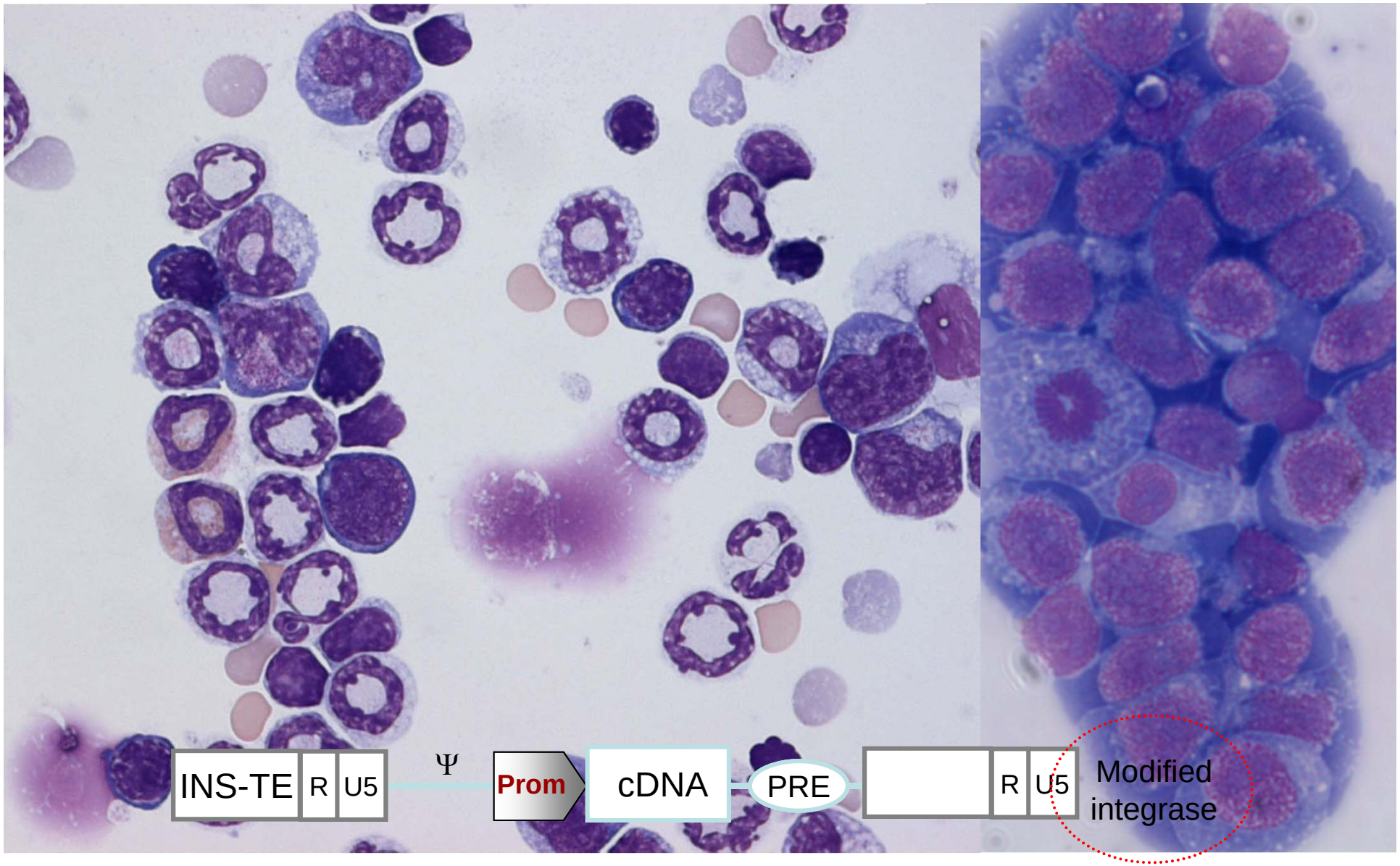

Preclinical assays to detect insertional transformation of hematopoietic cells

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Variable presentation of insertional mutagenesis

Mild molecular imbalance:

SCID-ADA (Aiuti et al., NEJM 2009)

Clonal dominance within 1 year, progression to myelodysplastic syndrome:

CGD (Stein et al., Nat Med 2010)

- milder presentation in murine model (Dinauer, Grez)
- not observed in primate model (Brenner et al., Mol Ther 2006)
- rapid selection of mutants in cultured cells
  (Du et al., 2005; Modlich et al., 2006)

Leukemia after >1 year:

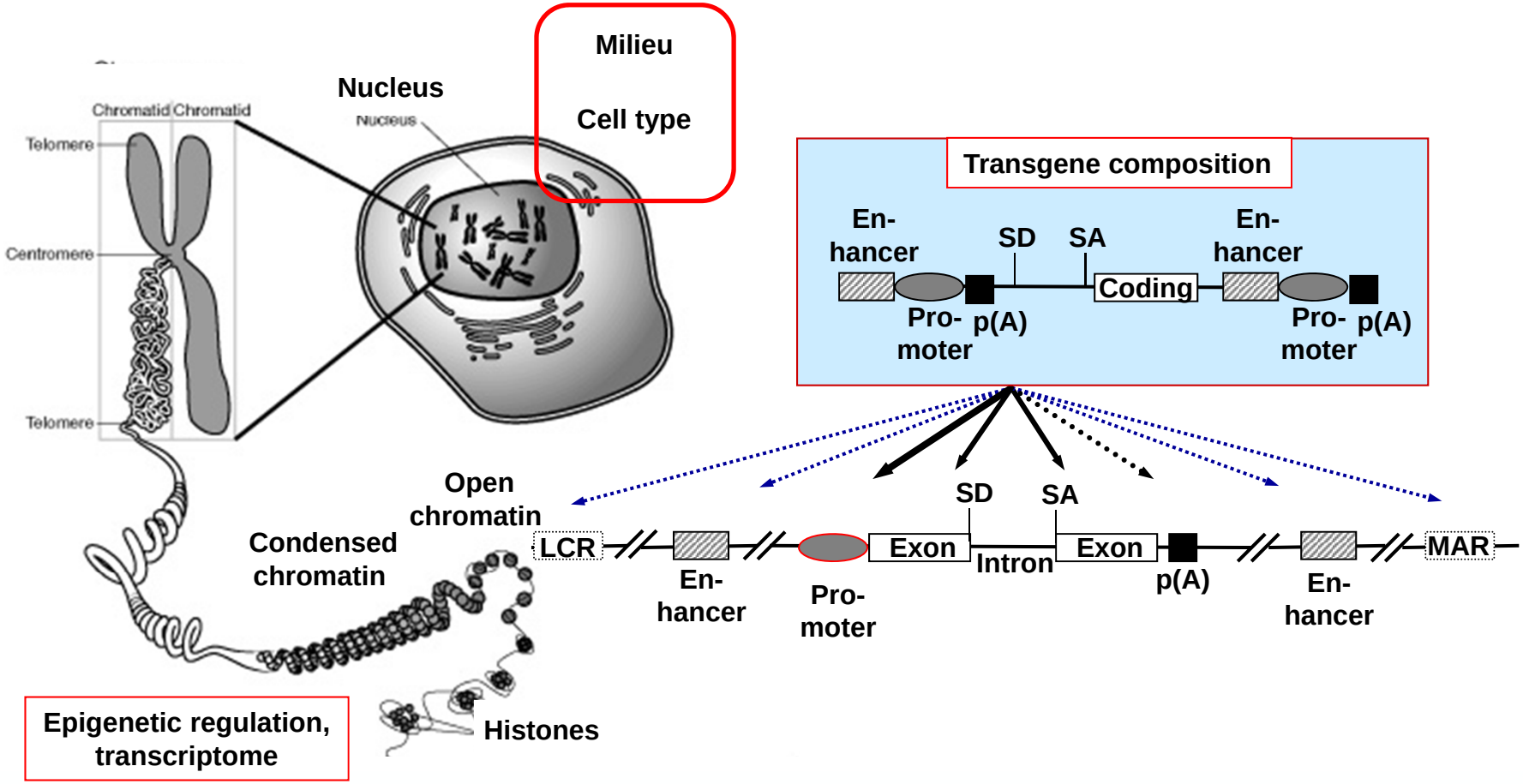
WAS (Boztug/Klein 2010)

