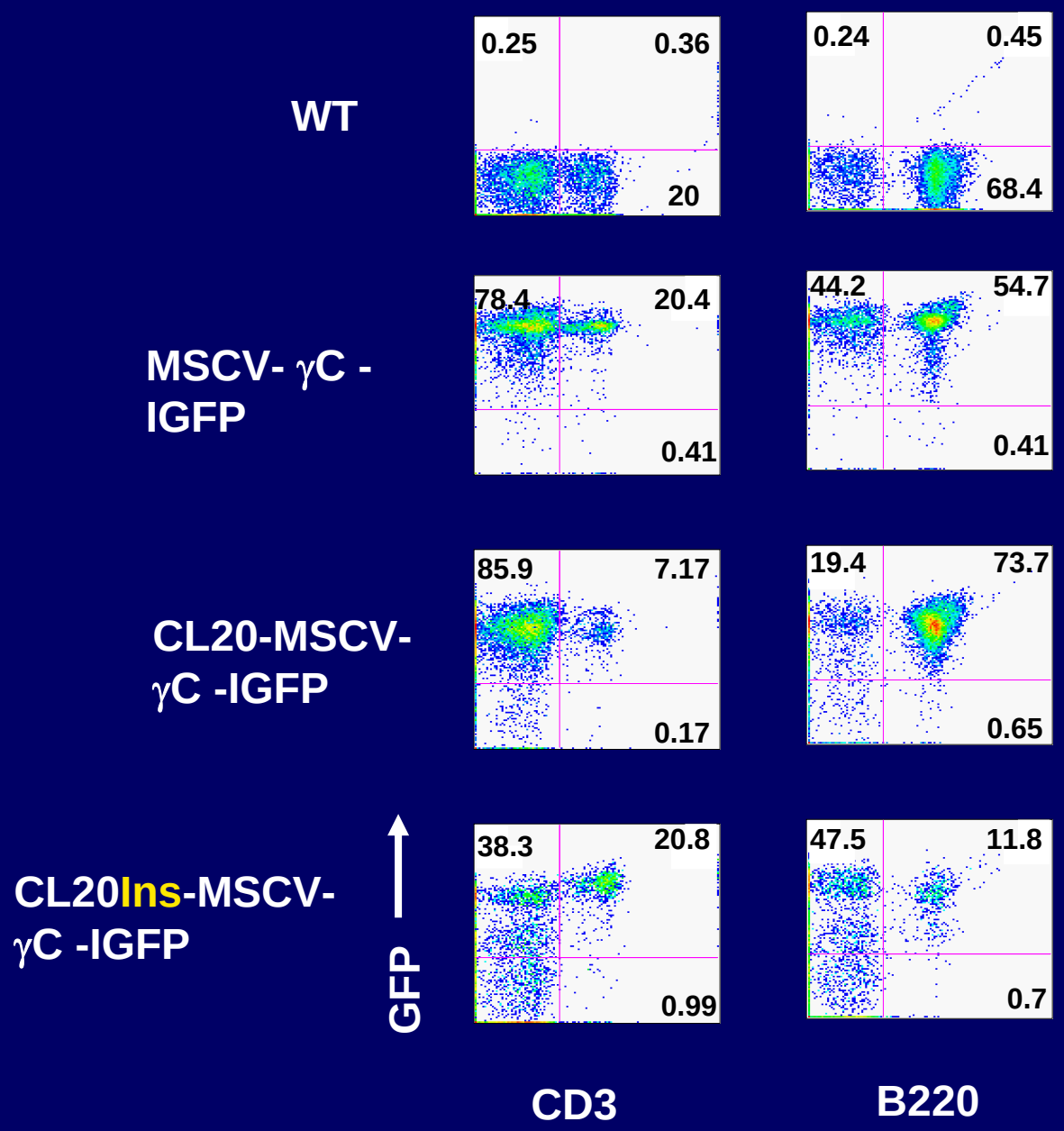
Lentiviral vectors for improved safety in gene therapy



Dunbar et al, PLOS, 2006

Lentiviral vector integrations have not been found in the MDS1/Evi Locus versus than seen with MLV vectors

Lentiviral vectors reconstitute T and B cells (8 weeks)



