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Chromatin Disorganization as a Regulator of Irradiation-Induced L1Md Expression and Hematopoietic Stem Cell Function

Yanís Pelinski

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Chromatin disorganization as a
regulator of irradiation-induced
LINE-1 expression and
hematopoietic stem cell function

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Synthèse de la thèse en Français

L'exposition à l'irradiation, comme lors des radiothérapies, affecte l'intégrité et la fonction des cellules souches hématopoïétiques (CSH). L'IR est donc associé au développement de maladies myéloïdes liées à la thérapie, telles que les syndromes myélodysplasiques (SMD) et les leucémies myéloïdes aiguës secondaires. Par conséquent, l'étude des mécanismes moléculaires qui contribuent à la perte de fonction des CSH induite par le stress, pourrait aider à identifier les patients à risque et à trouver éventuellement de nouvelles stratégies pour prévenir ces maladies.

Notre équipe a récemment découvert un nouveau mécanisme responsable de la perte de fonction des CSH murines suite à l'IR qui implique les L1Md, les sous-familles jeunes et actives des éléments LINE-1. Nous avons montré que l'expression des L1Md est augmentée suite à l'IR et que cela entraîne une accumulation de dommages à l'ADN et de défauts des CSH. Nous avons également montré que la thrombopoïétine (TPO), une cytokine de niche des CSH, prévient la perte de fonction des CSH induite par l'IR, l'accumulation de dommages à l'ADN et la dérépression des L1Md.

L'analyse de microarray a montré que la TPO induit un enrichissement des gènes de signalisation de l'IFN-I dans les CSH, dont beaucoup sont des facteurs de restriction virale. Au début de ma thèse, j'ai participé à une étude qui a montré que la TPO contrôlait l'expression des L1Md par cette voie de signalisation.

Les L1Md sont reconnues comme des contributeurs majeurs des réseaux de régulation des gènes. Leur expression est étroitement régulée par des mécanismes épigénétiques, tels que la marque répressive de l'histone H3K9me3.

Les principaux objectifs de mon projet de thèse sont donc de :

1. Comprendre les mécanismes par lesquels l'IR affecte l'épigénétique des CSH, et en particulier l'hétérochromatine.
2. Déterminer si, et comment, l'expression des L1Md peut affecter l'expression génique des CSH.
2. Déterminer si la TPO, via sa signalisation de type IFN, peut réguler la répression des L1Md par des mécanismes épigénétiques.

Nous avons réalisé des expériences ChIP-qPCR sur des CSH un mois après IR, et nous avons découvert que la dérépression de la L1Md est liée à une perte d'enrichissement de H3K9me3 au niveau de leurs promoteurs. Ces résultats ont été confirmés par des expériences ChIPseq qui ont montré qu'une grande majorité des loci L1Md présentaient une perte de H3K9me3 suite à l'IR par rapport à la condition non irradiée. Ce n'était pas le cas pour les sous-familles de rétroéléments plus anciennes, telles que les Lx5, ou pour les rétrovirus endogènes (ERV).

Les données RNAseq ont montré que l'IR dérégule fortement le transcriptome des CSH. Nous montrons également que les gènes réprimés lors de l'IR sont significativement plus susceptibles de contenir un L1Md dans leurs introns que par hasard ($p < 0,05$). Ceci est spécifique aux L1Md et aux gènes qui sont réprimés par l'IR. Certains de ces gènes sont impliqués dans l'oncogenèse ou la fonction des CSH. L'IR induit une perte de la signature du HSC ainsi que de la signature NFκB. Il est intéressant de noter que 55% des gènes appartenant à la signature HSC et qui sont réprimés lors de l'IR contiennent un L1Md dans leurs introns. L'orthologue humain de 75% des gènes réprimés lors de l'IR et hébergeant un L1Md, contient également un L1 jeune chez l'homme, suggérant une fonction conservée dans la régulation de l'expression des gènes hématopoïétiques.

Nous avons analysé plus en détail plusieurs gènes cibles, et validé une diminution de l'expression en IR accompagnée d'une perte de H3K9me3 au niveau de leur L1Md introniques respectif. L'analyse d'enrichissement des motifs, nous a permis d'observer que la perte de H3K9me3 au niveau des L1Md introniques des gènes réprimés par l'IR peut être liée à la perte de la signalisation NF-κB. De plus, nous avons montré que cette fonction de la voie NF-κB est conservée chez l'homme, et que l'IR peut affecter la fixation de NFκB1 au niveau des L1Mds introniques dans les gènes des CSH, entraînant leur perte de fonction.

Ces résultats montrent pour la première fois un lien entre l'IR et l'épigénétique des CSH, et suggèrent un rôle pour les L1Md dans la régulation de l'expression des gènes hématopoïétiques.

Nous montrons également que la TPO peut prévenir la perte globale de H3K9me3 aux L1Mds induite par l'IR, ainsi que 74% de la dérégulation transcriptomique induite par l'IR. Cependant, l'injection de TPO n'est pas suffisante pour empêcher la perte des signatures HSC ou NF-κB observées, ni pour empêcher la perte de l'enrichissement en H3K9me3 aux L1MD introniques que nous avons analysés par CHIP-qPCR. Ces résultats suggèrent que la TPO pourrait seulement induire qu'une restauration modérée de H3K9me3, mais pourrait fonctionner par d'autres mécanismes épigénétiques pour contrôler l'expression de L1Md et prévenir la perte de la fonction de CSH.

Les prochaines étapes de ce projet visent à étudier les mécanismes par lesquels l'expression des L1Md introniques pourrait spécifiquement conduire à la perte d'expression des gènes hôtes, ainsi qu'à comprendre les mécanismes par lesquels la TPO fonctionne pour mieux caractériser son rôle de modulateur épigénétique.

Index

Abbreviations.....	4
Table of illustrations.....	7
Acknowledgements.....	9
Introduction.....	12
.....	
Part I : Hematopoietic Stem Cells.....	12
1. The stem in the hematopoietic tree.....	13
2. Characterization of HSCs.....	14
a. Self-renewal vs. Differentiation.....	14
b. Phenotype of murine HSCs.....	16
c. Differences between human and murine HSCs.....	18
d. Cellular and molecular factors regulating HSCs.....	20
3. Thrombopoietin: an important cytokine in HSCs.....	21
a. Thrombopoietin (TPO) & its receptor MPL.....	21
b. TPO and the hematopoietic stem cell.....	23
c. JAK-STAT signaling.....	25
4. Epigenetics of HSCs.....	28
a. DNA methylation in HSCs.....	29
b. Histone acetylation.....	31
c. Histone methylation in HSCs.....	32
5. Threats to HSC function and stability.....	35
a. HSC aging.....	35
b. Mechanisms of HSC aging.....	36
i. DNA damage.....	36
ii. Reactive oxygen species.....	37
iii. Changes in polarity.....	38
c. Irradiation and aging of the hematopoietic system.....	38
d. Aging and epigenetics.....	39
Part II: Retroelements.....	41
1. Retrotransposable elements throughout (modern) history.....	42

2. Classification & function of transposable elements.....	42
a. LTR retroelements.....	43
i. LTR retroelements.....	43
ii. Endogenous retroviruses.....	44
b. Non-LTR retroelements.....	44
i. Short interspersed nuclear elements (SINE).....	45
ii. Long interspersed nuclear elements (LINE).....	46
3. Evolution of L1 elements.....	48
4. Impact of RTE expression and retrotransposition in the genome.....	50
a. Recombination.....	50
b. Transduction of 3' flanking sequences.....	51
c. Chimeric retrogene formation.....	51
d. Cis-regulation of gene activity.....	52
e. Alternative splicing.....	53
f. Alternative transcription termination.....	53
5. Regulation of retroelements.....	53
a. DNA methylation.....	54
b. Histone modifications.....	55
c. RNA interference.....	58
6. Retroelements: friend or foe in cancer?.....	58
PhD Project.....	62
Materials and Methods.....	65
Results.....	71
1. Irradiation leads to L1Md derepression and is correlated to loss of H3K9me3 at L1Md promoters.....	72
2. Irradiation induces major alterations in H3K9me3 in HSCs.....	74
3. Irradiation induces transcriptomic changes in HSCs.....	76
4. Intronic L1Md are associated to gene repression following IR.....	78
5. Loss of H3K9me3 at intronic L1Md may be linked to loss of host gene expression upon IR.....	80
6. Loss of H3K9me3 at intronic L1Md of IR-repressed genes may be linked to loss of NF- κ B signaling.....	82
Discussion.....	85

1. Irradiation induces epigenetic changes in HSCs.....	86
2. Irradiation induces transcriptomic changes in HSCs that are associated to the presence of intronic L1Mds.....	89
3. Does derepression of L1Mds by IR induce changes in gene expression <i>via</i> RNA interference?.....	91
4. The NF- κ B signaling pathway is downregulated upon IR and may be involved in loss of H3K9me3 at intronic L1Mds and loss of the HSC signature.....	94
5. Loss of Mecom expression may also lead to loss of the HSC signature.....	98
6. The role of TPO as an epigenetic modulator.....	100
7. Could TPO prevent loss of global H3K9me3 through its IFN-like signaling and thus repress RTEs?.....	104
Concluding remarks.....	107
Bibliography.....	108
Annex.....	148

Abbreviations

A:

ALCAM : Activated leukocyte cell adhesion molecule

AML : Acute myeloid leukemia

B:

BAAT : Bile Acid-CoA:Amino Acid N-Acyltransferase

BER : Base excision repair

BMT : Bone marrow transplantation

C:

C/EBP α : CCAAT Enhancer Binding Protein Alpha

CALR : Mutations in the calreticulin

CAMT : Congenital amegakaryocytic thrombocytopenia

CHIP : Clonal hematopoiesis of indeterminate potential

CYTOF : Cytometry by time of flight

5-hmC : Cytosine hydroxymethylation

5-mC: Cytosine methylation

D:

DDR : DNA damage response

DNMT : DNA methyltransferase

DNMTI : DNA methyltransferase inhibitors

DSB : DQouble-stranded breaks

E:

EBF1 : EBF Transcription Factor 1

EHMT1 : Euchromatic Histone Lysine Methyltransferase 1

EPO : Erythropoietin

ERK : Extra-cellular signal-regulated kinase

ERV : Endogenous retroviruses

ESC : Embryonic Stem Cells

ET : Essential thrombocythemia

Ezh2 : Enhancer of zeste

F:

FACS : Fluorescence-activated cell sorting

FOXO : Forkhead Box O

G:

GATA1-3 : GATA binding protein

GHR : growth hormone receptor

GTP : Guanosine triphosphate

H:

H3K4me3 : Trimethylation on lysine 4 of histone 3

H3K9me3 : Trimethylation on lysine 9 of histone 3

HDAC : Histone deacetylases

HERV : human endogenous retroviruses

HIF-1 α : Hypoxia-inducible factor 1 α

HoxB4 : Homeobox 4

HP1 : Heterochromatin protein 1

HSC : Hematopoietic stem cells

HSPC : Hematopoietic stem and progenitor cells

HUSH : Human silencing hub complex

I:

IAP : Intracisternal A particle

IFN : Interferon

IR : Ionizing radiation

ISC : intestinal stem cells

ISG : IFN-stimulated genes

J:

JAK : Janus kinase

K:

KAP1 : KRAB-associated protein 1

KO : knock-out
KRAB-ZFP : Krüppel-associated box zinc-finger proteins

L:
LINE-1 : Long interspersed nuclear element
LSH : Lymphoid-specific helicase
LTR : Long terminal repeat

M:
M-CSF : Macrophage colony stimulating factor
MAPK : Mitogen-activated protein kinase
MARL : Mammalian apparent LTR retrotransposons
MBD1 : Methyl-CpG Binding Domain Protein 1
MDS : m-Myelodysplastic syndrome
MeCP2 : Methyl-CpG Binding Protein 2
Meis1 : Meis homeobox 1
MLV : Murine leukemia virus
MNC : Mononuclear cells
MPLV : myeloproliferative leukemia virus
MPNs : Myeloproliferative neoplasms
MPP : Multi-potent progenitors
mRNA : Messenger RNA

N:
NFkB1 : Nuclear Factor Kappa B Subunit 1
NFκB : nuclear factor κB
NHEJ : non-homologous end-joining
NURF : nucleosome remodeling factor

O:
ORF : open reading frames

P:
Pax5 : Paired Box 5

PcG : polycomb group
PI3K : phosphatidylinositol-3
PLZF : promyelocytic leukemia zinc finger
PMF : primary myelofibrosis
PRC1 : polycomb repressive complex 1
PV : polycythemia vera

R:
RT-qPCR : Quantitative reverse transcription PCR
RISC : RNA induced silencing Complex
RNAi : RNA interference
ROS : Reactive oxygen species
RPN : Ribonucleoprotein particle
RTE : Retrotransposable elements

S:
SCF : Stem cell factor
SCLC : Small-cell lung cancer
SH2 : Src homology
SINE : short interspersed nuclear elements
SIRT7 : Sirtuin 7
SNS : Sympathetic nervous system
STAT : Signal transducer and activator of transcription
SUV39H1 : Suppressor Of Variegation 3-9 Homolog 1

T:
TBI : Total body irradiation
TE : Transposable elements
TET : Ten-eleven translocation
TFBS : transcription factor binding sites
TGFβ : Transforming Growth Factor beta
TPO : thrombopoietin
TPRT : target-primed reverse transcription

TRAF6 : TNF receptor associated factor

6

TRIM : Tripartite motif

TYK2 : Tyrosine Kinase 2

U:

UTR : untranslated region

W:

WRN : Werner protein

WS : Werner's syndrome

WT : wild-type

Table of illustrations

Introduction:

- Figure 1: Evolution of hierarchical models of hematopoiesis 14
- Figure 2: Phenotype of murine HSCs 17
- Figure 3: Hematopoietic hierarchy in humans and mouse 19
- Figure 4: Cellular and molecular constituents of the HSC niche 20
- Figure 5: TPO signaling pathway 23
- Figure 6: Signal transduction via JAK 26
- Figure 7: Biological significance of non-phosphorylated U-ISGF3-induced gene expression 27
- Figure 8: Epigenetic modifications and their effect on DNA accessibility 29
- Figure 9: DNA methylation and demethylation 31
- Figure 10: Histone modifications 33
- Figure 11: Regulation of HSC aging and rejuvenation 35
- Figure 12: Structure of an LTR retroelement 43
- Figure 13: Structure of Alu and SVA non-LTR retroelements 45
- Figure 14: Structure of LINE-1 elements 46
- Figure 15: Retrotransposition cycle of LINE-1 47
- Figure 16: Phylogenetic tree of mouse L1 families 48
- Figure 17: Different mechanisms of RTE influence on gene transcription and expression. 50
- Figure 18: Different mechanisms of RTE silencing 54
- Figure 19: Model of H3K27me3 acquisition following demethylation by Walter et al. 57
- Figure 20: Viral mimicry as described by Chiappinelli et al. 60

Results:

- Figure 1: Irradiation induces a global increase in L1Md expression which correlates to loss of H3K9me3 at L1Md promoter 72
- Figure 2: Irradiation induces a decrease of H3K9me3 mainly at L1Mds 74
- Figure 3: IR induces a strong deregulation of gene expression 76
- Figure 4: Intronic L1Md are associated with IR induced gene repression 78
- Figure 5: Gene repression upon IR is associated with loss of H3K9me3 at intronic L1Md 80
- Figure 6: Motif enrichment analysis show specific enrichment of NFkB motifs 82

Discussion:

- Figure 1: Schematic representation of the 'full-fragment' ABCA minigene constructed by Kaer et al.

- Figure 2: IR-induced L1Md derepression leads to transcription initiation at intronic L1Mds
- Figure 3. Schematic representation of the human Mecom locus.
- Figure 4: The role of TPO as an epigenetic modulator
- Figure 5: TPO can prevent loss of the NFkB signature only in genes downregulated upon IR that contain an intronic L1Md.

Concluding remarks

107

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INTRODUCTION

Part I

Hematopoietic Stem Cells

Hematopoietic Stem Cells

1. The Stem in the Hematopoietic Tree

In the late 1860s, Ernst Haeckel, a Darwinist scientist, coined the term “stem cell” to refer to a primordial unicellular organism from which all multicellular life “stemmed” from (Laurenti and Gottgens 2018). Later on, histopathologists applied this stem cell concept into normal and leukemic hematopoiesis, and the idea of a common progenitor for red and white blood cells, and for myeloid and lymphoid leukemic cells, emerged (Laurenti and Gottgens 2018). For the last 150 years, this stem cell concept has framed cellular development into a tree-like model, in which multipotent stem cells give rise to their progeny through an ordered series of branching steps. In line with this tradition, and like their name suggests, hematopoietic stem cells (HSCs), are effectively the stem cells of the hematopoietic system.

Throughout the 1950s, the first *in vivo* assay to characterize HSC function based on the rescue of lethal irradiation by bone marrow transplantation (BMT) was performed (Eldredge 1951). In 1961, the first estimations of HSC numbers were obtained by counting hematopoietic colonies in the spleens of transplanted mice (Till and McCulloch 1961). By the end of the 1980s, the emergence of fluorescence-activated cell sorting (FACS) had allowed for the purification of transplantable HSCs and introduced the concept of positive and negative selection (Spangrude et al. 1988; Laurenti and Gottgens 2018). Throughout history, HSC have been defined by two main characteristics: self-renewal and pluripotency, both of which can be verified using transplantation experiments. On the contrary, progenitors are characterized by the lack of prolonged self-renewal and a restricted lineage differentiation capacity, and are thus usually depleted 2/3 weeks post transplantation (Doulatov et al. 2010). Given the short lifespan of mature blood cells, HSCs are necessary to continuously give rise to progenitors and precursors. For this reason, the study of the hematopoietic system has been governed by the dogma that HSCs reside at the top of the hematopoietic cell hierarchy; and has been used as an archetype for the understanding of tissue stem cells origin and biology, and their involvement in aging, disease and oncogenesis (Orkin and Zon 2008).

Hematopoiesis is the process by which the cellular constituents of blood are continuously replenished throughout the lifetime of an organism. This process starts in the bone marrow, where it is estimated that an adult human produces over 1×10^{11} blood cells a day (Kaushansky 2006). Since the first enrichment attempts in HSCs, several surface markers have been discovered which have contributed to the characterization of hematopoiesis. Around the year 2000, characterization of progenitor populations downstream of HSCs provided bases for the first model of hematopoietic differentiation, which differentiated the lymphoid lineage from the myeloid, erythroid and megakaryocytic lineages at its first branching. As more surface

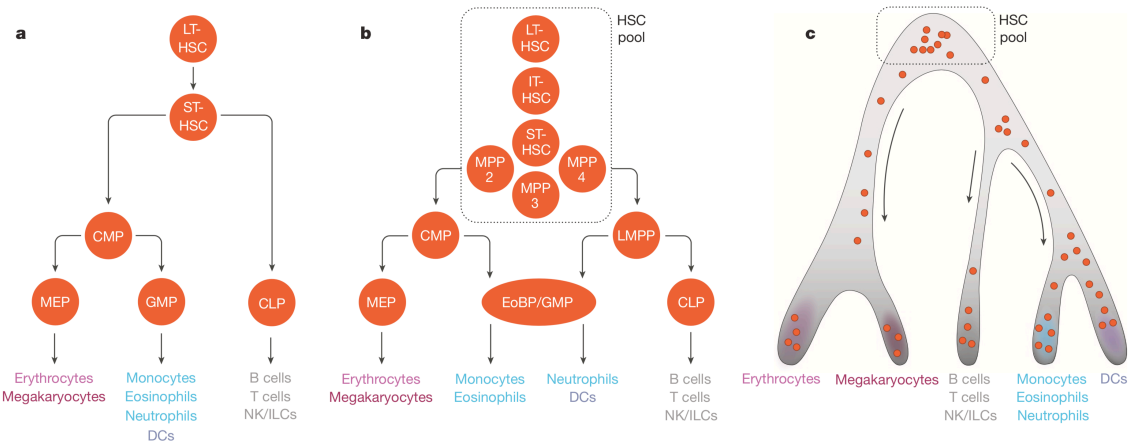


Figure 1: Evolution of hierarchical models of hematopoiesis. A) HSCs were originally represented as a homogeneous population, downstream of which the first lineage bifurcation separates the myeloid and lymphoid branches. B) the HSC pool is accepted to be more heterogeneous both in terms of self-renewal (vertical axis) and differentiation properties (horizontal axis), the myeloid and lymphoid branches remain associated further down in the hierarchy via the lymphoid-primed multipotential progenitor (LMPP). C) From 2016 onwards, single-cell transcriptomic snapshots indicate a continuum of differentiation. Each red dot represents a single cell and its localization along a differentiation trajectory. (Laurenti and Gottgens 2018)

markers were introduced, the tree evolved and introduced the subdivision of the multipotent progenitor compartments that included lymphoid and myeloid fates together, and early branching of megakaryocytes (Doulatov et al. 2010; Sanjuan-Pla et al. 2013; Pietras et al. 2015). In recent years the rise of single-cell transcriptomics has revealed major functional and molecular heterogeneity at the heart of the stem cell pool (Laurenti and Gottgens 2018). These new discoveries render the classic hematopoietic tree “*démodé*” and tend towards a continuum of differentiation of heterogeneous populations organized hierarchically, and which remain flexible to meet the ever-changing needs of blood demand (Laurenti and Gottgens 2018) (Figure 1). This new concept accommodates the fact that in an adult human the steady-state number of different HSCs is approximately 11000, which may divide only once every 3 months or once every 3 years, according to their phenotype (Catlin et al. 2011). Additionally, there are many more terminally differentiated erythroid cells than myeloid cells, and even less lymphoid cells, all of which have different turnover rates so the flux into each compartment must be highly regulated (Laurenti and Gottgens 2018).

2. Characterization of HSCs

a. Self-renewal vs Differentiation

Mature blood cells are abundantly produced in adults, however the HSCs from which they are derived are a very rare cellular population. These two characteristics present an interesting conundrum: how to maintain an adequate stem cell pool whilst meeting the organisms demand for blood products? (Seita and Weissman 2010)

At the steady-state, HSCs are mostly quiescent, meaning that they cycle very infrequently and are primarily in the G₀ phase of the cell cycle (Rossi, Seita, et al. 2007). They also maintain an autophagy-dependent, glycolytic state characterized by low mitochondrial activity and tightly regulated levels of protein synthesis, which are lower than those found in other types of hematopoietic cells (Simsek 2010; Takubo 2013; Warr 2013; Vannini 2016; Cabezas-Wallscheid 2017; Ho 2017). This is necessary to protect them against genotoxic insult, and is complemented by specific stress response mechanisms that preserve HSC integrity in case of DNA or protein damage, and/or metabolic stress (Laurenti and Gottgens 2018). However, it is important to bear in mind that these “HSC characteristics” are not absolute. HSCs can occasionally divide and get activated due to stress and enter, albeit transiently, the proliferative state (Laurenti and Gottgens 2018). In contrast, HSC progenitors are highly proliferative and metabolically active cells that depend on oxidative metabolism and mitochondrial function (Laurenti and Gottgens 2018). For these reasons, the bulk of hematopoiesis is actually ensured by downstream progenitors.

A carefully calibrated balance between self-renewal and differentiation must therefore be attained to ensure hematopoietic homeostasis and prevent aberrant HSC development and eventual disease initiation. For instance, excessive differentiation or insufficient self-renewal will result in the depletion of the HSC pool. On the other hand, insufficient differentiation, unrestrained self-renewal, or the existence of committed progenitors that retain self-renewal capacities can lead to myeloproliferative diseases or leukemia (Seita and Weissman 2010; Pinho et al. 2018).

At the cellular level, switching off self-renewal coincides with the “turning on” of lineage programs. Molecularly, however, recent studies have shown that the HSC transcriptome is shaped by a collection of metabolic and cellular properties that are not inherently linked to their pluripotency (Laurenti and Gottgens 2018). In fact, approximately 70% of the transcriptional differences between HSCs and early progenitors do not depend on lineage choice (Laurenti 2013). The same is true concerning chromatin accessibility and methylation (Ji 2010; Corces 2016; Farlik 2016).

The level of self-renewal capacity of an HSC is based on the number of symmetric divisions that an HSC can perform throughout its life. HSC engraftment outcomes and self-renewal are closely related (Benveniste et al. 2010). HSCs can be classified according to the sustainability and robustness of the graft response they produce in transplantation assays (Laurenti and Gottgens 2018). HSCs that can drive repopulation in primary and secondary transplantation assays from 16 to up to 44 weeks post-transplant and produce circulating monocytes, granulocytes, and lymphocytes are known as long-term HSCs (LT-HSC) (Benveniste et al. 2010). Like LT-HSCs, short-term HSCs (ST-HSC) can give rise to all hematopoietic cells, however their grafts peak after 2-3 weeks post transplantation, with circulating myeloid cells that

persist for 4-6 weeks and lymphoid cells that decay slightly slower (Benveniste et al. 2010). In recent years, a third kind of HSC, the intermediate-term HSC (IT-HSC), has been shown to produce clones that persist between 6-8 months and that are phenotypically different from LT-HSCs and ST-HSCs (Benveniste et al. 2010).

Heterogeneity in self-renewal capacity has been associated to how much time HSCs spend in quiescence (Laurenti and Gottgens 2018). Several studies have shown that the most dormant HSCs are capable of producing the most robust and prolonged repopulation responses post-transplant (Wilson 2008; Foudi 2009; Bernitz et al. 2016). Upon reception of mitogenic signals, HSCs must exit quiescence, enter the cell cycle and undergo division, which requires activating several pieces of metabolic and cellular machinery (Laurenti et al. 2015). Frequency of division and the time it takes for a single HSC to exit quiescence *in vitro*, are therefore two cell-cycle parameters that are inversely correlated to repopulation capacity (Laurenti and Gottgens 2018). In fact, the duration of a division starting from G_0 after stimulation, is shorter in ST- and IT-HSCs than in LT-HSCs (Benveniste et al. 2010). Dormant HSCs that are forced into activation by stress signals can return to dormancy, however it has been shown that HSCs that undergo four divisions in adulthood suffer an irreversible loss of self-renewal (Bernitz et al. 2016).

These findings suggest that the first step in HSC differentiation is not the loss of the ability to self-renew, but rather the loss of mechanisms that stabilize self-renewing behavior throughout stem cell divisions (Benveniste et al. 2010).

b. Phenotype of murine HSCs

The first official mouse HSC phenotype was proposed by Spangrude et al in 1988. By selecting the fraction of bone marrow cells which did not express the cell surface markers for B cells (B220), T cells (CD4 and CD8), granulocytes (Gr-1), and myelomonocytic cells (Mac-1), (lineage marker negative cells, Lin^-), they were able to isolate mouse HSCs. These cells are Lin^- but express the cell surface markers $Thy-1^{low}Sca-1^+$ and represented approximately 0.05% of total mouse bone marrow. These HSCs were capable of repopulating the bone marrow of lethally irradiated mice for a prolonged period (over 3 months) and produce myelomonocytic cells, B cells, and T cells (Spangrude et al. 1988).

Since then, an increasing number of cell-surface markers has been incorporated in order to further purify mouse HSCs (**Figure 2**). In the early 90s, the population isolated by Spangrude was further characterized and shown to include at least three different types of cells: LT-HSCs, ST-HSCs, and multi-potent progenitors (MPPs) which cannot self-renew (Morrison and Weissman 1994). In 1996 Ogawa et al showed that $Lin^-c-Kit^+Sca^+$ (LSK) cells contained primitive HSCs. LSK cells that are also $CD34^{low}$ were found to contain LT-HSCs. In fact, single-cell transplantation into lethally irradiated

Species	Name	Cell-surface phenotype	Self-renewal	Cell cycle properties	Differentiation
Mouse	LT-HSC	Lin ⁻ Sca1 ⁺ cKit ⁺ CD34 ⁻ CD150 ⁺ CD135 ⁻ CD48 ⁻ ± EPCR ⁺ ± Rho ^{lo}	High		
	IT-HSC	Lin ⁻ Sca1 ⁺ cKit ⁺ CD34 ^{lo} CD135 ⁻ Rho ^{lo} CD49b ^{hi}	Intermediate	Short G ₀ exit	
	ST-HSC/MPP1	Lin ⁻ Sca1 ⁺ cKit ⁺ CD135 ⁻ CD150 ⁻ CD48 ⁻	Low		
	α	NA	High	NA	Ly-deficient
	β	NA	High	NA	Balanced
	γ	NA	Intermediate	NA	My-deficient
	δ	NA	Low	NA	My-deficient
	MPP2	Lin ⁻ Sca1 ⁺ cKit ⁺ CD135 ⁻ CD150 ⁺ CD48 ⁺	Low	Similar	Ly-deficient
	MPP3	Lin ⁻ Sca1 ⁺ cKit ⁺ CD135 ⁻ CD150 ⁻ CD48 ⁺	Low		Balanced
	MPP4	Lin ⁻ Sca1 ⁺ cKit ⁺ CD135 ⁺ CD150 ⁻ CD48 ⁺	Low		Ly-biased

Figure 2: Phenotype of murine HSCs. Phenotypic or functionally defined murine HSC subsets based on combinations of cell-surface markers and/or function (Adapted from Laurenti and Gottgens 2018)

recipients showed that one in three LSK-CD34^{low} cells were capable of long-term reconstitution (Ogawa 1993). By the early 2000s, Christensen and Weissman showed that expression of the Flk2/Flt-3 receptor tyrosine kinase was inversely proportional to the duration of the reconstitution capacity, and thus LT-HSCs had an Flk2⁻ phenotype compared to ST-HSCs and MPPs (Christensen and Weissman 2001). Today LSK, CD34, and Flk2 are still commonly used markers to isolate HSCs.

The incorporation of novel cell surface markers has contributed largely to the reshaping of hematopoietic hierarchy. In particular expression of CD34 has helped discern HSC subpopulations relative to their degree of self-renewal, where LT-HSCs are LSK-Flk2⁻-CD34^{-/low} and ST-HSCs are LSK-Flk2⁻-CD34⁺ (Yang et al. 2005).

In 2005, the SLAM family of receptors, and in particular CD150, CD244 and CD48, were shown to be differentially expressed among hematopoietic progenitors in a way that correlates with stemness (Kiel et al. 2005). Kiel et al proposed an alternative enhancement of HSC purification by using the phenotype LSK CD150⁺CD48⁻. 47% of single cells with this phenotype yielded long-term multilineage reconstitution following transplantation into lethally irradiated recipients (Kiel et al. 2005). One important advantage of using CD150 is that it is less affected by mouse age and genetic background than other HSC markers (Schroeder 2010).

Since the introduction of IT-HSCs, the CD49b marker has been used to distinguish them from LT-HSCs, where LT-HSCs are CD49b⁻ and IT-HSCs are CD49b⁺ (Benveniste et al. 2010; Schroeder 2010).

HSCs are the most comprehensively analyzed adult mammalian stem cell type. Nevertheless, even after decades of research additional levels of complexity continue to surface and even the most purified HSC subsets still contain a large proportion of cells that do not possess HSC potential (Schroeder 2010).

c. Differences between human and murine HSCs

Hematological research is no exception to the phenomenon of murine model dominance. Since the emergence of HSCs in scientific literature in the 60s, the overwhelming majority of research on HSC and hematopoiesis has been carried out in mice (Parekh and Crooks 2013). To study human hematopoiesis, long-term reconstitution experiments are carried out using xenotransplantation models in mice. The dominance of murine studies has often led to the assumption that findings in mice can be universally applied to all hematopoiesis. Constant development of humanized xeno-transplantation models and *in vitro* culture systems have shown that, overall, the hematopoietic differentiation program is largely conserved between humans and mice (Parekh and Crooks 2013). However, there are some critical differences in phenotype, function, and regulatory mechanisms of stem cell maintenance between human and murine HSCs (Parekh and Crooks 2013).

Like their murine counterparts, human HSCs were initially isolated on the basis of cell surface markers that represented different stages of differentiation coupled with functional assays to determine phenotype (Seita and Weissman 2010). Throughout the late 80s and 90s, the first cell surface marker used to purify human HSCs was CD34, which is expressed in blood cells in the human fetal liver, cord blood, and adult bone marrow (Seita and Weissman 2010). *In vitro* culture assays revealed that almost all CD34⁺ cells were pluripotent, although the population remained very heterogeneous (Seita and Weissman 2010).

Further purification of this CD34⁺ HSC population was achieved due to the differential expression of the cell surface marker CD38. Over 90% of CD34⁺ cells are also CD38⁺, however cells that are capable of producing multilineage colonies are actually found in the CD34⁺CD38⁻ fraction (Huang and Terstappen 1994; Miller et al. 1999). Additionally, CD34⁺CD38⁻ cells are highly enriched for long-term culture-initiating cells compared to CD34⁺CD38⁺ cells (Hao et al. 1996; Petzer et al. 1996). Human HSCs also express Flt3, CD90, and CD49f (Baum et al. 1992; Sitnicka et al. 2003; Notta et al. 2011), and are negative for CD45RA (Majeti et al. 2007) (**Figure 3**).

Besides the expression of the CD34 that is specific for human HSCs, several other phenotypical differences between human and murine HSCs exist. Murine HSCs do not express either CD34 or Flt3 but do express CD38, whereas human HSCs do not express Sca-1 or CD150 (Schroeder 2010). Cellular differences also exist, for instance murine HSCs divide on average once every 40 days, whereas human HSCs divide on average once every 260 days (Doulatov et al. 2010). Many of the genes that are specifically expressed in murine HSCs are also expressed in human HSCs. However, there are some differences in gene expression and function pertaining to the evolutionary differences in hematopoietic gene regulation between human and mouse (Parekh and Crooks 2013). The transcription factor HoxB4 is an example of this, where its

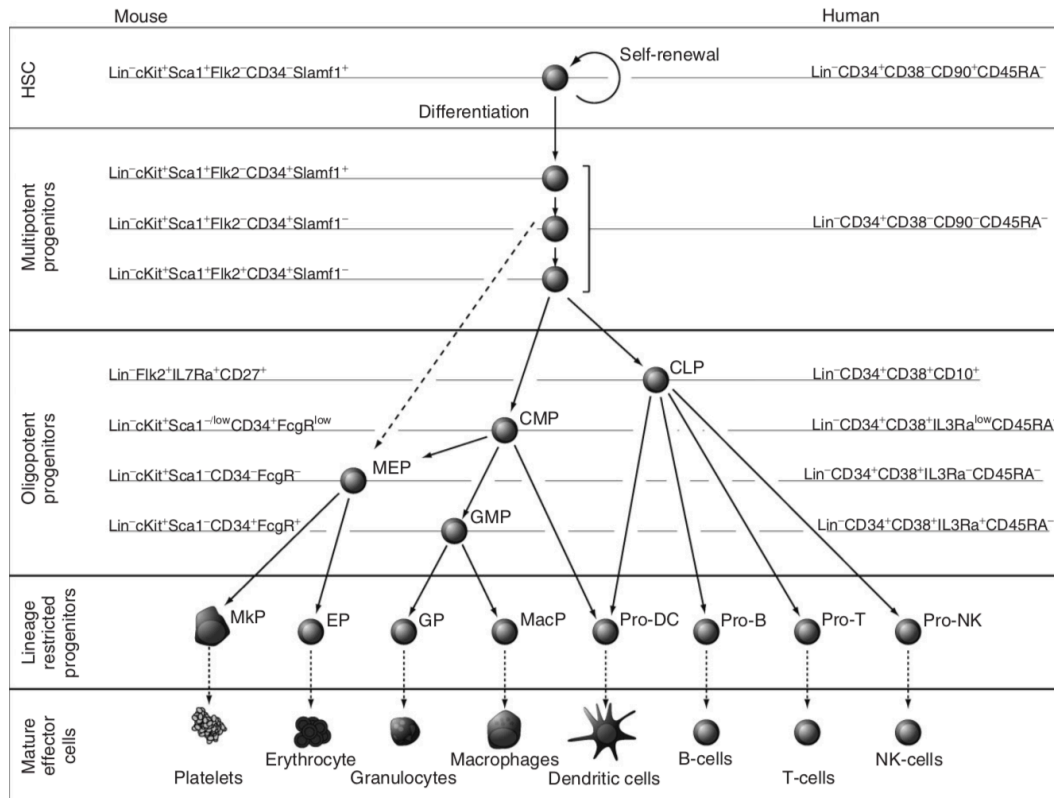


Figure 3: Model of the hematopoietic hierarchy comparing hematopoietic cell phenotype between mouse (left) and human (right) (Seita and Weissman 2018)

overexpression induces a 1000-fold expansion of murine HSCs, but only a 2-4-fold expansion in human HSCs (Doulatov et al. 2012). Additionally, HoxB4 can direct mESC towards a hematopoietic fate, whereas its human counterpart HOXB4 does not confer repopulating potential to hESC-derived hematopoietic cells (Doulatov et al. 2012).

Another important difference between human and murine HSCs concerns their ability to repair DNA damage. Mohrin et al. showed that murine HSCs have an enhanced DNA repair response compared to hematopoietic progenitors. Upon IR, HSCs exhibit an increased expression of prosurvival genes and a strong p53-mediated DNA damage response (DDR) (Mohrin 2010). On the contrary, upon IR human HSCs exhibit a delayed DDR and an enhanced proapoptotic response compared to progenitor populations (Milyavsky 2010). This data suggests that differences in self-renewal regulation, stem cell maintenance, differentiation, or microenvironmental interaction pathways exist between human and murine hematopoiesis (Schroeder 2010).

d. Cellular and molecular factors regulating HSC function

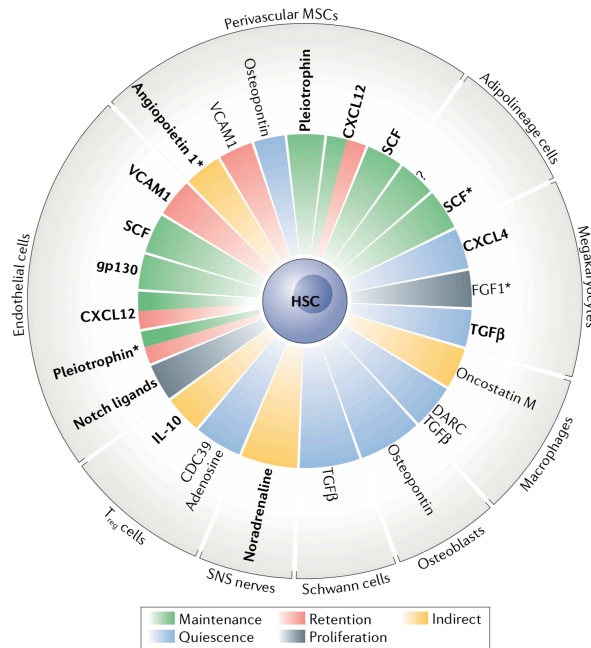


Figure 4: Cellular and molecular constituents of the HSC niche. Various cell types have been implicated in regulating HSC activity. The target plot illustrates how bone marrow niche cells contribute to HSC regulation indirectly or directly by synthesizing niche factors in the form of cell-bound or secreted molecules. The colour of the radial spokes indicates the HSC activity that is affected. (Pinho 2019)

HSC activity is regulated by a complicated network of cell intrinsic and cell extrinsic factors, and local cues from the bone marrow microenvironment (Figure 4).

The hematopoietic stem cell niche ensures hematopoietic homeostasis by controlling proliferation, self-renewal, differentiation and migration of HSCs and progenitor cells at steady-state and in response to insult or injury (Pinho and Frenette 2019). The niche consists of several non-hematopoietic and hematopoietic cells such as perivascular mesenchymal stem cells (MSCs), endothelial cells, osteoblasts, adipocytes, sympathetic nervous system (SNS) nerves, nonmyelinating Schwann cells, macrophages, megakaryocytes and regulatory T (Treg) cells (Pinho and Frenette 2019).

Several cytokines and their receptors have been shown to play essential roles in HSC function. Many hematopoietic cytokines were initially identified based on their abilities to support in-vitro formation of hematopoietic colonies from progenitors (Zhang and Lodish 2008). Some of these were also shown to bind directly to receptors on HSCs and regulate quiescence, self-renewal, differentiation, apoptosis, and mobility (Zhang and Lodish 2008). Under stress conditions, such as inflammation or infection, cytokines help increase the output of mature blood cells by affecting

survival, expansion and differentiation of lineage-committed progenitors (Metcalf et al. 2008; Rieger et al. 2009). Although lineage-specification is mostly mediated by intracellular regulators such as transcription factors, it has been shown that the cytokine macrophage colony stimulating factor (M-CSF) can directly induce the master regulator PU.1 and instruct myeloid cell-fate in murine HSCs, suggesting that cytokines can also play instructive roles in cell-fate determination (Mossadegh-Keller et al. 2013).

Two cytokines of remarkable importance in HSC function are stem cell factor (SCF) which binds to c-Kit, and thrombopoietin (TPO) which binds to Mpl. HSCs express high levels of both of these receptors (Ikuta and Weissman 1992; Solar et al. 1998). Both SCF and TPO can promote survival and proliferation of purified murine HSCs in serum-free culture medium at the single-cell level (Rossi, Seita, et al. 2007). In addition to SCF and TPO, many cytokines including IL-3, IL-6, IL-11, and Flt3 ligand have been reported to impact survival, proliferation and differentiation of HSCs (Seita and Weissman 2010).

Apart from classical hematopoietic cytokines, numerous signal transduction pathways have been shown to be relevant in adult HSCs. Notch ligands play an important role in lymphopoiesis and megakaryopoiesis and in the generation, maintenance, and expansion of HSCs (Chiba 2006). Non-canonical Wnt signaling is involved in generation and expansion of HSCs, although its role remains controversial as canonical Wnt-signaling is active in several types of leukemia (Nemeth and Bodine 2007). TGF- β /Smad and Ang-1/Tie-2 signaling are two pathways responsible for the maintenance of quiescence of HSCs (Zhang and Lodish 2008).

It is evident that a great majority of these cytokines and signaling pathways share redundant functions. Nevertheless, their actions reflect the balance of two essential HSC abilities: maintenance of self-renewal and differentiation.

3. Thrombopoietin: an important cytokine in HSCs

a. Thrombopoietin (TPO) & its receptor MPL

In 1958, Kelemen and Tanos came up with the term “thrombopoietin” to describe the “humoral substance responsible for platelet production” (Kelemen et al. 1958). Since then and all the way through the 80s, attempts at purifying TPO were largely unsuccessful (Hitchcock and Kaushansky 2014). Although seemingly unrelated at first, the discovery of the murine myeloproliferative leukemia virus (MPLV) and its oncogene *Mpl* in the early 90s, was actually the first step leading towards the purification of TPO (Hitchcock and Kaushansky 2014). In 1992, Vigon et al. cloned of *Mpl* for the first time and revealed that it encoded a member of the hematopoietic cytokine receptor family, however its ligand was unknown at the time (Vigon et al.

1992). In 1994, several groups identified quasi simultaneously that TPO was Mpl's ligand and that it was an important mediator of megakaryocyte differentiation (Bartley 1994; Lok et al. 1994; de Sauvage et al. 1994; Wendling et al. 1994).

TPO is a hormone that circulates in the blood and expressed in different tissues in the human body. The highest levels of TPO mRNA are found in the liver, where it is produced by hepatocytes (Lok et al. 1994). The kidneys, smooth muscles, and a number of other organs also express the gene (Bartley 1994; Lok et al. 1994; de Sauvage et al. 1994). Plasma concentrations of TPO vary with platelet count. Platelets express MPL, so the higher the platelet count, the more circulating TPO is eliminated, diminishing its plasma concentration and stimulating synthesis of more TPO. In patients with thrombocytosis, the steady-state level of TPO production is exceeded by platelet-mediated TPO metabolism, so TPO levels are low. On the contrary, in thrombocytopenic patients, most of the TPO that is produced is not eliminated by platelets due to their scarcity, and TPO plasma levels are high (Kuter and Rosenberg 1995; Hitchcock and Kaushansky 2014).