SCID-X1 (Howe et al., JCI 2008; Hacein-Bey-Abina et al., NEJM 2010)

- not observed in mouse models of SCID-X1
  (Cavazza-Calvo, Sorrentino, Alexander, Williams)
- observed within 1-2 years post BMT in BL6 mice
  (Modlich et al., Leukemia 2008)

Disease-specific cofactors
Stress hematopoiesis
Probability to acquire additional mutations

Factors involved in insertional mutagenesis



Integration preferences

	MLV	HIV	ASLV	random
near promoters	23%	8%	8%	5%
CpG	14%	2%		2%
active TUs	40%	66%	43%	35% (CD34+)

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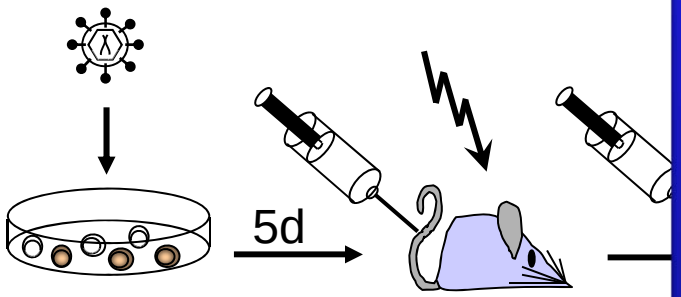
Vector-induced transformation: The C57BL6 serial BMT model

PEI
EMA
FDA
(NTP)

Vectors encoding cell surface markers, fluorescent proteins or therapeutic genes



Low dose versus High dose

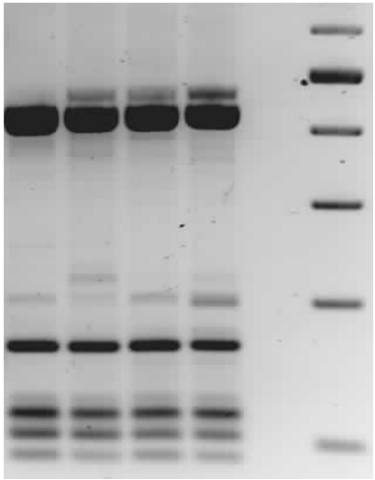
up to 11 mo

Evi1
Prdm16
Fli1
Bcl2-related
Ras-related
Lmo2
Ebf1
…

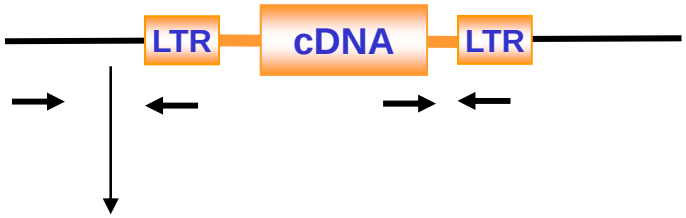
Leukemia
Clonal dominance
Integromics (IDDb)
"Phenotoxicity"

Li et al., Science 2002;
Kustikova et al.,Science 2005;
Blood 2007; Mol Ther 2009
Modlich et al., Blood 2005;
Leukemia 2008
Wicke et al., Mol Ther 2010
Maetzig et al., in revision

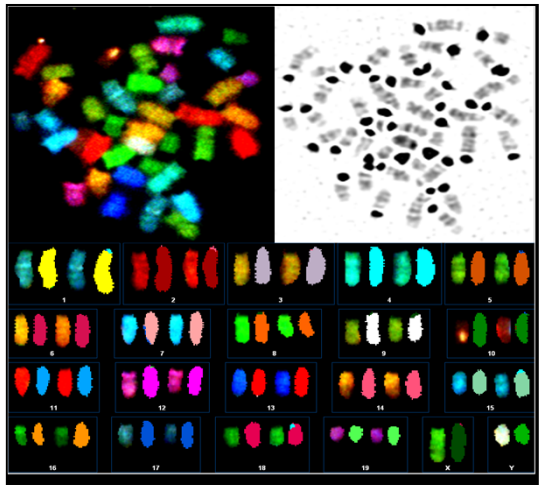
Rare but many forms
<2/10^7 cells
ALL
AML
HS
MPD
MDS
„Blastosis"