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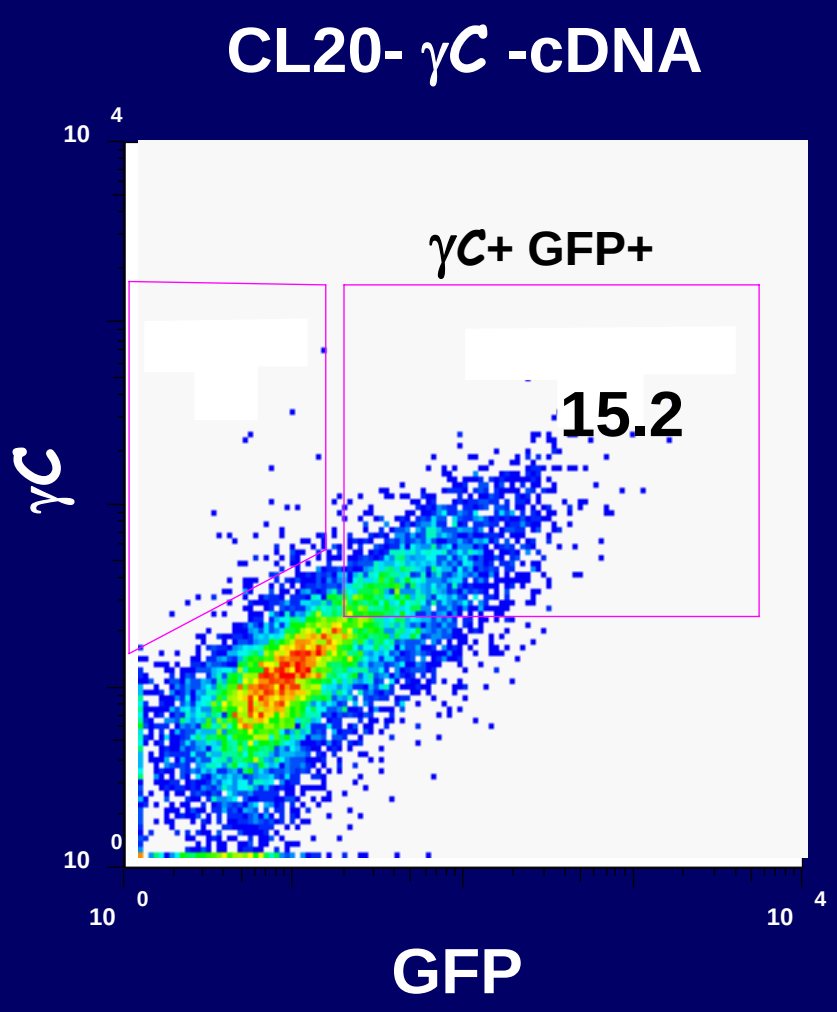