The TPO receptor, MPL, is 635 amino acids in length and contains three functional domains: an extracellular domain that contains its cytokine binding site, a transmembrane domain, and a cytoplasmic domain (Varghese et al. 2017). Mpl is a member of the type I cytokine receptor family along with interleukins (IL-2 to IL-7, IL-9, IL-13, IL-15 and IL-21), colony-stimulating factors (G-, and GM-CSF), erythropoietin (EPO), and the growth hormone receptor (GHR) (Fujii 2007; Hitchcock and Kaushansky 2014). To ensure signal transduction, MPL requires ligand-mediated tyrosine phosphorylation of the receptor and associated proteins in response to hematopoietic cytokine stimulation. TPO acts by binding to the extracellular domain of MPL, which leads to the dimerization of the receptor, thereby starting a cascade of signaling events within the target cell (Varghese et al. 2017) (**Figure 5**). MPL lacks intrinsic kinase activity, so it uses the Janus kinase (JAK) family of proteins to transduce signals from its extracellular domain to the cell. JAK2 and TYK2, both members of the JAK family, constitutively associate with the cytoplasmic domain in MPL and phosphorylate upon TPO binding. Despite the fact that both proteins phosphorylate MPL, only phosphorylation of JAK2 is essential for TPO signaling (Drachman et al. 1999). Activation of JAK2 leads to the phosphorylation of the distal portion of MPL and leads to the recruitment the signal transducer and activator of transcription (STAT) proteins, in particular STAT3 and STAT5, through their SH2 domains (Tortolani et al. 1995; Sattler et al. 1997). Mpl activation can also lead to the activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 (PI3K) pathways. Early work by our lab has shown that TPO drives megakaryocytic differentiation via a strong and prolonged activation of MAPK, that is specifically involved in the transition from proliferation to maturation of megakaryocytes (Rouyez et al. 1997; Fichelson et al. 1999). Our lab has also shown that TPO activates MAPKs

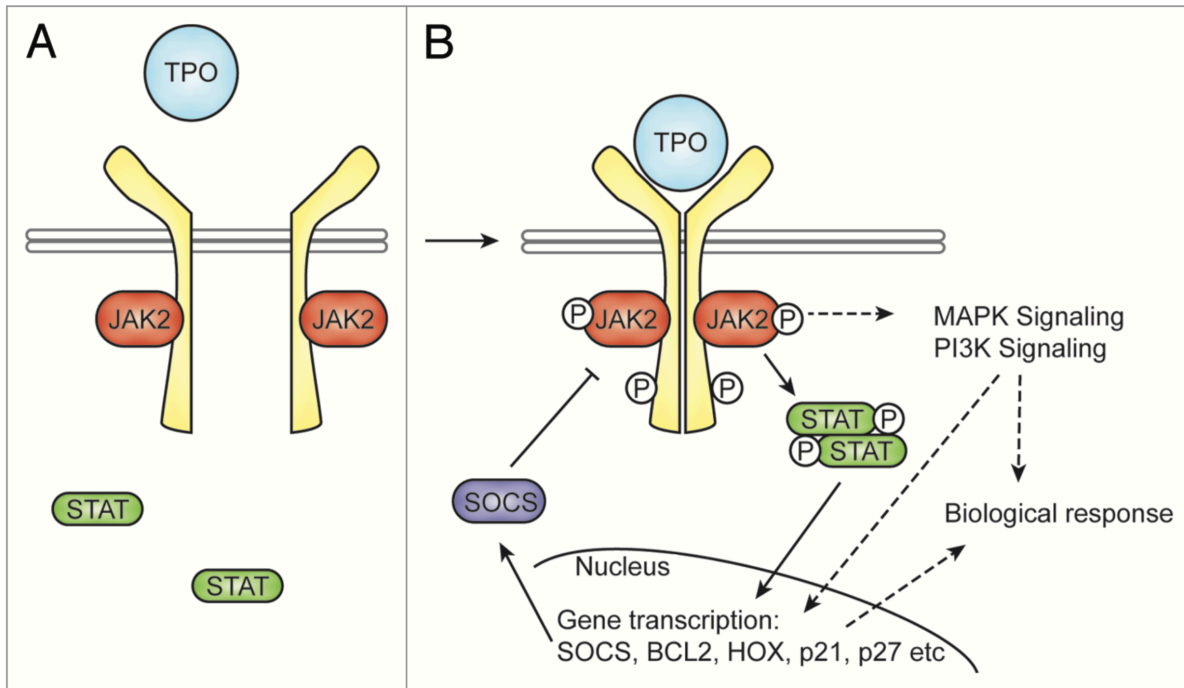


Figure 5: TPO signaling pathway. (A) Mpl (in yellow) exists as a monomer, and signaling molecules like JAK2 and STAT are inactive. (B) Upon TPO binding, Mpl dimerizes and this conformational change results in JAK2 phosphorylation which leads to Mpl phosphorylation. This leads to phosphorylation of STAT5 and STAT3, resulting in their dimerization and entry into the nucleus, where they activate downstream signaling pathways like the MAPK and PI3K pathways. STATs also induce transcription of the SOCS proteins which are generally not expressed and which act as negative feedback regulators off JAK2 and MPL activation (de Graaf and Metcalf 2011).

through the small GTPases Ras and Rap1, which act in concert to regulate the duration of MAPK signaling (Garcia et al. 2001).

MPL is expressed predominantly in megakaryocytes, platelets, hemangioblasts and HSCs. This expression patterns indicates two main functions for TPO: the regulation of platelet production and the maintenance of HSCs (Varghese et al. 2017). In TPO and MPL knock-out (KO) mice showed an 85% decrease in platelet and megakaryocyte count whereas other mature lineages were less affected (Gurney et al. 1994; Sauvage et al. 1996). TPO promotes the proliferation of megakaryocytic progenitors early in lineage commitment and increases ploidy of megakaryocytes, although it does not affect platelet shedding (de Graaf and Metcalf 2011).

b. TPO and the hematopoietic stem cell

In vitro studies showed that TPO could increase survival and proliferation of CD34⁺ hematopoietic progenitors (Ku et al. 1996; Sitnicka et al. 1996). TPO has been also shown to increase HSC interaction with the osteoblastic niche and to support HSC quiescence and expansion posttransplantation (Fox et al. 2002; Yoshihara et al. 2007). The emergence of KO mouse models that do not express TPO or MPL provided

evidence that TPO has an essential role in HSCs. These mice have only 10-20% of LT-HSCs of the normal number of LT-HSC in the basal state, and display increased HSC cycling and age-progressive loss of HSCs (Solar et al. 1998; Qian et al. 2007; Yoshihara et al. 2007). Transplantation of HSCs into TPO KO mice exhibited a 15/20-fold decrease in HSC expansion compared to wild-type (WT) controls (Fox et al. 2002). Transplantation MPL KO HSCs led to a decrease in hematopoietic progenitors of all lineages, as well as a decrease of 80-90% of total HSCs (Alexander et al. 1996; Solar et al. 1998). These studies highlight two contrasting functions for TPO. Whilst TPO maintains HSCs in quiescence to preserve them with age, it also serves to promote HSC expansion in times of crisis, such as post-transplantation (de Graaf and Metcalf 2011). Nevertheless, both functions show that TPO is a regulator of cell-cycle transition in HSCs.

TPO also plays a role in HSC maintenance in humans: congenital amegakaryocytic thrombocytopenia (CAMT), caused by MPL inactivating mutations, leads to a severe hypomegakaryocytic thrombocytopenia during the first years of life that develops into a pancytopenia in later childhood with increased risk of developing leukemia, suggesting a general defect in hematopoiesis (Ballmaier et al. 2001). Bone marrow transplantation is the only curative therapy for CAMT so far.

Myeloproliferative neoplasms (MPNs) are clonal disorders that affect hematopoietic stem cells (HSCs) and leading to the pathologic expansion of the myeloid lineage, mainly platelets in essential thrombocythemia (ET), red blood cells in polycythemia vera (PV), or platelets with collagen fiber formation in primary myelofibrosis (PMF) (Vardiman et al. 2009). The hallmark of these disorders is the constitutive activation of the JAK-STAT pathway (Vainchenker and Constantinescu 2013). The substitution of a stretch of 5 amino acids (W515L tryptophan-to-leucine substitution and W515K tryptophan-to-lysine) that normally prevent spontaneous MPL activation are found in ET and PMF patients (Pietra et al. 2011). Mutations in the calreticulin (CALR) also lead to constitutive activation of MPL (Chachoua et al. 2016). This highlights the important role of TPO signaling in HSCs and the major consequences that ensue upon its deregulation.

In recent years, our team has uncovered an important role for TPO in the regulation of the DNA damage response (DDR) in HSCs following genotoxic stress, such as ionizing radiation (IR). They showed that MPL KO mice displayed increased radiosensitivity due to impaired DNA repair and that TPO stimulates DNA repair *in vitro* and *in vivo* by increasing DNA-PK-dependent non-homologous end-joining (NHEJ) efficiency (de Laval et al. 2013). The team has also shown that TPO, which is a non-inflammatory cytokine, triggers Erk and the nuclear factor κ B (NF κ B) in HSCs and progenitor cells, and that these two pathways cooperate to induce and activate the early-response gene *Iex-1* upon DNA damage. *Iex-1* forms a complex with pERK and the catalytic subunit of DNA-PK, which is necessary and sufficient to promote TPO-increased DNA-PK

activation and NHEJ DSB repair in both mouse and human HSPCs (de Laval et al. 2014).

These results highlight that HSC environmental factors can control the DSB repair machinery to ensure chromosomal integrity and limit their long-term residual injury in response to IR. A single injection of TPO one hour prior to 2Gy total body irradiation (TBI) was enough to prevent the accumulation of DNA damage and restore reconstitution capacity post-transplantation in murine HSCs (de Laval et al. 2013). This study opened up new perspectives for the clinical use of TPO agonists prior to therapies involving DNA damaging agents to minimize the risks of HSC loss of function and the development of secondary leukemias. Interestingly, Wang et al. have shown that TPO administration post irradiation improved survival rates in lethally irradiated mice and increased bone marrow cell density and LSK cell numbers in the mice after sub-lethal irradiation primarily by promoting residual HSC proliferation (Wang et al. 2015). In humans, a recent study showed that the used of the synthetic small molecule mimetic of TPO, eltrombopag, specifically activates the C-NHEJ DNA repair mechanism, resulting in enhanced genome stability, survival and function of primary human HSPCs (Guenther et al. 2019).

c. JAK-STAT Signaling

The JAK-STAT signaling pathway was initially discovered in the investigation of interferon (IFN) gene induction. It is now considered one of the major evolutionary conserved signaling pathways employed by a diverse panel of cytokines, IFNs, and growth factors (O'Shea et al. 2015).

JAK kinases belong to a family of intracellular, non-receptor tyrosine kinases. JAK-mediated phosphorylation of STAT proteins activates them, allowing their dimerization and their entry into the cell nucleus where they can directly bind to DNA and affect gene expression (**Figure 6**) (O'Shea et al. 2013). There are four JAKs (JAK1-4), and Tyk2, which can selectively bind different receptor chains, explaining their different *in vivo* roles. For instance, JAK1 and JAK2 are activated by type II IFN signaling, JAK1 and Tyk2 are activated by type I IFN signaling, JAK2 and Tyk2 by TPO signaling, and JAK2 by EPO signaling.

Seven different STAT family members have also been identified that are activated as a result of diverse extracellular stimuli. For instance, type II IFN primarily activates STAT1, creating a STAT1-STAT1 homodimer that localizes to the nucleus to exert downstream effects. Type I IFNs phosphorylate STAT1 and STAT2, leading to their dimerization and subsequent entry into the nucleus where they bind IRF9 to form the ISFG3 complex. TPO activates primarily STAT5 and STAT3 (O'Shea et al. 2015). Although each cytokine tend to activate a specific STAT over another, activation of a STAT proteins can also occur by different promiscuous cytokines (O'Shea et al. 2015).

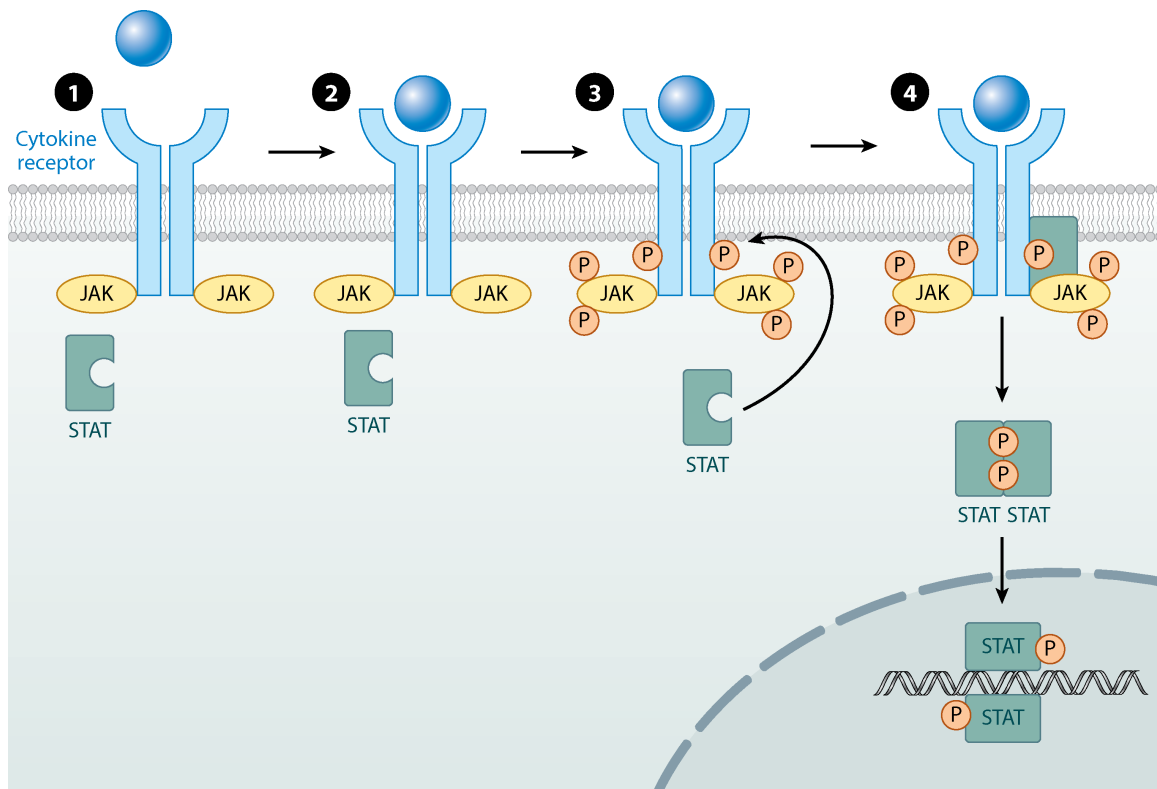


Figure 6: Signal transduction via JAK. (1) When a cytokine engages its receptor, JAKs become activated and phosphorylate each other, as well as the intracellular tail of their receptors. (2) This creates a docking site for STATs, which are now able to bind to the cytoplasmic domain of the receptor. (3) The STATs, in turn, are phosphorylated and activated, which allows them to dimerize. (4) The STAT-STAT dimer translocates to the nucleus, where it can directly bind DNA and regulate gene expression. (O’Shea 2015).

STAT proteins, like other transcription factors, can bind tens of thousands of genomic loci and regulate transcription of thousands of protein-coding genes, miRNAs and long non-coding RNAs (O’Shea et al. 2015).

Although standard functioning of the JAK-STAT pathway requires the phosphorylation of STAT, a number of non-canonical functions have been ascribed to non-phosphorylated STATs. For instance, exposure to a high dose of Type I IFN induces activation of IFN-stimulated genes (ISGs) mediated by the phosphorylation of STAT1/2 in a rapid manner, however, Cheon et al. showed that a second prolonged wave of ISG induction is mediated by non-phosphorylated STAT1 and STAT2 (Figure 7) (Cheon et al. 2013). This phosphorylation independent second response is activated even by low levels of IFN and provides the host cell with prolonged anti-viral functions even after IFN synthesis subsides and phosphorylation is reversed (Cheon et al. 2013).

Recent studies have also shown that STAT proteins have important roles in the regulation of chromatin structure and enhancer activity of differentiating cells.

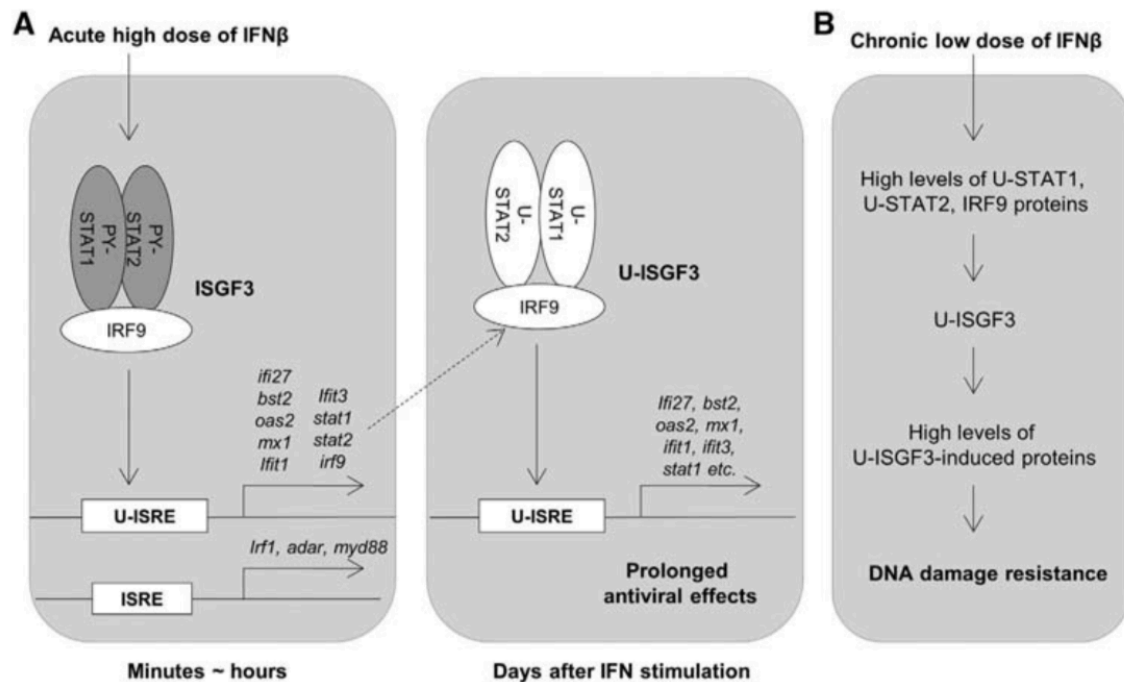


Figure 7: Biological significance of non-phosphorylated U-ISGF3-induced gene expression (A) U-ISGF3 prolongs anti-viral effects. IFN β induces the expression of a large number of ISGs quickly through the action of ISGF3 which binds to standard ISREs in ISG promoters. ISGs induced by an initial treatment with IFN β include STAT1, STAT2, and IRF9, and the encoded proteins accumulate in their unphosphorylated forms for days after IFN stimulation. The accumulated STAT1, STAT2, and IRF9 proteins form U-ISGF3, which selectively binds to the distinct ISREs in promoters of a subset of ISGs, most of which are anti-viral genes. The expression of these genes is prolonged, whereas the expression of the genes induced by ISGF3, but not by U-ISGF3, is terminated rapidly. (B) U-ISGF3 induces resistance to DNA damage. Chronic exposure to low doses of IFN β increases the levels of U-ISGF3 and the U-ISGF3-induced proteins, with no increase in STAT phosphorylation. The proteins induced by U-ISGF3 increase resistance to DNA damage. (Cheon 2013).

Constitutive activation of JAK2 can lead to development of hematopoietic malignancies in humans, and of hematopoietic tumor-like masses in *Drosophila* (Silver-Morse and Li 2013). The discovery of roles for JAK and STAT in heterochromatin regulation, began with a genetic screen in *Drosophila* to identify genes which affect the formation of these tumor-like masses that identified chromatin modifiers such as heterochromatin protein 1 (HP1) and the histone methyltransferase SUV39H1 (Silver-Morse and Li 2013). These are essential heterochromatin components involved in H3K9me3-mediated silencing. In 2006, Shi et al showed that JAK overactivation disrupts heterochromatic gene silencing in this species, and allows the derepression of genes that are not direct targets of STAT (Shi et al. 2006). More recently, Sousa-Victor et al. observed that intestinal stem cells (ISCs) induce the Argonaute family protein Piwi, which plays a critical role in heterochromatin maintenance, in response to JAK-STAT signaling induced by proliferation, genotoxic and viral stress (Sousa-Victor et al. 2017). Hu et al. have shown that non-phosphorylated STAT5A acts as a tumor suppressor in human colon cancer cell lines by binding to HP1 and stabilizing heterochromatin (Hu et al. 2013). These studies suggest that these non-canonical

functions of the JAK-STAT pathway to regulate heterochromatin may be conserved amongst species and thus be of more importance than originally thought.

4. Epigenetics of HSCs

Hematopoiesis is a process that involves the tight regulation of gene expression that guard the balance between self-renewal of HSCs and their differentiation into mature blood cell lineages. Genes involved in differentiation pathways must be repressed in HSCs, while self-renewal genes are repressed upon differentiation. Epigenetic mechanisms are responsible for regulating changes in global chromatin conformation that mediate this process.

Epigenetics refers to changes in gene expression that do not involve changes to the underlying DNA sequence, resulting in a change in phenotype without affecting the genotype (Kramer and Challen 2017). In this way, they act as a blueprint and maintain cell identity by modifying the chromatin landscape and directing which genes are expressed in which cell, when, and to what level. Epigenetic modifications are reversible and can also be altered in response to environmental signals (Kramer and Challen 2017). Reflecting the dynamic character of chromatin, recent studies suggested that both the amount of euchromatin, as well as the spatial distribution of heterochromatin regulate the transition between stem and progenitor cells (Ugarte et al. 2015; Koide et al. 2016). Ugarte et al. showed that differences in chromatin distribution affect the transcriptional capacity of a cell and are the most permissive in ESC, then move onto an intermediate state in HSCs, and culminate in a highly restrictive chromatin environment with the silencing of developmental programs in mature cells ¹. In particular they showed that the amount of euchromatin decreases while the layer of heterochromatin at the nuclear envelope increases during the differentiation of HSCs (Koide et al. 2016).

Epigenetic modifications involve active or repressive histone modifications, DNA methylation, and small RNAs (**Figure 8**). Histone tails can undergo major covalent and reversible modifications. Two of the most common modifications are the acetylation /deacetylation by histone acetyltransferases, and methylation/demethylation by histone methyltransferases (Sharma and Gurudutta 2016). Histone modifications impact gene expression by altering chromatin structure to make it more or less accessible to transcription factors and machinery (Kramer and Challen 2017). DNA methylation requires binding of a methyl group at carbon-5 position (5-mC) of a cytosine base by a DNA methyltransferase (DNMTs). This occurs primarily at CpG dinucleotides and CpG islands in gene promoters and mostly leads to long-term repression (Sharma and Gurudutta 2016). Recently, however, DNMT3b-mediated DNA methylation in gene bodies has been shown to act a positive regulator of gene expression in ESCs and colorectal carcinoma cell lines (Yang 2014; Baubec et al. 2015). Small RNAs can modify chromatin structure and silence transcription by guiding

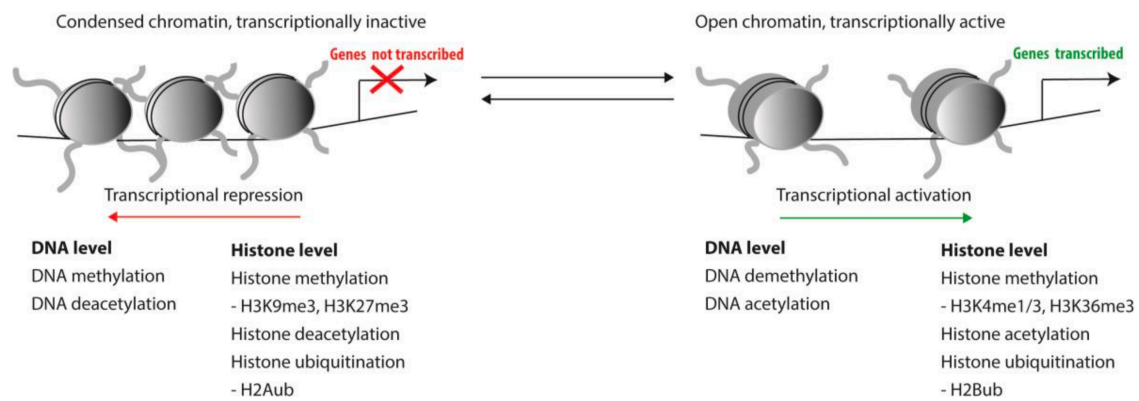


Figure 8: Epigenetic modifications and their effect on DNA accessibility. (Left) Condensed chromatin is transcriptionally silent and characterized by DNA methylation and repressive histone modifications. (Right) Open chromatin is associated to active transcription and characterized by DNA demethylation and activating histone modifications (Buisman 2019).

Argonaute-containing complexes to complementary nascent RNA scaffolds and then mediating the recruitment of histone and DNA methyltransferases (Holoch and Moazed 2015).

Cross-talk between DNA methylation and histone modifications exists, for instance the H3K9me3 methyltransferase SUV39H1 has been shown to interact with the DNA methyltransferases DNMT3A and DNMT1 (Fuks 2003). Loss of H3K9 methylation in *Suv39h*-knockout ESC decreases *Dnmt3b*-dependent DNA methylation at major centromeric satellites (Lehnertz et al. 2003). Moreover, it has also been shown that methyl-CpG-binding-domain proteins, such as MeCP2 and MBD1, might favor H3K9 methylation in the vicinity of the methylated genes regulated by these proteins (Fuks 2003). The distribution patterns of DNA methylation and histone methylation have a great influence on the binding of regulatory proteins to target sequences in the genome, affecting subsequent expression levels of downstream genes (Kramer and Challen 2017). This can help orchestrate physiological processes such as stem cell differentiation, and pathologically contribute to oncogenesis.

a. DNA methylation in HSCs

DNA demethylation occurs during early embryonic development and later during hematopoietic differentiation (Sharma and Gurudutta 2016). Presence of repressive epigenetic modifications, such as H3K9me3 or DNA methylation at promoter regions has been observed in partially reprogrammed iPS cells and correlates with failure to produce the robust reactivation of pluripotency genes (Calvanese et al. 2012).

DNMT3a and DNMT3b are two of the most important enzymes responsible for *de novo* DNA methylation. They act in synergy and are responsible for the establishment of new DNA methylation patterns during HSC development and throughout differentiation, and are associated to the silencing of self-renewal genes (Kramer and Challen 2017). Consistent with the latter, loss of DNMT3a results in expansion of the HSC population due to the upregulation of self-renewal genes such as Runx1 and Gata3, to the detriment of differentiation genes. Deletion of DNMT3a and DNMT3b further enhances HSC self-renewal by activating β -catenin signaling (Challen et al. 2014). Moreover, DNMT3a is one of the most frequently mutated genes in myeloid malignancies such as AML and myelodysplastic syndromes (MDS) (Kramer and Challen 2017). In contrast, DNMT3b is rarely mutated in these pathologies, but has been linked to tumor suppressor function (Schulze et al. 2016).

DNMT1 is a methyltransferase responsible for maintaining DNA methylation patterns in daughter cells upon cell division. Trowbridge et al. showed that DNMT1-deficient HSCs exhibited impaired self-renewal, as well as inefficiency to produce multilineage progenitors. In particular, they showed that deletion of DNMT1 induced enhanced cell cycling and inappropriate expression of mature lineage genes in myeloid progenitors (Trowbridge et al. 2009). These results suggest that DNA methylation plays an essential role in the progression of HSCs into MPPs and into lineage-restricted myeloid progenitors. DNMT1 has been shown to be upregulated in certain AML patients (Trowbridge et al. 2009). This result is supported by the fact that inhibition of DNMT1 impairs leukemogenesis and inhibits stem cell function in a mouse model of MLL-AF9-driven AML (Trowbridge et al. 2009).

Cytosine hydroxymethylation (5-hmC) is another important epigenetic modification that arises due to the oxidation of 5-mC by the ten-eleven translocation (TET) family of enzymes. 5hmC is considered to be an initial step in active demethylation mediated by TET enzymes (Tahiliani et al. 2009). Active demethylation involves cytosine replacement via the base excision repair (BER) pathway, which removes the methylated cytosine and creates a basic site that is then further processed by other enzymes (Li 2013). In contrast, passive demethylation can also occur during successive rounds of replication in the absence of functional DNA methylation maintenance machinery (**Figure 9**).

Mutant mouse model and patient genome sequencing studies have revealed important roles for the TET enzymes in hematopoiesis (Kramer and Challen 2017). Together with DNMT3a, TET2 is one of the most frequently mutated genes in myeloid malignancies (Cancer Genome Atlas Research Network et al. 2013). Interestingly, in all studies of clonal hematopoiesis of indeterminate potential (CHIP) across aging humans, the two most commonly variant genes are also DNMT3A and TET2 (Jaiswal et al. 2014; Zink et al. 2017). CHIP is associated with aging and an increased risk of developing hematologic malignancies and other aging-associated pathologies such as

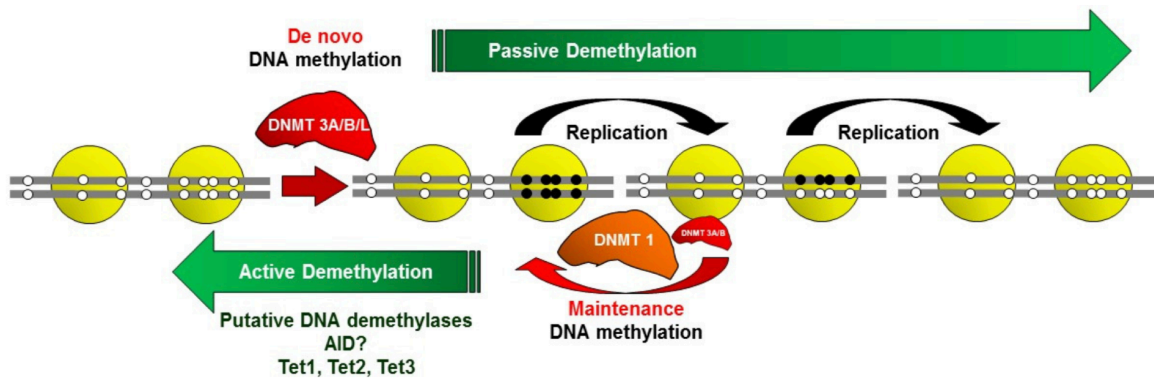


Figure 9: DNA methylation and demethylation. De novo DNA methylation patterns are established by DNMT3a and DNMT3b. This process is enhanced by catalytically active DNMT3L. During replication the original DNA methylation pattern is maintained by DNMT1. DNA methylation can be erased by active or passive demethylation. Active demethylation occurs by enzymatic replacement of a methylated cytosine by an unmethylated residue without cell division, and is mediated by TET proteins and AID. Passive demethylation occurs during successive replications when maintenance of DNA methylation is abrogated. Black circles represent methylated CpG sites and open circles represent unmethylated CpG sites (De Carvalho 2010).

atherosclerosis and cardiovascular disease (Jaiswal et al. 2017; Rauch et al. 2018). Tet2 KO and DNMT3a KO mice have increased self-renewal and competitive repopulation capacity, and increased myeloid cell output compared to their wildtype counterparts (Challen et al. 2011; Moran-Crusio et al. 2011). This could suggest that clonal hematopoiesis is a necessary evil to compensate for the diminished potential of aging HSCs (Kramer and Challen 2017).

b. Histone acetylation in HSCs

Acetylation of histones is associated with relaxed chromatin and promotes gene expression activation. Acetylation regulates self-renewal, proliferation and differentiation into hematopoietic progenitors. Acetylation functions through the transferring an acetyl group from acetyl-CoA to specific lysine residues and in doing so changes the charge of the lysine residue and affects DNA-histone association (Sharma and Gurudutta 2016). As a result, the chromatin structure is dispersed, and becomes accessible to transcription factors. Histone deacetylases (HDACs) are extensively involved in multilineage development, including the hematopoietic stem cell HSC-progenitor lineage, granulocyte-monocyte lineage, erythropoietic lineage and lymphoid lineage (Wang et al. 2020). During hematopoiesis, HDACs participate in the formation of a variety of transcriptional complexes where the reciprocal regulation between HDACs and TFs or other cofactors regulate histone acetylation levels, TF activity and functions of transcriptional complexes, which in turn modulate expression of various hematopoietic-related genes (Wang et al. 2020). For instance, simultaneous deletion of HDAC1 and HDAC2 results in the loss of HSCs and, consequently, early hematopoietic progenitors, which are associated with the

deregulated expression of genes linked to stem cell survival and maintenance, such as *Dmkn*, *Nurcks1* and *Tpt1* (Yamamura et al. 2006).

Consistent with a loss of “closed” chromatin that is observed during aging, HDACs such as the Sirt family proteins have been shown to protect HSCs from aging (Rimmelé et al. 2014). Poplineau et al. have also observed a clear increase in H3K27ac, marked at enhancer regions in aged myeloid progenitors, and that restriction of H3K27ac by the promyelocytic leukemia zinc finger (PLZF) can act as a gatekeeper to hematopoietic aging (Poplineau et al. 2019).

In line with this concept, histone deacetylase inhibitors are used to treat leukemia to induce chromatin remodeling and permit the re-expression of silenced tumor suppressor genes in leukemic stem and progenitor cells. This leads to the differentiation of leukemic stem cells and hinders their proliferation and self-renewal properties (Muñoz et al.). Valproic acid, another histone deacetylase inhibitor, is used in the treatment of acute promyelocytic leukemia and inhibits proliferation of leukemic cells by increasing the expression of death receptors and their ligands (Walasek et al. 2012).

c. Histone methylation in HSCs

Unlike acetylation, histone lysine methylation does not affect the charge of the lysine residues and thus has minimal impact on DNA-histone association. Rather, the different methylation status of specific histone lysines can serve as a unique platform for recruiting methylation “reader” proteins that activate or repress genes’ transcriptional activity. In general, histone H3 lysine 4 (H3K4), H3K36, and H3K79 methylation are gene activation marks, whereas H3K9, H3K27, and H4K20 methylation are generally gene-repressive modifications (Gu and Lee 2013) (**Figure 10**).

Trimethylation on lysine 4 of histone 3 (H3K4me3) is a chromatin mark associated with protein coding genes and sites of active transcription. H3K4me3 protects promoters from DNA methylation and maintains chromatin in an open state to promote transcription (Kramer and Challen 2017). Through ChIP-seq analysis, Sun et al. showed that aged HSCs exhibited broader H3K4me3 peaks across HSC identity and self-renewal genes, and also exhibited increased DNA methylation at transcription factor binding sites associated with differentiation-promoting genes (D. Sun et al. 2014). There is a strong correlation between altered H3K4me3 levels and transcriptional activity, where genes whose expression increases with age acquire H3K4me3 (D. Sun et al. 2014).

H3K27me3 is a silencing mark that is mediated by polycomb group (PcG) proteins and chromatin modifiers such as the enhancer of zeste (*Ezh2*). KO mice for *Ring1B*, a member of the polycomb repressive complex 1 (PRC1), exhibit hematopoietic

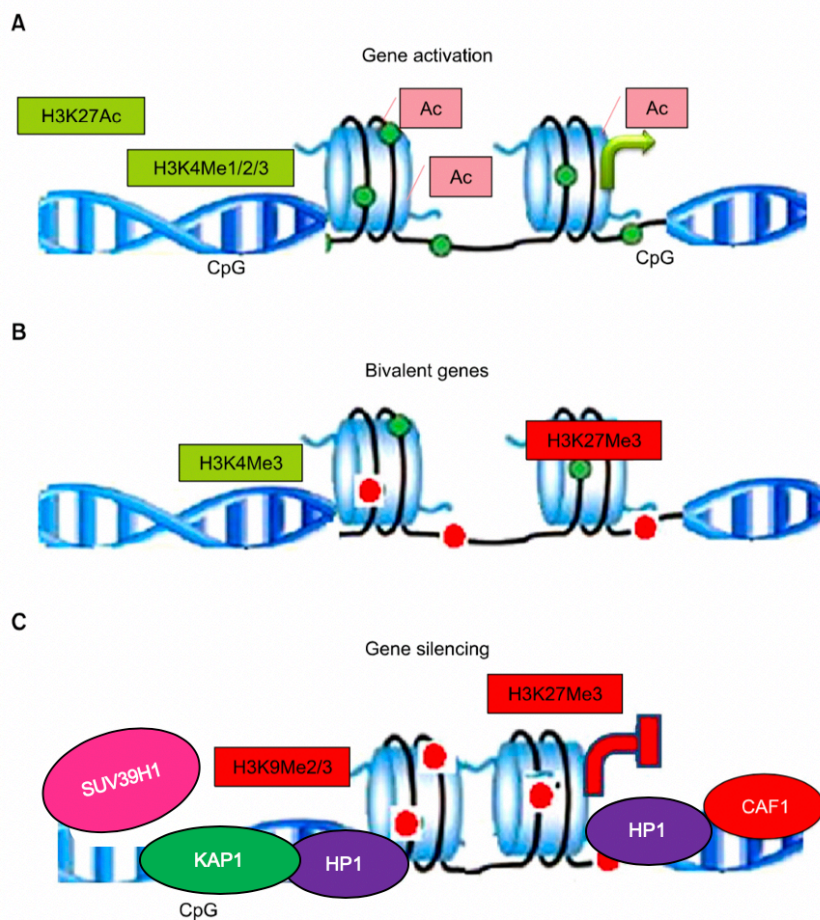


Figure 10: Histone modifications. A) Active genes: Open chromatin structure of transcriptionally active gene with loosely spaced nucleosomes. Acetylation of lysine neutralizes the positive charge, reducing affinity between histone and DNA, which functions as platforms for the recruitment of transcription factors or chromatin remodelers, thus histone modifications directly effects nucleosomal architecture. H3K4me3 is enriched around transcription start sites. H3K4me1 is enriched around enhancers and downstream. H3K27ac is enriched around active enhancers and transcription start sites. B) Bivalent genes: In undifferentiated stem cells, both H3K4me3 and H3K27me3 (active and inactive marks, respectively) are enriched around transcription start sites on many genes. The multiple coexisting histone modifications are associated with activation and repression. C) Inactive genes: H3K9me3 is broadly distributed on inactive regions. H3K27me3 and H3K9me3 are usually not colocalized in the same region of genes. Proteins associated for transcription silencing are SUV39H1, SETDB1, KAP1, HP1-Heterochromatin protein and CAF-1-Chromatin assembly factor1. (Modified from Sharma and Gurudutta 2016).

abnormalities (Eskeland et al. 2010). Bmi1, another member of the PcG family, has been shown to play important roles in self-renewal and differentiation by repressing transcription of genes involved in cellular senescence (Iwama et al. 2004). Bmi1 is frequently overexpressed in hematopoietic malignancies and its inhibition has been associated with impaired self-renewal and expansion in leukemic stem cells (Sharma and Gurudutta 2016).

In the hematopoietic system, HSCs possess the highest frequency of bivalent promoters, which declines throughout differentiation as lineage-restriction occurs (Weishaupt et al. 2010). Genes with bivalent promoters are generally not expressed, or at their expression is very weak, as they possess both the H3K4me3 activation mark as well as the H3K27me3 repressive mark (Kramer and Challen 2017). In addition to H3K4me3/H3K27me3-mediated bivalency, other histone modifications may also poise genes for rapid transcription. For example, trophoblast and endodermal stem cells demonstrate a pattern of bivalent H3K4me3/H3K9me3 modifications on lineage-specific genes that function independently of H3K4me3/H3K27me3 to regulate differentiation (Rugg-Gunn et al. 2010). Similarly, in mesenchymal stem cells, H3K4/H3K9me3 bivalent chromatin at several key adipogenic genes was demonstrated to keep lineage-specific genes poised, but repressed, until commitment to adipogenesis took place (Matsumura et al. 2015). Bivalent domains in HSCs include many master transcription factors involved in fate commitment, such as C/EBP α , Ebf1, Pax5, Meis1, Gata2 and Gata3. (Kramer and Challen 2017). With age, there is an increase in the number of bivalent domains, which can occur due to the acquisition of H3K27me3 at promoters that already possessed H3K4me3, or via the acquisition of both marks at an entirely new promoter (D. Sun et al. 2014).

H3K9me3 is a repressive chromatin mark that is usually found in heterochromatic regions and is mainly catalyzed by the methyltransferases SUV39H1, SUV39H2, and SETDB1 (Koide et al. 2016). H3K9me3 is recognized and bound by members of the heterochromatin protein 1 (HP1) family and associated critical adaptor proteins, such as KAP1, that coordinate chromatin compaction by self-association and recruitment of histone deacetylases, DNA methyltransferases, and structural RNAs (Maison and Almouzni 2004). Consistent with the crucial role of heterochromatin during differentiation and development, Miyagi et al. showed that deletion of KAP1 in mice induced cycling and apoptosis of HSCs, leading to rapid depletion of the stem cell pool in the bone marrow (Miyagi et al. 2014). Interestingly they also showed that KAP1 deficient HSCs exhibited ectopic expression of non-hematopoietic genes, and a significant decrease of HP1 levels, suggesting that the KAP1-HP1 axis is a critical repressor of genes that are not normally activated in the hematopoietic compartment (Miyagi et al. 2014). In turn, Koide et al. showed that deletion of SETDB1, a methyltransferase that works in cooperation with KAP1 and HP1, leads to depletion of HSCs and progenitor cells, as well as leukemic stem cells (Koide et al. 2016). They also showed that, like KAP1, SETDB1 restricts the activation of non-hematopoietic genes to preserve HSCs and progenitors (Koide et al. 2016). These results highlight the importance of H3K9-mediated silencing in HSCs as essential to preserve the specific transcriptional signature of HSCs (Miyagi et al. 2014; Koide et al. 2016).

SUV39H1-mediated H3K9me3 silencing has been shown to regulate lineage commitment by repressing lineage-inappropriate genes in early mouse development, and has an important role in the maintenance of cellular fate following commitment

to the T helper 2 lymphocyte lineage (Allan et al. 2012; Djeghloul et al. 2016). Deletion of *Suv39h1* in mice has been linked to the development of late onset B cell lymphomas (Peters et al. 2001). Djeghloul et al. recently showed that expression of SUV39H1 decreases with age in both human and mouse HSCs, leading to a global reduction in H3K9 trimethylation and perturbed heterochromatin function, which results in decreased B lymphopoiesis and a myeloid-biased differentiation output (Djeghloul et al. 2016). SUV39H1 was also shown to regulate resistance to Transforming Growth Factor beta (TGF β) in AML progression (Monaghan et al. 2019). TGF β is a signaling pathway frequently deregulated in hematopoietic malignancies with anti-proliferative and differentiation signaling demonstrated to directly arrest growth and inhibit colony formation in the leukemic stem cell population (Monaghan et al. 2019). p15 and p21, major tumor suppressors and targets of TGF β , are silenced in AML by pathways involving SUV39H1, giving the leukaemic cells an advantage to proliferate. Loss of SUV39H1 in AML cells leads to the reactivation of hypermethylated p15 (Lakshmikuttyamma et al. 2010).

5. Threats to HSC function and stability

a. HSC aging

In 2013, López-Otín et al. published a comprehensive review describing the hallmarks of aging in different organisms with special emphasis on mammalian aging, one of these was stem cell exhaustion (López-Otín et al. 2013). Stem cell exhaustion refers to the gradual loss of function of tissue-specific stem cells which hinders their capacity to maintain homeostasis in the tissue in which they reside (De Haan and Lazare 2018). Similarly to other adult stem cells, HSCs are vulnerable to age-related stress, and gradually lose their self-renewal and regeneration capacities (Lee et al. 2019) (**Figure 11**).

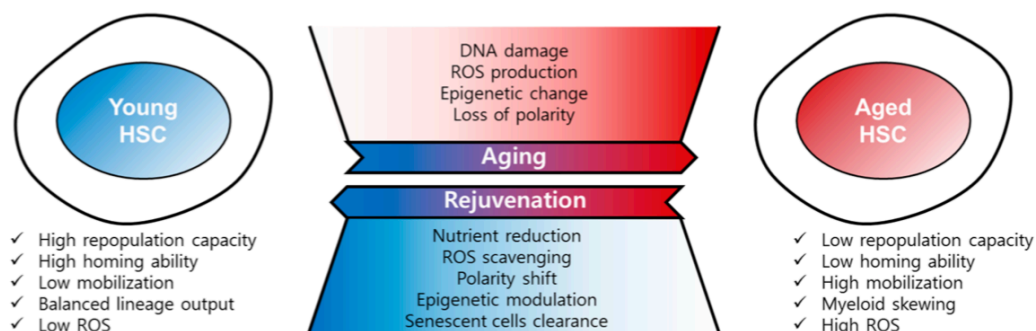


Figure 11: Regulation of HSC aging and rejuvenation. Aged HSCs have the hallmarks of low repopulation capacity, low homing ability, high mobility, myeloid skewing, and high ROS, among others. HSC aging is driven by DNA damage, ROS scavenging, polarity shift, epigenetic modulation, and senescent cells clearance (Lee 2019).

Many mouse transplantation experiments have proven that this is the case. For instance, HSC harvested from aged donor mice and transplanted into young recipients have a much lower self-renewal capacity compared to HSCs from young donor mice (Dykstra et al. 2011). At the single-cell level, young HSCs are capable of producing more mature hematopoietic cells and are in general functionally superior to old HSCs in all respects (Dykstra et al. 2011; Verovskaya et al. 2013). A recent study by Bernitz et al. has shown that the most primitive of LT-HSCs undergoes 5 divisions throughout its lifetime and that the age-dependent phenotype of HSCs only emerges after said 5 divisions (Bernitz et al. 2016).

The fact that old HSCs remain functionally impaired after transplantation into young recipients suggests that HSC aging is driven mainly by cell-intrinsic mechanisms. However the hematopoietic niche and microenvironment-derived cell-extrinsic factors also play important roles in HSC aging (Latchney and Calvi 2017).