Southern blot
LM-PCR

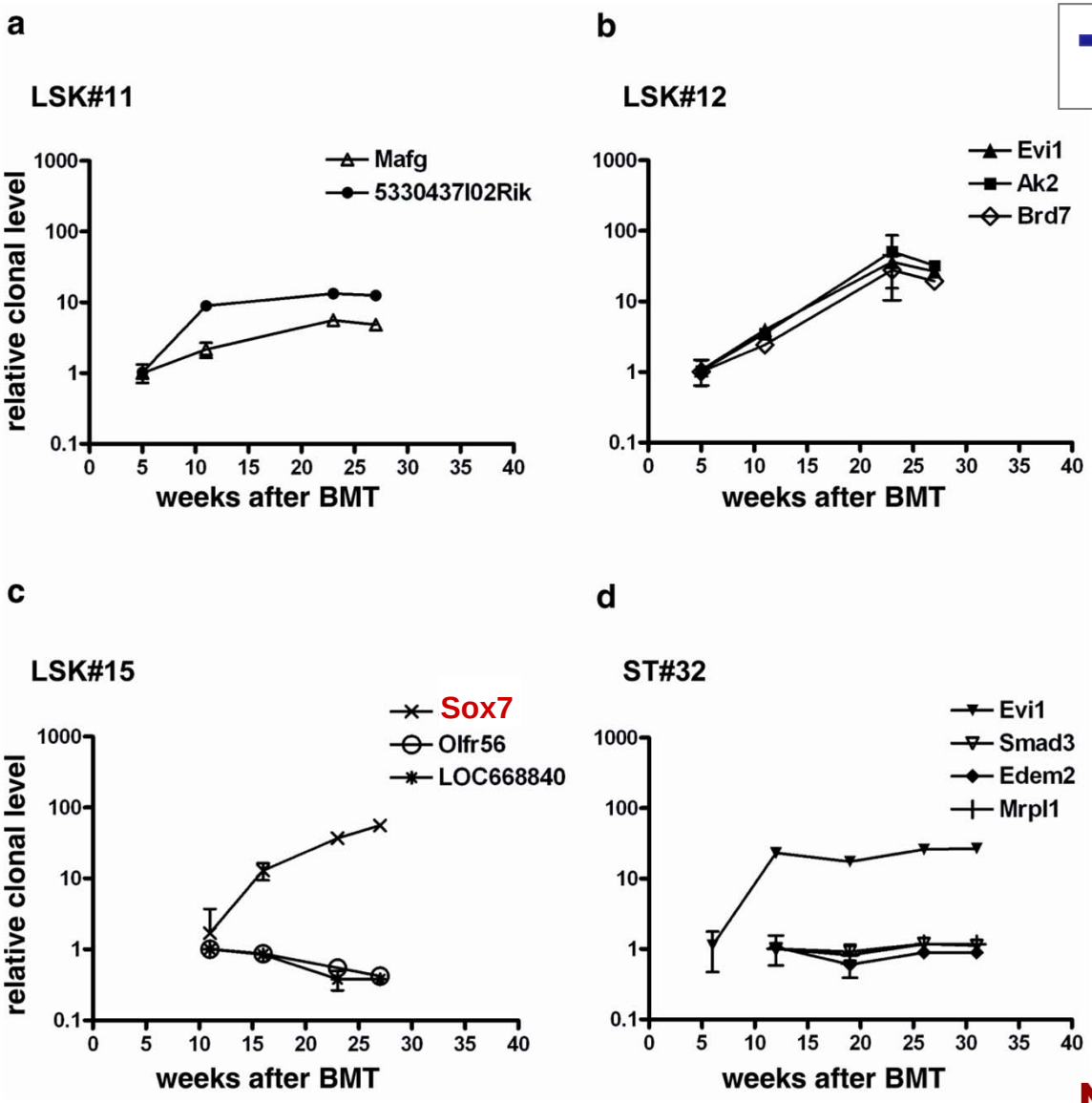


Flanking sequence, HTISA

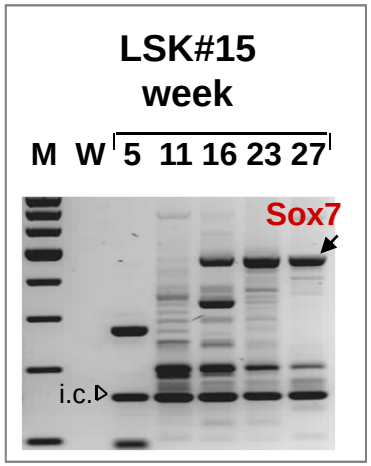


Shorter observation with molecular surrogates: Clonal dominance occurs within 6-24 weeks post BMT