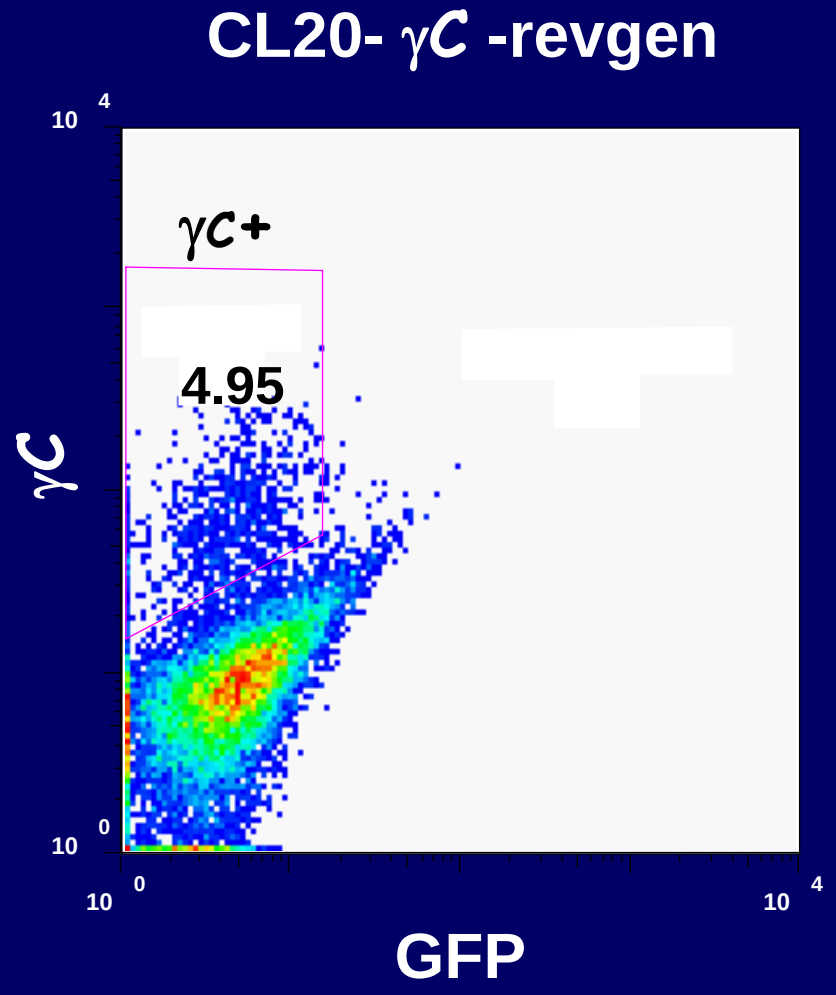