Multiple studies have reported that aging, in human and mice, leads to a myeloid-skewed hematopoiesis, which has been associated with many diseases such as both myeloid and lymphoid leukemias, anemia, declining adaptive immunity, and increased susceptibility to viral infection (Warren and Rossi 2009; Kuranda et al. 2011; Geiger et al. 2013). A 2-10 fold increase in phenotypic HSCs in the bone marrow has also been observed with age (De Haan et al. 1997; Rossi et al. 2005). This phenomenon could be explained as a compensatory mechanism for the loss of function that occurs with age, or by the fact that old HSCs are less quiescent than young HSCs and therefore undergo more cell divisions, or both (Pang et al. 2011; Geiger et al. 2013).

b. Mechanism of HSC aging

Several cell intrinsic factors, such as DNA damage, reactive oxygen species (ROS), changes in cell polarity, and epigenetic modifications, have been reported to contribute to the age-associated loss of function of HSCs, and are likely to be interconnected (De Haan and Lazare 2018).

i. DNA damage

The most irreversible cause of HSC aging is the accumulation of DNA damage (De Haan and Lazare 2018). In somatic cells, loss of genomic integrity compromises cellular viability and threatens information passage between parent and daughter cells (Jackson and Bartek 2009). Because they are long-lived, accumulating DNA damage is a greater risk for HSCs (Naka and Hirao 2011). Intrinsic factors that may induce DNA damage in HSC include an excessive amount of ROS, a byproduct of metabolism that acts as a potential source of chronic, persistent DNA damage in all cells; replication errors; and programmed double-stranded breaks (DSB) as during V-D-J and class-switch recombination in B cell development (Lombard et al. 2005). Extrinsic DNA

damage to HSC may be caused by ionizing radiation (IR) or genotoxic drugs such as those used in chemotherapy, among others (Lombard et al. 2005).

DNA damage can result in immediate termination of transcription and replication; mutagenesis, chromosomal aberrations, and cellular cytotoxicity. Several studies have isolated HSCs and progenitors from both humans and mice, and have found that older subjects exhibit a higher number of γ -H2AX foci, a surrogate marker thought to be indicative of DNA damage (Rossi, Bryder, et al. 2007; Beerman et al. 2014; Moehrle et al. 2015). Human CD34+ progenitors also accumulate DSBs during the normal aging process (Rube et al. 2011).

DDR is involved in cell-cycle regulation, cell death, senescence, transcriptional regulation, and chromatin remodeling (Li et al. 2016). Mouse models deficient in DNA repair factors, exhibit premature aging features, supporting the suggestion that defects in DNA repair accelerate aging (Vogel et al. 1999; de Boer 2002; Friedberg and Meira 2003). Several studies have shown that HSCs from mice deficient in nucleotide excision repair, telomere maintenance, and NHEJ, did exhibit not deplete stem cell reserves with age, but instead stem cell functional capacity was severely affected under conditions of stress, leading to loss of reconstitution and proliferative potential, diminished self-renewal, increased apoptosis and, ultimately, functional exhaustion (Nijnik et al. 2007; Rossi, Bryder, et al. 2007; Rube et al. 2011).

Similarly, humans suffering from progeroid syndromes exhibit phenotypes of premature aging as a results of faulty DNA repair. One of these is Werner's syndrome (WS), which occurs due to an inactivating mutation in the WRN gene, important for replication, repair and transcription (Goto 1997). WS patients develop an aging phenotype characterized by atrophic skin, thin gray hair, osteoporosis, type II diabetes, cataracts, arteriosclerosis, and cancer two to three decades prematurely (Goto 1997).

Taken together, these studies strongly suggest that appropriate functioning of DNA repair mechanisms is essential for HSC maintenance, and is an important tool to prevent functional decline due to aging (Lee et al. 2019).

ii. Reactive oxygen species (ROS)

HSCs reside under hypoxic conditions in the hematopoietic niche in order to ensure their protection from oxidative stress and preserve their self-renewal functions (Lee et al. 2018). Given their quiescent state, HSCs have low metabolic activity and therefore generate low levels of ROS. Despite this, ROS levels accumulate with age and can lead to ROS-induced oxidative stress in HSC, resulting in increased proliferation, differentiation, migration and apoptosis (Lee et al. 2019). Transplantation experiments showed that HSCs harvested from the osteoblastic niche where they are exposed to very low levels of ROS, had higher self-renewal capacities than HSCs harvested from

FOXO-deficient mice, which have high levels of ROS (Jang and Sharkis 2007). Additionally, reducing mitochondrial stress in aged HSCs can reverse their loss of function (Mohrin et al. 2015).

HSCs possess several mechanisms to regulate their amounts of ROS. The Forkhead O (FOXO) subfamily of transcription factors, as well as the Hypoxia-inducible factor 1 α (HIF-1 α) both regulate ROS in HSCs. For instance deletion of HIF-1 α , which is usually highly expressed in HSCs and is responsible for switching HSC metabolism from mitochondrial respiration to glycolysis, induces ROS and hinders long-term reconstitution capacity (Wheaton and Chandel 2011; Lee et al. 2018) .

Mitochondrial stress can play an important role in the maintenance of HSCs. In fact, Mohrin et al. have shown that reducing mitochondrial stress via the upregulation of SIRT7 can reverse loss of stem cell functioning (Mohrin et al. 2015). Nevertheless, the molecular causes of age-dependent metabolic remain unclear (De Haan and Lazare 2018).

iii. Changes in polarity

A cell is said to be polarized when organelles, proteins, mRNAs, and miRNAs inside it are distributed and maintained in a non-symmetrical organization (Florian and Geiger 2010). The small RhoGTPase Cdc42 (Cdc42) oscillates between an active state (GTP-bound) and an inactivate state (GDP-bound) and regulates actin and tubulin organization, cell-cell and cell-extracellular matrix adhesion, as well as cell polarity indicated by a particular distribution of the histone mark H4K16ac (Florian and Geiger 2010; Florian 2012). Florian et al. showed that Cdc42 is a new marker of HSC aging. GTP-bound active Cdc42 was found increased in aged HSCs and correlated with a loss of polarity (Florian 2012). Constitutive activation of Cdc42 led to premature HSC aging and induced the depolarization of Cdc42 itself, of tubulin, and of H4K16ac. On the contrary, inhibition of Cdc42 was found to restore polarity in aged HSCs and improve their functioning post-transplant (Florian 2012). Grigoryan et al. have shown increased activation of Cdc42 in aged HSCs induced repression of the nuclear envelope protein Lamin A/C and correlates with alteration in chromosomal architecture, nuclear shape and volume, and distribution of H4K16ac (Grigoryan et al. 2018). They also showed that these changes were reversible by decreasing Cdc42 activity, effectively reverting alterations of the epigenetic architecture in aged HSCs (Grigoryan et al. 2018). These results suggest that at least some aspects of HSC aging process seem to be reversible to a certain extent.

c. Irradiation and aging of the hematopoietic system

The impact of ionizing radiation (IR) on longevity was thoroughly studied in the late 1940s and all through the 1960s following the drop of the atomic bombs in Hiroshima and Nagasaki. Upton et al. reported accelerated aging and shortened life span in mice

that received a single large and non-lethal dose of gamma IR (Upton et al. 1960). However, the mechanisms driving the accelerated aging features observed post IR remained unknown for a long time thereafter. Early research suggested that effects of IR, contrary to aging, appeared to be restricted to genetic damage affecting dividing cells and were almost always confined to the development of neoplasms (Richardson 2009).

IR-mediated aging was initially associated to ROS-mediated damage, DSBs, and apoptosis, leading to deleterious and cumulative damage to DNA, lipids and proteins (Harman 1956). Exposure to total body ionizing radiation (TBI), like in radiotherapy, affects HSC integrity and results in the accumulation of DNA damage, loss of self-renewal ability, and myeloid-skewed differentiation. These alterations are also characteristic of preleukemic syndromes and found in aging subjects. Detection of clonal hematopoiesis after chemotherapy or radiotherapy for a nonmyeloid neoplasm has also been reported (Gillis et al. 2017). As a consequence, TBI is also associated with the development of myelodysplastic syndromes (MDS) and secondary acute myeloid leukemias (Little et al. 1999).

Several studies on atomic bomb survivors and childhood cancer survivors , have reported an enhanced inflammatory state and premature aging features as a consequence of exposition to IR (Neriishi et al. 2001; Hayashi et al. 2003; Oeffinger et al. 2006; Hsu et al. 2010; Ness et al. 2013). In fact, IR exposure is now associated to many age-associated chronic diseases such as coronary disease, stroke, digestive diseases, and respiratory diseases and frailty(Richardson 2009). Chronic low-grade inflammation is considered one of the hallmarks of aging and age-associated diseases, such as cancer and cardiovascular disease (Bektas et al. 2018). A recent study by Yoshida et al. reported an association between radiation exposure in atomic bomb survivors and monocytosis (Yoshida et al. 2019). Given the “short” lifespan of monocytes and their precursors, and the fact that many years have passed since radiation exposure, LT-HSCs are pointed at as the main culprits responsible for radiation-associated monocyte increase (Yoshida et al. 2019).

d. Aging and epigenetics

In the same way as mutations accumulate throughout a person’s lifetime, epigenetic alterations have been reported to increase upon aging. Analysis of DNA methylation and histone acetylation levels performed on monozygotic twins (aged 3-74 years old) revealed that epigenetic difference between twins was significantly higher in older twins than in younger ones (Fraga et al. 2005). This suggests that, over the course of a lifetime, our epigenetic landscape diverges from the original one.

Age-related loss of HSC function has been ascribed to telomere shortening, accumulation of DNA damage, and variations in gene expression. However, the

drifting epigenetic landscape of HSCs over time is also responsible for much of the age-related dysfunction of old HSCs.

Chambers et al. carried out comparative analysis between young and old HSCs and showed that genes associated with inflammation and stress response were upregulated, whereas genes involved in DNA damage repair and chromatin silencing were downregulated with age (Chambers et al. 2007). Several studies have also shown that genes involved in mediating high-order chromatin structures, chromatin remodeling and epigenetic regulation are deregulated in HSCs during aging (Rossi et al. 2005; Chambers et al. 2007).

In 2014, Sun et al. carried out a comprehensive genomic study comparing highly purified young and old HSCs. They observed that with age, there is a decreased expression of epigenetic regulators such as DNA and histone methyltransferases (Dnmt3a and Ezh2), and DNA demethylation proteins TET1 and TET3. In line with that result, they also observed reduced 5-hmC with age and a concomitant increase in DNA methylation (D. Sun et al. 2014). Aging-associated changes in histone marks included an increase of the activating H3K4me3 at genes associated with stem cell self-renewal and loss of differentiation capacity, suggesting that H3K4me3 changes may be linked to the functional changes that occur in aged HSCs (D. Sun et al. 2014). As was previously described in section 4, Djeghloul et al. have also observed a global loss of H3K9me3 in murine and human HSCs with age that impacts B lymphoid differentiation (Djeghloul et al. 2016).

Beerman et al. performed DNA methylome analysis of HSCs during aging and reported site-specific alterations of DNA methylation that occur at genomic regions associated with hematopoietic lineage potential and selectively target genes expressed in downstream progenitor and effector cells (Beerman et al. 2013). Recently, Adelman et al. have shown that human HSCs undergo age-associated epigenetic redistribution of DNA methylation and reductions in the activating H3K27ac, H3K4me1, and H3K4me3 (Adelman et al. 2019). They suggest that this reprogramming globally targets developmental and oncogenic pathways that are found similarly altered in AML patients of all ages and that encompass loss of active enhancers, promoters, and the deregulation of several epigenetic modifiers (Adelman et al. 2019).

All these studies depict several global epigenetic changes that occur in HSCs upon aging and that may result in altered transcriptional profiles and, consequently, in impaired HSC function. This can be exemplified by clonal hematopoiesis in the elderly, in which the three most commonly mutated genes are the epigenetic regulators DNMT3a, TET2, and ASXL1, and which is associated with an increased risk of developing hematologic malignancies and age-associated diseases such as atherosclerosis.

INTRODUCTION

Part II

Retroelements

Retrotransposable Elements

1) Retrotransposable elements throughout (modern) history

A large fraction of eukaryotic genomes is made up of transposable elements (TEs). First discovered by Barbara McClintock in the 1950s in maize DNA, TEs have since been found in genomes of almost all organisms. McClintock observed that some DNA fragments could move from one genomic location to another and that this phenomenon was responsible for the variegated color pattern of maize kernels (McClintock 1956). Initially, TEs were thought of as 'junk' DNA that, in spite of representing a high proportion of genomes, had no evident cellular or biological function (Muñoz-Lopez et al. 2016). In the 1980s, Doolittle and Sapienza, described TE DNA as a by-product of natural selection in eukaryotes, with no function other than to ensure their 'survival' in the species (Doolittle and Sapienza 1980). At the same time, Orgel and Crick coined the notion of 'parasitic DNA' due to the repetitive nature of TEs. They proposed that these sequences had the potential to propagate at a faster rate than host genomes, and thus be extremely disruptive (Orgel and Crick 1980).

Eventually, their high number and ubiquitous distribution throughout the genome stimulated the interest of the scientific community about their evolutionary origin and their potential biological functions (Leib-Mösch and Seifarth 1995). By the end of the 80s the notion of retrotransposable elements (RTE) was described as 'repeated DNA sequences with organizations similar to those of retrovirus DNA' (Temin 1985 Nov) and the term 'retrotransposition' referred to the RNA-mediated movement of genetic information from one locus to another (Finnegan 1989; Leib-Mösch and Seifarth 1995).

Over the last decades, TEs have been thoroughly studied and classifications pertaining to their origin, structure, and function have been established. Today, we know that TEs make up at least 50% of the human and mouse genome, and that although most of them are ancient and have lost their functions (infection or retrotransposition), certain subsets still retain activity (Furano 2000; International Human Genome Sequencing Consortium 2001; Mouse Genome Sequencing Consortium 2002; de Koning et al. 2011). It has now become undeniable that TE sequences have had a fundamental role during genome evolution, and still have important implications in the origin and evolution of many genomic disorders (Muñoz-Lopez et al. 2016).

2) Classification & function of transposable elements

Due to their deep evolutionary origins and their continuous diversification, there is a wide variety of TEs. The first classification of TEs was introduced by Finnegan in 1989, and distinguished TEs based on their transposition intermediate (RNA for Class I TEs or RTEs, and DNA for Class II TEs) (Finnegan 1989). Later, these were subdivided into several subclasses, according to their mechanism of chromosomal integration

(Bourque et al. 2018). In 2007, Wicker et al proposed a hierarchical classification system based on Finnegan’s original classification but that also included structural characteristics and mode of replication (Wicker et al. 2007). In particular, he included the notion of autonomous and non-autonomous into the classification, which can be found within both classes of TEs. An autonomous TE is one that possesses the necessary proteins and sequences to be able to transpose by itself. Non-autonomous TEs, on the other hand, do not possess all the necessary machinery to retrotranspose by themselves, and in most cases are mobilized by an autonomous TE (Kajikawa and Okada 2002).

Class I RTEs, also referred to as retrotransposons, mobilize as an RNA-intermediate using a ‘copy-paste’ mechanism. They effectively create new copies of themselves and then use reverse transcription to reintegrate back into the genome. RTEs usually attain a high copy number over a relatively short evolutionary period due to their replicative nature and continuous accumulation (Platt et al. 2018). This introduction will be focused on RTEs, and in particular on LINE-1 elements, which are highly expressed in hematopoietic stem cells and which are the subject of my research project.

On the basis of the presence, or lack thereof, of 100-300bp direct long-terminal repeats (LTR), class I RTEs fall into two major categories: the LTR and non-LTR retroelements.

a) LTR Retroelements

This category combines diverse elements that possess an LTR in their sequence and it is comprised by LTR retroelements and endogenous retroviruses (ERV). LTR elements can range in size up to 10Kb, and their structure is similar to that of retroviruses (Platt et al. 2018) (Figure 12).



Figure 12 : Structure of an LTR retroelement. The structure of LTR retroelements resemble that of exogenous retroviruses, where gag, pol and env proteins are flanked by two long-terminal repeats (Adapted from Muñoz-Lopez, 2016).

i) LTR Retroelements

Autonomous LTR retrotransposons contain at least a *gag* gene that encodes a viral particle coat, and a *pol* gene that encodes a reverse transcriptase, a ribonuclease H, and an integrase, providing the enzymatic machinery allowing them to reverse transcribe and integrate into the host genome (Makałowski et al. 2019). These are flanked by LTRs containing multiple regulatory elements and several open reading frames (ORFs)

encoding proteins necessary for retrotransposition. One of the main differences between LTR retrotransposons and retroviruses is that the former lack envelope genes and genomic components necessary to build a viral capsule (Deininger 2002). Three major families of LTR retrotransposons exist in vertebrates: Ty1-Copia and Ty3-Gypsy LTR retrotransposons, which occur in virtually all eukaryotes, and BEL families, which have only been found in animals. All families of LTR retrotransposons use a similar transposition mechanism (Wicker et al. 2007).

ii) Endogenous Retroviruses (ERVs)

ERVs are believed to be descendants of ancient germ-cell viral infections. They have been found in all vertebrate genomes and constitute about 8-10% of human DNA (Seifarth et al. 2005). The fact that they contain the *env* gene technically gives ERV infectious potential to spread between cells and individuals. However, most of the ERV sequences have undergone several deletions and mutations, rendering them transcriptionally silent and unable to retrotranspose (Gogvadze and Buzdin 2009). In the human genome, human endogenous retroviruses (HERVs) make up nearly 10% of the genome, but they are mostly inactive, despite the fact that they may still be able to produce gag, pol and env proteins (International Human Genome Sequencing Consortium 2001). Murine ERVs make up 8-10% of the genome, but in contrast to human ERVs, they can actively move using a 'copy-paste' mechanism and have a high mutagenic potential. They are represented by several subfamilies such as the intracisternal A particle (IAP), MusD elements, mammalian apparent LTR retrotransposons (MARLs), and non-autonomous early transposons (ETns) (Goodier et al. 2012).

b) Non-LTR retroelements

As their name suggests, non-LTR retroelements do not possess an LTR in their structure. The most prevalent forms of non-LTR are long interspersed nuclear elements (LINE), and short interspersed nuclear elements (SINE). SINEs are non-autonomous elements, and can only transpose using LINE enzymatic machinery (Ohshima et al. 1996).

i) Short interspersed nuclear elements (SINE)

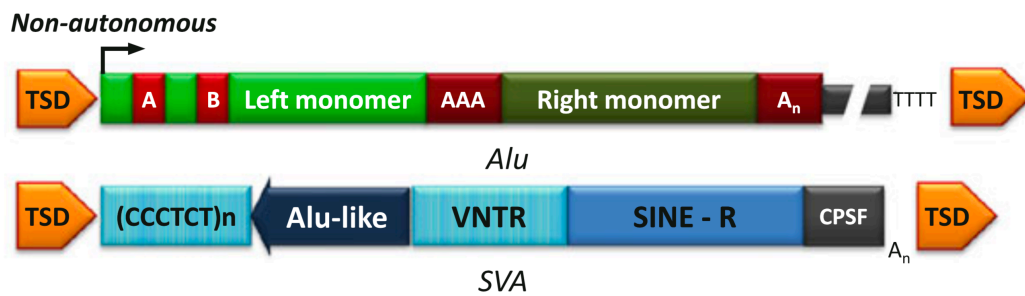


Figure 13 : Structure of Alu and SVA non-LTR retroelements. Alu elements contain an RNA Pol III promoter (A and B in red), left and right 7SL monomers separated by an adenosine-rich region. SVA elements harbor a variable number of tandem repeats, a SINE-r domain and a cleavage polyadenylation-specific factor. (Adapted from Muñoz-Lopez, 2016).

SINEs are small (80-500 bp) RTEs and comprise about 12% of the human genome (Wicker et al. 2007). SINEs possess an internal RNA polymerase III (Pol III) promoter at their 5' end, and retrotranspose by using an RNA Pol III transcript template and LINE1 (L1)-supplied endonuclease and reverse transcriptase for integration into the host genome (Boeke 1997). SINE sequences typically contain a poly(A) tail, or another A-rich stretch at their 3' end (Gogvadze and Buzdin 2009). They originated from the retrotransposition of small RNAs such as tRNA, 7SL RNA and even 5S RNA (Häsler and Strub 2006) (**Figure 13**).

The most abundant members of the SINE family in humans are the Alu repeats, which contain a cleavage site for the AluI restriction enzyme, after which it is named (Houck et al. 1979). Alu, and their murine counterpart B1, are the one of the most abundant RTEs in the human (10%) and mouse (7%) genome respectively (Tsirigos and Rigoutsos 2009). They have emerged over 65 million years ago and following the primate-rodent split, copies of Alu and B1 elements amplified independently in the two genomes, and acquired mutations that rendered them different from each other and from their ancestral 7SL RNA (Tsirigos and Rigoutsos 2009). With over a million copies in each genome, they can be considered as one of the most successful types of RTEs, although their current amplification rate is over 100-fold lower than when they emerged (Roy-Engel 2002). Alu elements have been shown to modulate gene expression at the post-transcriptional level and through insertional mutagenesis, and may be involved in alternative splicing, RNA editing and translation regulation (Häsler and Strub 2006).

A second class of parasitic SINE is the SVA, a hominid-specific element derived from three other repeats (SINE-S, VNTR, and Alu). Their structure and functions are much less well understood and remain difficult to categorize according to existing schemes due to their chimeric nature (Ostertag 2000). SVAs are the youngest RTE family, originating only 25 million years ago, and comprise only about 3000 copies in the

human genome (1%). SVAs exhibit classic hallmarks of L1 retrotransposition and constitute a highly active family of RTEs (Raiz et al. 2012). Recent studies have shown that SVA elements may be capable of transducing genomic sequences upon their mobilization and can also have promoter activity (Xing et al. 2006).

ii) Long Interspersed Nuclear Elements (LINE)

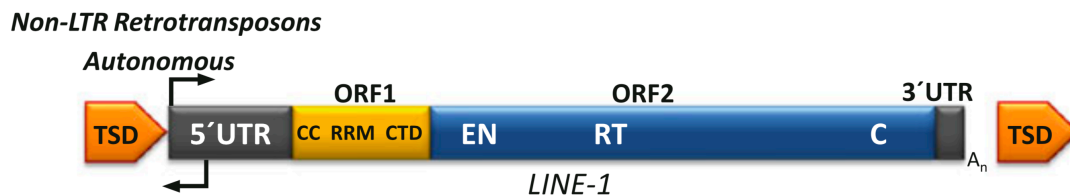


Figure 14 : Structure of LINE-1 elements. LINE-1 elements contain a 5'UTR region that harbors a sense and antisense promoter, two open reading frame proteins (ORF1 and ORF2), a coiled-coil motif, and RNA recognition motif, a carboxyl-terminal domain, a C-rich domain, and a poly-A tail. (Adapted from Muñoz-Lopez, 2016).

LINE-1 (L1) elements are the major class of autonomous retrotransposons and are present in abundance in all mammalian genomes. They comprise about 17-22% of the human genome and 19% of the murine genome (International Human Genome Sequencing Consortium 2001; Mouse Genome Sequencing Consortium 2002). The L1 subfamily is the only one that still remains active in the human genome (Mills et al. 2007). However, L1s mostly exist as 5'-truncated inactive copies, with only about 80-100 elements still capable of retrotransposition (Brouha et al. 2003).

L1 elements usually range in size between 6-7 kb, contain a 5' untranslated region (UTR), two open reading frames (ORFs, ORF1 and ORF2), a 3'UTR and a poly(A) tail. The 5'UTR in L1 elements harbors several transcription factor binding sites and serves as an internal promoter for transcription by RNA Pol II (Figure 14).

ORF1 encodes a 40 kDa RNA-binding protein that contains zinc finger, leucine zipper and coiled-coil motifs and display chaperone activity (Martin et al. 2005). ORF1 phosphorylation is required for L1 retrotransposition (Moran et al. 1996; Goodier et al. 2007; Cook et al. 2015).

The ORF2 is a large 149 kDa protein that contains endonuclease and reverse transcriptase activities, as well as a cysteine-rich domain necessary for retrotransposition (Moran et al. 1996). ORF2 makes a single-stranded nick at a recognized target site in genomic DNA and then the reverse transcriptase uses the nicked DNA to prime reverse transcription of the LINE RNA from the poly-A stretch at their 3' end (Makałowski et al. 2019). This is referred to as target-primed reverse transcription (TPRT). The use of a poly A tract as the priming target is likely to be a critical factor in allowing the nonautonomous elements, such as SINEs, to take

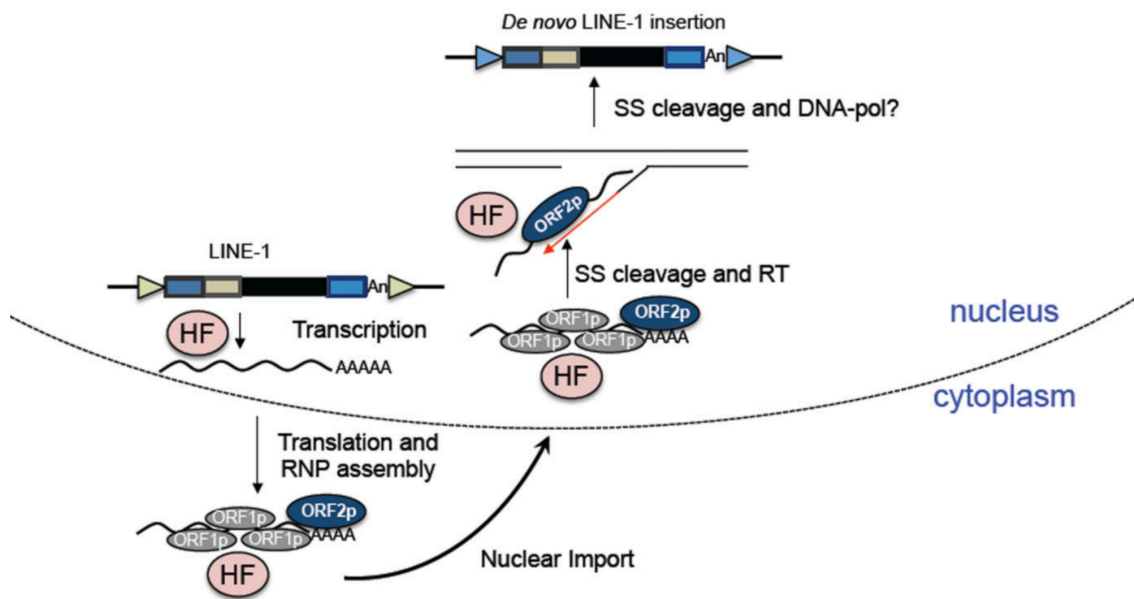


Figure 15 : Retrotransposition cycle of LINE-1. Full-length active L1 RNA is transcribed and transported into the cytoplasm where pORF1 and pORF2 are translated. These proteins bind to encoding mRNA and generate a ribonucleoprotein particle that is imported into the nucleus. Endonuclease activity of ORF2 generates a single-stranded nick in genomic DNA that is used by the reverse transcriptase activity of ORF2 to generate the first strand of cDNA. How second strand synthesis occurs remains unanswered. (Adapted from Garcia-Perez, 2018).

advantage of this L1 mechanism and be able to retrotranspose through the use of SINE A-rich regions to prime reverse transcription by L1 reverse transcriptase (Deininger 2002).

Novel L1 copies can be generated through retrotransposition of an active L1. Bicistronic L1-mRNA is transported into the cytoplasm following translation of several ORF1 proteins and a single molecule of ORF2 (Wei et al. 2001; Dmitriev et al. 2007) (Figure 15). These proteins will bind to the L1 mRNA to form a ribonucleoprotein particle (RNP), which will enter the nucleus and perform TPRT in order to retrotranspose. Following TPRT, cDNA synthesis occurs, although the mechanism by which this occurs which is not yet fully understood. This results in a *de novo* L1 insertion.

In most cases, new L1 insertions are a dead end due to 5' truncation. In addition, given that most insertions serve no function to the host, they accumulate mutations at a neutral rate. For this reason, younger L1 families are less divergent than older ones (Sookdeo et al. 2013). Despite the fact that the total number of L1 elements in the mouse genome is in the order of hundreds of thousands, the number of full length L1s that contain a promoter is estimated to be less than 20 thousand (Sookdeo et al. 2013; Penzkofer et al. 2016).

homologous promoters (Sookdeo et al. 2013). 5'UTRs of L1Mds are characterized by a special tandem structure, made up of individual repeating sequences called monomers (Loeb et al. 1986; Padgett et al. 1988). It is not clear how L1Mds have acquired repeating monomers in their promoters throughout evolution, but it is known that multiple monomers increase the transcription ability of an L1Md (Severynse et al. 1991; DeBerardinis and Kazazian 1999).

The coexistence of multiple active L1Md families is characteristic of L1Md evolution for the last 13 million years of mouse evolution. For instance, overlap in the pairwise divergence of six different L1Md families suggests that, between 1-2.5 million years ago, they were all active in the mouse genome (Zhou and Smith 2019). Sookdeo et al. propose a phylogenetic tree of L1Md evolution. The branching order of the tree is consistent with the age of the families.

Sookdeo reclassified L1Md elements and named according to their promoter types, which is determined by the type of monomers contained in the promoter. For this reason, his nomenclature sometimes differs from other consensus annotations such as the Repbase. While older families carry an Lx promoter, the most evolutionary recent L1Md lineages are the L1Md_A, L1Md_Tf, and the L1Md_Gf characterized by the A and F promoters respectively. Each of these lineages contains three subfamilies, the L1MdA- and the L1MdTf-I, II, and III respectively; and the L1MdGf_I and II (Sookdeo et al. 2013). These 3 monomer types are active in terms of transcription, and may therefore confer L1Mds the ability to retrotranspose (Zhou and Smith 2019). A striking observation from the tree is that families with resembling 5'UTRs do not form monophyletic groups, for instance the L1MdGf_I and II. This suggests that L1 families have adopted novel 5'UTRs, possibly due to recombination events between family members, or from ancient L1 families or from other unknown sources (Sookdeo et al. 2013).

Concerning the structure of L1Md, the protein coding sequence of the ORF2 is the most conserved region of L1. There are very limited amino acid changes, especially in the endonuclease and in the reverse transcriptase domains (Moran et al. 1996). In contrast, the coiled-coil domain of the ORF1 proteins bears the signature of adaptive evolution, possibly as a response to host repression. This adaptive evolution apparently correlates with the replicative success of L1 families (Boissinot and Furano 2001).

4) Impact of RTE expression and retrotransposition in the genome

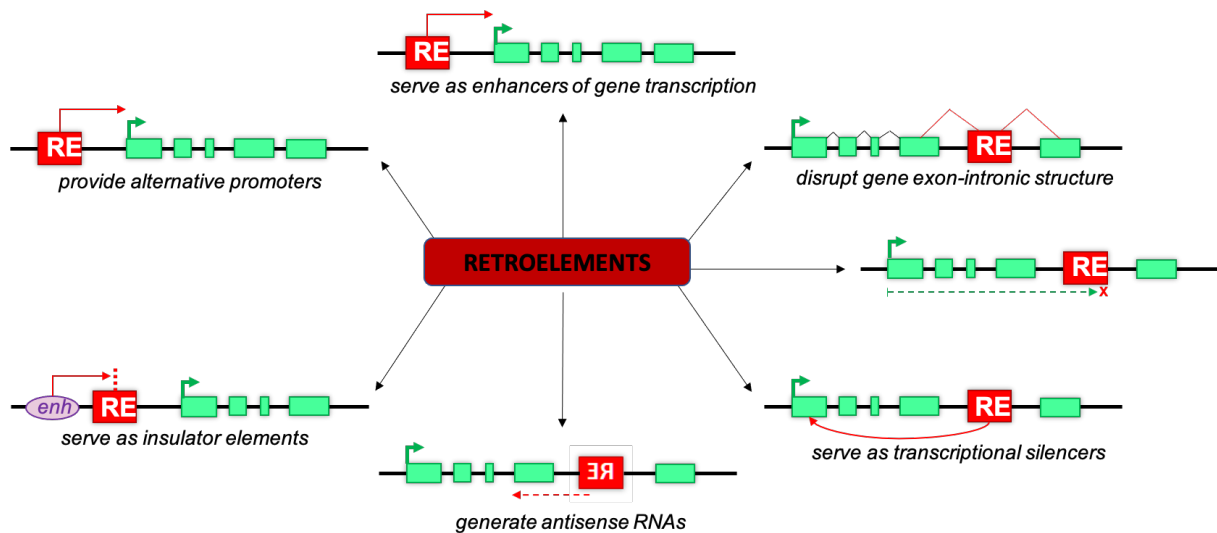


Figure 17 :Different mechanisms of RTE influence on gene transcription and expression. Red boxes represent RTE, green boxes are gene exons, green arrows represent transcription start sites, pink ovals are enhancers. (Adapted from Gogvadze and Buzdin, 2009).

Retrotransposition is a powerful means for genome expansion. Despite this, it is eventually counteracted by the removal of DNA via deletion, so balance between these two processes acts as a major driver in the evolution of genome size in eukaryotes (Bourque et al. 2018). Given that insertion and deletion of RTEs is often faulty, these processes can have an impact on surrounding host sequences. Some of these events occur at a high enough frequency that they result in copious amounts of duplications and reshuffling of host sequences, including genes and their regulatory elements (Bourque et al. 2018).

Apart from rearrangements that occur due to retrotransposition, L1, and RTEs in general, can promote structural variation long after they have lost the ability to retrotranspose (Carvalho and Lupski 2016) (Figure 17). RTEs can impact gene regulation by inserting their own regulatory elements (promoters, cryptic splice sites, terminators, enhancers, and insulators) into new genomic loci. These regulatory elements can in turn disrupt gene expression and structure of host and neighboring genes (Mita and Boeke 2016). These mechanisms of RTE- induced or RTE-enabled structural variation are independent of retrotransposition and have largely contributed to genomic evolution.

a) **Recombination**

Recombination is an essential player in evolution that produces genetic variability by means of pre-existing blocks of biological information (Makalowski 2000). Owing to their high copy number and sequence similarity, RTEs are the perfect substrates for

ectopic recombination. Recombination events can take place between the highly homologous regions of dispersed RTEs at distant genomic loci and result in major deletions, duplications and inversions (Deininger et al. 2003; Han et al. 2008; Ade et al. 2013). The possibility of this occurring depends on the number of homologous sequences and their length (Boissinot et al. 2006; Song and Boissinot 2007). The products of this illegitimate recombination may be deleterious, advantageous, or have no effect.

A lot of human diseases originate from recombination between RTEs. For instance, glycogen storage disease (Burwinkel and Kilimann 1998), Alport syndrome is due to recombination of L1 elements (Segal et al. 1999), and complete germ cell aplasia is due to HERV-1 recombination (Kamp et al. 2000). Alu elements have also been implicated in nearly 50 disease by causing recombination events (Belancio et al. 2008).

b) Transduction of 3' flanking sequences

L1 elements have a relatively weak polyadenylation signal, which may sometimes allow their RNA polymerase to continue onto other polyadenylation sites downstream, resulting in the transduction of sequences past the 3' end of the L1 sequence (Gogvadze and Buzdin 2009). L1-mediated transduction occurs through an RNA intermediate, does not require homologous recombination, and allows dispersal of non-L1 sequences to new genomic sites (Moran 1999). Shuffled sequences could be promoters, enhancers, or exons, and their dispersal could lead to the creation of new genes or alter the expression of existing genes. Furthermore, because L1 retrotranspositions are often 5' truncated, some transductions may lack an L1 sequence. Approximately 20% of all L1 inserts contain transduced DNA at their 3' ends, varying from a few nucleotides to stretches of over 1Kb. However, when taken together, these bits of transduced DNA make up 0,6-1% of the human genome (Gogvadze and Buzdin 2009).

c) Chimeric retrogene formation

Reverse transcriptase has the ability to change templates during cDNA synthesis, which is a well-known feature of retroviruses. The reverse transcriptase can jump from one place to another of the template, which is necessary for the synthesis of retroviral LTRs (Temin 1993).

Template switches can also occur during L1 reverse transcription. Bi- and tri- partite chimeric retrogenes have been identified in mammal and fungal genomes (Buzdin et al. 2003; Gogvadze et al. 2007). Chimeric retrogenes are composed of DNA copies of different cellular transcripts that are either fused together or, more frequently, fused to the 3'-end of an L1 (Gogvadze and Buzdin 2009). Cellular transcripts found in these chimeric elements correspond to mRNAs, rRNAs, small nuclear RNAs, 7SL RNAs and

Alu RTEs. Both parts of the chimeric retrogene are fused in the same transcriptional orientations, have a poly-A tail on their 3' end and are flanked by short direct repeats (Gogvadze and Buzdin 2009). The presence of chimeric elements in evolutionary distinct organisms suggests that template switching during L1 reverse transcription is a mechanism of genomic rearrangement that is conserved amongst species (Gogvadze and Buzdin 2009).

d) Cis- regulation of gene activity

The most prevalent type of reported cis-regulation by RTEs is their function as alternate promoters. ERVs contain cis-regulatory sequences and RNA Pol II promoters in duplicate within each of their flanking LTRs; L1 elements contain an internal Pol II promoter in their 5'UTR, as well as an antisense promoter also in the 5'UTR but downstream of the sense promoter; and SINE elements are transcribed by Pol III and thus contain internal sequence motifs to recruit it (Chuong et al. 2017). They can affect the level of gene transcription, drive the expression of novel transcripts that encode part of the coding region, modify the tissue specificity of its expression, and possibly contributing to disease risk by modulating the expression of adjacent genes (Garcia-Perez et al. 2016; Chuong et al. 2017). Truncated or extended proteins can also arise as a by-product of these processes, potentially allowing the host to acquire new functions (Garcia-Perez et al. 2016). In some cases, RTEs are actually the only known gene promoter. For instance, the liver-specific *BAAT* gene responsible for hypercholanemia has an ancient LTR as promoter in human, but not in mouse (Carlton et al. 2003).

Whole-genome analyses has shown that nearly 25% of all human promoters contain RTEs in their sequence (van de Lagemaat et al. 2003). 7-10% of characterized transcription factor binding sites (TFBS) were shown to be derived from RTEs (Polavarapu et al. 2008). TFBS that originate from RTEs evolve faster than non-repetitive TFBS, and are likely to contribute to the evolutionary process by driving species-specific regulation of gene expression (Gogvadze and Buzdin 2009).

In addition to their promoter activity, TFBS within RTEs can act as host gene enhancers in specific tissues or developmental contexts (Garcia-Perez et al. 2016). Conserved non-exonic RTEs tend to cluster within 1Mb of developmental genes and transcriptional regulators in the human genome, suggesting a common mechanism for RTEs to impact development (Lowe et al. 2007).

An example of RTEs acting as tissue-specific enhancers can be found in the innate immune system, where they can act to mediate IFN signaling in response to infection. Specifically, RTEs are enriched close to interferon stimulated genes (ISGs) (Chuong et al. 2016). One of the most enriched of these RTEs, the MER41B ERV, contains TFBS for STAT1, which mediates a type 2 IFN response (Chuong et al. 2016). The ERV RLTR30B in mice also contains STAT1 TFBS and it is enriched near functionally annotated

immunity genes (Chuong et al. 2016). This suggests that the ancestors of these ERVs potentially also contained STAT1 TFBS in order to exploit the hosts immune system to promote their own transcription (Chuong et al. 2016).

e) Alternative splicing

Alterations of splicing broaden the diversity of the protein repertoire that can be produced by a genome (Garcia-Perez et al. 2016). Retrotransposition can affect splicing via exon skipping, alternative donor or acceptor splice sites, intron retention, and exonization (Huda and Bushel 2013; Ayarpadikannan et al. 2015). L1s have been shown to contain numerous functional splice donor and acceptor sites. Internal splice sites can yield various processed L1 transcripts as well as hybrid transcripts between the L1 and its host gene (Gogvadze and Buzdin 2009). L1 mRNA processing through splicing that renders the splice product inactive has been proposed as a host mechanism to protect against excessive L1 transcription that could have important consequences for the cell (Thornburg et al. 2006).

f) Alternative transcription termination

It has recently been shown that, on top of the poly-(A) tail in their 3' end, L1s also possess an additional transcription termination site in the 3' of their ORF2 in an antisense orientation (Wheeler et al. 2005). This means that, regardless of the genomic orientation of the L1 in a gene, they have the potential to terminate transcription and produce a truncated RNA. It is estimated that about 8% of all poly(A) sites are associated with RTEs (Lee et al. 2008). Human poly(A) sites that are not conserved in mouse were shown to be much more associated to RTEs than conserved ones, suggesting that RTEs may be involved in the creation or modulation of poly(A) sites throughout evolution (Gogvadze and Buzdin 2009).

5) Regulation of retroelements

RTE activity and mobilization comes at a high risk to the host genome, thereby setting up an arms race between RTEs and host defense mechanisms (Jacobs et al. 2014; Friedli and Trono 2015). To persist in evolution, RTEs must attain a carefully calibrated balance between expression and repression; one that ensures that there is enough expression to promote amplification, but not too much that it leads to fitness disadvantage to the host (Bourque et al. 2018). RTE activity is thus tightly regulated in mammals to control the number of insertions that accumulate in the genome. Mechanisms that restrict RTE expression and mobilization are important in somatic cells, germ cells and pluripotent stem cells in early embryos that act as germ cell precursors. RTE insertions in the latter can potentially be passed onto progeny and increase RTE copy number throughout evolution (Crichton et al. 2014).

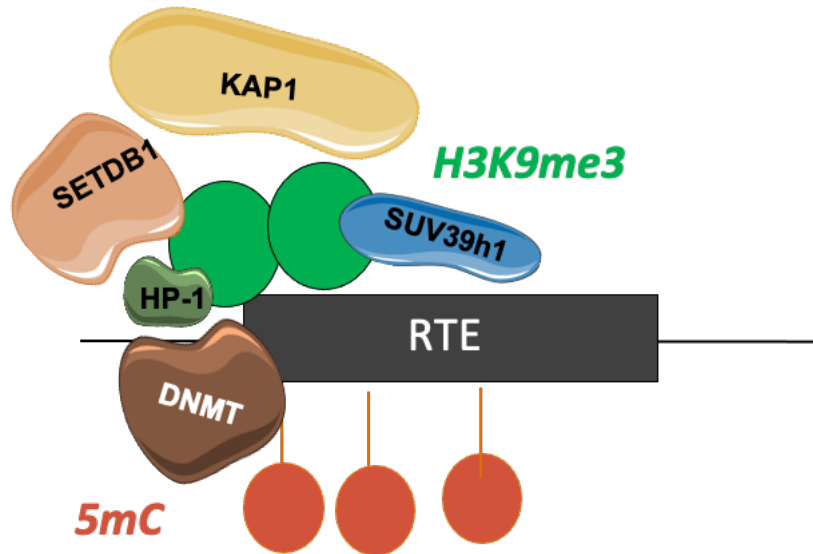


Figure 18 : Different mechanisms of RTE silencing. Different kinds of RTEs are silenced by different mechanisms, such as DNA methylation (orange) mediated by DNMT enzymes, or H3K9me3 (green), whose deposition at RTEs can be mediated through different methyltransferases (SUV39H1, SETDB1) and co-repressors such as KAP1.

DNA methylation, histone modifications, and RNA interference are some of the mechanisms that cells have developed to control RTEs (Gogvadze et al. 2007) (**Figure 18**). The epigenetic mechanisms that control RTEs may also be “moved” during the retrotransposition process, or spread onto flanking DNA sequences and thus may affect nearby gene expression and the epigenetic control of retrotransposition targeted loci (Fedoroff 2012).

a) DNA methylation

DNA methylation regulates both L1s and ERVs (Bourc’his and Bestor 2004). It is a complex process controlled by several interacting factors, and has been shown to be important to mammalian male germline development and in somatic cells (Goodier 2016; Mita and Boeke 2016). In fact, hypomethylation waves throughout embryonic development have been associated with high rates of retrotransposition (Saleh et al. 2019).

In mammals, DNA methylation occurs at cytosine residues in the context of CpG dinucleotides. Most of the genome, including RTE sequences, is heavily methylated. DNA methylation is mediated by three major enzymes that may work in cooperation. DNMT1 is the most abundant methyltransferase in mammals and is responsible for maintenance DNA methylation. It uses the parental strand as a methylation template to modify the newly synthesized daughter strand (Crichton et al. 2014). DNMT3A/B are involved in *de novo* methylation of unmethylated CpGs (Goodier 2016). DNMT3L, a member of the DNMT3 family, also exists and has been reported to be necessary for maternal methylation imprinting, possibly by interacting with DNMT3a and/or

DNMT3b (Hata et al. 2002). DNMT3L can also enhance the DNA methylation activity of DNMT3a and DNMT3b, but not that of DNMT1 (Suetake et al. 2004). These two enzymes have non-overlapping roles at certain genomic sequences. For instance, IAP elements have been found slightly hypomethylated in *Dnmt3b*^{-/-} embryos, but not in *Dnmt3a*^{-/-} embryos; although IAP hypomethylation was more pronounced in double knockout embryos, suggesting a degree of redundancy between the two enzymes at these loci (Okano et al. 1999; Crichton et al. 2014). Studies have also shown that embryonic stem cells use DNA methylation-independent mechanisms to silence RTEs. Knocking-out the three major DNA methylating enzymes in these cells did not yield a significant expression? repression of RTEs, except for in the case of IAP elements (Matsui et al. 2010; Karimi et al. 2011).