Clone-specific qPCR



➡ **quantitative HT method?**



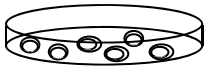
Kustikova et al. 2009

Olga Kustikova
Maike Stahlhut
Martijn Brugman

Hannover Medical School

Quantifying insertional mutagenesis in the IVIM assay

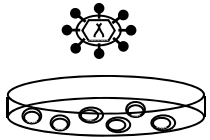
day 0



Primary
Lin.neg.
BM cells

2 days expansion

day 2- 5

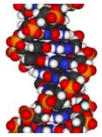


Transduction
100.000 cells
4x MOI 10

Incidence of mutants

4 days expansion

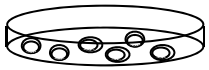
day 9



DNA Prep.
Mean copy no.
Facs analysis

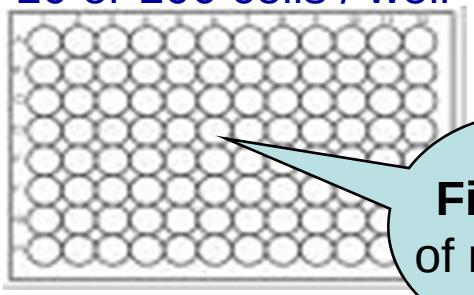
IMDM, 10%FCS,
SCF, Flt3L, IL11, IL3

10 days expansion



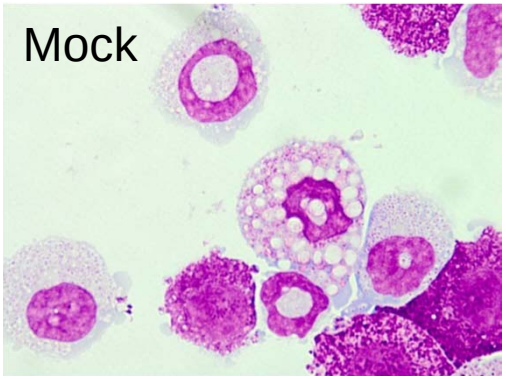
14 days replating

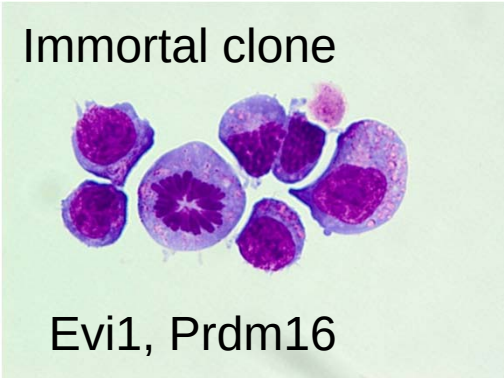
10 or 100 cells / well



Fitness of mutants

Limiting Dilution





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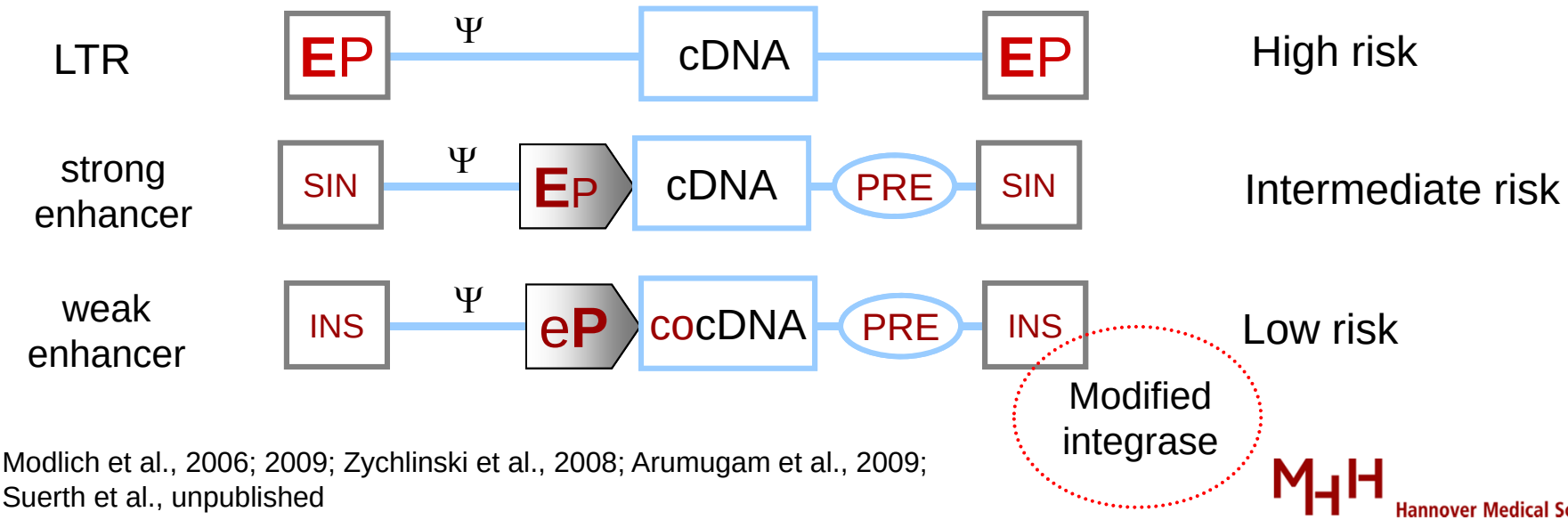
Vector modifications reduce the insertional risk

Weakening the **enhancer** (by mutation or using insulators) reduces the **fitness** of insertional mutants

Promoters lacking strong enhancers are relatively safe: incidence of mutants below detection limit
(improve RNA processing to enhance expression)

Risk of **integration profile** gamma:lenti:alpharetroviral ~ 9:3:1



Modlich et al., 2006; 2009; Zychlinski et al., 2008; Arumugam et al., 2009; Suerth et al., unpublished

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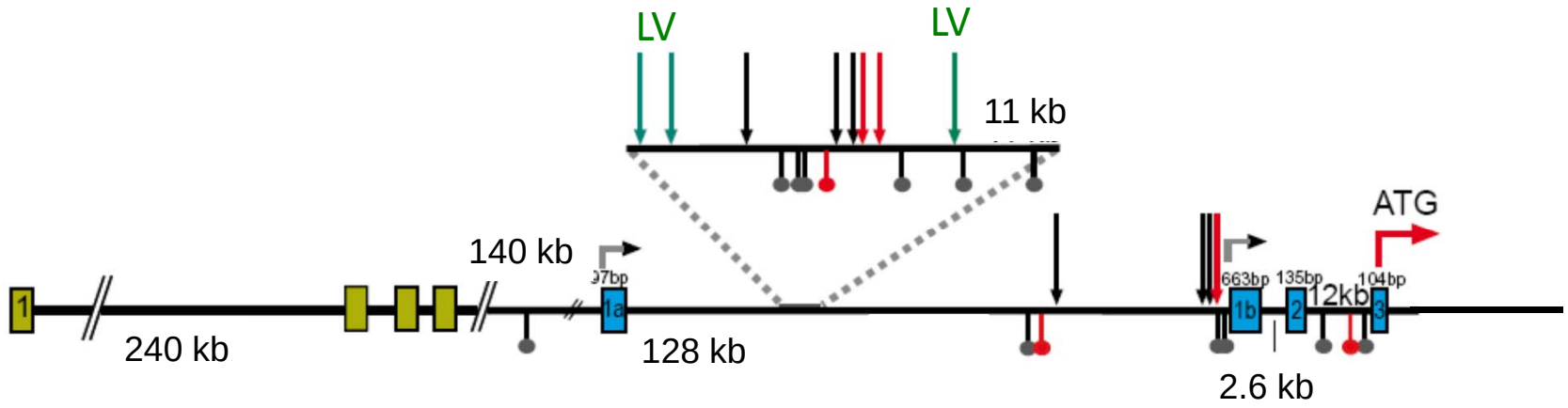
IVIM Assay: Evi1 = Lvi1 = Super-CIS (for gamma, lenti and alpha)

Arrows: insertions detected *in vitro*
Lollipops: insertions detected *in vivo*

Black, red: Gammaretroviral
Green: lentiviral

Evi1 locus:
Clinically relevant
Susceptible for TSS-proximal and intronic insertions
Highly preferred CIS in the IVIM assay
In vitro clusters mirror in vivo clusters

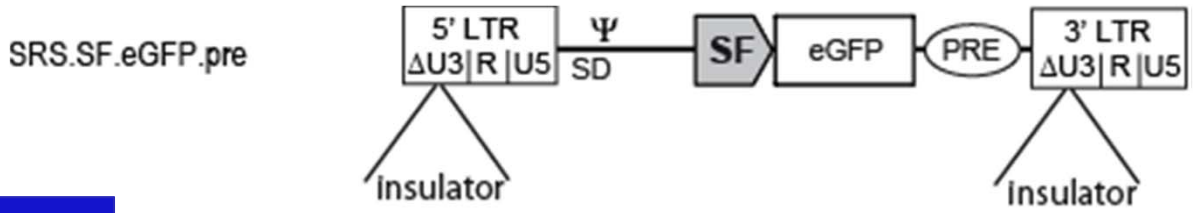


Mds1

Ute Modlich
Julia Suerth
Axel Schambach
Olga Kustikova
Martijn Brugman
Tobias Maetzig