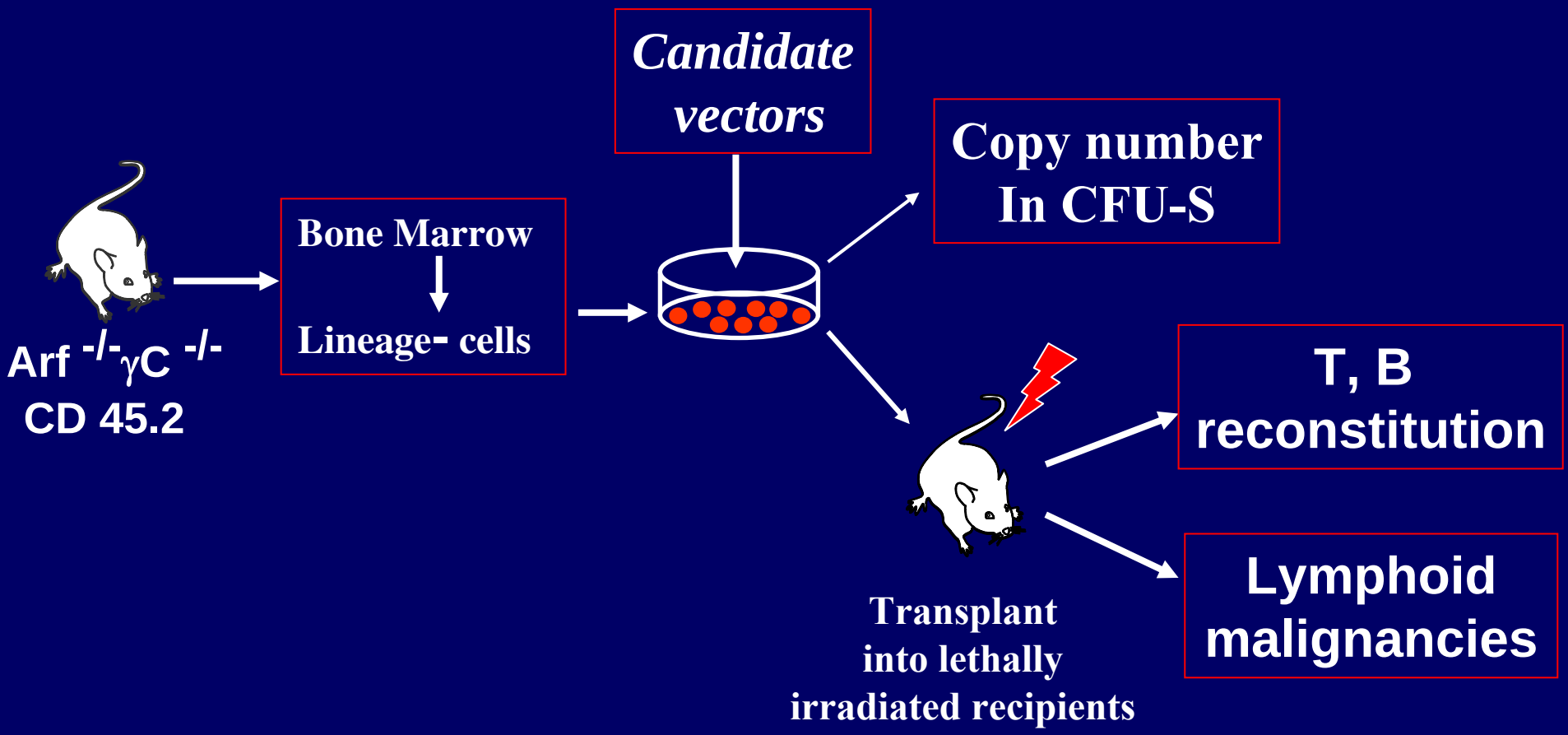
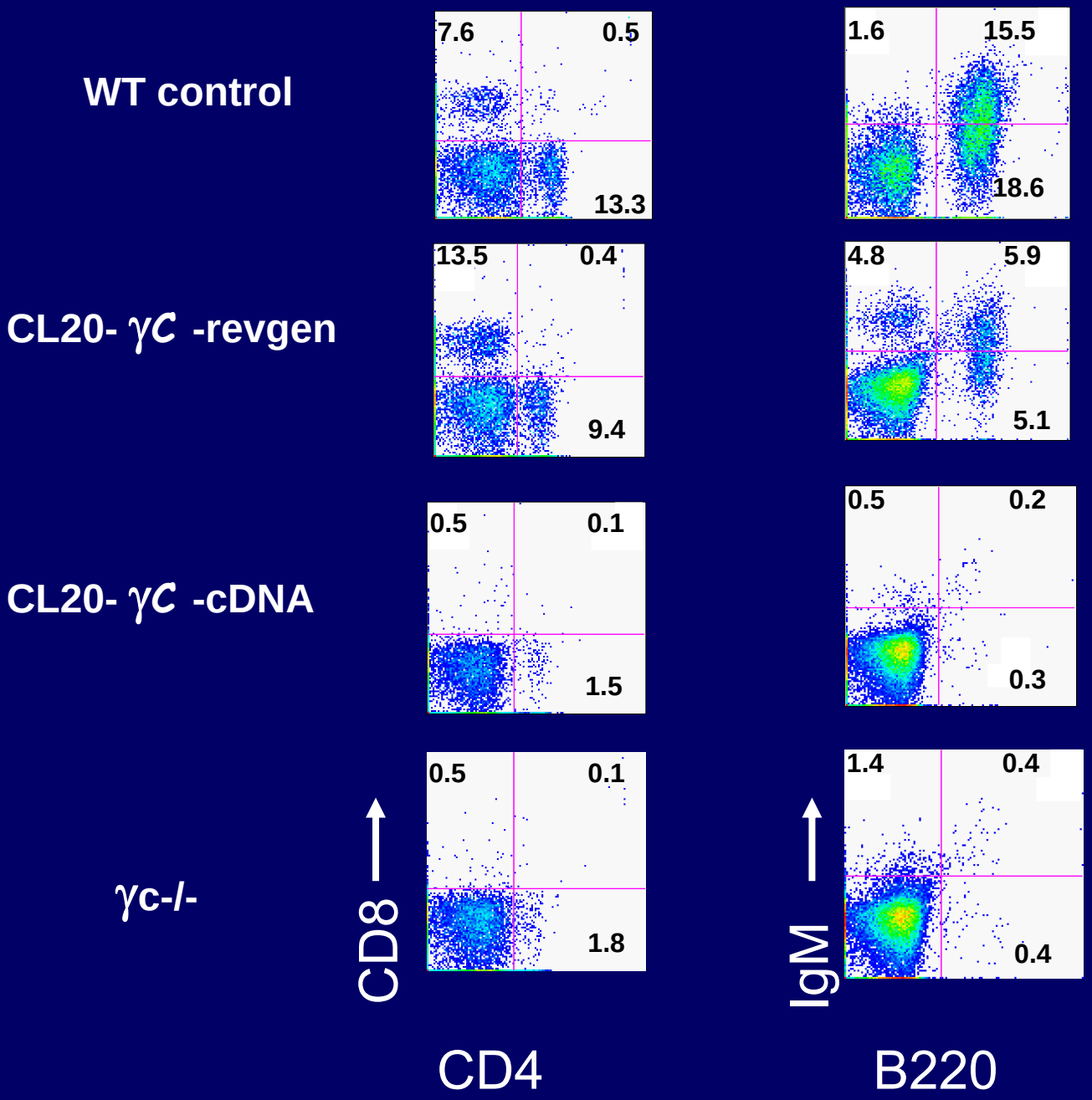