50% of the CpG islands in the human genome are located in repeats, of these 25% of them within Alu elements and 12% within L1s (Rollins et al. 2006; Xie et al. 2009). CpG islands in gene promoters are usually hypomethylated if the gene to allow gene expression. In contrast, L1 5'UTR are heavily methylated in somatic cells to suppress L1 expression (Crowther et al. 1991; Hata and Sakaki 1997). Hypomethylated CpG islands introduced following retrotransposition influence hypomethylation of nearby CpG islands, which is known as 'slopping shores'. This phenomenon can influence nearby gene expression of flanking regions in somatic cells (Mita and Boeke 2016). The opposite however has also been shown, for instance Salvador-Palomeque et al. have shown that following *de novo* L1 insertion in pluripotent stem cells, the L1 is rapidly recognized and repressed through dynamic DNA methylation changes (Salvador-Palomeque et al. 2019). Interestingly, younger ERVs, like the ERVK family, have a higher CpG density than older ERV families, suggesting the RTE age could play a role in methylation status of RTEs (Ohtani et al. 2018).

b) Histone modifications

Histone modifications also play an essential role in the RTE transcriptional repression. In particular, histone H3 tri-methylation at Lysine 9 (H3K9me3) has been pointed as the major player in RTE repression in mouse embryonic stem cells (Matsui et al. 2010; Karimi et al. 2011). H3K9me3 can be deposited at RTE sequences either by SETDB1 or by the SUV39H1 and SUV39H2 methyltransferases. H3K9me3 is recognized and bound by the heterochromatin protein 1 (HP1) family and coordinates heterochromatin compaction.

The SUV39H histone methyltransferases has been shown to be recruited specifically at evolutionary young LTR RTEs and at young, intact, and full-length L1 elements in ESC (Bulut-Karslioglu et al. 2014). Age-associated loss of SUV39H1 in HSCs has been correlated to an increase in expression of L1 elements (Djeghloul et al. 2016). In mice, deletion of *Suv39h1/2* resulted in a decrease of both H3K9me3 and DNA methylation at major satellite repeats in pericentric heterochromatin, whereas deletion of *Dnmt3a*

and *Dnmt3b* had no effect on H3K9me3 in the same area (Lehnertz et al. 2003). These findings suggest that SUV39H can act independently and upstream of DNA methylation to repress repetitive sequences in certain genomic contexts (Karimi et al. 2011). Interestingly, once SUV39H1 binds to H3K3me3, its N-terminus chromo domain facilitates recruitment of other methyltransferases, in particular DNMT3b, and leads to the establishment of heterochromatin and the local characteristic spreading of H3K9me3 and DNA methylation (Monaghan et al. 2019).

H3K9me3 can also be deposited at RTE loci by SETDB1. In mouse and human embryonic stem cells (ESCs), Krüppel-associated box zinc-finger proteins (KRAB-ZFP) bind to specific DNA sequences through an array of zinc fingers. There, they recruit the co-factor KRAB-associated Protein 1 (KAP1 or TRIM28) and mediate the docking of KAP1 at RTEs (Karimi et al. 2011). KAP1 serves as a scaffold for a heterochromatin-inducing complex involving SETDB1, the histone deacetylase-containing NuRD complex, and HP1 and triggers the deposition of H3K9me3 at the RTE sequence (Schultz et al. 2002). SETDB1-mediated H3K9me3 silencing in ESCs has been associated particularly with ERV repression (Matsui et al. 2010). This suggests that different H3K9me3-based mechanisms can be utilized to silence different RTE subfamilies (Walter et al. 2016). KAP1-mediated ERV silencing rapidly induces the recruitment of *de novo* DNA methyltransferases that methylate CpG dinucleotides, thereby establishing a more permanent silencing mechanism that does not depend on specific DNA sequences like KRAB-ZFPs (Rowe and Trono 2011). It has been recently shown that KAP1-mediated ERV silencing also takes place in most adult tissues (Turelli et al. 2014; Fasching et al. 2015).

Robbez-Masson et al carried out a study to determine if the human silencing (HUSH) complex, which is composed by TASOR, MPP8 and periphilin 1 and is recruited to genomic loci rich in H3K9me3, was necessary to repress RTEs and whether it collaborated with KAP1 to do so (Robbez-Masson and Rowe 2015). They showed that KAP1 and TASOR interact at both ERVs and at evolutionary young L1 elements (<5 million years), which reside in "leaky" heterochromatin areas (Robbez-Masson and Rowe 2015). However, TASOR is mainly required to induce H3K9me3-mediated silencing at young L1s, whereas KAP1 is necessary for both. Liu et al. have also shown that the HUSH complex and the MORC2 protein can selectively bind evolutionarily young, full-length L1s located within transcriptionally permissive euchromatic environments, and promote deposition of H3K9me3 for transcriptional silencing. Notably, these silencing events often occur within introns of transcriptionally active genes, and lead to the downregulation of host gene expression in a HUSH-, MORC2-, and L1-dependent manner (N. Liu et al. 2018). These results contribute to the notion that the chosen mechanism of epigenetic silencing of RTEs depends greatly on the nature and the evolutionary status of each RTE.

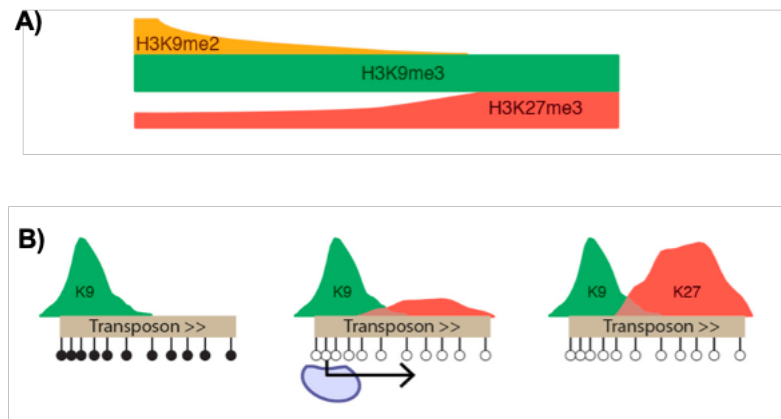


Figure 19: A) Model for the acquisition of H3K27me3 following genome-wide demethylation conditions at L1 and ERV. B) Upon loss of DNA methylation, H3K27me3 appears at GC-rich regions lacking H3K9me3. Line-1 elements exhibit H3K9me3 enrichment at their 5' end and gain H3K27me3 at their 3' end. (Adapted from Walter 2016)

Other repressive histone modifications, notably H3K9me2 and H3K27me3, have also been associated with RTE silencing (Karimi et al. 2011). H3K9me2, *via* its methyltransferases G9a (EHMT1) and GLP (EHMT2), has been shown to repress a class L ERV (MERV1) in mouse ESC; and polycomb-mediated H3K27me3 controls endogenous murine leukemia virus (MLV) elements (Walter et al. 2016). By recreating a global demethylation environment in a culture medium using 2 small kinase inhibitors and vitamin C in ESCs, Walter et al. showed that upon loss of DNA methylation, various RTE families were efficiently re-silenced. Whereas under demethylating conditions H3K9me3 remained stable, H3K9me2 globally disappeared and H3K27me3 started accumulating at L1 and ERVs (Walter et al. 2016) (**Figure 19**). H3K9me3 and H3K27me3 do not usually occur concomitantly. However, Walter et al show that this can occur in L1s and young ERVs, where H3K9me3 tends to occupy the 5' end and H3K27me3 the 3' end (Walter et al. 2016). Their results suggest that the pattern of H3K27me3 distribution corresponds to a compensation for the lack of DNA methylation, and suggest that polycomb-mediated silencing can be involved in RTE regulation to ensure longstanding genome stability (Walter et al. 2016).

Ohtani et al. showed that in several cancer cell lines such as HL60 cells, treatment with a DNA methylation inhibitor upregulated ERVs that were considerably younger than those upregulated by the knock-down of H3K9me3 or H3K27me3 methyltransferases (Ohtani et al. 2018). Their results suggest that the primary mechanism of suppression for young ERVs is DNA methylation, whereas intermediate and older ERVs are silenced via histone modifications. This puts forward the idea of an “epigenetic switch” from DNA methylation to histone-mediated silencing throughout host genome evolution (Ohtani et al. 2018). Interestingly, an increase of H3K9me2/3 was observed at LTRs following treatment with a methylation inhibitor, suggesting that “epigenetic switching” can also occur to ensure silencing after loss of DNA methylation and invoking a synergy between the two silencing mechanisms (Ohtani et al. 2018).

c) RNA interference

The existence of several different types of RNA interference (RNAi) pathways to repress RTEs suggests RNAi control was “exaptated” and rewired by the host genome in response to RTE activity (Mita and Boeke 2016).

Piwi-interacting (pi-RNAs) can be transcribed from RTE clusters and then exported into the cytoplasm where they are used as guides to degrade complementary RTE RNA by endonucleolytic processing (Aravin et al. 2008; Munoz-Lopez et al. 2008). Interestingly, in *Drosophila*, pi-RNA-mediated RTE silencing can spread to neighboring DNA and requires SETDB1, suggesting an intertwining of RTE expression and host cell chromatin regulation (Rangan et al. 2011; Lee 2015).

The microprocessor complex, composed by DGCR8 and Drosha, has been shown to recognize RNA domains present in L1, Alu and SVA RNAs and possibly control their expression levels by processing their transcripts (Heras et al. 2013).

DICER can also process sense and anti-sense RNA from L1 promoters and produce repeat-associated small interfering RNAs (rasiRNAs) that can be used by the RISC complex to degrade L1 mRNAs (Ciaudo et al. 2013). Moreover, it has been showed that double-stranded L1 RNA generated *in vitro* can be processed into functional siRNAs by DICER and suppress retrotransposition in cell culture assays (Yang and HH 2006).

6) Retroelements: friend or foe in cancer?

Owing to their “parasitic” reputation, RTEs have long been associated with mutant phenotypes and disease. RTE are well-documented to cause disease mainly *via* insertional mutagenesis and chromosomal rearrangements, however, even RTEs that are fixed in the human genome but encode dormant regulatory sequences, can also contribute to pathogenesis (Chuong et al. 2017). For instance, significantly higher RTE transcript levels have been found in several cancers and other diseases (Kassiotis 2014; Rodic´ et al. 2014). Recent studies suggest that environmental stimuli, such as infection and cellular stress; or biological processes, like aging and senescence, can induce disorganization of the epigenetic marks that repress RTEs in the genome, and thus trigger their spontaneous transcriptional activation (Kuy1 2012; Mourier et al. 2014; Van Meter 2014; Chuong et al. 2016).

Over the last few years many different studies have shown that in early tumorigenesis, derepression of RTEs can facilitate onco-exaptation, retrotransposition, mitotic errors, and sterile inflammation, all of which can severely impact genome integrity (Ishak et al. 2018). In fact, whereas promoter hypermethylation is quite frequent in cancer to silence of genes functioning in pathways such as DNA repair, cell cycle regulation,

promotion of apoptosis or control of key tumor-relevant signaling networks; hypomethylation occurs concomitantly at intergenic regions rich in repetitive sequences (Rauch et al. 2008; Berman et al. 2011). For instance, in epithelial tumors, hypomethylation of replication-competent L1s could explain the higher rate of transposition in tumor tissue compared to matched healthy tissues; and *de novo* L1 insertions are capable of activating oncogenic pathways in hepatocellular carcinoma and colorectal cancer (Lee et al. 2012; Shukla et al. 2013; Scott 2016). Moreover, ectopic activation of cis-regulatory sequences in RTEs can affect the expression of neighboring or host genes and result in the pathogenic activation of certain genes or pathways in some cells (Chuong et al. 2016). Reactivated RTEs can also induce expression of several noncoding RNAs, and although most of these transcripts are likely non-functional, some of them do have oncogenic properties (Chuong et al. 2016).

Through mutagenesis and loss of regulatory mechanisms that control RTE expression, host genomes can domesticate and profit of RTEs as regulators of gene transcription and genome organization (Ishak et al. 2018). This is a process that can be selected for during tumorigenesis, and is known as “onco-exaptation”, where RTEs are derepressed and acquire a fitness advantage over other cells as a result of oncogene expression. This in turn, favors clonal proliferation of cells where the RTE is derepressed and helps perpetuate tumor growth (Babaian et al. 2015). An example of this can be found in the MET oncogene in bladder cancers, where the hypomethylation of an L1 promoter enhances oncogene expression and leads to chimeric transcripts that translate into fusion proteins with oncogenic properties (Wolff et al. 2010). Additionally, cancer cells can also usurp RTEs to drive the deregulation of transcriptional networks and further promote genomic instability (Ishak et al. 2018).

Aging is another factor that can influence the epigenetic mechanisms that drive RTE repression. De Cecco et al. recently showed that in late senescence, L1 derepression, accompanied by a loss of H3K9me3 and H3K27me3, induces a chronic inflammation state mediated by IFN and increases susceptibility to age-associated disorders (De Cecco et al. 2019). It is therefore of paramount importance to characterize the deregulation of epigenetic mechanisms at RTEs, as this can reveal new epigenetic targets to bolster treatment options for cancer and age-associated diseases.

Upon disruption of RTE silencing, host cells employ what is known as the “viral mimicry” response. Transcripts derived from derepressed RTEs generate double-stranded RNAs (dsRNA) that are detected by a cytosolic dsRNA-sensing pathway that triggers an IFN-I response (Chiappinelli et al. 2015) (**Figure 20**). This in turn could lead to apoptosis and sensitization to immune checkpoint therapy (Chiappinelli et al. 2015). The discovery of this phenomenon highlights a potential vulnerability of cancer cells and opens the window to therapeutic strategies that could acutely activate repeats to increase tumor immunogenicity and thereby hinder cancer cell fitness (Cuellar et al. 2017; Ishak et al. 2018).

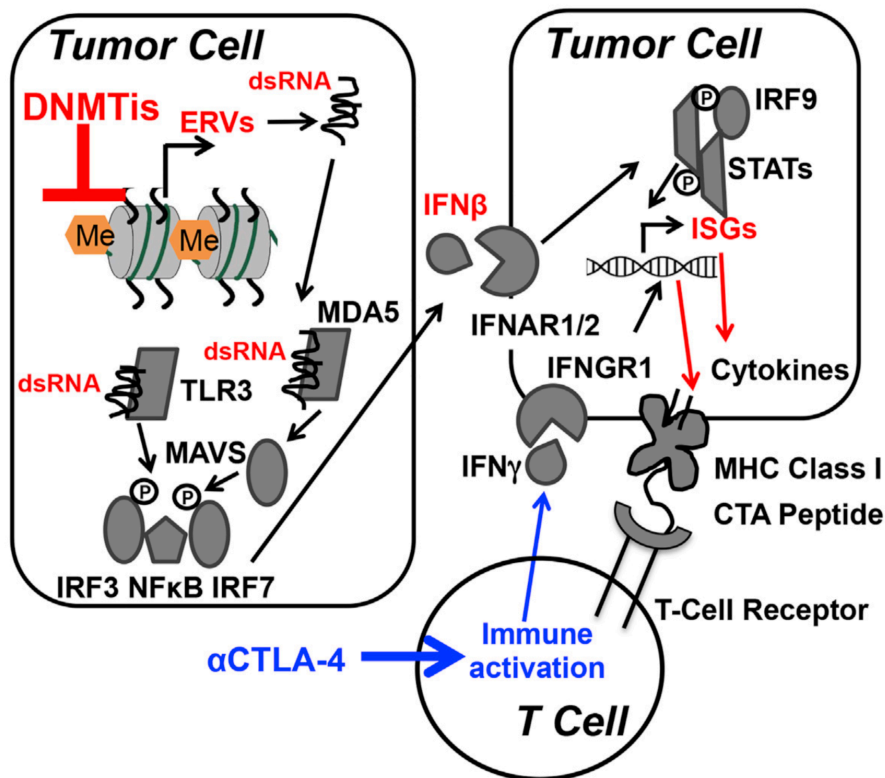


Figure 20 : Viral mimicry as described by Chiappinelli et al. DNMTis upregulate ERVs in tumor cells to induce a growth-inhibiting immune response. High expression of the genes associated with the antiviral response potentiate a response to immune check-point therapy. (Chiappinelli, 2015).

DNA methyltransferase inhibitors (DNMTis), such as 5-aza-2'-deoxycytidine (5-aza-CdR), are effective cancer therapies that have a significant success in the treatment of hematological malignancies such as myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Liu et al. 2016). 5-aza-CdR incorporates into DNA of proliferating cells during the S-phase and inhibits DNA methylation by trapping DNMTs onto the DNA, leading to their proteolysis. Demethylation then occurs naturally as DNA continues to replicate (Egger et al. 2004). Recent studies have suggested that DNMTis can induce viral mimicry in melanoma and colorectal cancer cells (Chiappinelli et al. 2015; Roulois et al. 2015). Nevertheless, primary and secondary resistance to epigenetic therapies is very common, and likely due to the fact that epigenetic mechanisms are reinforced by several positive and negative feedback loops that are capable of ensuring that silencing is maintained (Wrangle 2013). For instance, effectiveness of DNMTis can be limited by rapid remethylation once the treatment is stopped, or by the involvement of alternative silencing mechanisms such as histone modifications (Yang and HH 2006; Lay et al. 2014). Therefore, new therapy strategies to induce viral mimicry may involve the combination of DNMTis and inhibition of methyltransferases to prevent "compensatory" silencing through histone modifications.

Liu et al have shown that, in ovarian cancer, combination treatment between 5-aza-CdR and inhibition of the H3K9me2 methyltransferase G9a induces synergistic antitumor effects via the upregulation of ERVs leading to cell death through viral mimicry (M. Liu et al. 2018). In addition, Cuellar et al showed that inhibiting SETDB1 in AML cell lines diminishes H3K9me3 and results in upregulation of L1s, ERVs, and an antiviral response (Cuellar et al. 2017). Given that loss of DNA methylation has been shown to increase H3K27me3, disruption of facultative heterochromatin might be of particular interest to induce viral mimicry (Walter et al. 2016; Ishak et al. 2018). In particular, H3K27me3 can non-redundantly silence RTEs in somatic cells and EZH2 inhibitors have been shown to promote dsRNA formation in small-cell lung cancer (SCLC) cells, suggesting that EZH2 inhibition could be an ideal candidate for the induction of viral mimicry in different cancers (Ishak et al. 2016; Cañadas et al. 2018).

Nevertheless, induction of RTEs remains a double-edged sword. For instance an increase in a particular subset of ERVs has been linked to chemo- and radiotherapy resistant mesenchymal tumor subpopulations in SCLC. This subpopulation has low levels of EZH2 and upregulated ERVs, which induce dsRNA formation and are poised to undergo positive feedback signal amplification due to an antisense localization in the 3'UTR of some ISGs. This leads to an effective maintenance of the "mesenchymal state" to which treatment resistance is attributed (Cañadas et al. 2018). On the contrary, in non-small-cell lung cancer, therapy resistance can also be acquired by selective retention of silencing marks on RTEs. Drug-tolerant persisters that survive to otherwise lethal drug exposure are characterized by H3K9me3-mediated silencing at young L1s (Guler et al. 2017).

These studies highlight the role of RTEs as drivers of both, tumorigenesis and therapy response. In particular, they shed a light on the importance of the epigenetic mechanisms that regulate RTE expression and how they can be exploited in the context of novel cancer therapies.

PhD PROJECT

PhD Project

Upon my arrival at the lab, the team had recently uncovered a new mechanism that was responsible for HSC loss of function. They had shown that one month post TBI, there was an increase in expression of L1Md. This was correlated to an accumulation of DNA damage and a loss of reconstitution capacity post-transplantation. TPO injection one hour prior to IR could prevent both effects.

The team had performed microarray experiments on HSCs that were cultured with and without TPO for one hour prior to being irradiated. Results revealed that HSCs that had received TPO showed an enrichment of genes in the IFN-I innate immune response. Numerous IFN-stimulating genes (ISGs) are viral restriction factors and can sense DNA damage. Thus, they hypothesized that this signaling pathway could be linked to the ability of TPO to restrain L1Md in HSCs.

Throughout the first part of my PhD, I participated in this project by trying to determine if the TPO-mediated control of L1Md expression post TBI was dependent on the IFN-I signaling pathway.

IFN-I signaling requires activation of both STAT1 and STAT2. We showed that TPO cannot prevent increased RE expression or the accumulation of DNA damage following TBI in Stat1 and Stat2-KO mice. This shows that TPO-induced IFN-I signaling is required for TPO's ability to restrain TBI-induced L1Md expression and thereby persistent DNA damage in HSCs. Using blocking antibodies against IFNAR1, the IFN-I receptor, we showed that TPO-mediated ISG expression and IFN-like signaling is independent on the IFNAR receptor and therefore of IFN-I. In addition, we also showed that, like TPO, a single injection of IFN-I one hour prior to TBI can also control IR-induced long-term L1Md expression, further confirming the role of IFN signaling in the restriction of L1Md expression in HSCs.

These results show that TPO mimics IFN-type I signaling and that this signal is required to protect HSC from IR-induced L1Md expression. As the expression of TPO receptor (MPL) is restricted to the hematopoietic system in comparison to the ubiquitous expression of IFNAR receptor, these results also suggest that TPO could act as an IFN-specific to HSCs. Finally, they show that IFN-I has a novel protective role in HSC by restraining L1md-mediated damage.

These results were published in the Journal of Experimental Medicine in 2018, in an article in which I am co-first author . **“Thrombopoietin protects hematopoietic stem cells from retrotransposon-mediated damage by promoting an antiviral response”** Daniela Barbieri*, Emilie Elvira-Matelot*, Yanis Pelinski*, Laetitia Genève, Bérengère de Laval, Gayathri Yogarajah, Christian Pecquet, Stefan N.

Constantinescu, Françoise Porteu. This article is included as an annex to this manuscript.

L1Md expression is tightly regulated by epigenetic mechanisms such as the repressive H3K9me3 mark and 5mC DNA methylation. Previous studies have highlighted the importance of epigenetic modifications at LINE-1 elements in several types of cancers (Hur et al. 2014). Epigenetics also plays an important role in maintaining HSC function, sustained by the fact that epigenetic factors are frequently mutated in hematological malignancies. We therefore hypothesize that IR may induce epigenetic modifications that could lead to chromatin disorganization and thus be responsible for uncontrolled expression of L1Md.

L1Md expression in the genome can sustain major consequences for genomic stability. Their insertion can lead to the formation of double-stranded breaks and genomic rearrangements; Furthermore, L1Md are also recognized as important contributors to gene regulatory networks and thus may deeply impact the cell transcriptome (Faulkner et al. 2009). Aberrant activity of their sense and anti-sense promoters can affect the expression of neighboring or host genes or lead to the formation of L1Md-gene chimeric transcripts. This could also result in HSC loss of function .

In fly and human, STAT proteins have been shown to stabilize heterochromatin after genotoxic stresses via its association to heterochromatin protein 1 (HP1) (Shi et al. 2006). In particular, nuclear non-phosphorylated STAT1 and STAT2 play a role in resistance to viral infections and DNA damage via the induction of a long-lasting antiviral response that is non-toxic to cells (Cheon et al. 2013). On the other hand, in intestinal stem cells in fly, the JAK/STAT pathway induces chromatin remodeling and limits retroelement activity following infections or DNA damage, suggesting that this pathway activity may be conserved in somatic stem cells and/or amongst species, and that STAT proteins may be involved in L1Md control via epigenetics (Sousa-Victor et al. 2017).

The main objectives of my PhD project are thus to:

1. Understand the mechanisms by which IR affects HSC epigenetics, and in particular heterochromatin.
2. Determine if, and how, L1Md expression may impact HSC gene expression.
3. Determine if TPO, via its IFN-like signaling, may regulate L1Md repression through epigenetic mechanisms.

MATERIALS AND METHODS

Mice strains and treatments

Wild type (WT) C57BL/6J mice (6-8 week-old) were from the Envigo Laboratories. All the mice were housed in a specific pathogen-free environment. All procedures were reviewed and approved by the Animal Care Committee (CEAA N°26, registered n° 2013_080 and #2019_078_23286). Mice were injected retro-orbitally with TPO (Miltenyi) (16 µg/kg body weight) before sublethal TBI (2 Gy) (RX irradiator X- RAD 320).

Cell harvest and culture

Bone marrow was harvested from femur, tibia and hip bones in mice. Total bone marrow was depleted of differentiated hematopoietic cells (lineage-positive cells) using Mouse Hematopoietic Progenitor (Stem) Cell Enrichment Set (BD). Magnetically sorted Lineage-negative (lin⁻) cells were kept overnight at 4°C in IMDM medium supplemented with 10% FBS (HyClone) and 1% penicillin-streptomycin (ThermoFisher). Staining was performed for 20min at room temperature (RT) using Ly-6A/E (Sca-1) - PeCy7 clone d7 (558161, BD), CD117 (c-Kit) – PE clone 2B8 (553355, BD), CD34 (Mucosialin) – FITC clone RAM34 (560238, BD), CD135 (Flk2) – BV421 clone A2F10.1 (562898, BD), CD3ε (Lin) – APC clone 145-2C11 (553066, BD), TER-119 (Lin) – APC (557909, BD), CD45R/B220 (Lin) – APC clone RA3-6B2 (553092, BD) and CD11b (Lin) – APC clone WT.5 (562102, BD). Lin⁻Sca⁺c-Kit⁺ cells (referred to as LSK or HSPCs), HSCs (LSK CD34^{low}Flk2⁻) were sorted using ARIA3, ARIA Fusion or an Influx cell sorter (BD Franklin Lakes, NJ, USA) and collected in Stem Span (StemCell).

The BM-HPC cell line were a kind gift from Prof. Leif Carlsson (Umea Centre for Molecular Medecine, Sweden). BM-HPC cells are immortalized growth factor-dependent hematopoietic progenitor/stem cell lines that can generate erythroid, myeloid, and lymphoid cells upon transplantation into lethally irradiated mice (Pinto do Ó et al. 2002). BM-HPC cells were cultured in IMDM medium supplemented with 10% FBS, 1 mM glutamine, 100 ng/ml SCF, 10 ng/ml IL-6, 1.5x10⁻⁴M Monothiolglycerol (Sigma-Aldrich) and 1% penicillin-streptomycin. Where indicated, cells were stimulated using 1000U/ml Interferon (IFN-α) (Miltenyi) 1h prior to irradiation (2Gy).

Preamplification and Quantitative RT-PCR

1000 HSCs were lysed in Tri-Reagent (Zymo Research) and stored at -80°C until used. Total RNA was extracted using the Direct-Zol RNA microprep kit (Zymo research) and reverse-transcribed with EZ Dnase VILO (Invitrogen). 1.25ul of cDNA was preamplified for 14 PCR cycles in a multiplex reaction using Preamp Master-Mix (100-5580 - Fluidigm) and primer mix (200µM of each primer). To rule out primer dimerization or hairpin formation in the preamplification mix, primer sequences were previously analyzed using MFE3.0 PCR Primer Quality Control Software (Wang, 2019). Real-time PCR was performed using the SYBR pPCR premix Ex Taq (Takara)

on a 7500 real-time PCR machine (Applied Biosystems). Samples were tested for qPCR before reverse transcription to rule out detection of contaminating DNA. qPCR primers used were designed in different exons so as to minimize possible gDNA amplification. All data were normalized to the mean expression of RPL32, PPIA, and/or HPRT. Primer sequences are shown in Table S1.

ChIP-qPCR

0.1×10^4 HSCs or 0.3×10^5 to 1.5×10^6 BM-HPC cells were harvested in 1ml IMDM medium supplemented with 10% FBS and cross-linked using 1% formaldehyde (Invitrogen) for 10 min at RT. ChIP-qPCR experiments were performed using the True Micro-ChIP Kit (for 10000 or 300000 cells) or the iDeal ChIP-qPCR Kit (Diagenode) (for 1500000 cells) (Diagenode) according to manufacturer's instructions. Cells were sonicated using the Bioruptor Pico (Diagenode) sonication device for 10 cycles (20s ON/40s OFF or 30s ON/30s OFF for HSCs and BM-HPCs, respectively). Chromatin was incubated overnight at 4°C using 0.25µg of H3K9me3 (C15410193-Diagenode) per IP. ChIP DNA was eluted and purified using the MicroChIP Diapure Columns (Diagenode).

Subsequent qPCR Real-time PCR was performed using SYBR pPCR premix Ex Taq or LUNA Universal qPCR Master Mix (NEB) on a 7500 real-time PCR machine (Applied Biosystems). ChIP-qPCR primers for intronic L1Md were designed such that one primer is located in the 5' region of the L1Md, and the other primer is located in the intron of the host gene to allow the amplification of unique and specific product. Primer sequences are shown in Table S1.

Statistical analysis

Results were statistically evaluated using either the one-way ANOVA or unpaired t-test using GraphPad Prism™ version 8.0 software (GraphPad Software Inc., San Diego, CA, USA). The results are displayed as the means and SEM. The value of *P<0.05 was considered as significant, and **P<0.01 or ***P<0.001 as highly significant.

RNA-seq and ChIP-seq reads quality

Quality of RNA-seq reads was assessed with Fastqc v0.11.8, Fastq-screen(Wingett and Andrews 2018) v0.13.0 and MultiQC (Ewels et al. 2016) v1.7.

RNA-seq

RNA quantification. Salmon (Patro et al. 2017) tool v0.14.1 was used to quantify mm10 NCBI RNA reference sequences (O'Leary et al. 2016) (RefSeq Curated, last updated 2017-11-16) downloaded from UCSC Table Browser (Karolchik et al. 2004). Salmon was launched with the following parameters : --numBootstraps 60 --libType A --validateMappings.

Differential gene expression analysis. Statistical analysis was performed using R v3.5.1. Transcript expression levels were aggregated in Gene expression levels using tximport Bioconductor package (Soneson et al. 2015) v1.13.16. Deseq2 (Love et al. 2014) v1.22.2 method was used to identify differentially expressed genes between groups with a p-value threshold of 0.05. Genes were considered as expressed when at least 2/3 triplicates > 0 and as not expressed when all triplicates = 0.

Permutation test To create the list of genes hosting or neighboring an L1Md, BED files containing L1Md genomic localizations (from D. Bourc'h's lab) were annotated using HOMER software (Heinz et al. 2010; Lin et al. 2012; Duttke et al. 2019) to determine if L1Md were intronic or intragenic as well as the closest TSS. To obtain host gene names, the genomic localization of L1Mds were crossed to genomic localizations of genes using Refseq. Permutation test (n=10000) between list of genes hosting or neighboring an L1Md and differentially regulated genes (IR vs NIR), or random genes (from Refseq without differentially regulated genes) was performed using R studio and considered significant if $p < 0,01$.

Motif Enrichment Analysis was performed using BaMM! web interface (Siebert and Söding 2016; Kiesel et al. 2018) and *de novo* and motif scan modules. For *de novo* analysis, query motif was matched to known motifs using the GTDR mouse database (Yevshin et al. 2019). Motif search was also performed using the FIMO module of the MEME suit (Grant et al. 2011).

GSEA Analysis was performed using Hallmark Gene Sets V7. To plot graphs, $-\log_{10}$ pValue is set to 4 when $p < 0,0001$.

ChIP-seq

Alignments. Human sequences were found in Mouse ChIP-seq reads. The contamination was removed with Xenome (Conway et al. 2012) v1.0.0. After contamination removal, ChIP-seq sequence reads were mapped to the Mouse genome build mm10 by using Burrows-Wheeler Aligner MEM algorithm (Li and Durbin 2009) (BWA v0.7.17). The read group ID was attached to every read in the resulting alignment file (bam file) with the -R parameter, and shorter split hits were marked as secondary with -M. Samtools (Li et al. 2009) fixmate v1.9 was used to check mate-pair information between mates and fixed if needed on a name sorted bam file. The duplicate reads were tagged by samtools markduplicates using a position sorted bam file. Secondary alignments and unmapped reads have been filtered out and only properly paired reads have been kept. Two types of downstream analysis have been performed, with multimapped reads (mapping quality score ≥ 0) and one with uniquely mapped reads (mapping quality score ≥ 1). Cross-correlation scores (NSC and RSC) have been calculated by phantompeakqualtools package (Kharchenko et al. 2008; Landt et al. 2012) v1.2. DeepTools (Ramírez et al. 2016) bamCoverage v3.3.0 has been used to generate normalized bigwig files with the following parameters : --

binSize 1 --normalizeUsing BPM --extendReads --ignoreDuplicates. Then deepTools bigwigCompare was used to subtract input signal from chip signal.

Peak calling. Areas in the genome enriched with aligned reads (also called peaks) were identified with MACS2 (Zhang et al. 2008) callpeak v2.1.2 with the following parameters : -f BAMPE -g mm10 -q 0.05 --broad --broad-cutoff 0.05 for H3K9me3 broad mark.

IDR (Irreproducible Discovery Rate) analysis. To measure the reproducibility between replicate experiments, we used the IDR method (Li et al. 2011) v2.0.4.2 with the following parameters : --rank q.value --random-seed 12345 --plot. Peaks with a global IDR score < 0.05 were selected and used for downstream analysis.

Differential binding. To identify differences in DNA binding, the Bioconductor package Diffbind (Ross-Innes et al. 2012) v2.10 was used in R v3.5.1. Paired-end mode was activated for read counting step with SummarizeOverlaps method. The default mapping quality threshold (mapQCth) was modified in 0 for multimapping analysis or 1 for unique mapping analysis. DBA_DESEQ2_BLOCK method was used to consider unwanted variable during normalization and differential binding identification with an adjusted p-value threshold of 0.05.

RTE genome coverage. To generate RTE genome coverage, bedtools package v2.27.1 was used. -bga option on the genomeCoverageBed tool was used. The bedGraph generated were then converted to bigwig files using the bedGraphToBigWig tool.

Heatmaps. To plot heatmaps of H3K9me3 enrichment at peaks, deeptools package v3.2.0 was used in R v3.5.1. The peaks (IDR<0.05) files obtained for NIR and IR conditions were first fused using bedops. A matrix was then built using ComputeMatrix tool in the scale-regions mode between the generated fused bed file and the corresponding normalized bigwig files after input subtraction. A body length of 2.5kb (mean size of the peaks) was selected, as well as a 4kb distance upstream and downstream of the start and the end of the peak.

A file sorted region was generated and used to assess the presence or the absence of RTE after computing a matrix with the RTE genome coverage bigwig.

Primer Lists:

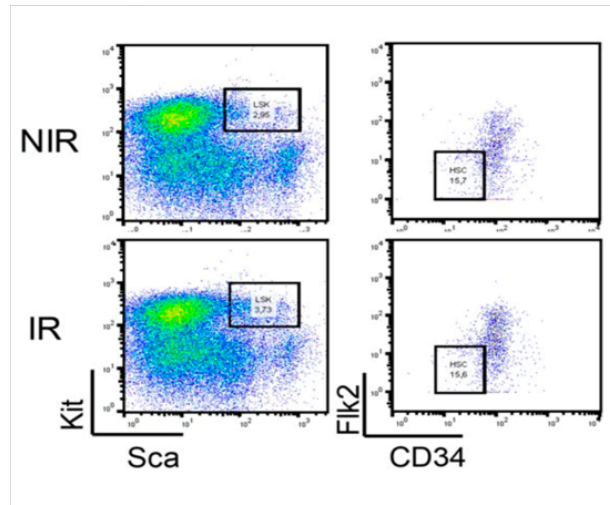
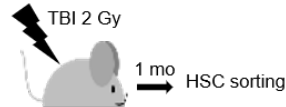
RT-qPCR

RT-qPCR			ChIP-qPCR		
Alcam	F	CGA TAC CCT GCC TGT GTC AT	L1Md_T Snx27	F	AGCCACTATACCCAGCTACATA
	R	GAA AAG GAA GGG CTG GAC CG		R	GACGGTGGCTGGCTGT
Ghr	F	TGG ATA CCC TAC TGC ATC AAG C	L1Md_F2 Rbms3	F	GTC CCG GAA CCA AGG TGG
	R	CTC CAC GAA TCC CGG TCA AA		R	GGT AAT TGC CAC ATT AAC AGA CA
HPRT	F	TGATTATGGACAGGACTGAAAGA	L1Md_T Ghr Fw	F	GGA GGG ATG CAA AGG CAG ATA
	R	AGCAGGTCAGCAAAGAACTTATAG		R	CTT AGG CCA TGG TGG TCT GAT
L1Md_A	F	GGATTCCACACGTGATCCTAA	L1Md_T Mapre2	F	GAA GCT CTG CGA CTC TCT CG
	R	TCCTCTATGAGCAGACCTGGA		R	CAG CTT AGT GTC GGT TTG TGG
Mapre2	F	CCA GGA GAC TAT GAG CAG GC	L1md_A Alcam	F	CGATTTCGCATTCTTCTACACG
	R	TAA TAC AGC CCG GGA AGA GC		R	ACACCTGCACAGGATTCCC
Mpl	f	AAA CCA GGC ACA AGT CAC CA	L1md_F2 Rmdn2	F	TGT GTT GTC AAG CTG CTA GTG
	r	GCT TCT CTC CTC GGT AGG CA		R	CAC CAG GGT GTG CTA AGT GAT
Mecom	F	CCA GAC CCA CTC CCT TCT TC	L1Md_T Mecom	F	CCT GTG CCA CAA ACC TCT CAG
	R	AAA GCT TCA AGC GGG TCA GT		R	AGT ATA CCC CCA TTG TCA CCC A
PPIA	F	GGC CGA TGA CGA GCC C	Spi1_TSS	F	TGG GTG CTG GAC TTA GAA CCT TGA
	R	TGT CTT TGG AAC TTT GTC TGC AA		R	AGA CAC AGA CCC TGG CTT TCA TGT
Rmdn2	F	TTC CAC TCG GCC ATC CTC T	L1Md_A	F	GGATTCCACACGTGATCCTAA
	R	GGT ACC AGA AGG CCA GCA AG		R	TCCTCTATGAGCAGACCTGGA
Rpl32	F	GAA ACT GGC GGA AAC CCA	RNA 5s	F	TACGGCCATACCACCCTGAA
	R	GGA TCT GGC CCT TGA ACC TT		R	CCTGCTTAGCTCCGAGATCA
Stat1	F	TCACAGTGGTTCGAGCTTCAG			
	R	GCAAACGAGACATCATAGGCA			
Stat2	F	GATCTCTGGAAGGATGGGCG			
	R	ATAGAGGAAGCGAGTGGGT			
Snx27	F	GCA AAG GTT GGC ATG GAC AG			
	R	CTT GCG AAT GGT CAG GCA AG			

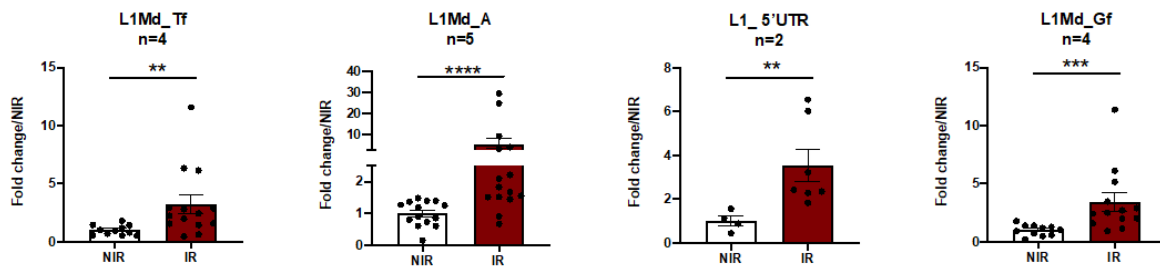
RESULTS

Figure 1

A)



RT-qPCR



B)

ChIP-qPCR H3K9me3

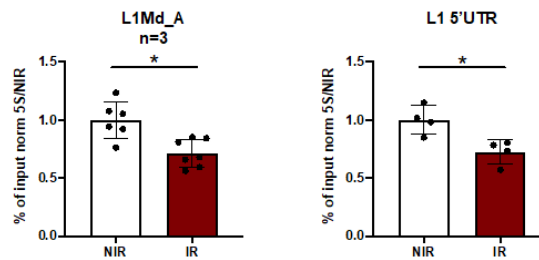


Figure 1. Irradiation induces a global increase in L1Md expression which correlates to loss of H3K9me3 at L1Md promoter. A) Experimental setup. B) Top row: L1Md expression quantified by RT-qPCR (n=1-5 independent experiments, each dot represents an individual mouse) Ct values were normalized to β -actin. Results are expressed as fold change from the mean value of the NIR condition and represented as means \pm SEM. (****p<0,0001 Mann-Whitney). Bottom row: H3K9me3 enrichment at L1Md promoters analyzed by ChIP-qPCR (n=1-3 independent experiments, each dot represents pools of 3 (NIR) or 4 (IR) mice) Ct values normalized % of input of negative control repetitive 5S ribosomal RNA. Results are expressed as fold change from the mean value of the NIR condition and represented as means \pm SEM (*p<0,05 Mann-Whitney).

Irradiation leads to L1Md derepression and is correlated to loss of H3K9me3 at L1Md promoters

Due to their repetitive nature, RTE expression can be a major source of genomic instability. We have previously shown that in the basal state, HSCs (Lin⁻ Sca^{high} Kit⁺CD34⁻ Flk2⁻) exhibit high levels of evolutionary recent and active RTEs compared to progenitors. This was observed by RT-qPCR using primers recognizing the promoters of the majority of the L1Md subfamilies (L1-5'UTR), and also for L1_A, L1_Gf, and L1_Tf subfamilies. Exposure to total body irradiation (TBI) leads to an increase in expression of these L1Mds one month post-IR (**Fig. 1A**) which is linked to active retrotransposition and to the accumulation of DNA damage in HSCs (Barbieri et al. 2018). Heterochromatin, through DNA methylation and trimethylation of lysine 9 of histone H3 (H3K9me3) plays a major role in repressing repeated sequences. It has been shown that in murine and human HSCs, there is a global decrease of H3K9me3 with age that correlates to an increase of RTE expression (Djeghloul et al. 2016). We were therefore interested in knowing if changes in RTE expression following IR could also be related to a loss of H3K9me3 enrichment at their promoters. We performed ChIP-qPCR in HSCs and analyzed H3K9me3 enrichment following IR globally at the L1Md promoters (L1Md-5'UTR) and more precisely at the promoter of the L1Md_A subfamily. Results showed a decreased H3K9me3 enrichment at 5'UTR and L1_A promoters following IR (**Figure 1B**) that correlates to the increase in L1Md expression (**Figure 1A**).