Hannover Medical School

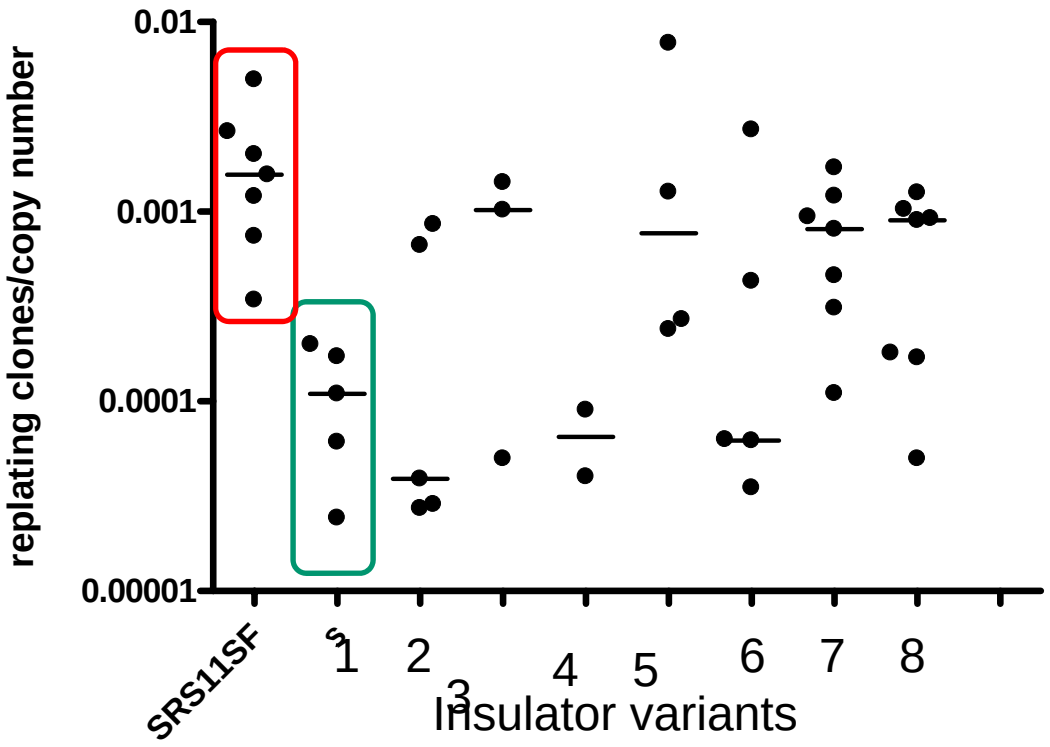
Insulators in the IVIM assay



Insulators impair the fitness of the mutants

Most insulators are not potent enough to eliminate the transforming potential of the SFFV enhancer-promoter

Considerable variability of results caused by genetic instabilty of some variants?



Daniela Zychlinski
Axel Schambach
Nicolas Mermod
Jacques Lenz
Ute Modlich

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IVIM assay summary

Max. sensitivity 1-2 mutants/10^5 cells with SF91EGFPpre (pos. control)

Quick and quantitative (incidence, fitness)

Strong impact of culture conditions and cell density (SOP)

Mutants have insertions in Evi1, Prdm16 or Ras-related genes

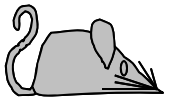
Established in multiple labs

Successfully performed in preparation of Phase 1 clinical trials

SCID-X1	gamma-SIN
WAS	lenti-SIN
CGD	gamma-SIN, lenti-SIN
SCA/Thal	lenti-SIN

Modlich et al., 2006; 2009; Zychlinski et al., 2008; Arumugam et al., 2009

Cell-intrinsic component:
Insertional transformation preferentially in HSC



CD45.2 BM

↓

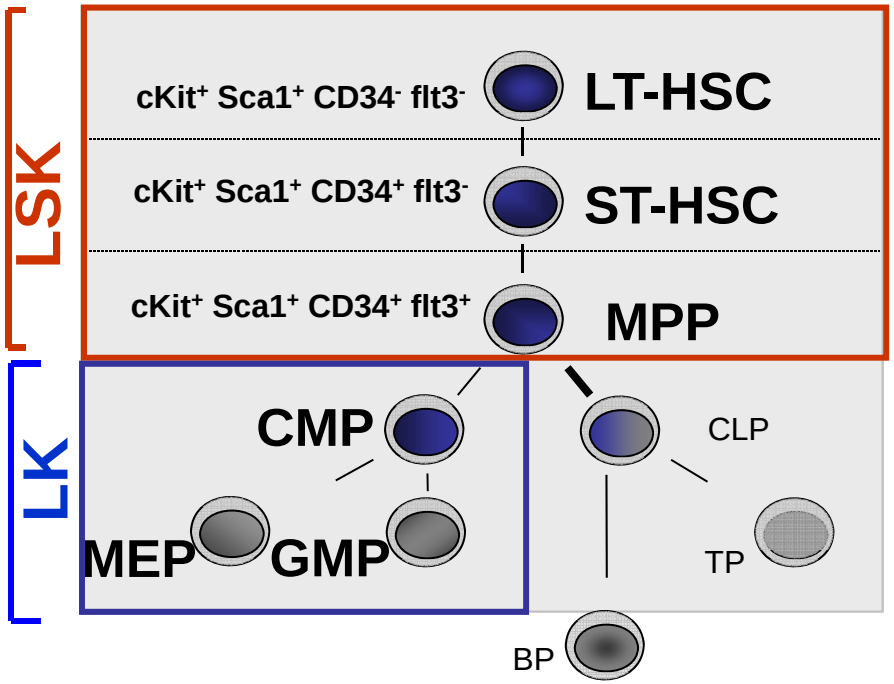
Lin-, Sca1+, cKit+
1% of Lin-

Lin-, Sca1-, cKit+
20% of Lin-

↓

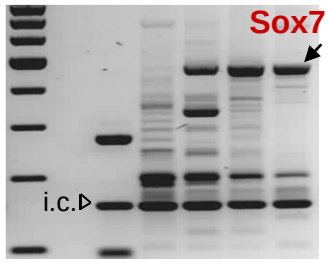
SCF, IL3, IL11, Flt3L

gammaretro

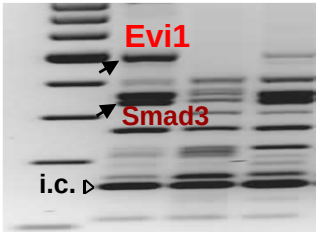


Frequency of Evi1 clones *in vivo:*
< 1 in 1,400,000 LK
1 in 75,000 LSK
1 in 4,000 ST-HSC