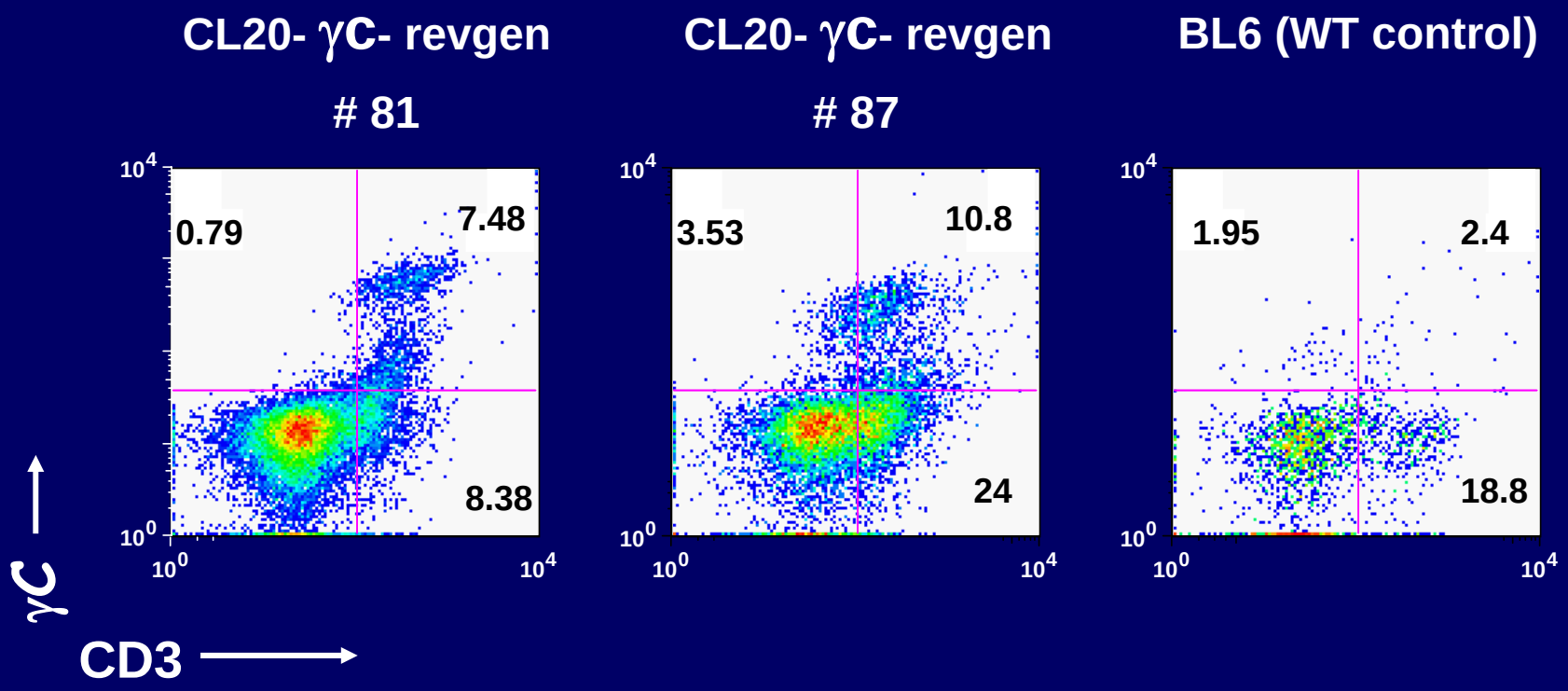
Experimental design



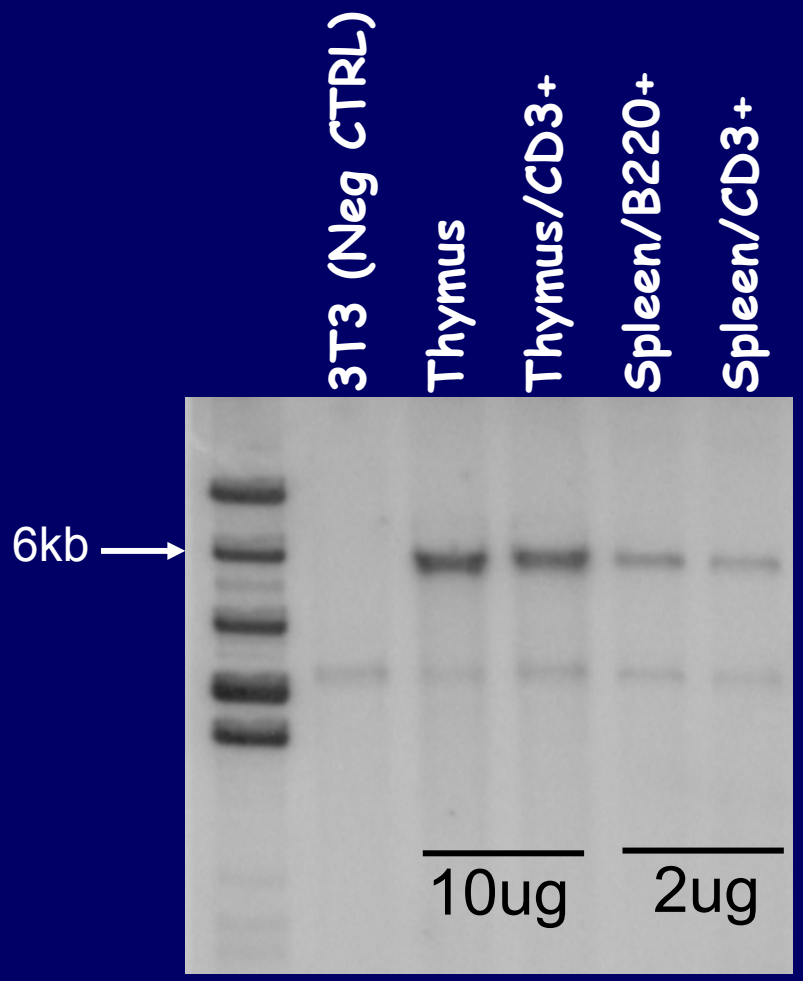
Peripheral blood T and B cell reconstitution at 8 weeks



Reconstituted T lymphocytes express human γC



CL20-γc-revgen vector is present in T and B lymphocytes in other organs of reconstituted mice