Figure 2

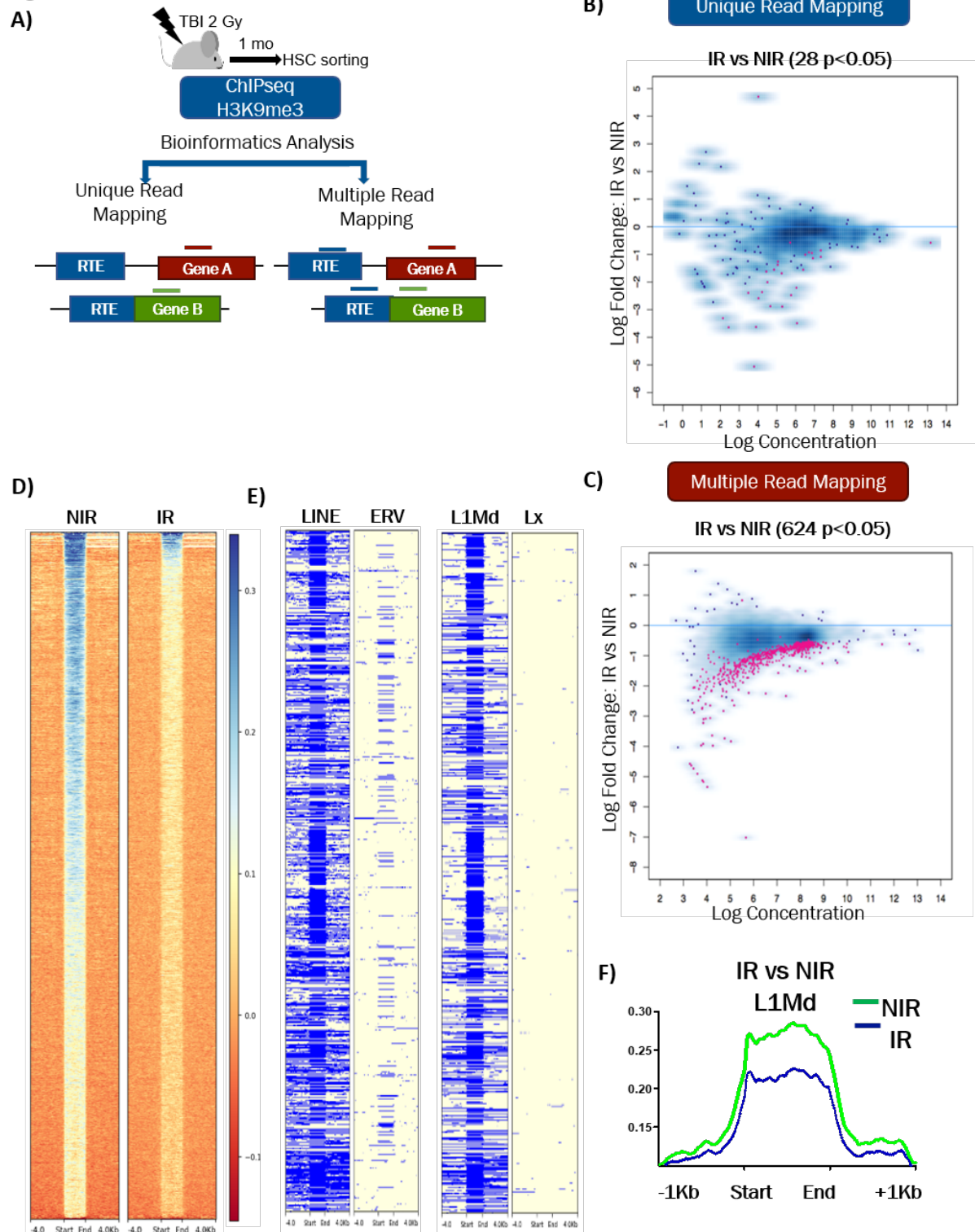


Figure 2. Irradiation induces a decrease of H3K9me3 mainly at L1Mds A) Mice were subjected to 2Gy total body irradiation (IR), or not (NIR). 20000 HSCs were sorted from pools of 4/5 mice. B-D) Quantitative analysis of H3K9me3 enrichment at peaks was performed both on unique (B) and multiple (C) mapping reads. MA-plots analysis shows a major decrease in H3K9me3 enrichment at peaks (blue dots). Significantly ($p < 0.05$) and differentially enriched peaks are represented by the pink dots. (D) Heatmap of H3K9me3 enrichment at peaks in NIR and IR conditions. Heatmaps show H3K9me3 enrichment 4kb upstream and downstream of the peaks, and in the 2.5kb scaled peak regions (2.5kb being the mean size of the peaks) E) genomic coverage at RTEs. Blue = presence and yellow=absence of the RTE at the genomic locus.) F) Plot profile representing H3K9me3 enrichment 1kb upstream and downstream as well as all along the L1Md sequences in IR (blue) vs NIR (green) conditions.

Irradiation induces major alterations in H3K9me3 in HSCs

To further characterize the global effects of IR on HSC heterochromatin, H3K9me3 ChIP-seq was performed (**Figure 2A**). Classical analysis of ChIP-seq data discards the reads mapping to multiple loci, leading to a loss of information concerning RTEs. We therefore chose to analyze our data in two ways: in the classical manner on reads mapping to unique loci (i.e.: genes); and on reads mapping to both unique and repetitive loci (i.e.: RTEs and genes). Reads mapping to multiple loci are arbitrary assigned to only one locus, as previously described by Bulut et al (Bulut-Karslioglu et al. 2014). Therefore read localization is not precise, but gives global information about H3K9me3 enrichment at RTE subfamilies. Peak calling was performed to identify genomic regions significantly enriched for H3K9me3 compared to input. Quantitative analysis of H3K9me3 enrichment at H3K9me3 peaks between IR and NIR conditions revealed that only 28 peaks are significantly ($p < 0.05$ – pink dots) and differentially enriched in the unique mapping read analysis (**Figure 2B**), compared to 624 peaks in the multiple mapping read analysis (**Figure 2C**). This suggests that the majority of H3K9me3 variation occurs at repetitive sequences such as RTEs. On these 28 and 624 differential peaks, only one was found significantly upregulated in IR, showing that IR induces an overall decrease in H3K9me3 (**Figure 2B and C**). Heatmap analysis of the H3K9me3-enriched peaks confirms the global loss of H3K9me3 upon IR (**Figure 2D**). Crossing the heatmap data with RTE genomic coverage further shows that H3K9me3 peaks are particularly enriched in LINE-1 elements compared to ERVs, and more precisely in young LINE-1 elements such as L1Mds compared with older Lx LINE-1 (**Figure 2E**). Plot profile analysis on the L1Md sequences confirmed the global decrease of H3K9me3 enrichment at L1Mds (**Figure 2F**).

Figure 3

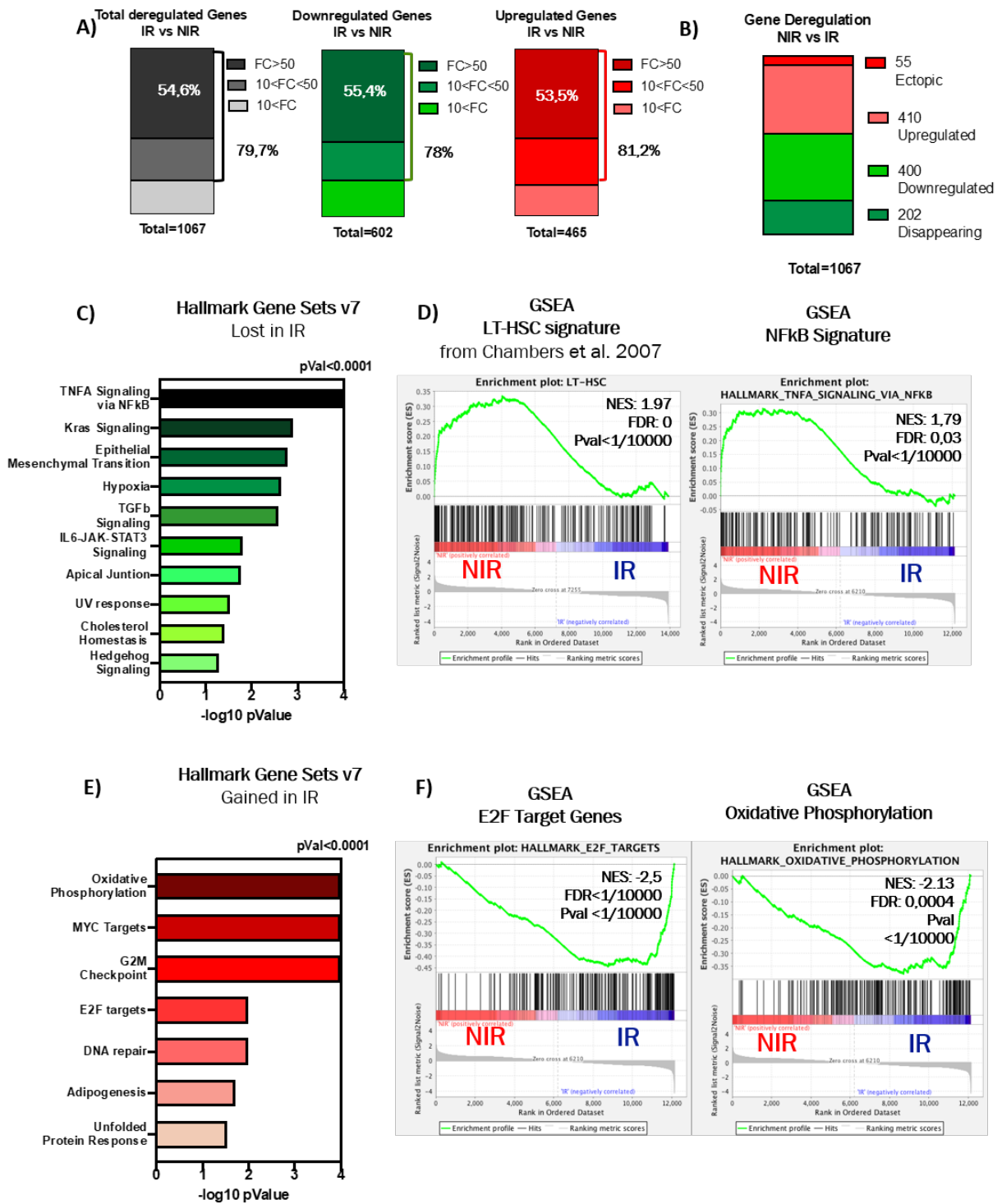


Figure 3. IR induces a strong deregulation of gene expression A) Repartition of differentially expressed genes in IR compared to NIR according to their fold change. All genes ($p < 0.05$) (grey), downregulated genes (green) and upregulated genes (red). B) Number of genes down- or upregulated, disappearing and appearing in IR. C-F) GSEA analysis using Hallmark Gene Sets V7 $-\log_{10}$ pValue is set to 4 when $p < 0.0001$. Gene sets lost (C, D) or gained (E, F) in the IR condition compared to NIR. D) Enrichment plots showing gain of NFkB and HSC signatures upon IR. F) Enrichment plot showing gain of oxidative phosphorylation and MYC targets signatures upon IR.

Irradiation induces transcriptomic changes in HSCs

Global changes in H3K9me3 brought about by IR can affect RTE expression. RTE derepression can in turn induce transcriptional modifications. We therefore performed RNA-seq analysis one month after irradiation to determine the impact of IR on the HSC transcriptome. Results revealed that 1067 genes were differentially expressed ($p < 0.05$) between IR and NIR conditions. Among them, 602 and 495 are respectively down- and upregulated. Deregulation of gene expression is very strong, since almost 80% of the genes present a fold change of at least 10, and 55% present a more than 50 fold change between IR and NIR conditions. The same remains true for downregulated and upregulated genes separately (**Figure 3A**). Interestingly, 11.8% ($n=55$) of upregulated genes are genes that are not expressed (transcript per kilobase million (TPM) < median of IR for at least 2 triplicates) in the basal state and become ectopically expressed upon IR (TPM > median of IR for at least 2 triplicates). On the other hand, 33.5% ($n=202$) of genes show a complete disappearance of their expression upon IR (TPM < median of NIR for at least 2 triplicates). (**Figure 3B**).

Gene set enrichment analysis (GSEA) revealed that IR induces a significant loss of several signaling pathways involved in HSC function and quiescence, including NF- κ B, TGF β , hypoxia, and JAK-STAT3 (**Figure 3C**). NF- κ B signaling has been shown to play a role in HSC self-renewal and quiescence (Fang et al. 2018), and loss of the HSC signature was observed in mice deleted for the NF- κ B subunit p65/RelA (Stein and Baldwin 2013). An altered NF- κ B activity is also involved in HSC loss of function upon aging (Chen et al. 2019), and contributes to leukemia progression (Kagoya et al. 2014) suggesting that loss of this signature could explain the impaired function of HSCs upon IR. Interestingly, GSEA analysis revealed a significant loss of the HSC signature (Chambers 2007) upon IR (**Figure 3D**). Hallmark gene set analysis also revealed that signaling pathways regulating oxidative phosphorylation, MYC and E2F targets, DNA damage checkpoint, and DNA repair were upregulated upon IR (**Figure 3E-F**), which is consistent with the accumulation of DNA damage we and others have observed following IR exposure (de Laval et al. 2013; Barbieri et al. 2018).

Figure 4

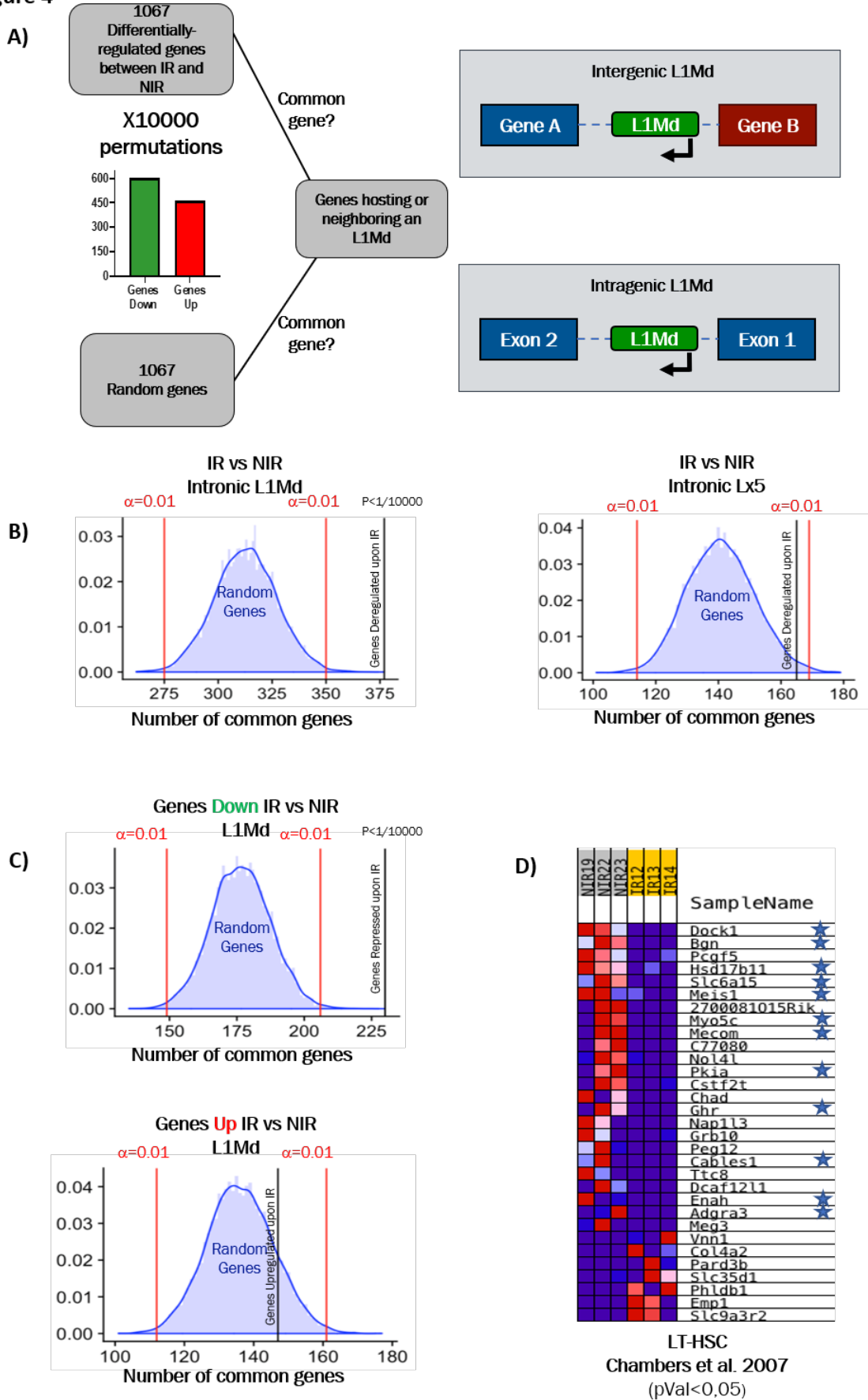


Figure 4. Intronic L1Md are associated with IR induced gene repression. A) Lists of n deregulated genes or 10000 lists of n random genes were crossed with the lists of genes either hosting or neighboring an L1Md. B-C) Blue: Distribution of the number of genes found in common between 10000 lists of random genes and genes hosting an L1Md. The black vertical line represents (B) the number of genes found in common between deregulated genes upon IR and genes hosting an L1Md (left) and genes hosting an Lx5 (Right), or (C) between genes downregulated (top) or upregulated (bottom) upon IR and containing an intronic L1Md. Significance bars ($p < 0.01$) in red. D) Heatmap of the expression of genes from the HSC signature that are significantly either up (red) or down (blue) regulated in IR vs NIR. Blue stars indicate the presence of an intronic L1Md in these genes.

Intronic L1Md are associated to gene repression following IR

The above data show that IR both induces a global loss of H3K9me3 that mainly affects L1Mds and can affect the HSC transcriptome. To establish if there is a correlation between these two phenomena, we looked for an association between gene deregulation and the presence of L1Mds in their neighborhood or in their gene body. To do so, we crossed the list of genes deregulated upon IR with a list of genes neighboring or hosting an L1Md (*see materials and methods*), and we did the same for 10000 lists of the same number of random genes (**Figure 4A**). We found that genes deregulated upon IR are significantly associated with intronic L1Mds ($p < 0.0001$ -permutation test) (**Figure 4B**) and not with neighboring L1Md (*data not shown*). This association seems specific as it was not found with the older L1 subfamily Lx5 (**Figure 4B**). Interestingly, the association to intronic L1Mds was specific for genes that are downregulated upon IR, as opposed to upregulated (**Figure 4C**). These results suggest that intronic L1Mds might play a role in regulating the expression of their host genes.

The human orthologous of 55% of the genes repressed upon IR and hosting an intronic L1Md, host young human or primate L1 (L1Hs/L1PA). This suggests a conserved functional role of young L1s in regulating hematopoietic gene expression. Consistent with this data, we found that 50% of the genes ($n=12/24$) belonging to the HSC signature and whose expression is repressed upon IR contained an intronic L1Md (**Figure 4D**). 9/12 (75%) of the human orthologous of these genes also contained an L1Hs/L1PA.

Figure 5

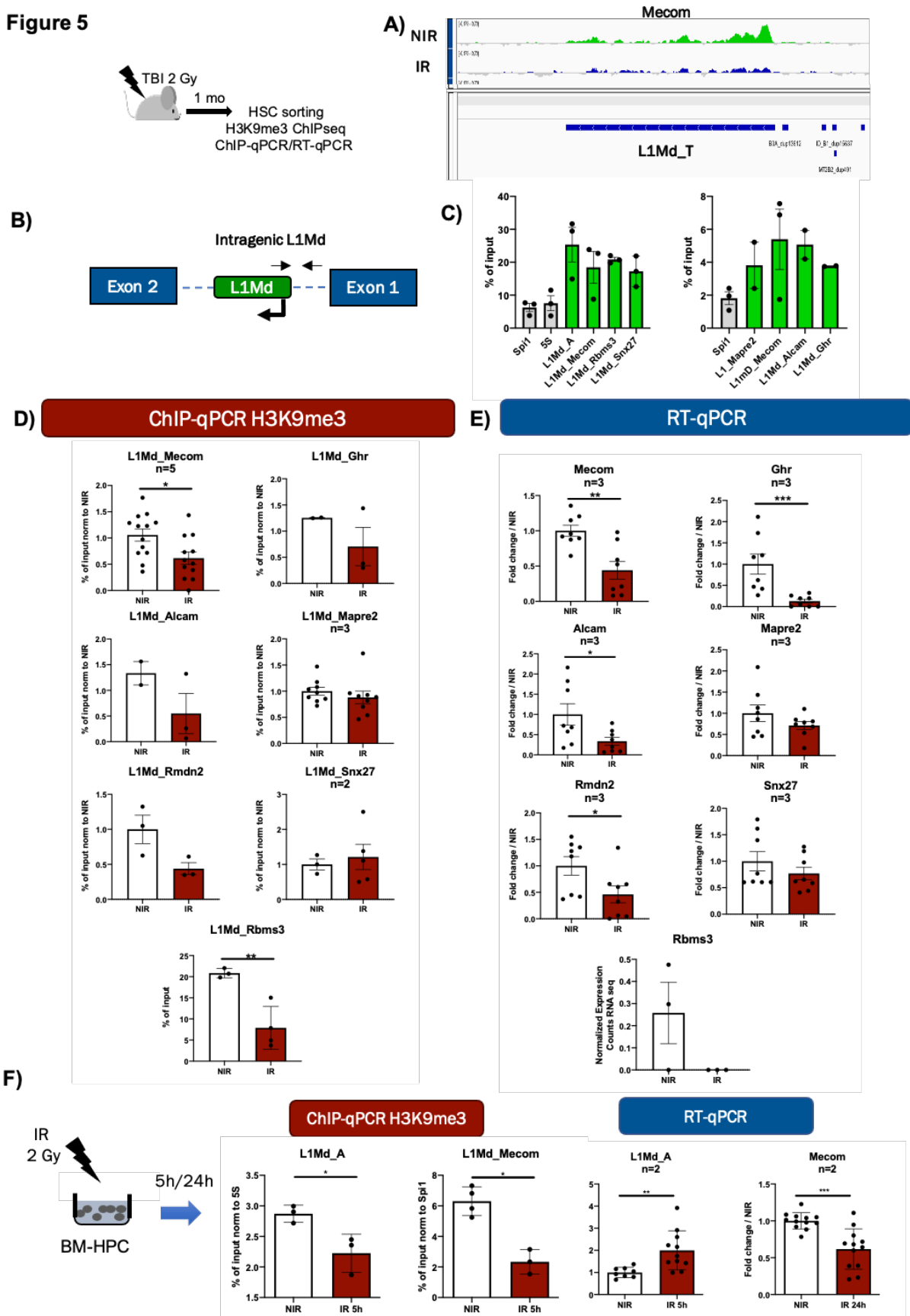


Figure 5. Gene repression upon IR is associated with loss of H3K9me3 at intronic L1Md. A) Integrative genomic representation of H3K9me3 enrichment at Mecom's intronic L1Md in NIR (green) and IR (blue) conditions. At the bottom are represented the RTE. B) Schematic representation of primer positioning for ChIP-qPCR experiments allowing the amplification of a unique and specific product (left). C) H3K9me3 enrichment at the basal state evaluated by ChIP-qPCR at intronic L1Md of HSCs of two independent experiments but representative of all. Each dot represents pools of 3 mice. Results are expressed as % input of the NIR condition. D) H3K9me3 enrichment evaluated by ChIP-qPCR at intronic L1Md of HSCs sorted one month post IR. (n=1-5 independent experiments, each dot represents pools of 3 (NIR) or 4 (IR) mice) Results are expressed as fold change from the mean of % input of the NIR condition. (*p<0,05 Mann-Whitney). E) Gene expression evaluated by RT-qPCR in the same cells one month post IR. (n= 3 independent experiments, each dot represents an individual mouse) Ct values were normalized to mean of RPL32 and HPRT. Results are expressed as fold change from the mean value of the NIR condition and represented as mean +/- SEM. Rbms3 expression is represented by normalized expression counts from RNAseq data. F) BM-HPC were submitted to 2Gy IR in vitro and collected either 5h or 24h after IR for subsequent H3K9me3 ChIP-qPCR (left) or RT-qPCR (right) experiments. Ct values were normalized to input (ChIP-qPCR) or to HPRT (qPCR). Results are expressed as fold change from the mean value of the NIR condition and represented as mean +/- SEM. L1Md_A expression and H3K9me3 enrichment at L1Md_A promoter (left) normalized to % of input of 5S. Mecom expression and H3K9me3 enrichment at Mecom intronic L1Md (right) normalized to % of input of Spi1. (**p<0,001; **p<0,001;p<0,01; *p<0,05 Mann-Whitney/ t-test).

Loss of H3K9me3 at intronic L1Md may be linked to loss of host gene expression upon IR

We next examined whether the loss of the repressive mark H3K9me3 occurs at intronic L1Mds of repressed genes upon IR, and whether this correlates to changes in host gene expression. We performed H3K9me3 ChIP-qPCR experiments to determine if there was a loss of H3K9me3 at the intronic L1Mds of several target genes that were repressed upon IR (**Figure 5A**). To do so, we designed qPCR primers that spanned the region between the start of the 5' end of the L1Md and the flanking intron. This allows for the amplification of unique and specific products (**Figure 5B**). We selected target and control genes for which the presence of an intronic L1Hs/L1PA was conserved in their human orthologous. Three of the target genes, *Mecom*, *Ghr* and *Alcam* are significantly downregulated in IR. They are involved in HSC functions and harbor L1Md_T, F2 or A in their introns, respectively. The two control genes, *Mapre2* and *Snx27*, harbor an intronic L1Md_T but their expression is not affected by IR ($0.7 < \text{fold-change} < 1.3$).

We observed that in the basal state, H3K9me3 is enriched at the specific intronic L1Md loci of target and control genes, as it is globally at L1Md_A promoters. In contrast, there was no H3K9me3 enrichment in expressed control loci such as the *Spi1* promoter or the the 5S rRNA gene in the basal state (**Figure 5C**). We also observed that IR induces a decrease of H3K9me3 enrichment at intronic L1Mds of *Mecom*, *Ghr* and *Alcam*, which correlates to a decrease in their mRNA levels following IR (**Figure 5D**). No variation in H3K9me3 enrichment at intronic L1Md or in the mRNA levels of the two control genes, *Mapre2* and *Snx27*, was observed in irradiated HSCs (**Figure 5E**). These data suggest that loss of H3K9me3 is not random and might be associated with gene repression upon IR.

To determine if the effect of IR on H3K9me3 at intronic L1Mds of these genes was direct, HSC intrinsic and short-term, we used an immortalized bone marrow (BM) derived hematopoietic progenitor/stem cell line (BM-HPC), a kind gift of L. Carlsson (Umea Medical Centre, Sweden). This cell line was used due to the rapid decrease in c-Kit and an increase in Sca-1 induced by IR (Simonnet et al. 2009), which makes sorting HSCs on these markers soon after IR not possible. We found that BM-HPC cells irradiated (2Gy) *in vitro* showed an increased expression of L1Md_A as quick as 5h post IR compared to non-irradiated cells. This was accompanied by a decreased enrichment of H3K9me3 at L1Md_A promoters. Enrichment of H3K9me3 was also decreased at the intronic L1Md_A in *Mecom* 5h post IR. This loss of H3K9me3 was followed by a decrease in *Mecom* mRNA observed 24h post IR (**Figure 5F**). These data suggest that IR directly and quickly affect HSPC heterochromatin, L1Md derepression and gene expression.

Figure 6

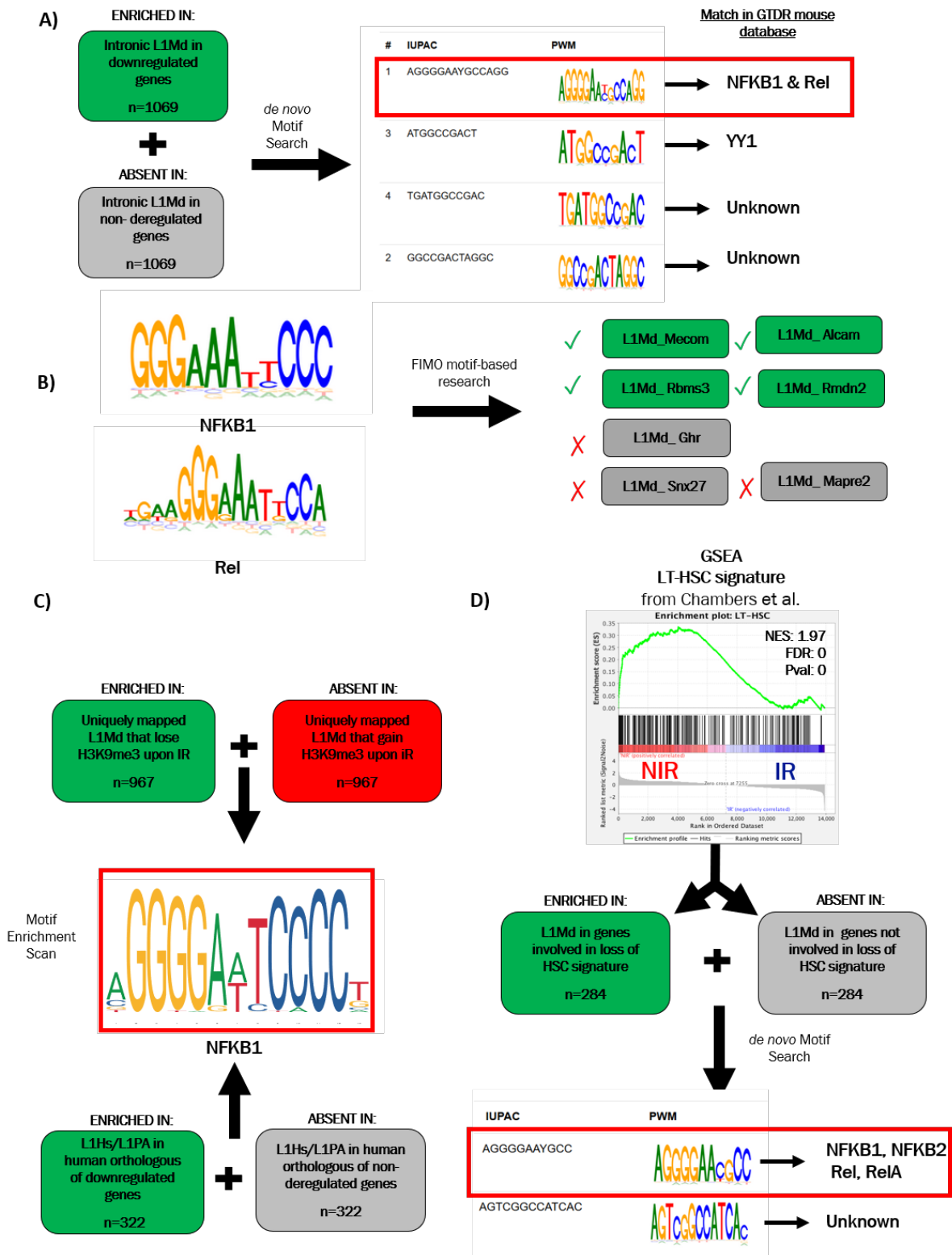


Figure 6. Motif enrichment analysis show specific enrichment of NFKB motifs. A) *De novo* motif enrichment analysis on L1Md sequences located in introns of A) downregulated genes vs nonderegulated genes performed using the BaMM tool. Enriched motifs are matched to known motifs using the GTDR Mouse Database. B) Motif-based FIMO analysis on L1Md from candidate downregulated genes and control genes studied in Fig.5. C) Motif enrichment scan of (top) L1Md sequences that lose H3K9me3 upon IR vs L1Md sequences that gain H3K9me3 upon IR, (bottom) L1HS/L1PA sequences of the human orthologous of downregulated genes vs L1HS/L1PA sequences of the human orthologous of non deregulated genes. D) *De novo* motif enrichment analysis of L1Md sequences in genes of the HSC signature involved in the loss of the HSC signature observed upon IR vs. L1Md sequences of genes not involved in the loss of the HSC signature.

Loss of H3K9me3 at intronic L1Md of IR-repressed genes may be linked to loss of NF- κ B signaling

We then wished to characterize the mechanism that could account for the specific loss of H3K9me3 observed at the intronic L1Mds of some of the HSC IR-repressed genes compared to L1Mds located in introns of genes randomly selected from the list of non-deregulated genes. For that, we performed a *de novo* motif research on the L1Md sequences using BaMM software (Siebert and Söding 2016; Kiesel et al. 2018). We verified that the mean size of the L1Mds located in deregulated and non-deregulated genes that were compared was similar. This research yielded four motifs specifically enriched in the L1Md sequences of downregulated genes compared to L1Md sequences in non-deregulated genes. These motifs were crossed with the Gene Transcript Regulation Database (GTDR) mouse database, to identify known transcription factor binding motifs. Two of the motifs did not match to any known transcription factors. Of the two remaining motifs one matched p50 nuclear factor (NFKB1) and to Rel, both belonging to the NF- κ B signaling pathway; and the other one to the transcription factor Ying-Yang 1 (YY1) (**Figure 6A**). Given the loss of the NF- κ B signature observed upon IR (**Figure 3C**), we chose to focus on the NFKB1 and Rel motifs. We then performed motif-based sequence analysis using the FIMO tool and found that the NFKB1 and Rel motifs were present in the intronic L1Mds of all of the selected target genes downregulated upon IR, except for *Ghr*. These motifs were absent from the intronic L1Mds of the two control genes whose expression is not affected by IR (*Mapre2*, *Snx27*) (**Figure 6B**). These data suggest that NF- κ B signaling may be involved in H3K9me3-mediated intronic L1Md repression.

Through motif comparison analysis, we observed that the NFKB1 motif was more accurately matched than the Rel motif to the motif we found enriched through *de novo* motif research. Therefore, we performed motif enrichment scan, and searched for enrichment of the NFKB1 motif on the L1Hs/L1PA of the human orthologous of genes downregulated upon IR against L1Hs/L1PA of human orthologous of randomly selected non-deregulated genes. We found that the NFKB1 motif was highly enriched on the L1Hs/L1PA of the human orthologous of genes downregulated upon IR (**Figure 6C**), suggesting that this NF- κ B pathway function involved in the repression of intronic L1Md is conserved in humans.

Using our ChIP-seq data, we analyzed H3K9me3 enrichment at unique reads mapping to L1Md loci in IR vs NIR conditions. We then compared L1Md sequences that specifically lose H3K9me3 upon IR against those that specifically gain H3K9me3 upon IR (p -val<0.05) and found that the NFKB1 motif was specifically enriched in L1Md sequences that lose H3K9me3 upon IR (**Figure 6C**). These results, together with the loss the TNFA-mediated NFKB signaling signature observed in GSEA, strongly suggest that the NF- κ B signaling pathway may be involved in H3K9me3 deposition at L1Mds in the introns of some HSC genes and could thus contribute to the regulation

of gene expression in these cells. Supporting this possibility, *de novo* motif research also showed the specific enrichment of NF- κ B motifs, including the NFKB1 motif, in L1Mds of genes involved in the loss of HSC signature detected by GSEA, compared to L1Mds in genes not involved in the loss of this signature (**Figure 6D**). These results suggest that IR-induced loss of NF- κ B signaling may affect NFKB1 binding at intronic L1Mds in HSC genes and therefore be involved in the loss of HSC function.

DISCUSSION

Discussion

RTEs represent an important, endogenous source of genomic instability for HSCs. Their expression can increase as a response to physiological stresses such as irradiation, viral infections, heat shock, and exposure to heavy metals (Li et al. 1999; Kale et al. 2005). We have shown that HSCs express high mRNA levels of several L1Mds, and that their expression further increases upon sublethal total-body irradiation (TBI) and remains high for at least a month in irradiated HSCs. Using L1-GFP transgenic reporter mice, it was shown that productive L1 retrotransposition occurs in HSCs *in vivo* and that it plays an important role in IR-induced HSC genomic instability and loss of function. These results were published in the article that is included as an annex to this manuscript.

1. Irradiation induces epigenetic changes in HSCs

The effects of exposure to IR can span several generations in animals and humans (Kovalchuk and Baulch 2008). Several studies have suggested that this could be due to epigenetics. Epigenetic mechanisms appear to play an important role in determining the physiological response of species to environmental stressors, such as IR. Studies on plants have shown that the epigenetic system mediates the regulation of gene expression changes in response to environmental changes; in animals, changes in the epigenome have also been reported as a response to environmental stress, such as nutrition or water deficiency, grazing, light or temperature alterations, social environment (Horemans et al. 2019). Mouse models have also shown that IR can induce dose-dependent, gender-, and tissue-specific effects on global hypomethylation (Tharmalingam et al. 2017). Filkowski et al. have shown that paternal irradiation leads to changes in the microRNAome and decreased expression of the *de novo* methyltransferase DNMT3a, leading to aberrant methylation of L1 and SINE B2 elements in the thymus tissue. Hypomethylation of L1 and SINE B2 elements was also found in their progeny, due to a significant decrease in lymphoid-specific helicase (LSH), which is essential to maintain DNA methylation, also brought about by inherited changes in the microRNAome. These results establish a link between irradiation and the epigenetic control of RTEs (Filkowski et al. 2010). Luzhna et al. have shown that exposure to low doses of IR induced hypomethylation of L1 ORFs resulting in increased expression and mobilization of L1s in mammary gland tissue (Luzhna et al. 2015). Similarly, Lee et al. showed that L1 methylation levels were lower in nuclear power plant workers than in control subjects (Lee et al. 2015). Maze et al. have shown that stress by repeated cocaine exposure induces decreases in H3K9me3 enrichment specifically at L1 repeats and is correlated to an increased expression of L1 in the nucleus accumbens (Maze et al. 2011).

HSCs represent the compartment that is the most susceptible to IR, however few things are known about how IR affects epigenetics and RTE control in these cells.

Miousse et al. have shown that a pool of Lin⁻ cells containing hematopoietic stem and progenitor cells (HSPCs) was more susceptible to epigenetic alterations than mononuclear cells (MNCs), and that exposure to low doses of IR was capable of causing long-term epigenetic aberrant DNA methylation due to a significant decrease in Dnmt3a expression and aberrant expression of L1 elements (Miousse et al. 2014). In a more recent study, the same group also showed a decrease in DNA methylation at L1 elements in HSCs 2 months post exposure to low dose IR (0,1/1 Gy) (Miousse et al. 2017).

Recent studies have shown that in somatic cells, repressive histone marks, such as H3K9me3, H3K9me2, and H3K27me3, can play a role in regulating RTE expression (Fasching et al. 2015; Ecco et al. 2016; Walter et al. 2016; Robbez-Masson et al. 2018). Chromatin state can also have an important influence on HSC identity and function. In fact, reorganization of heterochromatin is one of the main factors driving aging and oncogenesis. Sun et al. have shown that epigenetic alterations can occur in aged HSCs and may be linked to alterations in TET1, TET3, or DNMT3a gene expression (Deqiang Sun et al. 2014). Djeghloul et al. have shown that with age, young and active L1 (L1Md) expression increases and is correlated to a global loss of H3K9me3 (Djeghloul et al. 2016). Interestingly, this study observes increased expression of the same L1Mds that we have seen upregulated upon IR in HSCs. Nevertheless, the effect of IR on H3K9me3 in HSCs and RTE regulation has never been addressed before. As in aging, we also found that IR induces a global loss of H3K9me3 in HSCs following IR. Using ChIP-qPCR experiments we also showed that there is a loss of H3K9me3 enrichment at L1Md promoters one-month post TBI that correlates to an increase in L1Md expression in HSCs. In line with Djeghloul's results, our results suggest that the H3K9me3 mark plays an important role in the repression of L1Md expression in HSCs. However, it would be interesting to compare H3K9me3 ChIP-seq results in aged HSCs to those from irradiated HSCs to see if there are any differences in the epigenetic changes brought about by IR and aging.

Miousse et al., showed that upon IR there was a loss of up to 12% fold-change of DNA methylation in the 5'UTR of L1Mds (L1Md_A and L1Md_T) in HSCs of C57Bl6 mice. On our part, we have observed a loss of 31% enrichment of H3K9me3 at L1Md_A promoters and 28% loss at common L1Md 5'UTR regions by ChIP-qPCR upon IR (Miousse et al. 2017). Despite the fact that our experimental setups are different, (we irradiated at a dose of 2Gy vs. 0,1Gy/1Gy in their study), these two results show how two different epigenetic mechanisms such as DNA methylation and histone modifications are simultaneously affected by IR. Several studies have shown that different RTEs are silenced by different epigenetic mechanisms according to their evolutionary status, type, structure and tissue localization (Walter et al. 2016; Ohtani et al. 2018; Trizzino et al. 2018). Miousse et al. stated that the loss they observed on DNA methylation in HSCs was not L1-promoter type-dependent or evolutionary age-dependent, as they compared and found same effects on younger L1Md_A and

L1Md_T to the more ancient L1Md_Fanc(Miousse et al. 2017). On the contrary, we observed global H3K9me3 loss specifically at young L1Mds compared to older Lx or ERVs, suggesting that H3K9me3 enrichment is L1-promoter type-dependent and evolutionary age-dependent. We have not assessed H3K9me3 enrichment at L1Md_Fanc, so it is possible that, despite the fact that this subfamily is older than the other L1Mds that we have tested, that it there is also loss of H3K9me3 at L1Md_Fanc post IR. DNA methylation should be assessed at an even older L1 family, such as the Lx, to truly be able to see if changes in DNA methylation are age-dependent.

It would be interesting to study if changes in DNA methylation or other epigenetic marks such as H3K9me2 or H3K27me3 are also deregulated upon IR, and if so, if they also affect young L1Mds or other types of RTEs. Concerning DNA methylation, bisulfite sequencing experiments would be useful to evaluate changes in DNA methylation post IR. To study the impact of IR on other histone marks, we have already performed ChIP-seq experiments on H3K27me3 as well as on the activating histone mark H3K4me3. Their analyses are underway. The effect of IR on different epigenetic marks could also be done globally through IF or western blot analysis. We have also performed immunofluorescence experiments to study the global effect of IR on epigenetic marks. However, in the case of H3K9me3 we have obtained variable and contradictory results to our ChIP-seq data. It is possible that the H3K9me3 mark is hardly accessible to the antibody, and that this, coupled to epitope unmasking which could occur after IR, would lead to observed changes in H3K9me3 signaling without actual biological changes in H3K9me3. To try to bypass this issue, we performed western blot experiments, but we did not observe any differences in total H3K9me3 between non-irradiated and irradiated conditions. The issue with performing this type of experiments in HSCs is the very limited cell numbers, which complicates the quantification of small differences, so fine-tuning of these experiments is required in the future. The use of mass cytometry (CYTOF) technology would be a valuable tool to obtain a global picture of all the epigenetic changes induced by IR. The advantage of CYTOF is that it allows for the simultaneous analysis of several epigenetic marks, and could potentially elucidate compensatory epigenetic mechanisms that may be put in place following IR exposure (for example, if another silencing mark such as H3K27me3 would be enriched upon loss of H3K9me3). Additionally, mass cytometry also allows for the analysis of different cellular populations at the same time, therefore it would also be possible to observe differences in how IR affects epigenetics in HSCs compared to progenitor cells. This could provide additional clues in the understating of how IR can affect HSC function.

Given the difficulties of sorting freshly irradiated HSCs, we have analyzed L1Md expression and their association with the H3K9me3 mark one month after IR when the HSC markers are restored allowing their FACS sorting. To bypass this problem and determine whether the changes induced by IR are direct, HSC intrinsic, and short-term we used a bone-marrow derived hematopoietic progenitor (derived from LSKs) cell

line (BM-HPC) (Pinto do Ó et al. 2002). With this cell line we were able to observe that changes in H3K9me3 and L1Md_A induction following IR occur as fast as 5h after irradiation *in vitro*. This shows that the effect of IR is direct rather than resulting from selection *in vivo* of a particular population of cells with low H3K9me3 levels. Moving forwards, validation of these results through *in vitro* experiments in HSCs are required.

The understanding of the complex epigenetic regulation of different RTEs is an essential step in the use or the conception of epigenetic drugs that can induce or repress RTE sequences, either to potentiate their cytotoxic effects or to restrain their potential for genomic insult. For example, the development of epigenetic drugs that could protect HSCs against the deleterious effects of IR could be of interest for patients undergoing radiotherapies for solid tumors in hopes of preventing the unwanted deregulation of L1 elements that could threaten genomic integrity and HSC function, thereby hindering the chances of developing MDS or myeloid leukemias with age and preserving HSC function .

2. Irradiation induces transcriptomic changes in HSCs that are associated to the presence of intronic L1Mds

RTEs occupy a large portion of human and mouse genomes, and 20% accounts for L1 elements only (Mouse Genome Sequencing Consortium 2002). Of these, nearly 7000 full-length L1 elements have been reported to be evenly distributed between intronic and intergenic regions in humans (Khan et al. 2006). Faulkner et al. have shown that 6-30% of mouse and human transcripts initiate within repetitive elements (Faulkner et al. 2009). RTEs may affect gene regulation in a variety of ways, therefore sought out to determine if the global changes in H3K9me3 induced by irradiation could impact RTE expression and the HSC transcriptome.

Our RNAseq data show that IR induces strong transcriptional deregulation in HSCs, with downregulation being more prevalent than upregulation. The observed deregulation was very strong, with most expression changes surpassing a 50-fold change. Given that IR induced a global loss of H3K9me3, the fact that there are more genes downregulated than upregulated may seem contradictory. However, H3K9me3 is usually active in non-coding regions of the genome, rather than in the control of gene expression. This mark also has a known role in RTE repression We have observed that loss of H3K9me3 occurs at L1Md loci, and that this correlated to an increase of L1Md expression. Therefore we hypothesized that derepression of L1Mds could be linked in part to IR-induced changes in gene expression.

The above data show that IR both induces a global loss of H3K9me3 that mainly affects L1Mds and can affect the HSC transcriptome. To establish if there is a correlation between these two phenomena, we looked for an association between gene deregulation and the presence of L1Mds in their neighborhood or in their gene body.

To do so, we crossed the list of genes deregulated upon IR with a list of genes neighboring or hosting an L1Md (see *materials and methods*), and we did the same for 10000 lists of the same number of random genes (**Figure 4A**). We found that genes deregulated upon IR are significantly associated with intronic L1Mds ($p < 0.0001$ -permutation test) (**Figure 4B**) and not with neighboring L1Md (*data not shown*). This association seems specific as it was not found with the older L1 subfamily Lx5 (**Figure 4B**). Interestingly, the association to intronic L1Mds was specific for genes that are downregulated upon IR, as opposed to upregulated (**Figure 4C**). These results suggest that intronic L1Mds might play a role in regulating the expression of their host genes.

To analyze this hypothesis we looked for an association between gene deregulation and the presence of L1Mds in their neighborhood or in their gene body by crossing the list of genes deregulated upon IR with a list of genes neighboring or hosting an L1Md. Surprisingly, we showed that genes downregulated upon IR were significantly and specifically associated with intronic L1Md. Furthermore, no association was found with intergenic L1Md or with older RTEs such as the Lx5 family.