LSK#15 week



ST-HSC#32



Kustikova et al., Mol Ther 2009
Newrzela et al., Blood 2008

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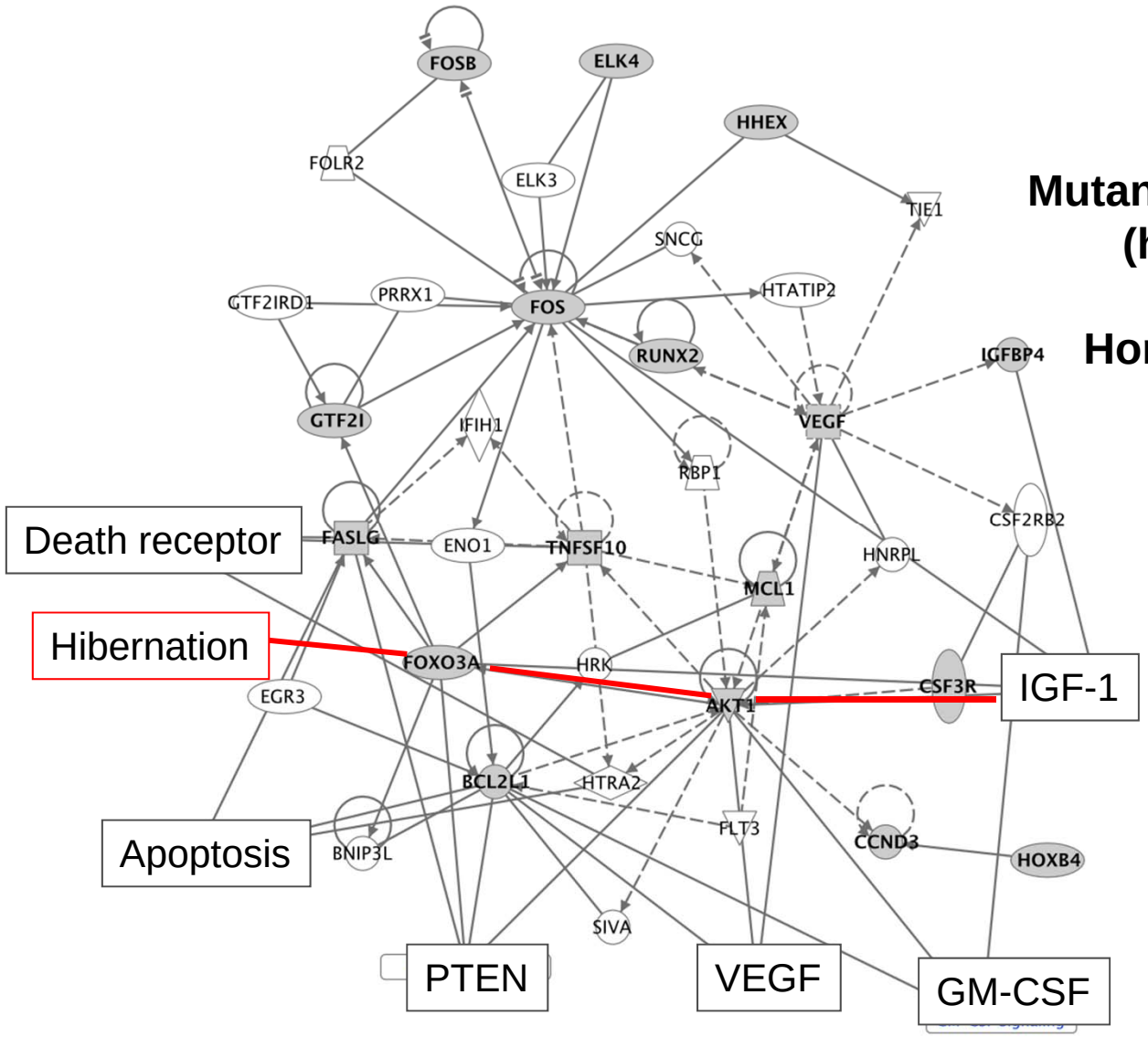
Ingenuity analysis of insertional dominance database



Hypothesis:
Mutants have a sleep disorder (hits in „hibernome“)

Homeostatic mechanism specific for HSC

SPP1230
Deutsche Forschungsgemeinschaft
DFG

MHH Hannover Medical School

Vulnerability of HSC…

What is the effect of improved cytokine conditions that support HSC expansion and polyclonal repopulation?

Deterministic model: conditions supporting HSC expansion also increase the risk of insertional transformation (more mutants, potential selection during prolonged in vitro expansion)

Stochastic model: Polyclonal hematopoiesis suppresses insertional mutants (homeostatic balance, fitter competitors)

Lentiviral transduction in HSC-expanding conditions

STIF (Zhang/Lodish)

SCF, TPO, IGF-1, FGF-1

Prestimulation → Post-transduction expansion

A) 9h → 14h
B) 9h → 168h
C) 48h → 14h
D) 48h → 168h

Lentiviral vector: LV SIN-SF-GFP

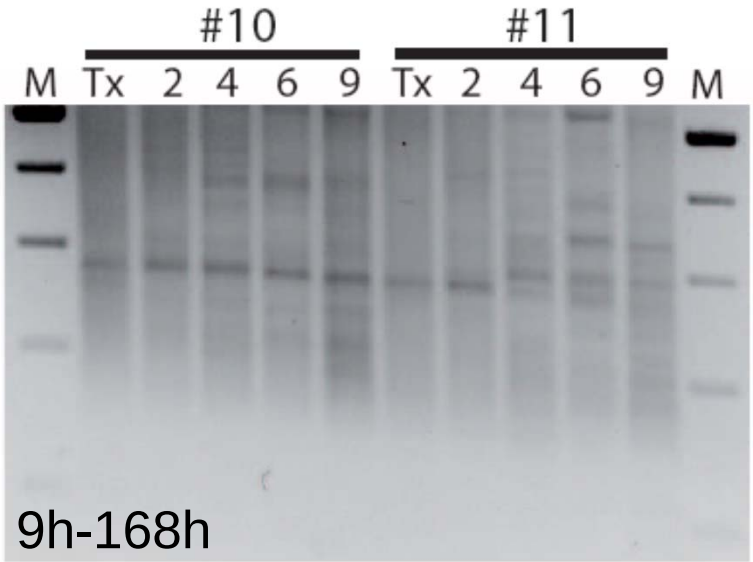
- strong SFFV enhancer-promoter
- insertional mutagen in IVIM assay

(Modlich et al, *Mol Ther* 2009)