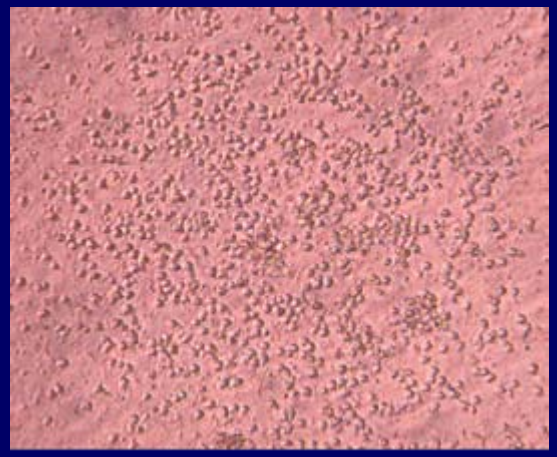


Full size, unreaggranged provirus band

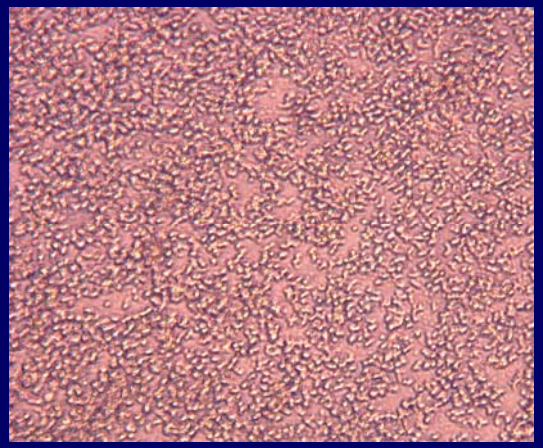
Southern blot
Bgl II digest
RRE as probe
Unrearranged vector size: 5.9kb