Intragenic RTE expression can lead to gene repression in many ways. It has been shown that intronic L1 elements can act as molecular “rheostats” by inhibiting elongation of RNA Pol II, and inducing premature termination and cryptic polyadenylation with L1 ORF1 and ORF2 sequences of the host gene transcript (Perepelitsa-Belancio and Deininger 2003; Han et al. 2004). Moreover, a study by Aporn Dewan et al. shows that in cancer cells, hypomethylation of intragenic L1s leads to the formation of dsRNA between the L1 mRNA and the complementary pre-mRNA of its host gene. This dsRNA then gets targeted by the RISC complex that detects and degrades complementary RNA molecules, leading to downregulation of host gene expression (Aporn Dewan et al. 2011). Recent studies have shown that the HUSH complex and the MORC2 protein specifically target intronic, long full length, young L1s both in mouse and human cells (L1Md, L1PA/Hs) and can mediate transcriptional silencing through H3K9me3 recruitment leading to downregulation of host gene expression (N. Liu et al. 2018; Robbez-Masson et al. 2018). However, it is not clear whether this mechanism also applies here. Indeed, the association we observed between genes downregulated upon IR and presence of intronic L1Mds does not necessarily concern full-length L1Mds that contain active promoters in their 5'UTR regions. In addition, this association was not affected when we separated intronic L1Mds by size. This suggests that the ability of an intronic L1Md to affect gene expression does not depend on the presence of an active promoter. This was further confirmed by experiments in genes such as *Ghr*, *Alcam*, or *Rmdn2*, which harbor small L1Mds in their introns (1.2-3.5Kb) that lack L1-promoters, where we observed a loss of gene expression post IR accompanied by a loss of H3K9me3 at their intronic L1Mds.

Whereas RTEs have also been shown to act as cis-regulators of gene expression *via* their promoter or enhancer activity, we did not find an association between genes that were

upregulated upon IR and intronic or intragenic L1Md. We did not experimentally analyze H3K9me3 enrichment by ChIP-qPCR at L1Mds of upregulated genes, so it may be possible that IR did not impact enrichment of H3K9me3 at these loci altogether and thus they remained repressed. An alternative would be that loss of H3K9me3 alone is not enough to induce alternative transcription or enhancer activity. Supporting this alternative, a very recent study by Cao et al. showed that RTEs with enhancer activity are in fact characterized by activating histone marks such as H3K4me1, H3K27ac, H3K4me2, and H3K4me3 (Cao et al. 2019). Ongoing ChIPseq analysis of the activating mark H3K4me3 will be useful to determine if irradiation can impact activating marks at L1Mds and whether this could be related to increased transcription of some upregulated genes.

3. Does derepression of L1Mds by IR induce changes in gene expression *via* RNA interference?

As was mentioned briefly in the previous section, L1 expression has been reported to affect gene expression in different ways. Recruitment of RNA Pol II to promoters has been generally established as a rate-limiting step in gene activation. Manipulation of the chromatin environment by affecting histone methyltransferases or histone deacetylases is associated to RNA Pol II stalling (Wu and Snyder 2008). Han et al. have shown that highly expressed genes have small amounts of L1s, whereas poorly expressed genes have large amounts of L1 sequence. They suggested that because RNA pol II gradually pauses and/or dissociates from the template as it encounters longer stretches of L1 sequence, L1 intronic insertions would attenuate expression of their host gene (Han et al. 2004). Additionally they also proposed that, even though L1 insertions in either orientation can attenuate the expression of host genes, premature polyadenylation would be expected to be more prevalent for antisense L1 insertions and transcriptional elongation defects for sense insertions (Han et al. 2004). Kaer et al. have shown that intronic full-length L1s can also induce intron retention and force exonization and cryptic polyadenylation upstream of L1s. They proposed that intronic L1s could interfere with elongation of Pol II by forcing it to slow down or dissociate from its template, resulting in prematurely terminated transcripts and thus a decrease in gene expression (Kaer et al. 2011). Both Han's team, and Kaer's team, showed that transcriptional interference was stronger when the L1 insertion was arranged in tandem orientation with respect to host gene transcription (Han et al. 2004; Kaer et al. 2011).

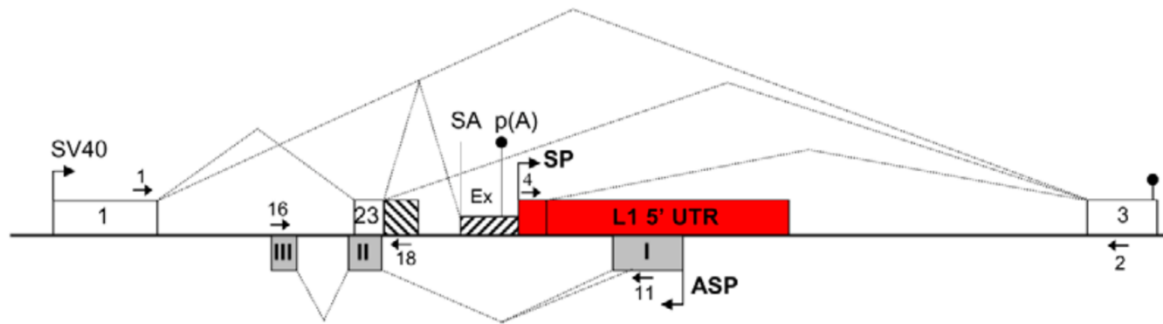


Figure 1. Schematic representation of the ‘full-fragment’ ABCA minigene constructed by Kaer et al. A 1036 bp genomic fragment containing intron 22, exon 23 and intron 23 of the ABCA9 gene and a 990 bp L1 5’ UTR was inserted into the exon trapping vector pSPL3 containing SV40 promoter. Splicing of exons observed in different transcripts is shown by diagonal lines. Deletions made in ABCA FI construct are marked with lines below the scheme. Intron retention to exon 23 is shown by hatched box with downward diagonals. Exonization of intronic sequence upstream to L1 59 UTR is shown with hatched box with upward diagonals and marked with Ex. In this exon cryptic acceptor splice site (SA) and polyA signal (p(A)) are marked. Additional SV40 polyA signal (marked with lollipop) is located in exon 3. L1 ASP drives transcription from the opposite strand and produces transcript containing exons I, II and III (grey boxes). (Kaer et al. – 2011)

Kaer et al. actually proposed a Russian-doll-like model, based on nested genes, for how genes could be generated from within genes as a result of a mobile element such as an L1 (Kaer et al. 2011). They performed an in silico genome-wide search for prematurely terminated transcripts bearing signatures of intron retention, forced exonization and cryptic polyadenylation at their 3’ ends upstream to the human intronic L1 retrotransposons (Kaer et al. 2011). From this analysis, they selected the ABCA9 gene which contained a full-length intronic L1PA3 and they constructed a minigene containing a portion of the ABCA9 gene that contained an L1 5’UTR and an SV40 promoter (**Figure 1**). They showed that Pol II from the L1 promoter acted as a “roadblock” by forcing the elongating SV40 Pol II to pause or dissociate from the template and thereby strongly interfering with SV40 transcription (Kaer et al. 2011). They also proposed that L1 Pol II from an antisense promoter could also affect SV40 Pol II elongation by colliding against it and decreasing its transcription efficiency (Kaer et al. 2011).

To study the mechanism *via* which IR-induced L1Md derepression could interfere with gene expression, we used *Mecom* as our candidate gene. The L1Md in *Mecom* we analyzed is full-length and in tandem orientation with respect to host gene transcription. We performed Ser5Pol-II ChIP-qPCR to assess initiating polII enrichment. Our preliminary results showed that upon IR there was an enrichment of Ser5 RNA Pol II at the promoter of *Mecom*’s intronic L1Md (**Figure 2**). Phosphorylation of Ser5 is a mark for transcriptional initiation, whereas phosphorylation of Ser2 complex promotes elongation (Kornblihtt 2006). Lorincz et al. suggest that the appearance of a Pol II phosphorylation mark that is typical of initiation at specific sites within a gene could be either the cause or the consequence of the generation of internal ‘roadblocks’ to elongation. Our preliminary results are consistent with initiation activity at *Mecom*’s intronic L1Md and suggests that L1Md transcription upon IR may

Figure 2

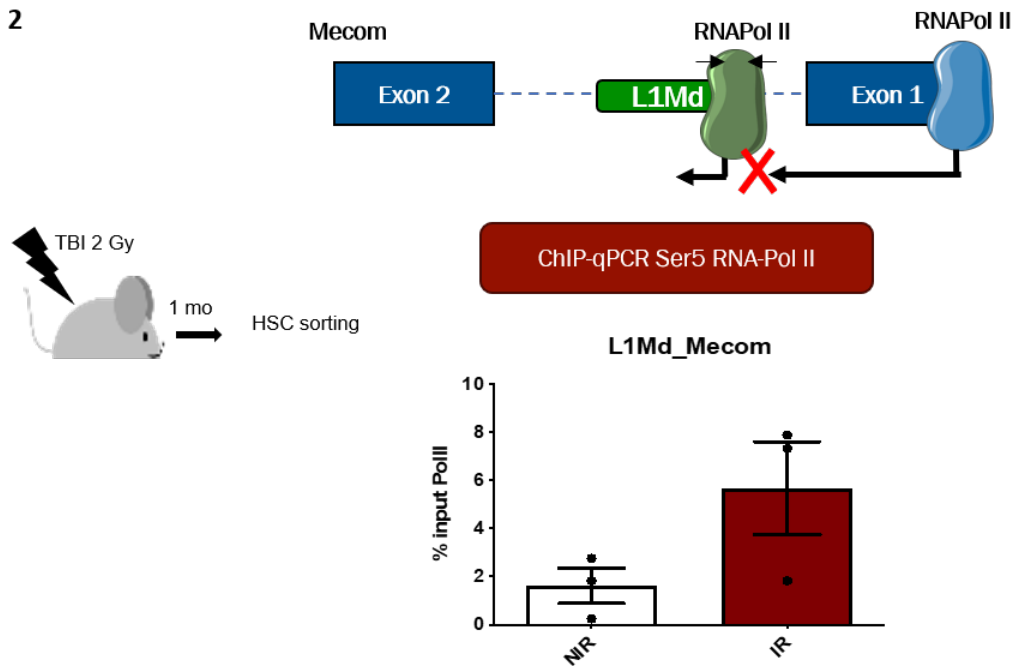


Figure 2. IR-induced L1Md derepression leads to transcription initiation at intronic L1Md. Ser5Pol-II enrichment at an intronic L1Md_T promoter in Mecrom analyzed by ChIP-qPCR of one independent experiment. Each dot represents a pool of 3 (NIR) or 4 (IR) mice. Ct values normalized to % of input. Results are expressed as fold change from the mean value of the NIR condition and represented as mean \pm SEM.

likely take place. We also observed that Ser5 phosphorylation occurs concomitantly to the loss of H3K9me3, but further research should be carried out to determine the causality of these events. Furthermore, it would be interesting to assess the ratio of Ser2 Pol II immediately upstream and downstream of the L1Md, which would help illustrate if there is accumulation of Pol II due to stalling.

Nevertheless, as was mentioned earlier we have also observed changes in gene expression following IR in genes that contain short L1Mds that likely lack promoters (Alcam, Ghr, and Rmdn2), as well as in genes that contain L1Mds in anti-sense orientation (Ghr and Rmdn2) with respect to host gene transcription. Han et al. show that cryptic poly-adenylation sequences can occur at sites scattered throughout the entire L1 sequences (Han et al. 2004). This suggests that despite of their lack of promoter, truncated, small intronic L1Mds could still have an impact on host gene expression by inducing premature termination of host gene transcription. Loss of H3K9me3 in the gene body can also affect RNA-Pol II elongation rate, as well as gene splicing, so regardless of intronic L1Md transcription, loss of H3K9me3 can still affect host gene expression. Alternatively, it is also possible that intronic L1Md sequences of genes whose expression is affected by IR could harbor alternative promoters in their 3'-end and that transcription could be triggered at these sites due to alterations in epigenetic and signaling pathways as a result of IR. Faulkner et al. have described a 3'

L1 promoter that is expressed in several tissues and were the bulk of L1 transcription initiation took place (Faulkner et al. 2009).

Due to the repetitive nature of L1Md, it is impossible to test the expression of a particular L1Md by RT-qPCR or classical RNAseq. Therefore, in order to truly be able to determine if L1 transcription is taking place, the use of new long-read nanopore RNA sequencing technology should be considered. The advantage of this technique over classical RNA-seq is that it allows for the identification of full-length transcripts, that are not fragmented or amplified, and that it allows for the easy characterization of antisense transcripts.

4. The NF- κ B signaling pathway is downregulated upon IR and may be involved in loss of H3K9me3 at intronic L1Mds and loss of the HSC signature

GSEA analysis showed that IR leads to the loss of the HSC signature as described by Chambers et al. Chambers et al. used a global gene expression profiling approach and characterized hematopoietic cells by defining a “fingerprint” consisting of 40-350 genes that were uniquely expressed in HSC as opposed to other progenitor cells (Chambers 2007).

We found that 50% of genes in the HSC signature and whose expression was decreased upon IR harbored an intronic L1Md, suggesting an involvement of L1Md in the IR-induced loss of the HSC signature. Moreover, 75% of the human orthologous of these genes also contain a young human and primate L1 (L1Hs/L1PA), which suggests that these evolutionary recent L1s could have a conserved functional role in the regulation of HSC gene expression.

To validate these results, we selected several target genes downregulated upon IR and which are all conserved in human and harbor different types of intronic L1Mds (L1Md_T, A and F2). We evaluated if changes in expression post IR were accompanied by changes in enrichment of H3K9me at their respective intronic L1Md.

Two of these genes belong to the HSC signature. One of them, *Mecom* was discovered in a model of retrovirus-induced acute myeloid leukemia, where it was overexpressed as the result of insertion of viral promoter and enhancer sequences (Bartholomew et al. 1989). *Mecom* harbors a large L1Md located in the first intron of the longer *Mecom* isoform. We also analyzed the Growth Hormone Receptor (*Ghr*), whose signaling has been implicated in several age-related hematopoietic phenotypes (Stewart et al. 2014). GHR is a class I cytokine receptor that, like TPO, signals via the JAK-STAT signaling pathway (Dehkhoda et al. 2018). GHR is expressed in most human leukemia forms (Manabe et al. 2006) but its role in HSC function and leukemogenesis has been less characterized than *Mecom*'s. *Ghr* contains 17 intronic L1Mds spread out throughout its introns.

One of the other targets we analyzed was the activated leukocyte cell adhesion molecule (ALCAM). Although ALCAM does not belong to the HSC signature that Chambers described, ALCAM is differentially regulated in adult hematopoiesis and is highly expressed in LT-HSCs where its level progressively increases with age (Jeannet et al. 2013). *Alcam* harbors 6 L1Mds in its introns. Jeannet et al. showed that *Alcam*^{-/-} HSCs had reduced long-term repopulating capacity *in vitro* and reduced long-term engraftment potential upon transplantation (Jeannet et al. 2013). A very recent study by Wei et al. shows that the *ALCAM* gene promoter was heavily methylated in placentas and peripheral blood of women suffering from preeclampsia suggesting that DNA methylation may play an important role in the regulation of *ALCAM* expression (Wei et al. 2020). These studies suggest that even though ALCAM expression is not specific of HSCs, it still plays an important role in regulating HSC function and that its expression is sensitive to epigenetic modifications.

Our results validate our RNA-seq data and showed that upon IR, expression of *Mecom*, *Ghr*, and *Alcam* was downregulated, and that it coincided with a loss of H3K9me3 at their respective intronic L1Mds. The same proved true for two other selected target genes, *Rmdn2* and *Rbms3*, which were found downregulated upon IR. On the contrary, we did not observe changes in mRNA expression, or H3K9me3 enrichment in genes which were not deregulated upon IR that also harbored intronic L1Md, such as *Mapre2* or *Snx27*. Importantly, in the basal state L1Mds from both *Mapre2* and *Snx27* genes are similarly enriched in H3K9me3. These results show that IR does not affect similarly all intronic L1s and rise an interesting question as to why.

GSEA analysis also revealed that the TNF- α /NF- κ B signaling pathway is one of the most downregulated upon IR. Cytokines belonging to the TNF family induce rapid transcription of genes regulating inflammation, cell survival, proliferation and differentiation, primarily through activation of the NF- κ B pathway (Hayden and Ghosh 2014). In HSCs, TNF- α activates the Notch and NF- κ B pathways to establish HSC fate, suggesting a requirement of inflammatory signaling in HSC generation (Espín-Palazón et al. 2014). A recent study by Yamashita et al. has shown that TNF- α promotes HSC survival and myeloid differentiation by activating a strong and specific p65-nuclear factor κ B (NF- κ B)-dependent gene program that primarily prevents necroptosis rather than apoptosis, induces immunomodulatory functions, and poises HSCs for myeloid cell production (Yamashita and Passegué 2019). The NF- κ B pathway can also be induced by a number of stimuli such as viral and bacterial infections, and physical or oxidative stresses (Gilmore 2006). It was also previously shown by our lab that IR can induce a moderate NF- κ B response (de Laval et al. 2014).

A recent study by Fang et al. has shown that TNF receptor associated factor 6 (TRAF6) is essential to regulate HSC homeostasis by preserving self-renewal and quiescence through basal activation of NF- κ B in the absence of inflammation (Fang et al. 2018).

Interestingly TRAF6 activity is regulated by intracellular homeostatic processes such as oxidative stress, and has been reported as upregulated in a subset of MDS and AML patients and in genetic mouse models, where it leads to hematopoietic defects (Fang et al. 2012; Fang et al. 2017). A possible explanation for the loss of NF- κ B signature may be that IR interferes with TRAF6 function and lead to a loss of NF- κ B signaling. Deletion of TRAF6 results in loss of HSC fitness, so this could contribute towards HSC loss of function upon IR.

To understand the mechanisms that direct the specific loss of H3K9me3 at intronic L1Md of genes downregulated in IR, we performed *de novo* motif enrichment analysis, which provides an unbiased approach to detect enriched motifs and then matches them to known motifs. Interestingly, L1Md sequences of genes downregulated upon IR were significantly enriched for a particular motif that was matched to the p50 subunit of NF- κ B motif (NFKB1) as well as for the transcription factor Rel, which also belongs to the NF- κ B pathway. Both the NFKB1 and Rel motifs were found in the intronic L1Mds of *Mecom*, *Alcam*, *Rmdn2* and *Rbms3* that exhibited loss of H3K9me3, and were notably absent from the intronic L1Md of control genes *Mapre2* and *Snx27*, which do not lose H3K9me3 upon IR. There was however one exception, *Ghr*, where we did observe a loss of H3K9me3 upon IR but were not able to detect enrichment of either the NFKB1 or Rel motifs. We were only able to perform this experiment on *Ghr* L1Md once, so loss of H3K9me3 at this intronic L1Md requires further validation. Additionally, the L1Md in *Ghr* on which we performed ChIP-qPCR is the smallest L1Md (1217bp) that was analyzed, so it is possible that the portion of the L1Md that contained the motif was truncated. We performed motif analysis on the 17 intronic L1Mds in the *Ghr* gene and actually detected enrichment for the NFKB1 motif in its one of them. It would therefore be possible to imagine that through L1 interaction loops, loss of H3K9me3 at one L1Md could also affect H3K9me3 at other L1Mds. Supporting this idea, none of the L1Mds present in the control genes *Snx27* and *Mapre2*, present the enriched NFKB1 motif. Further research is required to clarify this observation, but it would be interesting to perform ChIP-qPCR experiments on the L1Mds that contains the enriched NFKB1 in *Ghr* to see if it also loses H3K9me3 enrichment following IR.

We also performed *de novo* motif enrichment analysis and observed that the NFKB1 pathway was also enriched in the L1Md sequences of genes that lose H3K9me3 compared to the L1Md sequences of genes that gain H3K9me3; and in the L1Md sequences of the genes that contribute to the loss of the HSC signature compared to L1Md sequences of genes that do not contribute to the loss of the HSC signature. Furthermore, the L1HS/L1PA of the human orthologous of genes downregulated upon IR were also enriched for the NFKB1 motif compared to the L1HS/L1PA of the human orthologous of non-deregulated genes. Together with the loss of the NF- κ B signature observed in GSEA, these results strongly suggest that this pathway may be involved in H3K9me3 deposition at L1Mds of some genes. This could thus contribute to the

regulation of host gene expression in HSCs and thereby to the loss of the HSC signature. This function could be conserved in humans.

In line with this idea, the NF- κ B pathway transcription factors have been shown to interact with epigenetic mediators, such as histone deacetylases, and impact HSC function. Priel et al. have shown that the transcription factor CEPB α , which is essential for myelopoiesis, can activate the p50 subunit of NF- κ B (NFKB1) by displacing histone deacetylases leading to the induction of antiapoptotic genes (Paz-Priel et al. 2011). Interestingly, we found that *Cebpd*, another member of the CEBP transcription factor family that can form heterodimers with CEPB α (Kinoshita et al. 1992), is among the genes that contribute to the loss of the NF- κ B signaling following IR in our GSEA analysis. Furthermore, our RNA-seq data show a strong decrease of CEPB α upon IR. Fleenor et al. have also reported inhibition of CEPB α upon IR and loss of self-renewal in HSCs. These results also suggest that the loss of NF- κ B signaling could be related to the loss of HSC function we have observed.

Elsharkawy et al. have shown that NFKB1 recruits the histone deacetylase HDAC1 and acts as a negative regulator of inflammation (Elsharkawy et al. 2010). Moreover, Ea et al. have shown that the methyltransferase EHMT1 also interacts with NFKB1 and negatively regulates an important portion of NF- κ B target genes (Ea and Baltimore 2009). Together with Priel's results, these studies establish a connection between epigenetic mechanisms and the downregulation of NF- κ B signaling. If NF- κ B signaling is involved in the recruitment of repressive marks such as histone deacetylases or methyltransferases, it could also be imagined that a downregulation of this signaling pathway could also affect HSC epigenetics. However, presence of NFKB1 at L1 elements has not been reported as of yet.

These results suggest that transcriptional deregulation upon IR could possibly be a consequence of epigenetic deregulation at intronic L1Mds present in these genes; and may lead to the loss of overall cellular functionality. The fact that an important portion of genes in the HSC signature contains an L1Md opens up new perspectives in the understanding of the molecular mechanisms that can drive leukemogenesis or the establishment of the preleukemic state; it can also provide new therapeutic candidates in the treatment of hematopoietic malignancies.

The next steps of this project should be geared towards gaining an understanding of the mechanisms by which NFKB1 may control H3K9me3 deposition at specific L1Mds and how L1Md specifically mediate gene repression following IR, as well as the functional impact this has on HSCs. The deletion of intronic L1Md using CRISPR Cas9 technology and Cas9 expressing mice would be the best tool to fully understand the impact of a specific gene intronic L1Md derepression has on its expression. I have already designed guides targeting the intronic L1Md in *Mecom*. We expect that the deletion of its intragenic L1Md will lead to an increase of *Mecom* expression in NIR

mice. If gene repression upon IR is in fact mediated by intronic L1Md derepression, we also expect to see no change in *Mecom* expression upon IR in HSCs with L1Md deletion.

5. Loss of *Mecom* expression may also lead to loss of the HSC signature

An alternative explanation for the loss of the HSC signature upon IR could also come from the loss of *Mecom* expression. MECOM is highly expressed in the human embryo as well as in HSCs in the adult bone marrow. Yuasa et al. reported that HSCs in *EVI-1*-deleted mice are deficient in proliferation and repopulation, and also express low levels of GATA-2 which is crucial for the proliferation and differentiation of HSCs. Re-establishing *EVI-1* expression in these mice can restore the cell ability of proliferation and differentiation by upregulating GATA-2 expression, highlighting *EVI-1*'s role in the proliferation and differentiation of HSCs (Yuasa et al. 2005).

Most literature does not differentiate between MECOM and *EVI-1*. The *MECOM* locus actually gives rise to several alternatively spliced transcripts that code for at least 2 different proteins, *EVI-1* and MECOM. The MECOM protein contains an extra exon and encodes a PR domain (a sub-class of zinc fingers), but is otherwise identical to the *EVI-1* protein (Maicas et al. 2017). *EVI-1* possesses its own transcription start sites, on the contrary mRNA transcripts initiating at *MDS1*, located 0.5 Mb upstream of *EVI1*, can splice from exon 2 of *MDS1* into exon 2 of *EVI-1* (**Figure 3**) (Zhang et al. 2011). Whereas *EVI-1* is considered to be a potent oncogene associated with transformation and proliferation in multiple leukemias, expression of MECOM is frequently abrogated and a low MECOM/*EVI-1* expression ratio predicts an extremely poor prognosis for AML patients (Ivanochko et al. 2019). A recent study by Maicas et al. has shown that the MECOM form reduces the growth ability of hematopoietic progenitor cells, whereas the *EVI-1* form does the opposite. They suggest that the *EVI-1* protein acts by promoting tumor progression, while MECOM protein may function in a tumor-suppressive manner, highlighting opposite function of the two different proteins (Maicas et al. 2017). Pinheiro et al. have shown that MECOM can act as a specific H3K9me1 methyltransferase in human embryonic fibroblasts to initiate heterochromatin formation, which is then converted in the nucleus by the SUV39H enzymes into H3K9me3 (Pinheiro et al. 2012). Interestingly, Goyama et al. have reported that *EVI-1* interacts with the histone methyltransferases SUV39H1 and G9a, and that this interaction contributes to transcriptional repression and bone marrow immortalization by *EVI-1* (Goyama et al. 2010). Furthermore, Vinatzer et al. have also shown that MECOM can interact with histone deacetylases through its zinc-finger domain to mediate gene repression (Vinatzer et al. 2001). Taken together, these studies suggest that MECOM/*EVI-1* has a role in the regulation of epigenetic machinery to mediate gene repression. We observed that upon IR, there was a loss of expression of *Mecom*. It could therefore be possible that *Mecom* downregulation upon IR could play a role in the global loss of H3K9me3 we observe upon IR by impacting

methyltransferase activity, as well a contribute to the loss of the HSC signature. However, in this case scenario, loss of the HSC signature would not be a consequence of loss of H3K9me3 at intronic L1Md linked to the presence of NFKB1 motifs.

MDS1 and EVI1 complex locus (MECOM)

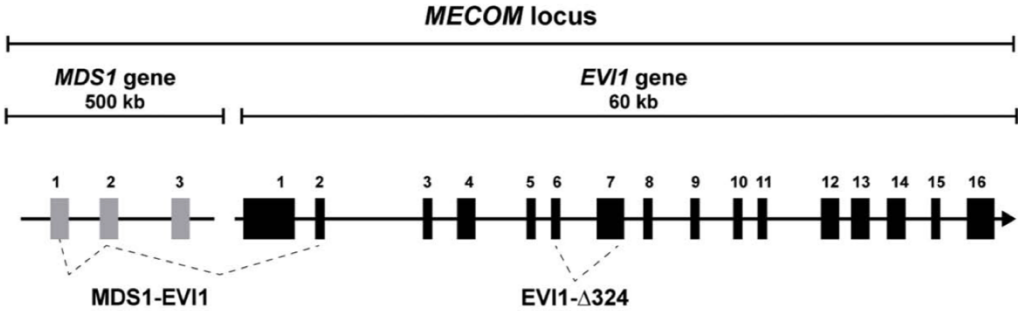


Figure 3. Schematic representation of the human Mecom locus. The alternative intergenic splicing between the second exon of *MDS1* and the second exon of *EVI1* is represented by dashed lines. The alternative intragenic splicing variants of *EVI1* are represented by lines. (Maicas et al. – 201

6. The role of TPO as an epigenetic modulator

L1Mds represent an important endogenous source of genomic instability for HSCs. We have shown previously that TPO can protect HSCs from loss of function and accumulation of DNA damage following IR. Through microarray experiments, the team showed that TPO induced an anti-viral IFN-like response. We showed that TPO could also protect HSCs from uncontrolled RTE expression and mobilization caused by IR. The fact that TPO injection is done prior to IR suggests that TPO acts as a protective mechanism rather than a corrective one and may therefore act through an epigenetic mechanism. In this section, I will discuss the results we obtained concerning the role of TPO as an epigenetic modulator in HSCs, and why we chose to exclude them from the results section of this manuscript.

To test the possibility that TPO may act as an epigenetic modulator, in parallel to IR we performed ChIP-seq experiments in HSCs from mice treated with one dose of TPO 1h before IR (**Figure 4A**). Interestingly, opposite to what was seen after IR where the majority of the differentially enriched peaks were downregulated, multiple mapping analysis shows that only 238 reads are significantly ($p < 0.05$) and differentially enriched in the IR+T condition compared to the NIR condition, and that 74 of the reads were up and 164 of them were down in IR+T (**Figure 4B**). These results suggest that TPO injection may be capable of preventing IR-induced loss of H3K9me3 at certain loci. Heatmap analysis of the H3K9me3 enriched peaks confirmed the global loss of H3K9me3 upon IR that is prevented by TPO injection (**Figure 4C**). ChIP-qPCR in HSCs that TPO injection could also prevent decreased H3K9me3 enrichment at 5'UTR and L1_A promoters following IR (**Figure 4D**). These results suggested that TPO may control L1Md expression through H3K9me3.

We also performed RNA-seq analysis to determine the impact of IR in the presence of TPO on the HSC transcriptome. Interestingly, results revealed that TPO could prevent 74% of the transcriptomic changes brought about by IR, but could only restore ($\text{TPM} > \text{median of IR for at least 2 triplicates}$) the expression of 6,8% of the genes which disappear ($\text{TPM} < \text{median of NIR for at least 2 triplicates}$) upon IR). However, TPO injection prior to IR also induced nearly as much transcriptional deregulation as IR, with 1034 specific differentially expressed genes (687 down- and 347 upregulated) in the IR+T condition compared to the NIR condition (**Figure 4E**).

We had observed a strong ($p < 0.0001$) and specific association between genes downregulated ($p < 0.05$) upon IR and the presence of intronic L1Mds. However, we found that genes that were downregulated in the IR+T condition were also significantly, although less strongly ($p = 0.01$), associated to the presence of intronic L1Mds (**Figure 4F**). Furthermore, when we analyzed what happened to the expression of genes that were downregulated in IR vs NIR in the IR+TPO condition,

Figure 4

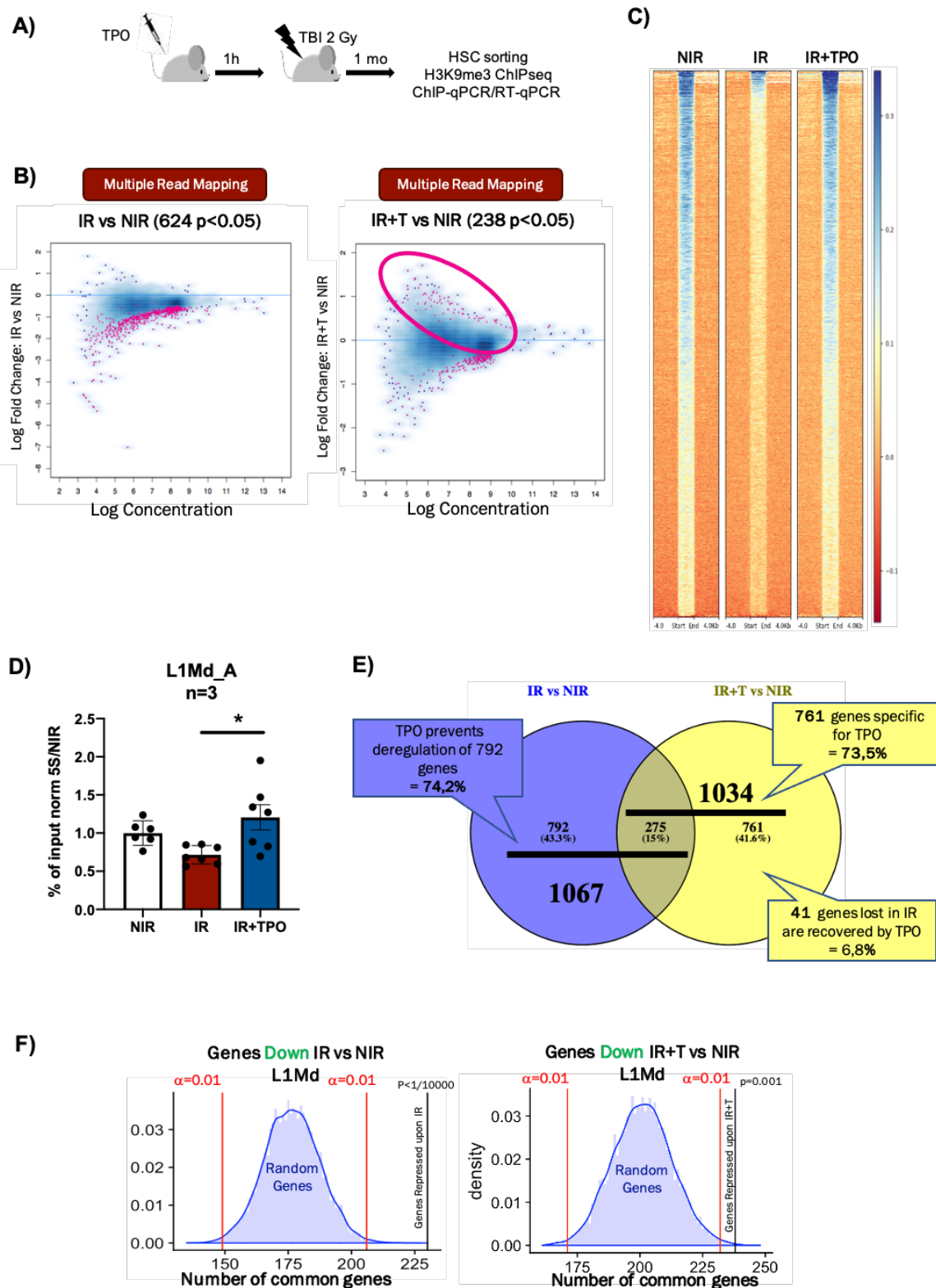


Figure 4. The role of TPO as an epigenetic modulator. A) Experimental setup. Mice were injected with TPO retro-orbitally 1h prior (or not) to 2Gy total body irradiation. HSCs were sorted from pools of 3-5 mice. B) Quantitative analysis of H3K9me3 enrichment at multiple mapping reads in IR (left) and IR+TPO conditions (right). MA-plots show significantly ($p < 0.05$) and differentially enriched peaks in pink dots. C) Heatmap of H3K9me3 enrichment at peaks in NIR, IR and IR-T conditions. Heatmaps show H3K9me3 enrichment 4kb upstream and downstream of the peaks, and in the 2.5kb scaled peak regions (2.5kb being the mean size of the peaks). H3K9me3 enrichment at L1Md_A promoters analyzed by ChIP-qPCR (n=3 independent experiments, each dot represents pools of 3 mice) Ct values normalized % of input of negative control repetitive 5S ribosomal RNA. Results are expressed as fold change from the mean value of the NIR condition and represented as means +/- SEM (* $p < 0,05$ one-way ANOVA). E) Venn diagram showing gene deregulation in the IR vs NIR (purple) and IR+TPO vs NIR (yellow) condition. F) Distribution of the number of genes found in common between 10000 lists of random genes and genes hosting an L1Md. The black vertical line represents the number of genes found in common between downregulated genes upon IR (right) and downregulated genes in the IR+T condition (left). Significance bars ($p < 0.01$) in red.

we observed that TPO could only moderately restore the expression of some of these genes regardless of whether or not they contained an intronic L1Md (**Figure 5A**).

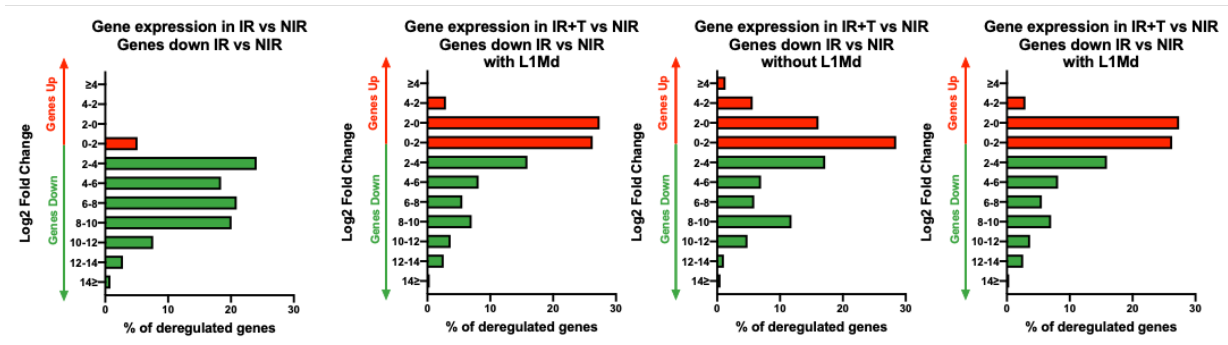
TPO injection was not able to prevent the loss of the HSC or NF- κ B signatures that we observed upon IR (**Figure 5B**). In line with these results, TPO did not prevent loss of H3K9me3 enrichment at any of the intronic L1Mds that we analyzed by ChIP-qPCR (*Mecom*, *Alcam*, *Ghr*, *Rbms3*, *Rmdn2*), and was only able to moderately prevent loss of gene expression post IR for some of them. (**Figure 5C**). These results suggest that TPO may only be able to induce a moderate restoration of H3K9me3, potentially due to the fact that it does not restore the NF- κ B signature. This may not be enough to prevent loss of host gene expression, and therefore could explain why loss of the HSC signature is not prevented by TPO.

These results suggest that despite rescuing the global loss of H3K9me3 at L1Mds induced by IR, TPO could function through other additional mechanisms, to control L1Mds and or gene expression; For example, it could act as a modulator of DNA methylation or other histone marks to further repress L1Md expression. Walter et al. have shown in ESCs that upon loss of DNA methylation, various RTE families were efficiently re-silenced through an epigenetic switch leading to an increase in H3K27me3 (Walter et al. 2016). Ohtani et al. also showed that an increase of H3K9me2/3 was observed at LTRs following treatment with a DNA methylation inhibitor, showing that “epigenetic switching” occurs to ensure silencing after loss of DNA methylation (Ohtani et al. 2018). It would therefore be imaginable that the opposite may also exist, and that TPO would promote the setup of other epigenetic mechanisms as a “back-up” response in order to protect HSCs from L1Md derepression upon IR-induced loss of H3K9me3.

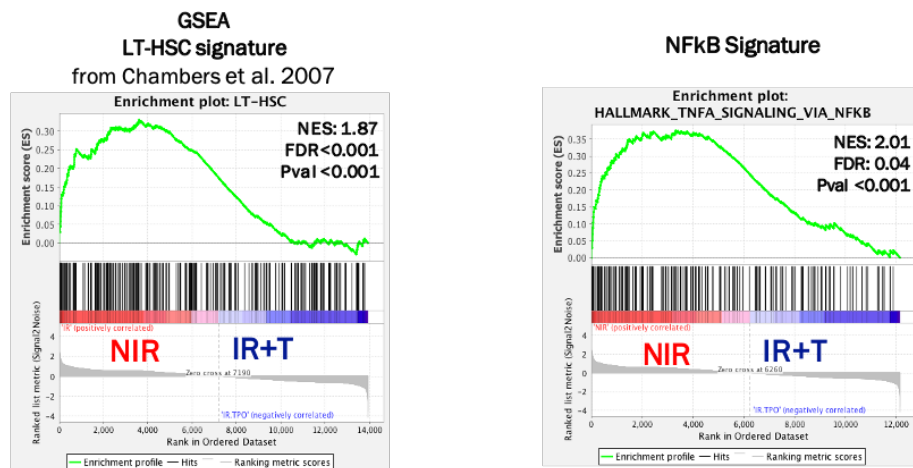
Further research is necessary to be able to understand the mechanisms by which TPO functions on and to better characterize its role as an epigenetic modulator. These should involve bisulfite sequencing at L1Md sequences and determine if IR and/or TPO affect DNA methylation. CYTOF technology and CHIP-seq would also be useful to see if TPO brings about any epigenetic changes that are different from those observed in IR, or if compensatory mechanisms for the loss of H3K9me3 are put in place by TPO, for example enrichment of another silencing mark such as H3K27me3.

Figure 5

A)



B)



C)

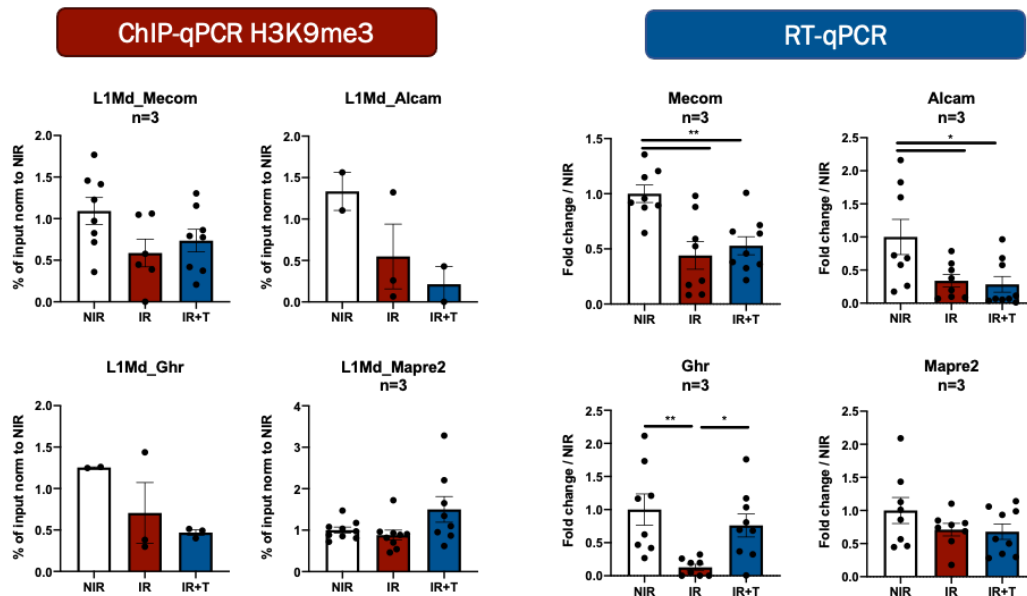


Figure 5. TPO can prevent loss of the NFkB signature only in genes downregulated upon IR that contain an intronic L1Md. A) (from left to right) Expression level of genes downregulated in IR (1), and in the IR+T condition (2), with (3) or without (4) intronic L1Md analyzed in the IR+TPO condition. y-axis represents fold change of expression and x-axis represents % of deregulated genes in IR+TPO condition. B) GSEA Enrichment plots showing loss of HSC (left) and NFkB (right) signature in the IR+TPO condition compared to NIR. C) (left) H3K9me3 enrichment evaluated by ChIP-qPCR at intronic L1Md of HSCs sorted one month post IR. (n=1-3 independent experiments, each dot represents pools of 3 (NIR) or 4 (IR) mice) Results are expressed as fold change from the mean of % input of the NIR condition. (right) Gene expression evaluated by RT-qPCR in the same cells one month post IR(+TPO). (n= 3 independent experiments, each dot represents an individual mouse) Ct values were normalized to mean of RPL32 and HPRT. Results are expressed as fold change from the mean value of the NIR condition and represented as mean \pm SEM Gene expression evaluated by RT-qPCR in HSCs one month post IR with and without TPO. (n= 3 independent experiments, each dot represents an individual mouse) Ct values were normalized to RPL32 and HPRT. Results are expressed as fold change from the mean value of the NIR condition. (** p<0,01; *p<0,05 One-way ANOVA).

7. Could TPO prevent loss of global H3K9me3 through its IFN-like signaling and thus repress RTEs?

In the previous study, I showed that TPO is capable of controlling RTE expression in a STAT1- and STAT2-dependent manner (Barbieri et al. 2018).

Non-canonical functions of the JAK-STAT pathway have been reported in *Drosophila* and mammals that affect cellular epigenetic status by globally modulating heterochromatin stability (Shi et al. 2006; Brown and Zeidler 2008). In *Drosophila*, a connection between JAK-STAT signaling and chromatin remodeling was suggested by the observation that the transcriptional repressor Ken recruits a nucleosome remodeling factor, NURF, to STAT/Ken mutual binding sequences to repress STAT-mediated transcription (Brown and Zeidler 2008). In 2006, Shi et al. first showed that *Drosophila* JAK was involved in the regulation of heterochromatin (Shi et al. 2006). In 2008, they showed that non-phosphorylated *Drosophila* STAT is involved in regulating heterochromatin stability via its association with HP1, where it acts as a mediator HP1 localization and H3K9me3 formation (Shi et al. 2008). In humans, Hu et al. have shown that unphosphorylated STAT5a binds to HP1 to stabilize heterochromatin, and that expressing unphosphorylated STAT5a or HP1 inhibited colon cancer growth in mouse xenograft models (Hu et al. 2013). Cheon et al. also reported that non-phosphorylated STAT1 and STAT2 can drive a secondary prolonged IFN response leading to a constitutive resistance to DNA damage (Cheon et al. 2013). These studies supported the hypothesis that TPO, through its STAT-dependent IFN-like signaling, may be able to control L1Md expression through epigenetic mechanisms.