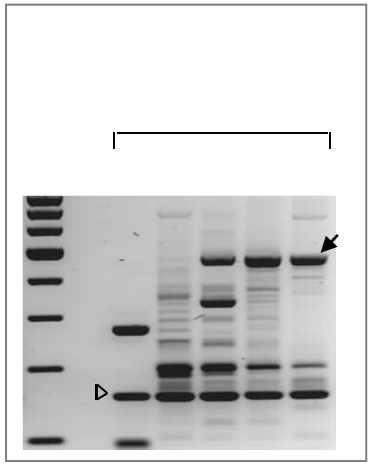
STIF supports polyclonal hematopoiesis

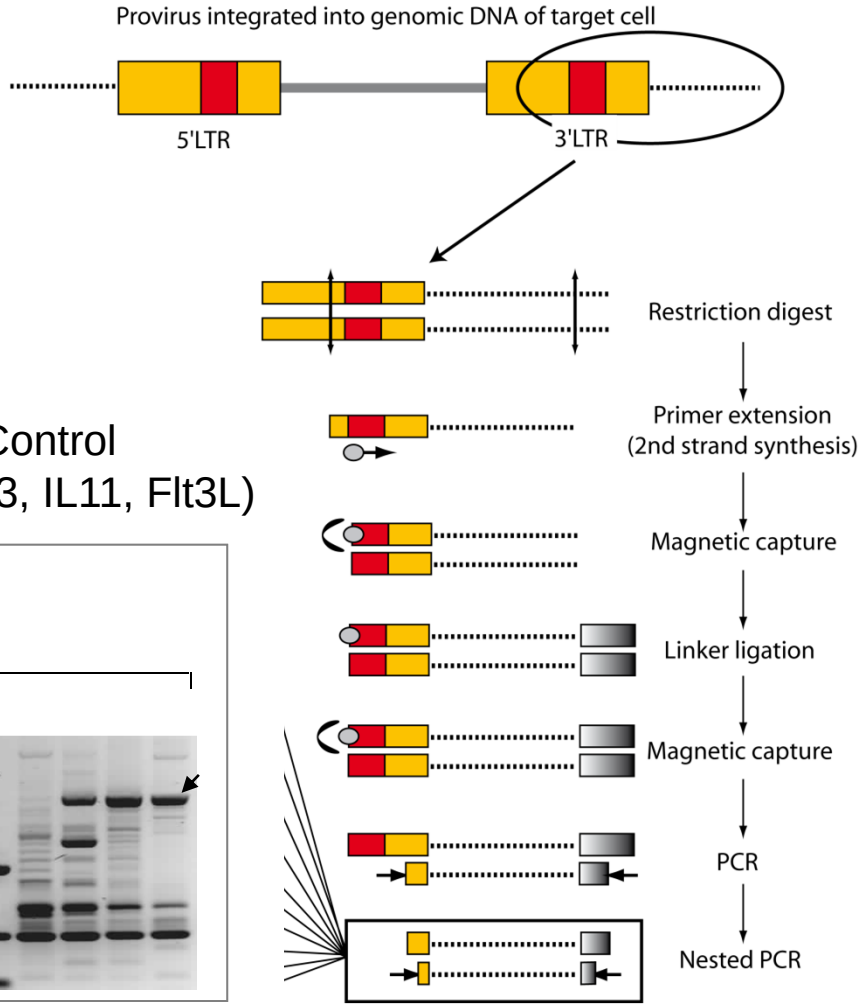
54 mice
Normal long-term hematopoiesis
Most >50% gene marking

STIF
(SCF, TPO, IGF2, FGF1)



Control
(SCF, IL3, IL11, Flt3L)





Kustikova et al, *Mol Ther* 2009

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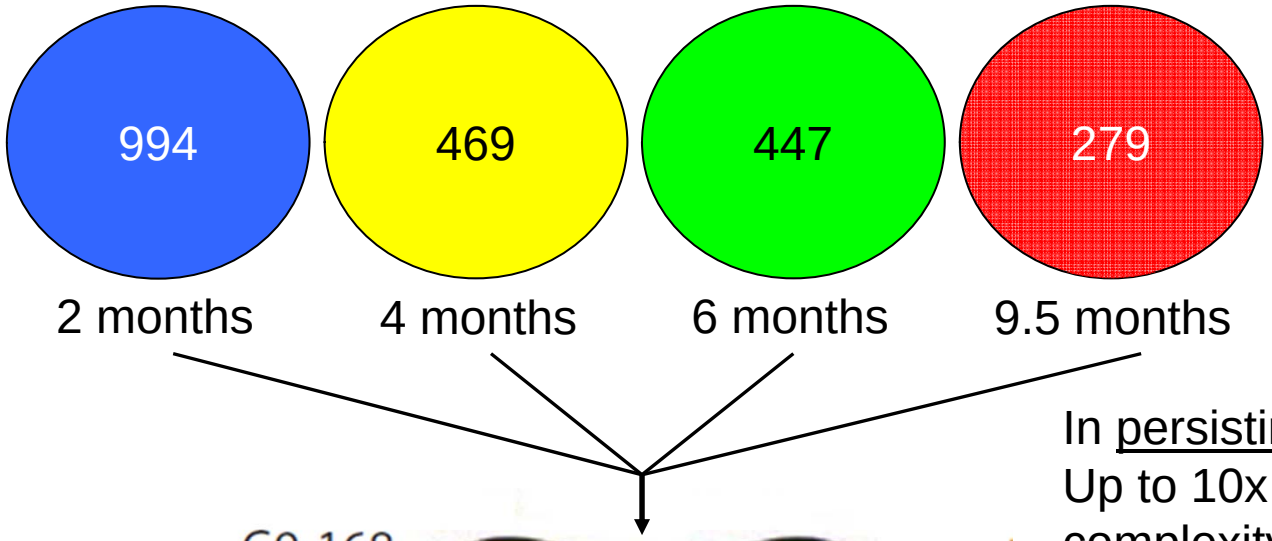
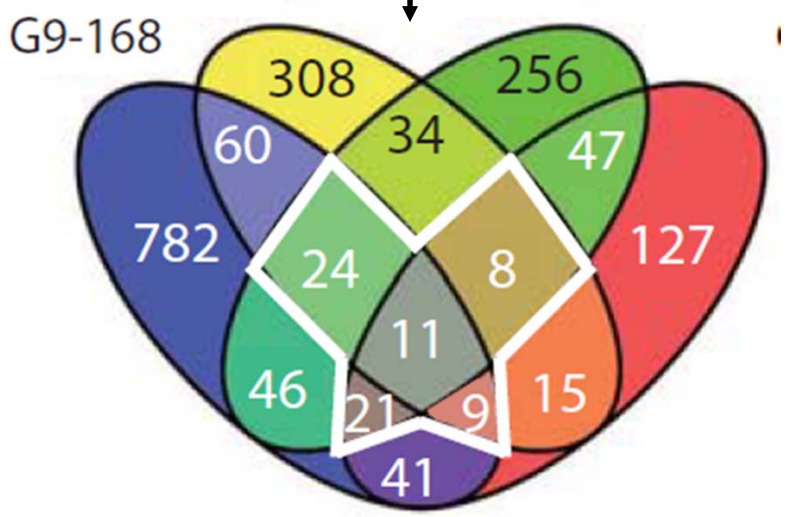
Polyclonally fluctuating hematopoiesis in C57Bl6 mice



numbers of unique integration sites: up to 195/sample



In persisting clones (inner field): Up to 10x increase of clonal complexity after ex vivo expansion. No enrichment of oncogene hits by post-transduction expansion. Data support stochastic model (suppression of mutants by fit competitors)