Reconstituted T lymphocytes respond to IL2 + ConA

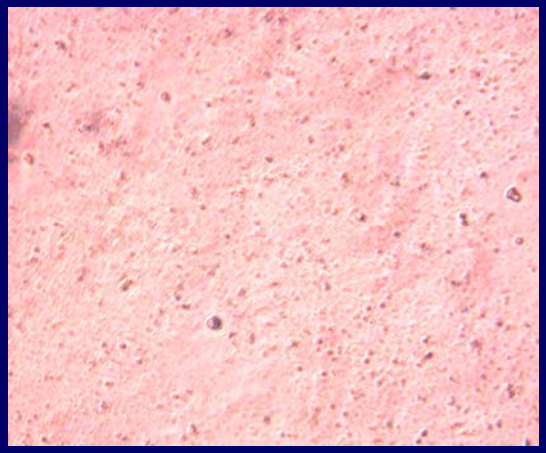
WT

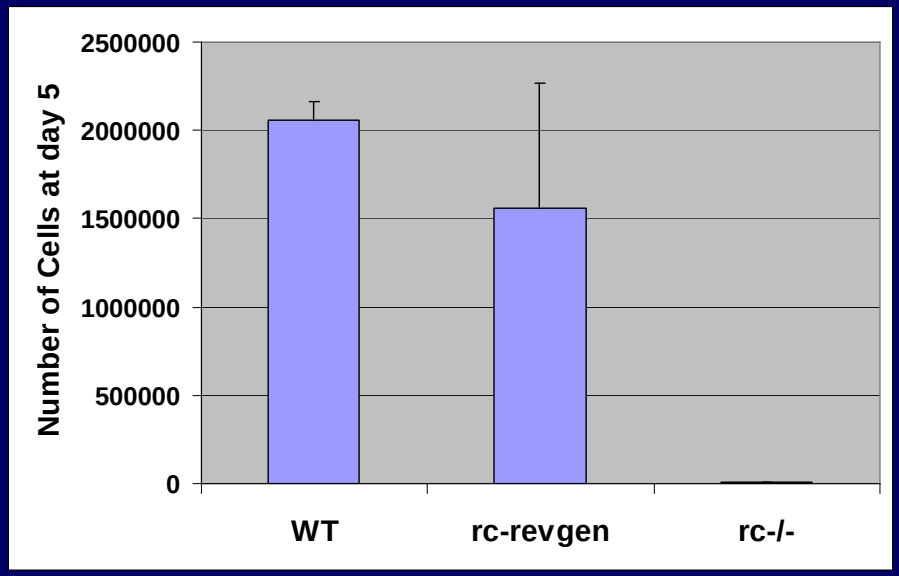


γc -/-
CL20- γc-revgen

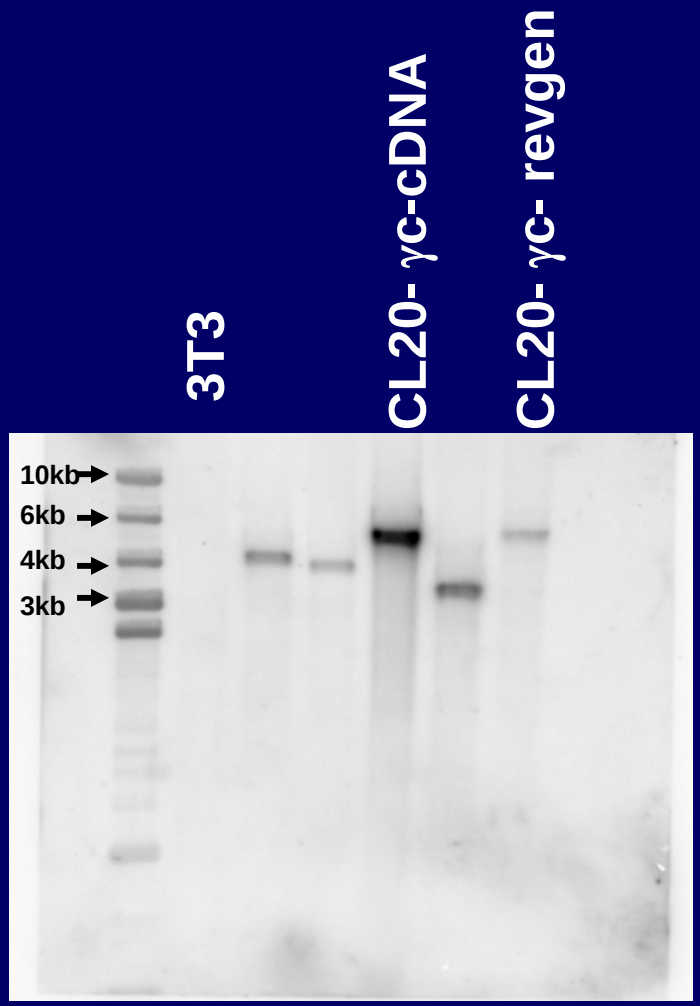


γc -/-





Low viral titer in CL20-γc-revgen vector



Virus titer:

CL20- γc-cDNA: 1x10^6/ml

CL20- γc-revgen: 1x10^5/ml

Southern of transduced 3T3 cells
RRE asprobe, Bgl II

Expected band size:

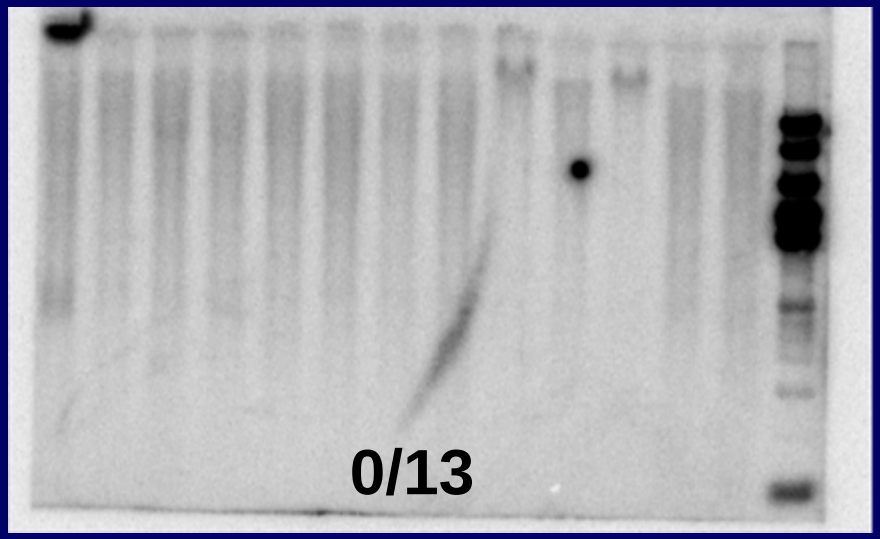
CL20- γc-cDNA: 5680bp

CL20- γc-revgen: 5912bp

Low transduction efficiency of CL20- γC -revgen vector in CFU-S due to low vector titer