Many viral restriction factors are IFN-regulated genes (Schneider et al. 2014). Several studies have reported that IFN- α stimulation or overexpression of IFN-stimulated genes (ISGs) reduces RTE expression and L1 propagation in cell lines (Koito and Ikeda 2011; Goodier et al. 2012; Zhang et al. 2014; Goodier et al. 2015). In particular Zhang et al. have shown that the IFN-regulated OAS-RNase L system can restrict mobility of L1 and IAP elements, as well as reduce L1 mRNA levels. Yu et al. have also shown that in human cell lines added IFN suppresses L1 retrotransposition, whereas endogenous IFN signaling appears to restrict the propagation of L1 and their activity in cultured cells through stimulation of the JAK-STAT signaling cascade (Yu et al. 2015).

We performed motif enrichment analysis using HOMER and observed that STAT1 and ISRE motifs (to which both STAT1 and STAT2 can bind) were present in the ORF1 and ORF2 of most L1Md subfamilies. Our initial hypothesis was that TPO could activate STAT1, which could interact with HP1 and specifically direct its localization towards L1Md, and thus prevent loss of H3K9me3 upon IR, which occurs mainly at these genomic loci. Unfortunately, answering this question was very challenging as

chromatin immunoprecipitation experiments on transcription factors require a high number of cells that is impossible to attain in HSCs. A possible solution would be to use the BM-HPC cell line model, which would solve the cell number issue. However, these cells do not express the TPO receptor MPL. Like TPO, IFN- α can activate STAT1 and also prevent IR-induced L1Md over expression, so an alternative would be to use IFN instead of TPO and assess STAT1 enrichment at L1Md promoters in cells stimulated with IFN prior to IR.

Interestingly, previous work by our lab has shown that IR induces a moderate NF- κ B response that is potentiated by TPO to increase DSB repair (de Laval et al. 2014). As was described in section 4, components of NF- κ B signaling have been shown to interact with epigenetic modulators. Several studies have shown that cross-talk between IFN- α and NF- κ B signaling plays an important role in controlling viral infection. For instance, Rubio et al. have shown that IFN- α and NF- κ B have overlapping functions and that enhancing NF- κ B activation can compensate eventual deficiencies in IFN- α signaling and contribute to fight viral infection. Moreover, other independent studies have shown that the NF- κ B pathway can induce ISGs *in vivo* and *in vitro* independently of IFN- α ISG production (Dixit et al. 2010; Balachandran and Beg 2011; Basagoudanavar et al. 2011; Hasan et al. 2013). These studies contribute to the idea that, through its IFN-like signaling and its ability to potentiate NF- κ B signaling, TPO may be able to control RTE expression by affecting HSC epigenetics, which is in line with our results showing that TPO prevents loss of the NF- κ B signature in genes downregulated upon IR who harbor an L1Md.

Another possible explanation would be that TPO, through its IFN-like signaling, induces the expression of ISGs that can themselves control L1Md repression. For instance, members of the tripartite motif (TRIM) family, of which many members are important regulators of IFN- α responses and are induced by TPO, have been involved in transcriptional silencing. An ideal candidate would be TRIM28, also known as KAP-1, who is known for mediating H3K9me3 deposition at ERVs via SETDB1 and KRAB-ZFPs (Fasching et al. 2015; Ecco et al. 2016). Furthermore, Robbez-Masson et al. have shown that TRIM28 can interact with the HUSH complex to co-repress young ERV and L1 elements (Robbez-Masson et al. 2018). However, they do show that TRIM28 and TASOR (a member of the HUSH complex) only bind L1 elements weakly compared to ERVs, suggesting that escaping repression is still possible (Robbez-Masson et al. 2018). We have not yet evaluated the impact of IR and TPO on expression of TRIM28 or proteins in the HUSH complex, but moving forward these would be some of the most interesting targets to study the mechanisms by which TPO could control L1Md. Furthermore, it would also be interesting to analyze the enrichment of members of the HUSH complex at L1Md. Our hypothesis is that TPO could induce TRIM28 expression which could cooperate with the HUSH complex and mediate H3K9me3 deposition at L1Md, leading to their repression. Finally, another potential target to study could also be TRIM33, which has been found induced by TPO by our

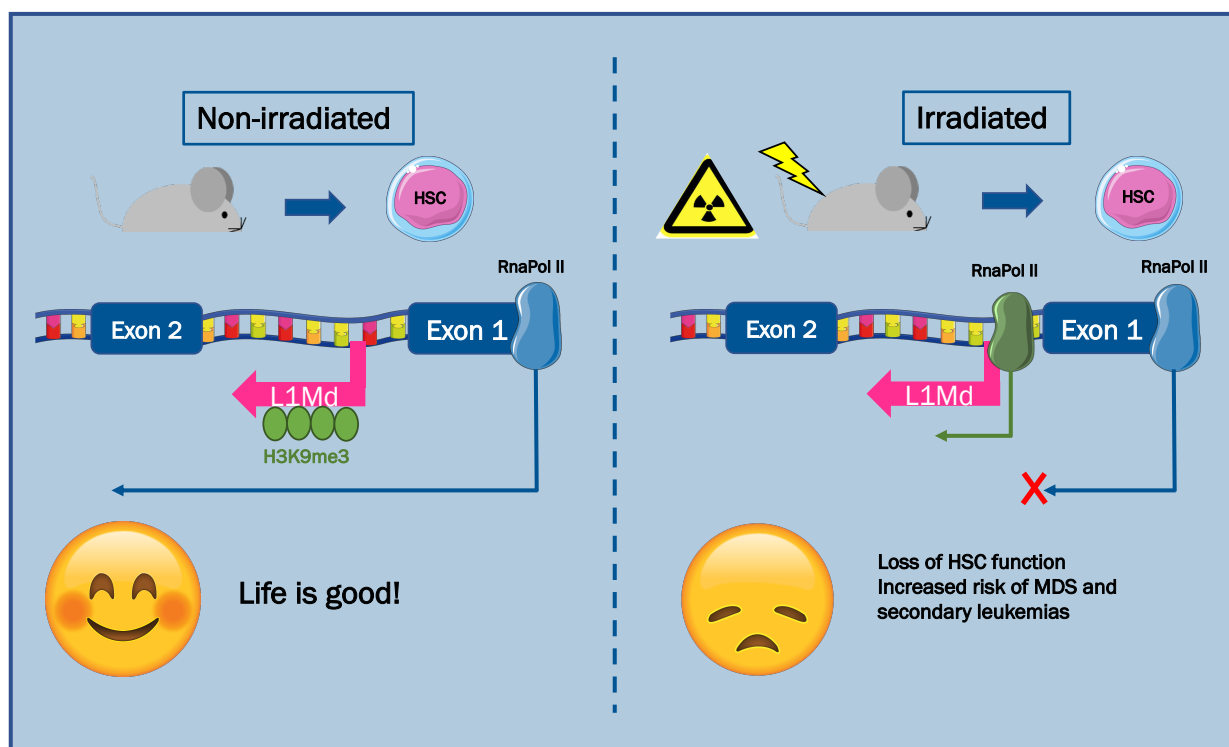
team, and has been shown to participate in the control of ERV expression in mouse liver and testis (Herquel et al. 2013; Isbel et al. 2015).

Given the emerging potential role for the NF- κ B pathway that we have observed, it would be interesting to study the relationship between IFN signaling and the NF- κ B pathway to be able to understand the mechanisms of action of TPO in RTE control.

Concluding remarks and summary diagram

The impact of IR on HSC epigenetics and RTE expression has been so far largely overlooked. In particular, the impact that heterochromatin dynamics and RTE deregulation can have on HSC function has never been addressed before. We show here for the first time that IR induces major epigenetic changes in HSCs, and that transcriptional downregulation upon IR is associated with intronic L1Md expression. We validated that genes that are downregulated upon IR, exhibit loss of H3K9me3 enrichment at intronic L1Mds and that this is not the case in genes whose expression is not affected by IR, highlighting the specificity of this mechanism. These data open up new perspectives to study mechanisms contributing to the establishment of the pre-leukemic context and the development of therapy-related myeloid malignancies. The fact that many epigenetic factors are frequently mutated in these pathologies, as well as in clonal hematopoiesis, also provides new targets for the development of novel therapies.

We showed that TPO, through its STAT1- and STAT2-dependent IFN signaling, is capable of controlling L1Md expression. We have also shown that TPO also plays a role as an epigenetic regulator of HSCs, however further studies are required to characterize the mechanisms by which this occurs.



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ANNEX

ARTICLE

Thrombopoietin protects hematopoietic stem cells from retrotransposon-mediated damage by promoting an antiviral response

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Maintenance of genomic integrity is crucial for the preservation of hematopoietic stem cell (HSC) potential. Retrotransposons, spreading in the genome through an RNA intermediate, have been associated with loss of self-renewal, aging, and DNA damage. However, their role in HSCs has not been addressed. Here, we show that mouse HSCs express various retroelements (REs), including long interspersed element-1 (L1) recent family members that further increase upon irradiation. Using mice expressing an engineered human L1 retrotransposition reporter cassette and reverse transcription inhibitors, we demonstrate that L1 retransposition occurs *in vivo* and is involved in irradiation-induced persistent γ H2AX foci and HSC loss of function. Thus, RE represents an important intrinsic HSC threat. Furthermore, we show that RE activity is restrained by thrombopoietin, a critical HSC maintenance factor, through its ability to promote a potent interferon-like, antiviral gene response in HSCs. This uncovers a novel mechanism allowing HSCs to minimize irradiation-induced injury and reinforces the links between DNA damage, REs, and antiviral immunity.

Introduction

Hematopoietic stem cells (HSCs) maintain homeostasis and replenish blood and the immune system throughout life. Maintenance of genomic integrity is crucial for the preservation of these functions. DNA damage in HSCs is associated with a reduced ability to reconstitute hematopoiesis and with an altered lymphoid/myeloid lineage potential (Nijnik et al., 2007; Rossi et al., 2007). The mechanisms underlying these effects are still poorly understood. This is, however, of major clinical concern. It is also crucial to understand why after radiotherapy or with age there is an accrued risk of developing bone marrow aplasia or secondary myelodysplastic syndromes. Double-strand breaks (DSBs), which are the most harmful form of DNA damage, can be generated by exogenous treatments such as ionizing radiations (IR) or internally by products of metabolism or as a result of genome replication or alteration of repair mechanisms (Rossi et al., 2007; Hoeijmakers, 2009).

Another highly dangerous, albeit poorly studied, source of endogenous DNA damage could come from the mobilization of retroelements (REs; Mita and Boeke, 2016). These sequences represent 30–50% of human and mouse genomes and can spread through an RNA intermediate using a “copy–paste” mechanism.

REs can be classified into two major groups: long terminal repeat (LTR) elements, which comprise endogenous retrovirus (ERV), and non-LTR elements. This latter group includes long interspersed element-1 (LINE-1 or L1) and short interspersed elements (SINES). ERVs exhibit relatively high activity in the mouse, whereas in humans, only the non-LTR elements are believed to be capable of retrotransposition. L1s continue to diversify genomes, on their own and through their ability to mobilize SINES. A full-length L1 element consists of a 5′-untranslated region (5′-UTR) containing an internal promoter and two open reading frames encoding ORF1 and ORF2 proteins. ORF1 has chaperone and nucleic acid binding properties and ORF2 carries the L1 endonuclease and reverse transcription activities.

Propagation of REs in the genome requires DNA disruption. L1s are particularly strong inducers of DNA damage. Indeed, even the ORF2 protein alone, or abortive retrotransposition, can induce widespread DSBs, chronic DNA damage, and senescence (Gasior et al., 2006; Belancio et al., 2010). Derepression and mobilization of REs can lead to deletions and translocations and represent an increasingly recognized source of genomic instability (Gilbert et al., 2002, 2005; Symer et al., 2002; Iskow et al.,

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2010; Erwin et al., 2016). They also have a profound influence on the transcriptome and contribute to the wiring of regulatory networks in a cell-specific fashion (Han et al., 2004; Faulkner et al., 2009; Xie et al., 2013; Elbarbary et al., 2016). Given this harmful potential, RE expression is under tight control. ERVs and L1s are highly expressed in embryonic stem cells (ESCs) and germ cells, and L1 retrotransposition occurs during embryogenesis (Martin and Branciforte, 1993; Garcia-Perez et al., 2007; Kano et al., 2009; Mita and Boeke, 2016). Recent studies have also described somatic expression of L1 mRNA, as well as de novo insertions, particularly during neuronal progenitor differentiation and in the human brain (Muotri et al., 2005, 2010; Coufal et al., 2009; Belancio et al., 2010; Baillie et al., 2011; Evrony et al., 2012). Furthermore, increased L1 expression and new somatic insertions have been detected in various tumors (Iskow et al., 2010; Lee et al., 2012; Solyom et al., 2012). Previous studies have also shown that genotoxic stress can induce RE mobilization in different cell lines (Ishihara et al., 2000; Hagan et al., 2003; Farkash et al., 2006).

Similar to HSCs from irradiated animals, aged HSCs display persistent DNA damage. We and others have recently demonstrated that aged human and mouse HSCs display up-regulated expression of L1, SINE, and intracisternal A-particle (IAP) REs (Sun et al., 2014; Djeghloul et al., 2016). However, in spite of its possible relevance to HSC genomic instability, the mechanistic link between RE expression/mobilization and the accumulation of DNA damage has not been addressed. We show here that HSCs express various REs, including young L1 elements that represent an important intrinsic source of DNA damage. Indeed, L1s can successfully mobilize in vivo in HSCs upon total body irradiation (TBI) and are responsible for the long-lasting DNA damage induced by this treatment. We have previously shown that thrombopoietin (THPO), a critical HSC self-renewal factor (Qian et al., 2007; Yoshihara et al., 2007), limits TBI-induced HSC DNA damage and injury by improving DSB repair (de Laval et al., 2013). In this study, we uncover a novel mechanism by which THPO can control HSC genomic stability by restraining RE expression and mobilization. This activity is mediated by its ability to trigger a potent antiviral and IFN-like, STAT1- and STAT2-dependent signaling in HSCs. IFNs are critical for the cellular defense against viruses and are produced abundantly mainly during infections. Thus, the ability of HSCs to mount an antiviral innate immune state in response to a self-renewal cytokine may represent an important constitutive means to resist to RE-induced threat.

Results

Irradiation increases retrotransposon expression in HSCs

REs can be viewed as stress response genes and have been linked to DNA damage. To determine whether they could be involved in HSC genomic instability after genotoxic stress, we first assessed expression of the various REs in HSCs and various progenitors sorted by FACS using quantitative reverse transcriptase (qRT)-PCR. Analyses using primers recognizing various regions of L1 elements (5'-UTR and ORF2) or specific for active evolutionary recent mouse L1 family members (L1_A, Tf, and Gf) showed that HSCs (LSK-CD34⁺Flk2⁻) express L1 mRNA

levels, including recent elements, at a significantly higher level than multipotent (LSK-CD34⁺Flk2⁺), common myeloid progenitors (CMP), and granulocyte-macrophage progenitors (GMP) myeloid progenitors or LSK cells, a mixed population of HSCs and progenitor cells (HSPCs; Fig. 1 A and Fig. S1 A). HSCs also express significant levels of two other movable REs, SINE B1 and IAP (Fig. S1 A). Quantitative PCR (qPCR) analysis performed on RNA samples before reverse transcription gave very low or undetermined cycle threshold (Ct) values, indicating that the higher RE expression found in HSCs was not a result of genomic contamination (data not depicted). In addition, expression of L1s and IAP was much lower in myeloid progenitor populations than in HSCs even though all the samples were prepared and tested together.

L1 fragments are frequently embedded into genes. The limited numbers of HSCs precluded us from performing Northern blot analysis. Thus, to confirm that HSCs express full-length L1 mRNA rather than truncated forms that would be transcribed from other genomic sites, purified RNA was reverse transcribed using a sense-strand L1-specific primer recognizing the 3' end of ORF2 (Fig. S1 B), allowing the detection of only sense-strand L1 RNA transcripts, as described (Wissing et al., 2012). ESCs, known to express high levels of L1 elements were used as a positive control. As shown in Fig. S1 B, primers detecting L1 5'-UTR and ORF2 could amplify a 2.3 kB fragment from both ESC and HSC ORF2-directed cDNAs. No band was amplified when the reverse transcription step was omitted. This suggests the presence of full-length L1 RNAs species in HSCs.

We next examined whether ionizing radiation could affect RE expression. Attempts to assess short-time effects of irradiation led to unreproducible results, probably as a result of stress-induced variations imposed by the culture conditions in vitro. It was also impossible to assess RE expression in vivo short term after IR because TBI, even at low doses, has been shown to induce a rapid decrease in c-Kit and an increase in Sca1 (Simonnet et al., 2009), impeding HSC sorting. Therefore, we chose to analyze RE expression 1 mo after mice were subjected to a single low dose of irradiation (2 Gy; Fig. 1 B), a time and dose at which expression of these markers has recovered. In addition, under these conditions, irradiated HSCs still present DNA damage, as shown by increased γ H2AX foci (Simonnet et al., 2009; de Laval et al., 2013). HSCs harvested 1 mo after TBI displayed a general increase in L1 elements, as observed with 5'-UTR and ORF2 primers and of recent L1_A, Tf, and Gf family members. TBI also increases IAP elements, as compared with nontreated cells. To confirm increased expression of active L1 elements in HSCs, we performed immunofluorescence (IF) analysis using specific antibodies directed against the mouse L1 ORF1 protein (ORF1p; Martin and Branciforte, 1993; Malki et al., 2014). This antibody was able to detect ORF1p in ESCs, but not in mouse embryonic fibroblasts (Fig. S1 C). Although few cells expressing low amounts of ORF1p with the characteristic punctate cytoplasmic staining (Malki et al., 2014) could be detected in LSK-CD34⁺Flk2⁻ HSCs, ORF1p expression was significantly increased 1 mo after TBI (Fig. 1 C). This indicates that TBI induces long-lasting change in expression of L1 elements in HSCs.

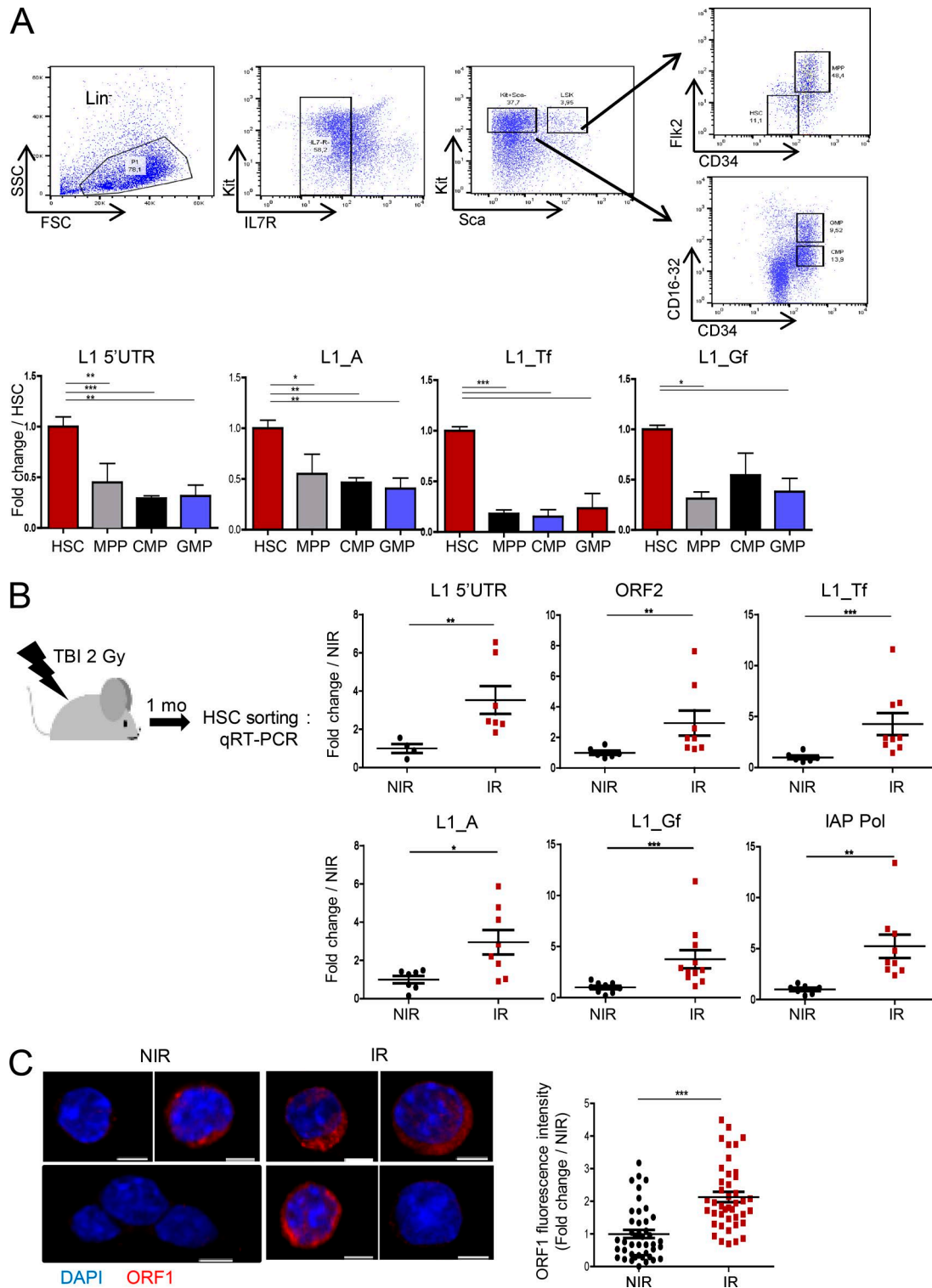


Figure 1. **Irradiation increases RE transcript levels in HSCs.** (A) qRT-PCR analysis of L1 expression in LSK-CD34⁻Flk2⁻ HSCs and progenitors. Ct values were normalized to β -actin. Results are expressed as fold change from the mean value of HSCs. Means \pm SEM, $n = 6$ (5'-UTR and L1_A) or 3 (L1_Tf and Gf) pools of six to eight mice; two independent experiments. One-way ANOVA with Dunnett's multiple comparison test. (B) Experimental design and RE mRNA expression in LSK-CD34⁻Flk2⁻ HSCs cells isolated 1 mo after 2 Gy TBI (IR) or nontreated (NIR). Results are expressed as fold change from the NIR mean value after normalization as in A. Each dot represents an individual mouse. Means \pm SEM from two (upper panels) and three (lower panels) independent experiments. Mann-Whitney test. (C) Representative images and quantification of L1 ORF1p staining in HSCs (LSK-CD34⁻Flk2⁻) isolated 1 mo after TBI or left untreated as in B. Bars, 3 μ m. ImageJ was used for quantification. For each cell the fluorescence intensity was normalized to the total cell surface and then to the mean intensity of NIR cells. Each dot represents a cell. Means \pm SEM from two independent experiments. Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

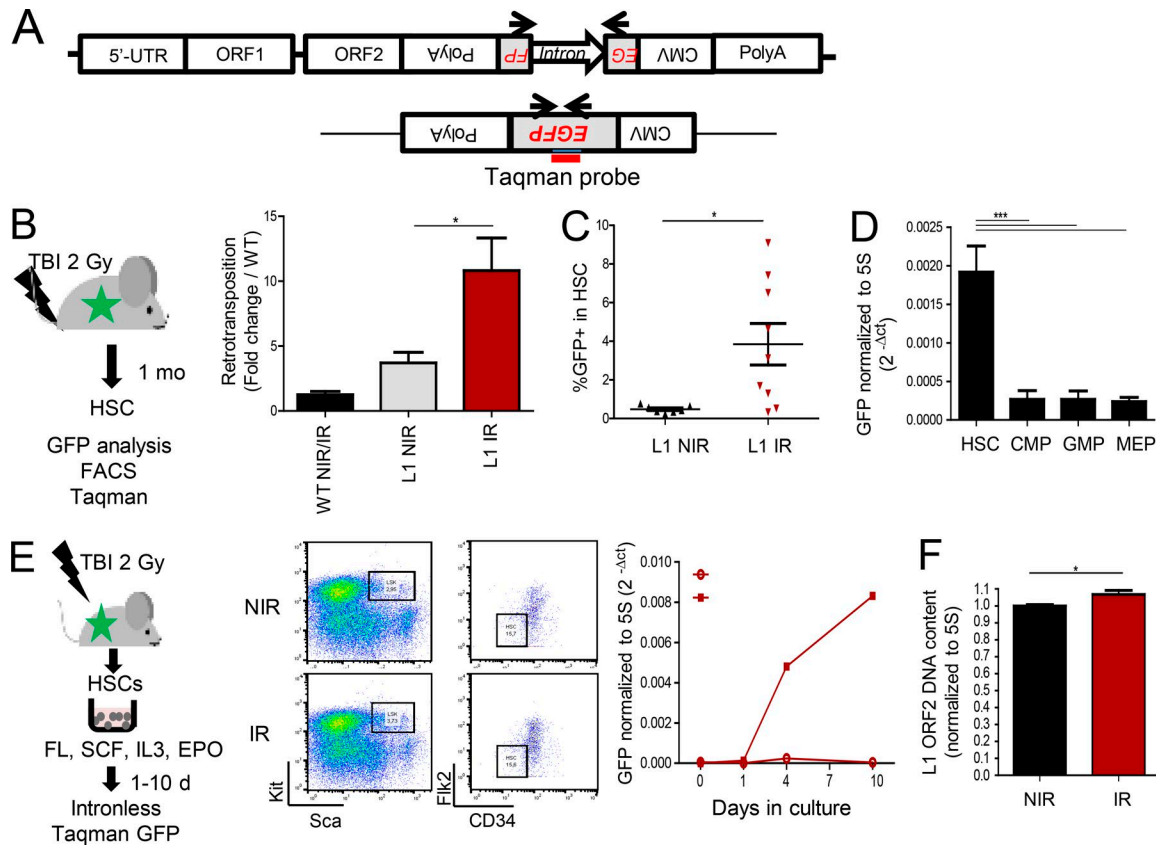


Figure 2. Irradiation promotes active retrotransposition in HSCs. (A) The huL1-GFP transgene and position of the primers used to detect retrotransposition. (B–D) Experimental design and analysis of L1 retrotransposition by Taqman (B and D) or FACS (C) in HSCs and progenitors of L1-GFP mice before (NIR; B–D) or after TBI (IR; B and C). (B) Means \pm SEM, $n = 3$ (WT), 7 (L1-GFP NIR), and 13 (L1-GFP IR) mice from three independent experiments. Mann-Whitney test. (C) Each triangle represents an individual mouse. Means \pm SEM from two independent experiments. Mann-Whitney test. (D) $n = 7$ (HSC, CMP, and GMP) and 6 (MEP) mice from two independent experiments; ANOVA with Dunnett’s multiple comparison test. (E) Kinetics of L1 retrotransposition after TBI by Taqman-based qPCR assay. Left, experimental design; middle, representative FACS images of HSC sorting just after TBI; right, GFP expression normalized to 5S rDNA. Means from two independent cultures. Open circles, NIR; closed squares, IR. (F) qPCR analysis of the number of genomic copies of L1 ORF2 in HSCs 1 mo after TBI (IR) or not (NIR), normalized to 5S rDNA. $n = 6$ (NIR) and 8 (IR) mice. Means \pm SEM from two independent experiments. Mann-Whitney test. *, $P < 0.05$; ***, $P < 0.001$.

Irradiation induces L1 retrotransposition in vivo in HSCs

The increase of both L1 mRNA and ORF1p, which is required for retrotransposition, suggests that L1 mobilization may take place in HSCs. To test this hypothesis in vivo, we made use of a transgenic mouse model that carries an engineered human L1 harboring a GFP-based retrotransposition reporter cassette (referred to here as L1-GFP), where the human L1 transgene is under the control of its native 5'-UTR promoter (Fig. 2 A). In this model, GFP is expressed only if L1 is transcribed, spliced, reverse transcribed, and reintegrated in the genome (Okudaira et al., 2011). Retrotransposition can be monitored using a Taqman qPCR assay with GFP exon-exon junction primers and probe (Fig. 2 A). Retrotransposition was detected in LSK-CD34⁺Flk2⁺ HSCs isolated from nonirradiated L1-GFP mice when compared with WT mice, albeit at low levels (Fig. 2 B). However, retrotransposition was greatly increased 1 mo after TBI (Fig. 2 B). Confirming these results, 1 mo after TBI, GFP could also be detected by FACS analysis in HSCs defined by the LSK-CD48⁺CD150⁺ phenotype (Fig. 2 C and Fig. S1 D) and in LSK cells (Fig. S1, D and E). As for L1 mRNA, the engineered human L1 retrotransposed at a higher level in HSCs than in myeloid

progenitors at the basal state (Fig. 2 D). To analyze the kinetics of retrotransposition despite the loss of HSC markers 1–10 d after irradiation, HSCs were sorted immediately after TBI and cultured in vitro (Fig. 2 E, left panel). Under these conditions, no change in Sca and Kit marker expression or LSK and HSC numbers and repartition were observed (Fig. 2 E, middle panel; and Fig. S1 F). Monitoring GFP by the sensitive Taqman assay at different times of the culture showed that retrotransposition events could be detected as early as 4 d after irradiation and increased by day 10 (Fig. 2 E, right panel). Likewise, the presence of GFP could be detected in the Lin⁻ cell population isolated 4 and 7 d after different times (Fig. S1 G). These results indicate that the increased retrotransposition is a result of a direct effect of irradiation on HSCs.

Finally, we determined whether endogenous L1 can also mobilize after TBI, using an established qPCR assay for detecting de novo RE insertion (Muotri et al., 2010). We found that HSCs isolated from WT mice 1 mo after TBI had increased ORF2 genomic copy number (Fig. 2 F), suggesting that endogenous L1 retrotransposition could be promoted in HSCs in vivo upon irradiation.

L1 retrotransposition is involved in persistent DNA damage and loss of function of HSCs upon irradiation

L1 mobilization in cell lines has been shown to induce widespread DSBs, as measured by the presence of γ H2AX foci and senescence (Gasior et al., 2006; Belancio et al., 2010). We and others have shown that TBI induces γ H2AX foci in HSCs that persist for several weeks. This is accompanied by a loss of HSC function (Simonnet et al., 2009; de Laval et al., 2013). Thus, we next analyzed whether L1-increased expression and retrotransposition could be involved in these phenomena. L1 mobilization requires its reverse transcription activity, which is carried by ORF2p (Mita and Boeke, 2016). This activity is sensitive to reverse transcription inhibitors (RTIs), including the nucleoside analogue 3'dideoxycytidine (ddC; Dai et al., 2011). Mice were subjected to TBI and treated with ddC or PBS daily for 1 mo (Fig. 3 A). As shown in Fig. 3 B, IR-induced persistent γ H2AX foci in HSCs were significantly decreased upon ddC treatment. ddC also partially restored the loss of proliferation in vitro of irradiated HSCs (Fig. 3 C). Similar results were observed upon treatment with a nonnucleoside RTI, Efavirenz (EFV; Fig. S2, A and B). To determine whether inhibition of active retrotransposition could also rescue HSC proliferation in vivo, competitive transplantation experiments were performed with ddC-treated bone marrow (Fig. 3 A). At the end of treatment (stage 1), the numbers of HSCs and progenitors and their repartition in the LSK compartment was greatly altered in the irradiated mice, as compared with their nontreated counterparts (Fig. S2, C–E). This is in agreement with previous data (Shao et al., 2014; Fleenor et al., 2015). However, no difference was observed between the PBS- and ddC-treated groups at that stage. In contrast, 15 wk after reconstitution, the absolute numbers (Fig. 3 D) and the relative frequencies (Fig. S2 F) of donor LSK and HSCs found in the BM were greatly improved in the ddC-treated group, when compared with the PBS group. Secondary transplants confirmed that the capacity of HSCs to reconstitute hematopoiesis after TBI could be restored by ddC treatment (Fig. 3, E–G; and Fig. S2 G). This shows that TBI-induced retrotransposition leads to persistent γ H2AX foci and negatively impacts HSC self-renewal.

Thrombopoietin restrains L1 expression and retrotransposition in HSCs

The above data suggest that HSCs may need means to protect themselves against the possible harmful potential of transcribed active transposable elements. We previously showed that one injection of THPO before irradiation could limit TBI-induced accumulation of γ H2AX foci and loss of HSC function (de Laval et al., 2013). To examine whether this could be linked to an effect on RE expression and/or mobilization, mice were injected with a single dose of THPO 1 h before TBI, and RE expression was assessed 1 mo later (Fig. 4 A). We found that this treatment prevented TBI-induced increased expression of recent L1 members and IAP in HSCs. Similar results were observed in LSK cells (Fig. S3 A). Conversely, the basal RE mRNA levels were significantly increased in *Mpl*^{-/-} HSCs deficient for the THPO receptor (Fig. 4 B) and in *Thpo*^{-/-} LSK cells (Fig. S3 B).

THPO could also inhibit in vivo L1 mobilization in L1-GFP mice, as shown by the significantly reduced level of retrotransposition

detected by Taqman assay 1 mo after TBI in HSCs from mice that had received THPO instead of PBS 1 h before TBI (Fig. 4 C). To determine whether this was a result of a direct effect of THPO on HSCs, HSCs were sorted immediately after TBI, with or without THPO treatment, and cultured in vitro in the presence or absence of THPO (Fig. 4 D, left panel). After 10 d, GFP Taqman assays showed that the highest levels of retrotransposition were detected in the progeny of HSCs isolated from irradiated mice cultured in the absence of THPO (Fig. 4 D, upper panel). THPO completely blocked TBI-induced retrotransposition, whether it was injected 1 h before TBI or added after TBI in the culture, demonstrating its direct effect on HSCs in vivo and in vitro. Similar results were observed by measuring GFP by FACS in the LSK compartment (Fig. 4 D, bottom panel). In contrast, THPO had no effect on the total cell number and the percentage of LSK cells and HSCs recovered at the end of the culture in vitro (Fig. S3, C and D).

L1 retrotransposition was also significantly increased in both LSK-CD34-Flk2⁻ and LSK-CD48⁻CD150⁺ HSCs from L1-*Mpl*^{-/-} (Fig. 4 E and Fig. S3 E) and L1-*Thpo*^{-/-} mice (Fig. S3 F), even under steady-state conditions. It was further enhanced in L1-*Mpl*^{-/-} HSCs 1 mo after TBI (Fig. 4 F). Thus, THPO signaling in vivo is required to limit RE expression and L1 mobilization in HSCs, under both steady-state conditions and irradiation stress.

THPO induces an early antiviral, IFN-like gene expression response in HSCs

We next thought to determine by which mechanism THPO could limit RE expression and activity in HSCs. Because only one injection of THPO 1 h before TBI is sufficient to protect HSCs from increased RE expression and retrotransposition, we hypothesized that THPO-mediated control of REs takes place at early time after THPO stimulation. We previously showed that a short preincubation of purified HSCs in medium containing THPO before irradiation in vitro could fully recapitulate the effect of THPO injection in vivo on HSC genomic stability and function (de Laval et al., 2013). To analyze whether the ability of THPO to prevent TBI-induced RE expression was a result of THPO-induced specific transcriptional changes during this preincubation time, purified LSK-CD34-Flk2⁻ HSCs were cultured in vitro for 45 min with or without THPO before irradiation, and microarray analyses were performed 45 min later (Fig. 5 A). 338 differentially expressed genes (Table S1; fold change \geq 1.5, P values \leq 0.05) specifically regulated by THPO were identified. Ingenuity pathway analysis revealed pathways related to IFN signaling and antiviral innate immunity as top significantly THPO-activated canonical pathways (Fig. 5 B). More than 60% of the up-with-THPO gene list (Fig. 5 C and Table S1) is composed of IFN-stimulated genes (ISGs) with characteristics of IFN type I response genes that are found in the Interferome database (Rusinova et al., 2013). Indeed, qRT-PCR assays showed that these genes are induced by IFN- α in LSK cells (Fig. S4 A).

qRT-PCR analyses on LSK-CD34-Flk2⁻ HSCs stimulated in vitro with THPO confirmed the microarray results (Fig. 6 A). Importantly, increased ISG expression was also detected in HSCs isolated 90 min after THPO injection in mice, showing that THPO could also induce an early IFN-like gene response in vivo (Fig. 6 B). The response is specific for THPO, as shown

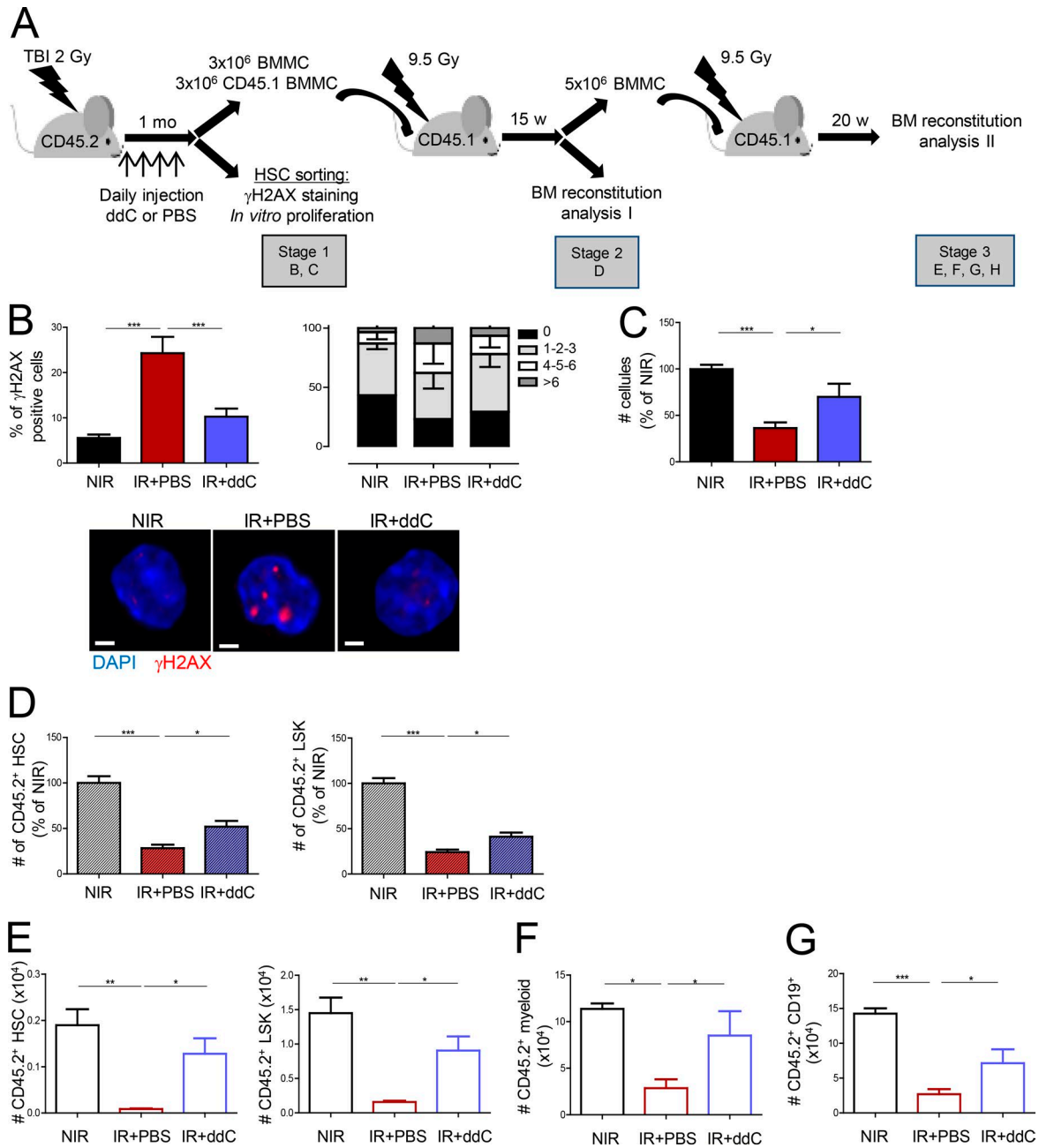


Figure 3. Retrotransposition induces HSC damage. (A) Experimental design for RTI treatment. BM, bone marrow; BMMC, BM mononuclear cell. **(B and C)** γ H2AX foci positive cells, foci repartition, and representative images (B) and ex vivo proliferation of LSK-CD34-Flk2⁻ HSCs isolated at stage 1 (C). Means \pm SEM, $n = 7$ (NIR and PBS) and 8 (ddC) mice from two independent experiments. ANOVA with Dunnett's multiple comparison test. Bars, 30 μ m. **(D)** Total CD45.2⁺ donor contribution in HSCs and LSK cells at stage 2 in mice transplanted with cells from mice irradiated and treated with ddC or not. Results were normalized to the total numbers of CD45.2⁺ HSCs or LSK cells found in the NIR controls and represent the means \pm SEM from 7 (NIR), 9 (IR+PBS), and 10 (IR+ddC) mice from two independent experiments. One-way ANOVA with Dunnett's multiple comparison test. **(E, F, and G)** Total CD45.2⁺ donor contribution at stage 3 in HSCs and LSK cells (E), myeloid CD11b⁺ and Gr1⁺ cells (F), and CD19⁺ B cells (G). Means \pm SEM, $n = 4$ (NIR and IR+PBS) and 5 (ddC) mice from one representative experiment out of two performed. One-way ANOVA with Dunnett's multiple comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

by the absence of significant ISG induction upon incubation of HSCs in medium without THPO, despite the presence of the other cytokines (Fig. S4 B). Furthermore, the induction of ISG expression by THPO was similar in irradiated and nonirradiated samples (Fig. S4 C). In agreement with the highest *Mpl* expression in HSCs (Qian et al., 2007), THPO-mediated ISG induction was more potent in HSCs than in LSKs (Fig. 6 A, right panel).

THPO was able to trigger ISG expression in vitro in HSCs from mice deficient for IFN-I receptor *Ifnar1* (Fig. S4 D), indicating that THPO-induced, IFN-like gene expression response does not result from increased IFN secretion or cross talk with IFN-type I receptor. Confirming these results, injection of MARI-5A3, an anti-IFNAR1 blocking antibody, could reverse IFN- α - but not THPO-induced ISG expression in HSCs in vivo (Fig. S4 E). Finally,

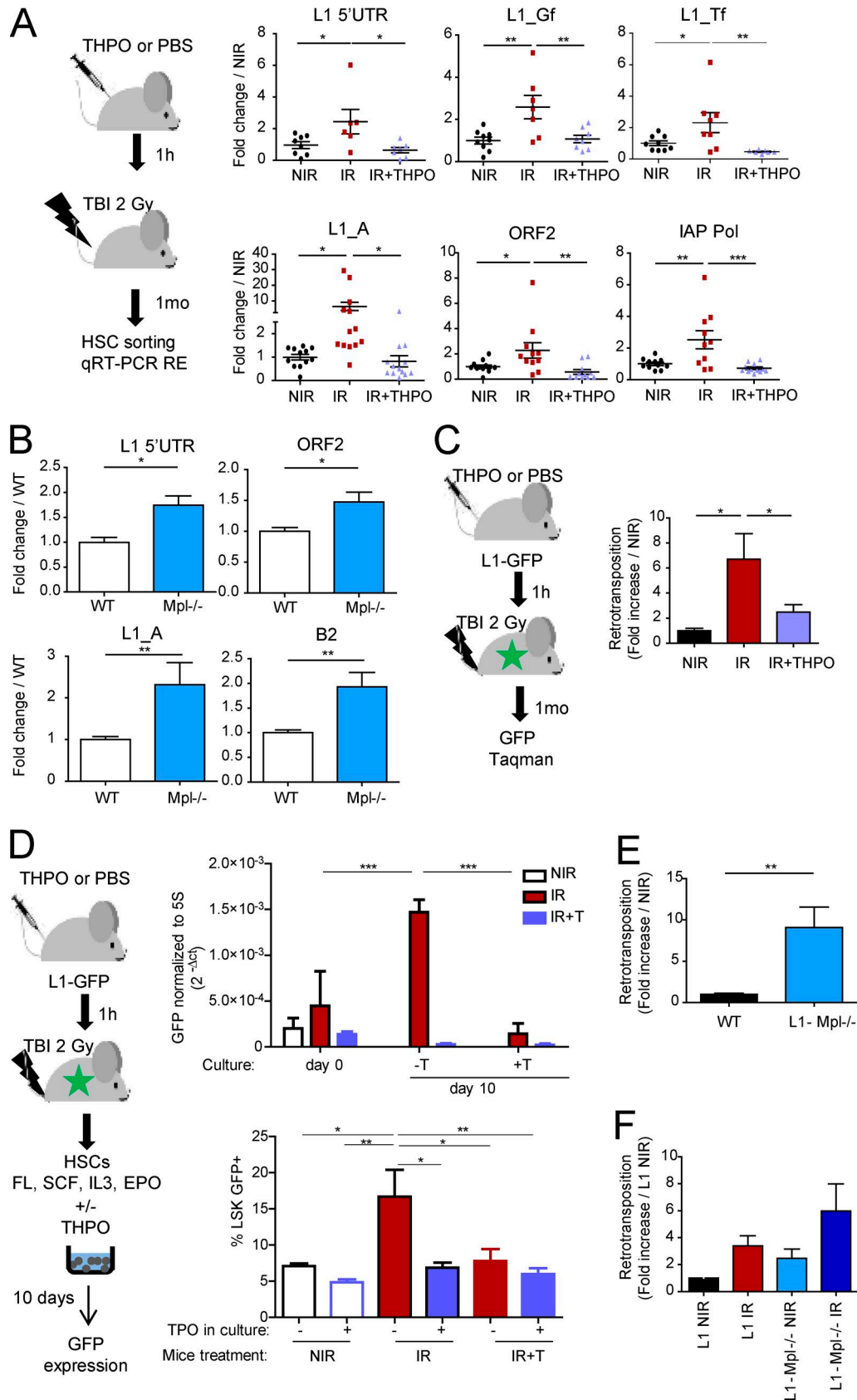


Figure 4. THPO signaling controls L1 expression and retrotransposition in vivo in HSCs. (A) Experimental design and RE mRNA expression in HSCs isolated 1 mo after 2 Gy TBI with (IR+THPO) or without (IR) THPO injection or nontreated (NIR). Each point represents an individual mouse. Results represent means \pm SEM and are expressed as fold change from the NIR mean value after normalization. Data are pooled from three (upper panels) or four (lower panels) independent experiments. One-way ANOVA with Dunnett's multiple comparison test. (B) RE expression in WT and *Mpl*^{-/-} HSCs. Means \pm SEM normalized to

confirming THPO's ability to induce an antiviral transcriptional response in vivo, *Mpl*^{-/-} HSCs displayed decreased levels of THPO-induced ISGs (Fig. 6 C). Altogether, these results show that THPO is a potent inducer of antiviral response genes in HSCs/HSPCs in vitro and in vivo and can behave as an IFN-like factor.