Consistent repopulation obtained with expanded, gene-modified HSC

Tobias Maetzig
Martijn Brugman
Stefan Bartels
Ute Modlich
Axel Schambach
Olga Kustikova

Maetzig et al., in revision

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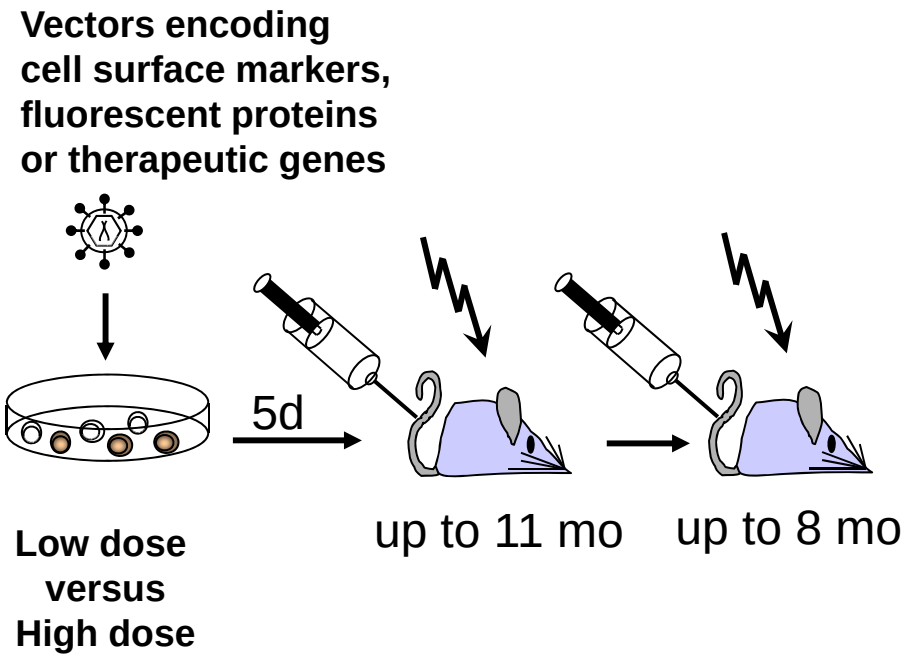
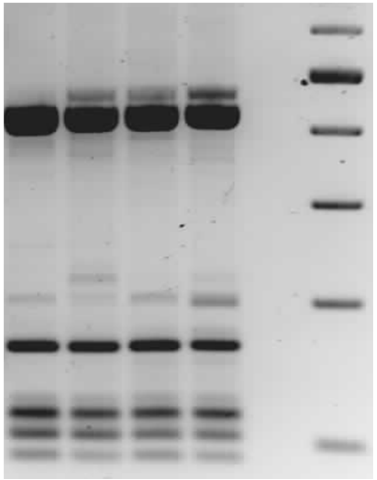
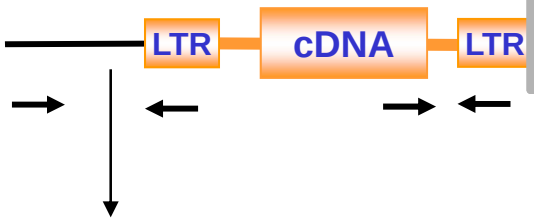
Insertional transformation: The C57BL6 serial BMT model





Southern blot
LM-PCR



Flanking sequence, HTISA

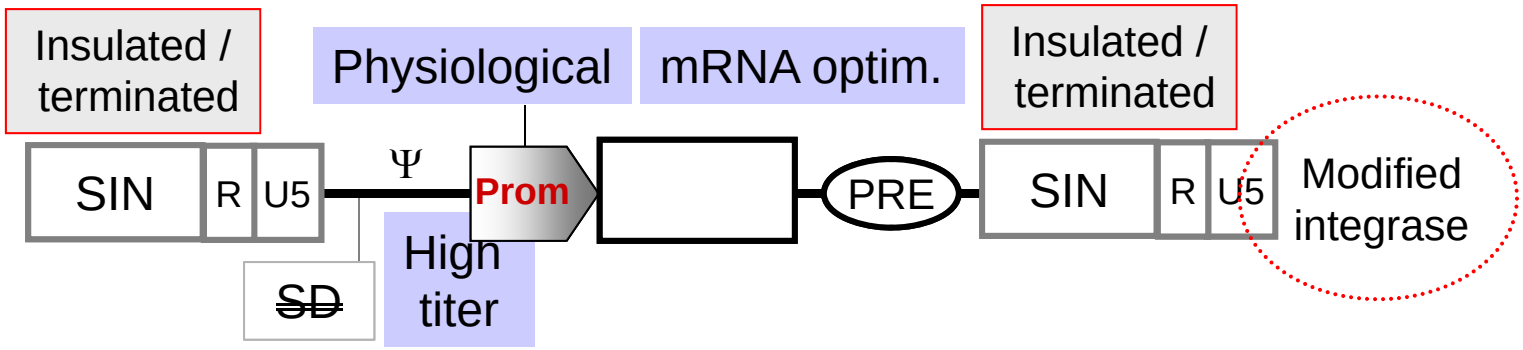
Strengths:
Broad range of phenotypes and associated mutations (incl. gene disruption)
Risk factors can be addressed (cell type, cytokines, „stress“)

Weaknesses:
Leukemia rare (<2 in 10^7 transduced cells)
Long-term observation (> 10 mo)
Expensive (up to 2000 €/mouse)

➡ **Increase sensitivity, reduce observation time and costs** using molecular surrogates

Vector design and transduction conditions for clinical trials

New vectors	Clinical trial
SCID-X1 (multicenter USA and EU)	2010
CGD (Grez et al., Frankfurt)	2011
WAS (Klein et al., Hannover)	2012
…	



It remains TBD which insertion profile is safest for vectors lacking strong enhancers

Improve transduction conditions
Ensure polyclonal repopulation

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Thank you and

Collaborators
Jürgen Bode
Toni Cathomen
Punam Malik
Manuel Grez
Adrian Thrasher
Boris Fehse
Hannes Klump
Christof von Kalle
David Williams
Hans Schöler
Timm Schröder
Brigitte Schlegelberger

Vectorology, iPSC: Melanie Galla, Christine Völkel, Johannes Kühle Tobias Mätzig, Niels Heinz, Daniela Zychlinski, Axel Schambach

Insertional mutagenesis, transformation, Mpl: Olga Kustikova, Martijn Brugman, Zhixiong Li, Adrian Schwarzer, Johann Meyer, Tamaryin Godinho, Ute Modlich

HSC expansion, ES-HC: Niels Heinz, Dietrich Lesinski, Birgitta Ehrnström, Bernhard Schiedlmeier

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