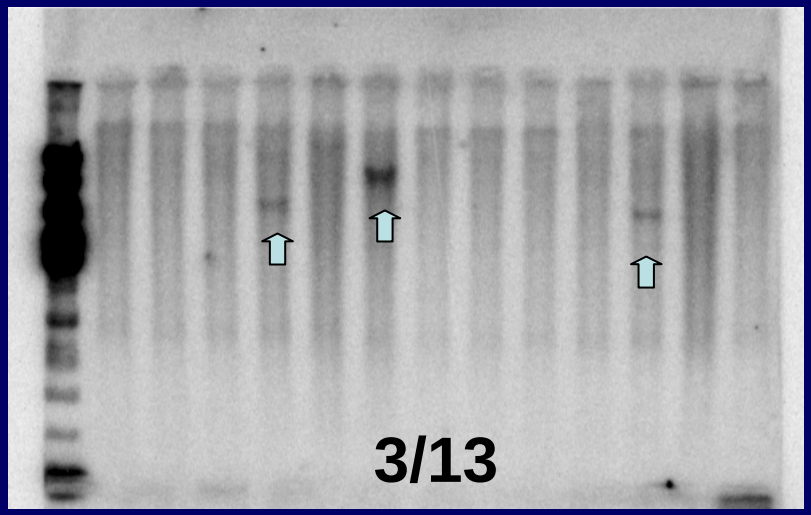
CL20- γC -revgen

MOI = 0.4



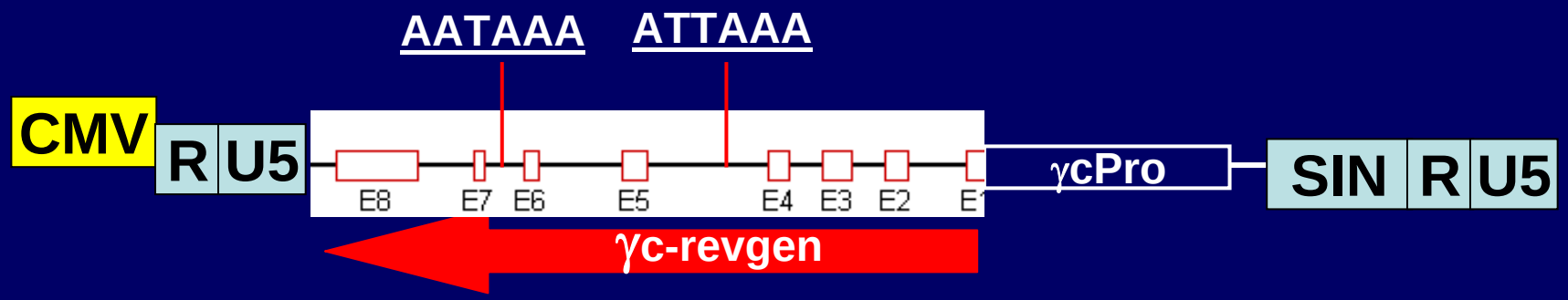
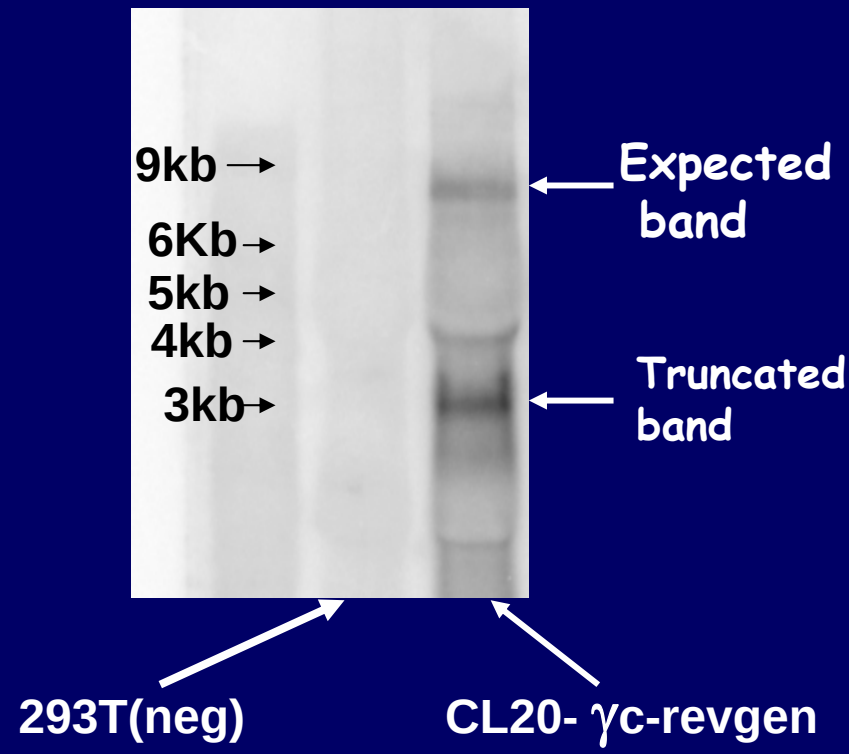
CL20- γC -cDNA

MOI = 4



Southern blot
RRE as probe
EcoR1, cut once in the vector.

Low virus titer is due to presence of cryptic polyadenylation signals on the antisense strand of γc



CL20- γc-revgen