THPO-induced, IFN-like signaling in HSCs is required to limit retrotransposon expression upon irradiation

Numerous ISGs are viral restriction factors (Schneider et al., 2014). Thus, we next examined whether this signaling could be linked to its capacity to restrain REs in HSCs. Like THPO, all IFNs signal via the JAK/STAT pathway. The main IFN type I signaling involves STAT1 and STAT2, which form a transcriptional complex with IRF9, called ISGF3, that binds to ISG promoters (Schneider et al., 2014). Fig. 7 A shows that THPO induces a rapid and sustained STAT1 phosphorylation in HSCs. We could not assess Stat2 activation in HSCs as a result of the lack of antibody recognizing mouse phospho-STAT2 in IF or cytometry. However, THPO could induce phosphorylation of both STAT1 and STAT2, together with ISG expression, in a human cell line expressing *Mpl* (Fig. S5, A and B). THPO-induced ISG expression was completely abolished in both *Stat1*^{-/-} and *Stat2*^{-/-} HSCs (Fig. 7, B, C, and D), supporting the possibility that THPO can mimic IFN type I signaling. No significant change in *Mpl* mRNA levels were observed in HSCs from these mice (Fig. S5 C).

Strikingly, THPO injection in *Stat1*^{-/-} mice could not prevent TBI-induced increase in L1 and IAP expression in HSCs (Fig. 8, A and B). Similar results were observed using *Stat2*^{-/-} LSK cells (Fig. 8, A and C). In agreement with a role of STAT1 and STAT2 in controlling THPO-mediated RE expression under basal conditions in vivo, HSCs from nontreated *Stat1*^{-/-} and *Stat2*^{-/-} mice, as *Mpl*^{-/-} HSCs, express slightly higher levels of IAP and L1 REs than WT HSCs. Furthermore, both *Stat1*^{-/-} and *Stat2*^{-/-} cells displayed decreased THPO-mediated resorption of γ H2AX foci at 1 mo after TBI (Fig. 8, D and E). These results show that THPO-induced IFN type I signaling in HSCs is required to prevent long-lasting accumulation of REs and RE-induced persistent γ H2AX foci upon irradiation. Interestingly, injection of IFN- α before TBI could also block irradiation-induced increased RE expression in HSCs (Fig. 9). This supports the possibility that IFN-type I signaling may have an unexpected protective role on HSCs upon genotoxic stress.

Discussion

Because of their lifelong potential, HSCs need to be protected from endogenous and exogenous insults that may trigger genomic instability to ensure their long-term functional activity and prevent their transformation. Intrinsic HSC-protective

mechanisms, including low metabolism, high xenobiotic efflux activity, quiescence, and activation of a strong DNA damage response, as well as environmental factors such as the hypoxic nature of the niche or the action of cytokine-controlling repair pathway activity have been shown to contribute to minimize accumulation of DNA damage and allow preservation of HSC potential (Bakker and Passegué, 2013). We show here that RE expression and mobilization can also constitute an endogenous source of HSC genomic instability that increases upon genotoxic stress. We described the ability of HSCs to mount a constitutive IFN-like antiviral response in response to THPO as a novel HSC intrinsic protective mechanism against this form of damage.

Recent data have highlighted the causal relationships between the L1- or LTR-containing RE activity and IFN-induced antiviral response. Indeed, many viral restriction factors are IFN-regulated genes (Schneider et al., 2014). Mutations or deficiencies in several of these genes that we found induced by THPO in HSCs, such as *Samhdi*, lead to abnormal RE accumulation and retrotransposition. Mutant *SAMHDI* of Aicardi-Goutières syndrome patients are defective in L1 inhibition, leading to abnormal L1 and Alu/SVA accumulation and mobilization (Zhao et al., 2013). REs in turn can serve as a source of endogenous signal that triggers type I IFN immune response, eventually leading to autoimmune diseases (Stetson et al., 2008; Zhao et al., 2013). Conversely, it has been recently reported that IFN- α stimulation or overexpression of several ISGs reduce RE expression and L1 propagation in cell lines (Goodier et al., 2012, 2015; Koito and Ishizaka, 2013; Zhang et al., 2014; Hu et al., 2015; Yu et al., 2015). This shows that IFN contributes to the immune control of both REs and exogenous pathogens. Using HSCs deficient for *Stat1* or *Stat2*, which are both required for IFN-I signaling and antiviral immunity (Durbin et al., 1996), we demonstrate here that this pathway is also crucial to confer on THPO the ability to regulate RE expression in HSCs and therefore limit HSC threat upon irradiation.

The mechanism underlying this inhibitory effect remains to be determined. Recent studies have shown that various ISGs can restrict L1 and IAP expression by different means (Pizarro and Cristofari, 2016). This includes interaction with ORF1p or L1 RNA in cytoplasmic stress granules, disruption of ribonucleoprotein particle integrity, RNA degradation, or processing by the RNA-induced silencing complex (Goodier et al., 2012, 2015; Zhang et al., 2014; Hu et al., 2015; Pizarro and Cristofari, 2016). This suggests that THPO-induced ISGs may be required to prevent L1 accumulation by acting mainly at the posttranscriptional level. At the transcriptional level, RE repression is controlled mainly by epigenetic mechanisms (Mita and Boeke, 2016). We have shown recently that H3K9-trimethylation catalyzed by Suv39h1 is required to repress both L1 and IAP expression in HSCs during the course of aging (Djeghloul et al., 2016). Differences in H3K9

the mean values of WT mice. *n* = 10 (5'-UTR, ORF2, and L1_A) and 7 (B2) pools of three WT or five *Mpl*^{-/-} mice from three to four independent experiments. Mann-Whitney test. (C) Experimental design and GFP Taqman assays in HSCs isolated from L1-GFP mice 1 mo after TBI with or without THPO injection. Means \pm SEM, *n* = 7 (NIR), 10 (IR), and 11 (IR+THPO) mice from two independent experiments. One-way ANOVA with Dunnett's multiple comparison test. (D) Experimental design and GFP expression in total cell progeny and in the LSK compartment (FACS) of L1-GFP HSCs isolated immediately after TBI with or without THPO injection and cultured for 10 d in vitro in the presence (+T) or absence (-T) of THPO; two (IR) and three (NIR and IR+THPO) independent cultures. Means \pm SEM. One-way ANOVA with multiple comparison tests. (E and F) L1 retrotransposition in HSCs and progenitors from L1-*Mpl*^{-/-}, either nontreated or 1 mo after TBI, as indicated. Means \pm SEM. (E) *n* = 12 (WT) and 13 (L1-*Mpl*^{-/-}) mice from four independent experiments. Mann-Whitney test. (F) *n* = 7 (L1 and L1-*Mpl*^{-/-} NIR), 9 (L1 IR), and 8 (L1-*Mpl*^{-/-} IR) mice from two independent experiments. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

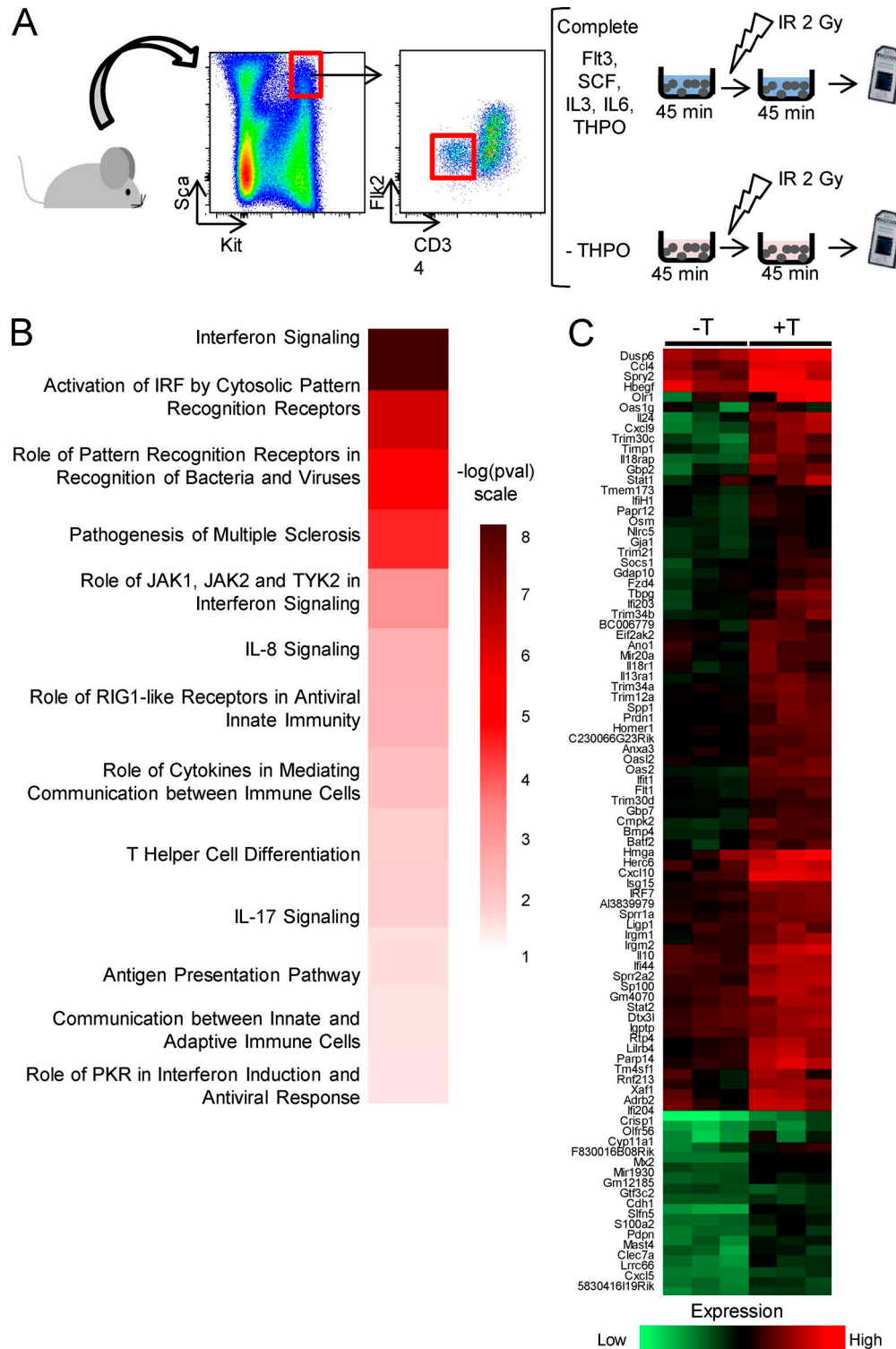


Figure 5. **THPO induces an IFN signature in HSCs.** (A) Experimental design for microarray analysis of THPO differentially regulated genes in HSCs. Gray objects represent Affimatrix Gene Chips (microarrays) used to measure gene expression in cells after treatments. (B) Heat map of ingenuity pathway analysis p-values for the top canonical pathways of differentially regulated genes, obtained using the Perseus software (MaxQuant). (C) Means of triplicate values for up-regulated genes calculated and clustered using the Perseus software.

methylation and chromatin condensation between HSCs and progenitors (Ugarte et al., 2015) could explain the high basal expression levels of L1 and IAP REs in the former. Interestingly, IFN- α has been shown to inhibit hepatitis B virus transcription

by inducing a STAT1/2-dependent epigenetic regulation (Belloni et al., 2012). ISGs involved in transcriptional silencing, such as *Trim33*, were found among THPO-up-regulated genes (Rajsbaum et al., 2008). Interestingly, *Trim33* has been shown to control

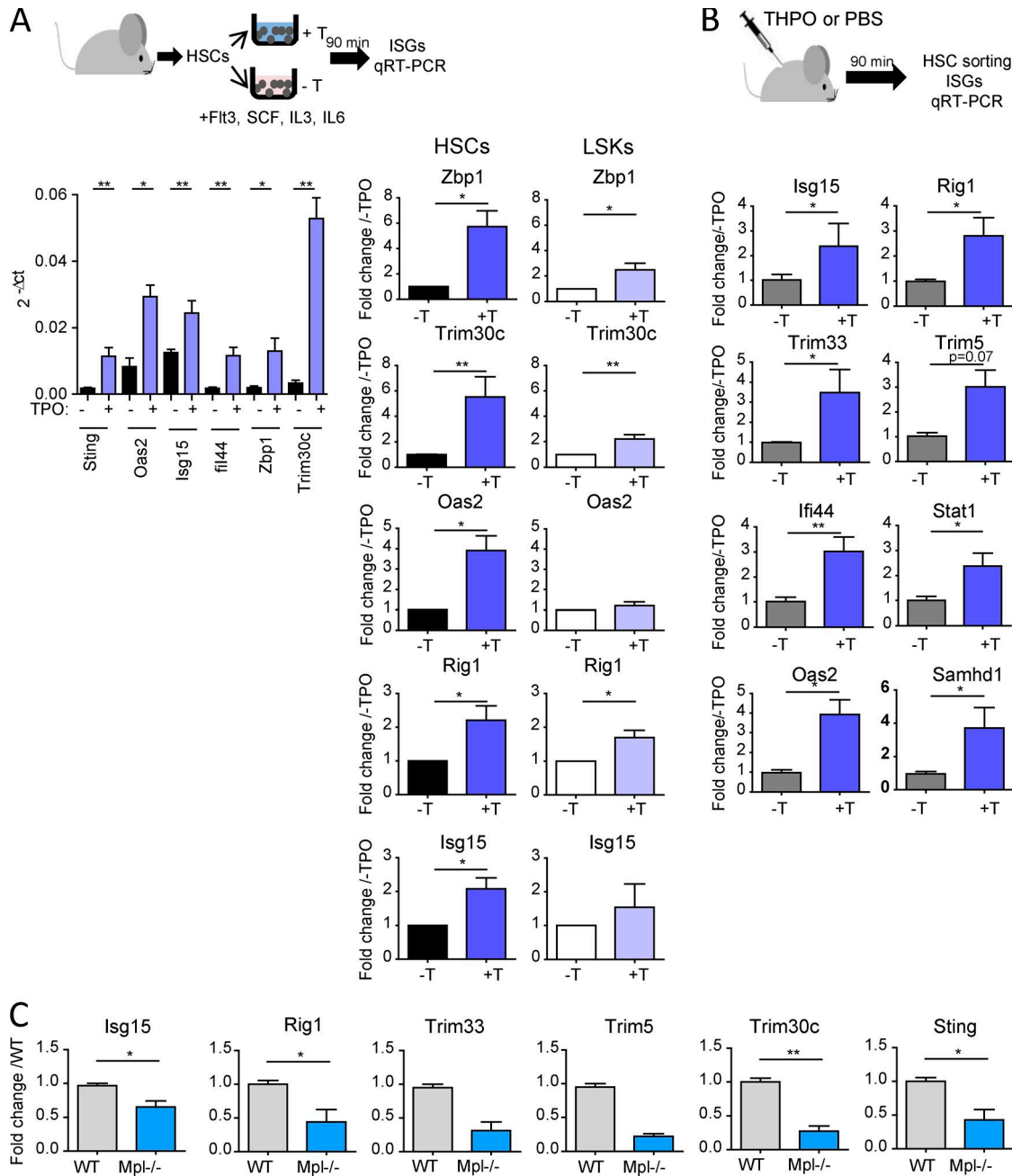


Figure 6. THPO induces IFN-stimulating gene expression in HSCs. (A) qRT-PCR analysis for THPO-up-regulated genes in HSCs and LSK incubated for 90 min in vitro in a medium containing THPO (+T) or not (-T). Data normalized to β -actin and/or Gapdh levels. Left, means \pm SEM of $2^{-\Delta Ct}$ values from $n = 4$ –5 pools of three to six mice in two independent experiments. Mann-Whitney test. Right, comparison of ISG expression in HSCs and LSK cells stimulated with (+T) or without (-T) THPO. Data were normalized as above and are presented on the same scale. Means \pm SEM, $n = 4$ –6 pools of mice for HSCs and 8–11 for LSK cells; three to five independent experiments. Paired t test. (B) qRT-PCR analysis in HSCs isolated from mice 90 min after THPO injection in vivo (+T) or not (-T). Data are expressed as fold change from the nontreated mice mean value after normalization. Means \pm SEM, $n = 5$ (-T) and 7 (+T) mice from two independent experiments. Mann-Whitney test. (C) Basal ISG mRNA expression in WT and *Mpl*^{-/-} HSCs. Means \pm SEM normalized to the mean of WT HSCs, $n = 3$ (Isg15, Rig1, Trim30c, and Sting) or 2 (Trim33 and Trim5) pools of three (WT) to six (*Mpl*^{-/-}) mice from three independent experiments. Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$.

expression of different LTR-containing REs (Herquel et al., 2013; Isbel et al., 2015). Thus, the THPO-induced STAT1/2 signaling pathway might restrain RE accumulation in HSCs through transcriptional regulatory mechanisms. This could explain the long-lasting effect of a single THPO injection on RE expression. Whether one specific or several ISGs

are required to control RE expression in HSCs requires further investigation.

In agreement with previous reports showing increased RE mobilization upon stress (Ishihara et al., 2000; Hagan et al., 2003; Farkash et al., 2006), we found that TBI triggers not only increased L1 expression, but also successful de novo L1

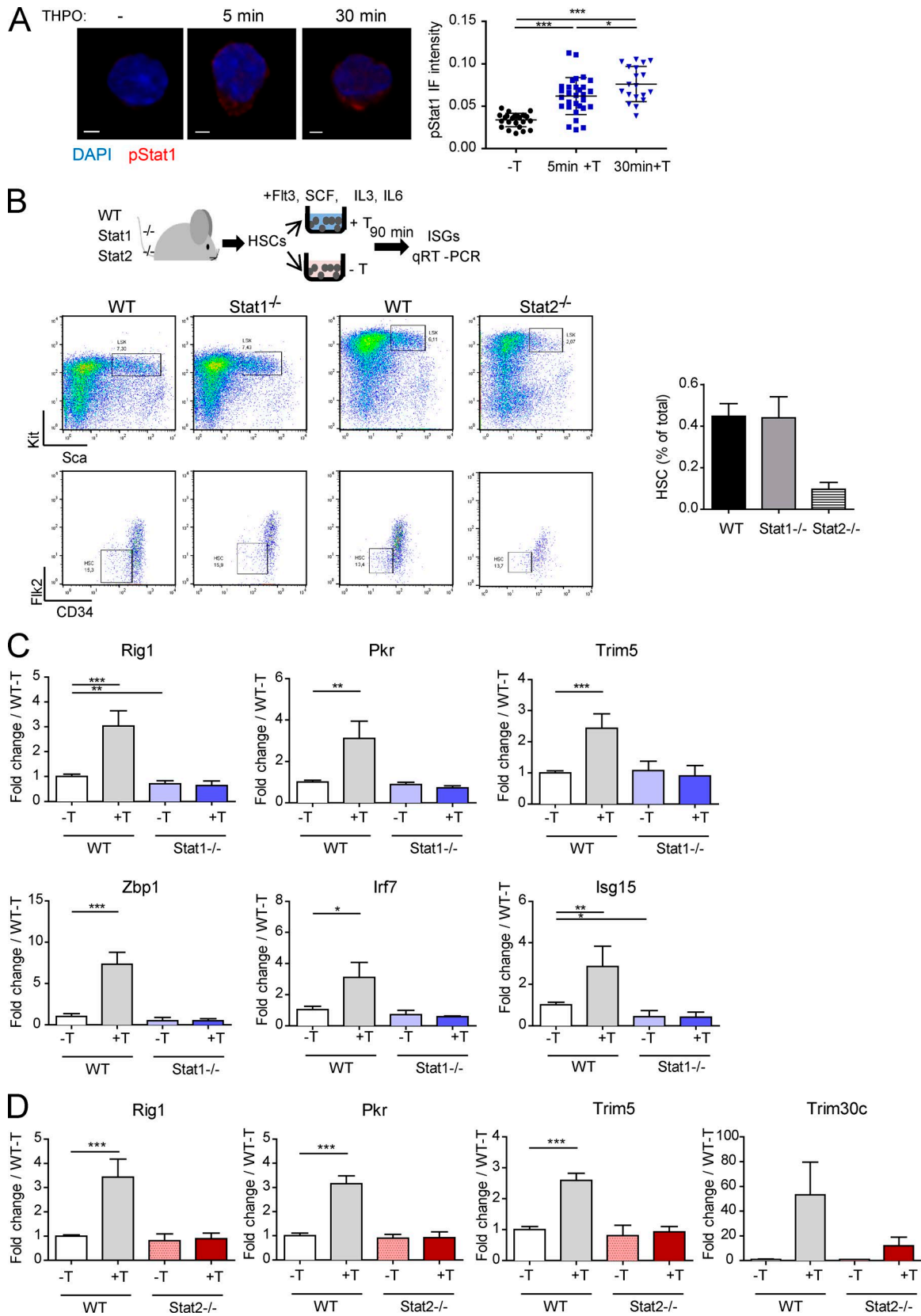


Figure 7. **STAT1 and STAT2 are required for THPO-mediated regulation of RE expression and DNA damage upon irradiation.** (A) Representative images and quantification of pSTAT1 staining in HSCs, with or without stimulation with THPO in vitro for the indicated times. Each point represents a cell. Means \pm SEM. One-way ANOVA with Dunnett's multiple comparison test. Bars, 30 μ m. (B) Experimental design and representative FACS images of WT, Stat1^{-/-}, and Stat2^{-/-} HSC sorting of the experiments shown in C and D. The graph on the right shows the total HSC frequency in WT, Stat1^{-/-}, and Stat2^{-/-} mice. Representative

retrotransposition in vivo in primary HSCs. Very recently, Macia et al. (2017) reported that human CD34⁺ cells do not express L1 and are unable to support L1 retrotransposition, suggesting that L1 mobilization in somatic healthy tissues is restricted to neuronal precursor cells. The discrepancy between this study and ours could be a result of differences in the cell population tested and/or to the treatment applied. Indeed, the CD34⁺ cells used by Macia et al. is a population composed mainly of progenitors, whereas we found higher L1 expression and retrotransposition levels in HSCs than in LSKs and progenitors. In addition, in the basal state, HSCs express low ORF1p and display low retrotransposition levels, both phenomena being greatly increased upon irradiation.

THPO injection before TBI reversed irradiation-induced L1 expression and L1 mobilization in HSCs. Addition of THPO in the culture after irradiation could also restrain L1 mobilization, demonstrating a direct effect of the cytokine on HSCs. In addition, L1 mobilization was enhanced in HSCs from L1-*Mpl*^{-/-} and L1-*Thpo*^{-/-} mice, showing that THPO signaling is required to control L1 retrotransposition in HSCs, in vivo, after genotoxic stress, as well as under steady-state conditions. Although ddC had no effect on TBI-induced changes in LSK and HSC numbers (Shao et al., 2014; Fleenor et al., 2015), its ability to rescue both γ H2AX foci accumulation and HSC reconstitution ability after TBI further demonstrates that endogenous retrotransposition plays a role in the long-lasting HSC injury induced by irradiation. This suggests that THPO-mediated L1 mobilization repression plays an important role in its ability to maintain HSC genomic stability. We have previously shown that THPO restrains HSC DNA damage by up-regulating the efficiency of the classical DNA-PK-dependent nonhomologous end joining (NHEJ) DNA repair (de Laval et al., 2013). These two mechanisms are not mutually exclusive. Indeed, RE insertion requires the creation but also the repair of broken DNA and is modulated by DNA repair pathways (Coufal et al., 2011; White et al., 2015). L1, but also SINE and IAP REs, can integrate at preformed DSBs and repair them (Lin and Waldman, 2001; Morrish et al., 2002; Onozawa et al., 2014). In the case of L1, insertions at preexisting DSBs are independent of ORF2p endonuclease activity and increase in cells deficient or mutated for NHEJ factors (Morrish et al., 2002). Interestingly, such repair is highly mutagenic and often shows features of alternative-NHEJ with the presence of short stretches of microhomologies (Onozawa et al., 2014), similarly to what is observed in irradiated cells in the absence of THPO signaling (de Laval et al., 2013). Thus, by modulating DNA repair, THPO may also regulate the extent and the type of RE insertions in HSCs and their ability to induce genomic instability. Because integration of viruses into host DNA induces DNA damage, some ISG products with antiviral activity, such as SAMHD1 and TREX, have developed the ability to regulate DNA damage and repair responses (Yang et al., 2007; Clifford et al., 2014). This suggests that the THPO-induced IFN-like response may constitute a positive retrocontrol pathway improving HSC DNA damage responses.

THPO has been shown to activate STAT1, STAT5, and STAT3 in cell lines and megakaryocytes (Drachman et al., 1997; Rouyez et al., 2005). We show that THPO also induces a rapid activation of STAT1 in HSCs. THPO also triggers STAT1 and STAT2 phosphorylation in a human cell line expressing Mpl. The requirement of both STAT1 and STAT2 for THPO-mediated ISG induction and RE repression suggests that THPO activates an IFN-I-like signaling in HSCs. This is to our knowledge the first demonstration of the involvement of STAT2 in THPO signaling. Although intriguing, this recalls previous studies showing that IFNs and THPO can induce similar transcriptional complexes and that IFN- α can trigger megakaryopoiesis (Rouyez et al., 2005; Haas et al., 2015). Chronic IFN-I exposure in vivo has been shown to induce transient HSC proliferation and/or apoptosis (Essers et al., 2009; Pietras et al., 2014), whereas THPO is required for HSC maintenance through regulation of survival, quiescence, or self-renewal divisions and favors transplantation (Qian et al., 2007; Yoshihara et al., 2007; Kovtonyuk et al., 2016). However, our results suggest that under some instances, such as genotoxic stress, IFN- α and THPO may similarly protect HSCs against REs. This function might be important to prevent DNA damage in HSCs during emergency myelopoiesis. In addition to STAT1/STAT2 activation, each cytokine also activates its own specific signaling pathways leading to unique gene and functional programs that may explain their different final effects on HSCs. For example, THPO stimulates integrin inside-out signaling and adhesion as well as pathways blocking oxidative stress that have been shown to be required for HSC function (Kirito et al., 2005; Umemoto et al., 2012). It also induces the expression of quiescence genes such as *p57* (Yoshihara et al., 2007), whereas IFN- α exposure induces cell cycle genes (Essers et al., 2009) or a transient decrease of quiescence regulators, including *p57* (Pietras et al., 2014). Although constitutive IFN-I secretion does occur in healthy mice, IFNs are secreted in abundance, primarily in response to viral infection. The ability of THPO to behave as a stronger inducer of ISGs in HSCs than in LSKs, together with its restricted expression in the hematopoietic system, suggests that it has evolved as a constitutive IFN, more specifically dedicated to HSCs and allowing their protection against RE-induced threat while maintaining their self-renewal ability. By inducing Stat1 and Stat2 expression, THPO may also prime HSCs to respond to IFNs upon infection.

Transcriptional derepression and increased mobilization of REs occur in the genome of aging somatic cells, including HSCs (De Cecco et al., 2013; Sun et al., 2014; Van Meter et al., 2014; Djeghloul et al., 2016). Interestingly, in HSCs, the same retrotransposon mRNA species were found to be increased with TBI and age, e.g. evolutionary recent active L1 family members (A, Tf, and Gf), as well as IAP retroviruses. This suggests that the deregulation of RE expression with age, as after irradiation, could be involved in HSC loss of function and persistent DNA damage. Flach et al. (2014) have recently shown that the γ H2AX foci present in old HSCs are resulting from residual replication stress on ribosomal DNA and colocalize with the nucleolus. We were not

experiment with cells from four (WT) and two pools of six mice (*Stat1*^{-/-} and *Stat2*^{-/-}). Means \pm SEM. (C and D) ISG mRNA expression in WT, *Stat1*^{-/-} (C), and *Stat2*^{-/-} HSCs (D) after stimulation for 90 min in vitro with (+T) or without THPO (-T). Data are normalized to the mean value of WT HSCs cultured in the absence of THPO. Means \pm SEM, *n* = 4 pools of five to six mice from two independent experiments. Paired *t* test. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

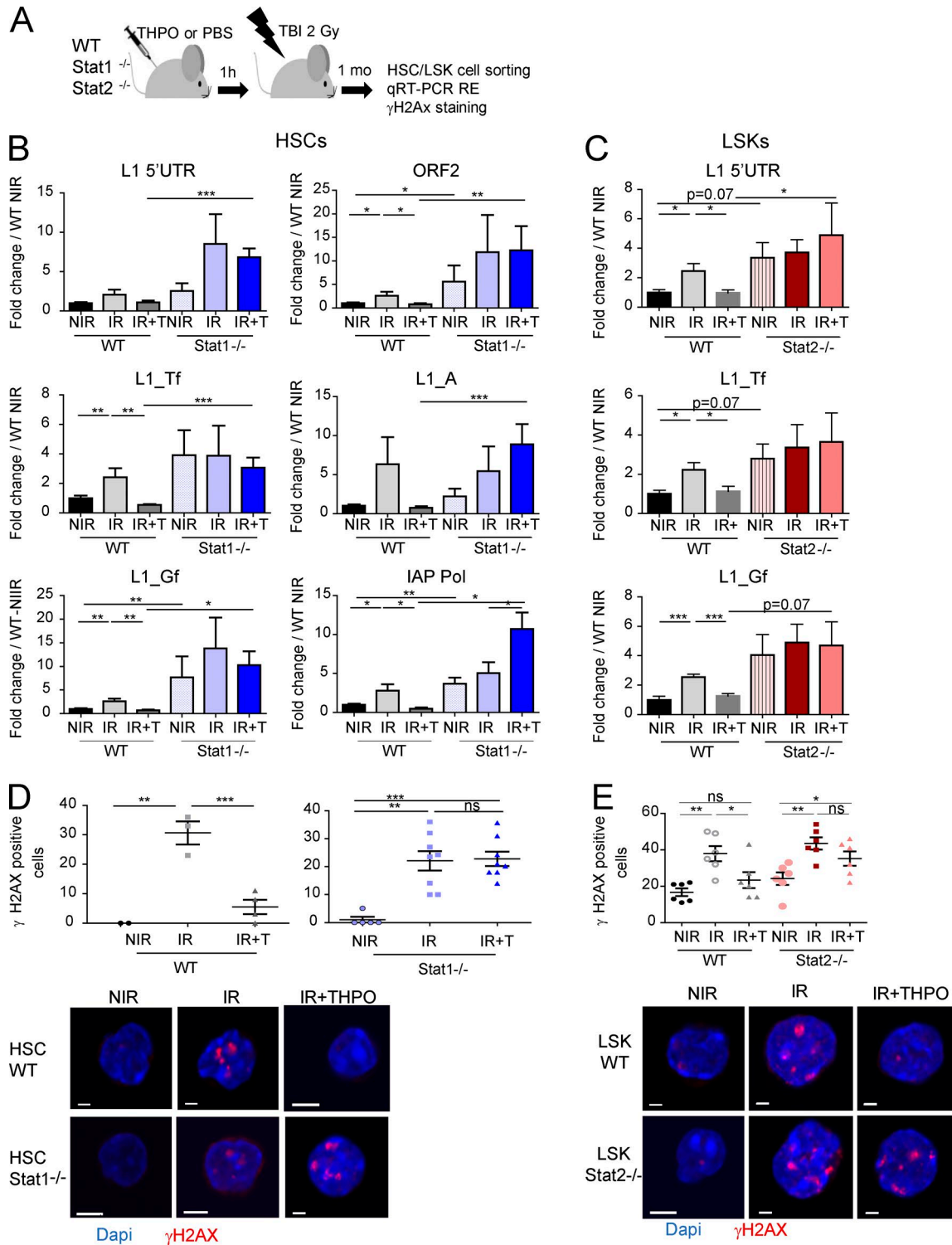


Figure 8. THPO-induced IFN type I signaling is required for THPO-mediated regulation of RE expression and DNA damage upon irradiation. (A) Experimental design for the experiments shown in B–E. (B and C) RE mRNA in WT, *Stat1*^{-/-} HSCs (B), and *Stat2*^{-/-} LSK cells (C) from nonirradiated mice (NIR) or 1 mo after TBI with (+T) or without THPO (-T) injection. Data are normalized to the mean values of nontreated WT mice. Means \pm SEM. (B) *n* = 8–12 mice. (C) *n* = 5–7 mice pooled two independent experiments. One-way ANOVA with Dunnett’s multiple comparison test and Mann-Whitney tests. (D and E) Representative images and quantification of WT, *Stat1*^{-/-} HSCs (D), WT, and *Stat2*^{-/-} LSK cells (E) expressing more than four γ H2AX foci 1 mo after TBI in the presence or absence of THPO. Each point represents an individual mouse. Means \pm SEM. One-way ANOVA with Dunnett’s multiple comparison test. (D) WT, representative experiments from three similar performed; *Stat1*^{-/-}, data pooled from two independent experiments. (E) Data pooled from two independent experiments. One-way ANOVA with Dunnett’s multiple comparison test. Bars, 30 μ m. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. ns, not significant.

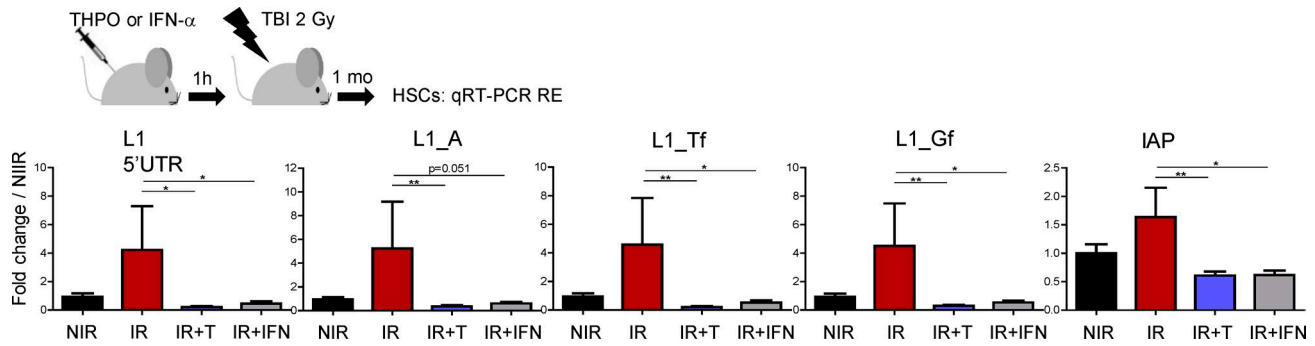


Figure 9. **IFN- α limits RE expression in HSCs upon irradiation.** Experimental design and RE mRNA expression in HSCs isolated 1 mo after 2 Gy TBI with prior injection of one dose of THPO or IFN- α as indicated. Results represent means \pm SEM and are expressed as fold change from the NIR mean value after normalization. $n = 6$ (NIR, IR, and IR+THPO) or 7 (IR+IFN) mice per condition, pooled from two independent experiments. Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$.

able to demonstrate such a nucleolar localization of γ H2AX foci persisting 1 mo after sublethal TBI (Fig. S5 D). Thus, whether the deregulation of REs plays a direct role in HSC aging remains to be investigated thoroughly.

Our results also suggest that maintenance of a certain level of THPO signaling may be required to prevent radiotherapy-induced myeloid malignancies. This is in agreement with the fact that patients with *MPL* loss-of-function mutations have an increased propensity of developing myelodysplastic syndromes (Maserati et al., 2008). These observations could also be highly relevant in the context of myeloproliferative neoplasms in which IFN-I administration is used as a treatment. IFN- α causes preferential depletion of *Jak2V617F*-mutated HSCs compared with normal HSCs (Hasan et al., 2013; Mullally et al., 2013). On the other hand, with progression to myelofibrosis, patients become resistant to this treatment. Our results showing that increased THPO signaling and ISG expression can protect HSCs from RE-induced DNA damage may explain this resistance. In that context, chronic IFN-I treatment and STAT1/2 induction were shown to induce chemotherapy resistance in certain cancers (Khodarev et al., 2004; Cheon et al., 2013). Because massive unleashing of REs has been shown to induce cancer stem cell apoptosis (Chiappinelli et al., 2015), strategies combining TBI and STAT1/2 blockade could also be considered.

Materials and methods

Mice and mice treatments

All mice are on a C57BL/6 background. *Mpl*^{-/-} and *Thpo*^{-/-} mice were described previously (de Laval et al., 2013). *Stat1*^{-/-} (Durbin et al., 1996) and *Stat2*^{-/-} (Park et al., 2000) mice were obtained from M. Muller (University of Veterinary Medicine, Vienna, Austria) and T. Kolbe (University Center for Biomodels, Vienna, Austria), respectively. WT C57BL/6J CD45.2 and CD45.1 mice were from Envigo and Charles River Laboratories, respectively. The transgenic mice expressing an engineered human L1 harboring a GFP-based retrotransposition reporter cassette (L1-GFP, strain 67; Okudaira et al., 2011) was from by T. Okamura and Y. Ishizaka (National Center for Global Health and Medicine, Department of Infection Diseases, Tokyo, Japan). L1-GFP mice were crossed with *Mpl*^{-/-} and *Thpo*^{-/-} (referred to

as L1-*Mpl*^{-/-} and L1-*Thpo*^{-/-} mice, respectively). All the mice were housed in a specific pathogen-free environment. All procedures were reviewed and approved by the Animal Care Committee no. 26 approved by the French Ministry for Research (agreement number no. 01773.03). Unless otherwise specified, mice of 6- to 10-wk of age were used. Mice were treated with one dose of THPO (16 μ g/kg body weight, i.v.) or IFN- α (50,000 U, i.v.), anti-IFNAR blocking monoclonal antibody (200 μ g; MAR1-5A3) before sublethal TBI (2 Gy; RX irradiator X-RAD 320). ddC, EFV (10 mg/kg weight, s.c.; Sigma) or their respective diluent alone (PBS for ddC and DMSO for EFV) were injected s.c. 1 h before TBI (2 Gy) and then daily for 1 mo.

Cells and cell culture in vitro

Lin⁻Sca1⁺Kit⁺ cells (referred to as LSK or HSPC), HSCs (LSK-CD34⁻Flk2⁻ or LSK-CD150⁺CD48⁻), multipotent progenitors (MPP, LSK-CD34⁺Flk2⁺), CMPs (Lin⁻Sca1⁻c-Kit⁺CD34^{hi}CD16/32⁻) and GMPs (Lin⁻Sca1⁻c-Kit⁺CD34^{low}CD16/32⁺) were sorted using a cell sorter (Influx; BD). HSCs were cultured in serum-free expansion medium (StemSpan; StemCell Technologies) supplemented with recombinant Flt3-Ligand (FLT3-L; 100 ng/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml), and stem cell factor (SCF, 100 ng/ml) in the presence (+THPO) or absence (-THPO) of 100 ng/ml THPO or 100 ng/ml IFN- α . All cytokines were from Peprotech. When the cells were irradiated in vitro, THPO was added to the medium 1 h before, as described (de Laval et al., 2013). For in vitro growth, duplicate samples of 100 HSCs were sorted in 96-well plates in proliferation medium: IMDM medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 1 mM glutamine and containing 25 ng/ml FLT3-L, 10 ng/ml IL-3, 25 ng/ml SCF, 25 ng/ml THPO, 4 U/ml erythropoietin (EPO), and 50 μ M β -mercaptoethanol. Cell numbers were evaluated at different times. UT7-Mpl cells were grown in α -MEM medium supplemented with 10% FCS and 2 u/ml EPO. The cells were stimulated by adding directly the THPO mimetic peptide GW395058, as described previously (de Laval et al., 2013).

Bone marrow reconstitution

For bone marrow reconstitution experiments, 3×10^6 CD45.2⁺ bone marrow cells isolated from mice subjected to TBI (2 Gy) and treated with or without ddC for 1 mo were injected in lethally

irradiated (9.5 Gy) C57BL/6 CD45.1 congenic mice, together with 3×10^6 competitor CD45.1⁺ cells. Bone marrows were collected 4 mo later. After red blood cell lysis, the cells were stained with antibodies against CD45.2, CD45.1, and HSC markers and analyzed by FACS. Secondary reconstitutions were performed by injecting 5×10^6 BM cells from the first recipients in lethally irradiated CD45.1⁺ recipients. BM was harvested 5 mo later.

qRT-PCR

Total RNA was extracted using the RNeasy plus micro kit (Qiagen) or the Direct-Zol RNA micro prep kit (Zymo Research; Proteogene) and reverse transcribed with superscript Vilo (Thermo Fisher). Real time PCR was performed using SYBR qPCR premix Ex Taq (Takara) on a real time PCR machine (7500; Applied Biosystems). Quantification was done using the $\Delta\Delta C_t$ -method with normalization on β -actin, Gapdh, and/or Hprt expression. For analysis of RE expression, the extracted RNAs were subjected to an additional treatment with RNase-free DNase (Ambion) according to manufacturer instructions, and the samples were tested for qPCR before reverse transcription to rule out detection of contaminating DNA. Only the samples giving Ct values close to the no template control were further analyzed. Primer sequences are shown in Table S1.

Retrotransposition assays

L1-GFP mice were treated with THPO or PBS and subjected to TBI (2 Gy) 1 h later. 1 mo after TBI, total bone marrow was stained with lineage (CD11b, Gr1, CD19, CD3, and Ter119)-APC, Kit-Perpcy5.5, Sca-PECy7, CD48-PB, and CD150-PE antibodies, and GFP was analyzed in the LSK- and HSC-gated populations. Alternatively, the presence of GFP was assessed by Taqman-based qPCR assay using primers and probe spanning the GFP exon-exon junction (Iijima et al., 2013). In brief, 700–1,000 cells were FACS-sorted directly in 10 μ l of prepGEM tissue extraction buffer (ZyGEM) and lysed according to the manufacturer's instructions. A nested PCR using GFP-specific primers was used to preamplify the L1 transgene, and the presence of the exon-exon junction was then assessed by qPCR using primers and an exon-exon junction probe specific for GFP (Table S1). Data were normalized to RNA 5S DNA. To test retrotransposition in vitro, 700 HSCs were isolated from L1-GFP mice immediately after TBI (2 Gy) or THPO injection and TBI treatment and cultured at 37°C in IMDM medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 1 mM glutamine, and containing 25 ng/ml FLT3-L, 10 ng/ml IL-3, 25 ng/ml SCF, 25 ng/ml THPO, 4U/ml EPO, and 50 μ M β -mercaptoethanol supplemented, or not, with 25 ng/ml THPO. Cells were harvested at different times and DNA was extracted as above. The presence of GFP was analyzed by exon-exon Taqman assay. At day 10 of culture, the cells were stained with Kit-Prpcy5.5 and Sca-PECy7 antibodies and GFP expression was analyzed by FACS, gating on the LSK cell compartment.

Microarray analysis

Triplicate samples of 10,000–15,000 LSK-CD34-Flk2⁻ HSCs were sorted and incubated in vitro for 45 min in complete medium, with or without THPO before irradiation at 2 Gy. RNA was

purified 45 min later using the RNeasy Plus micro kit (Qiagen) and were hybridized onto whole genome arrays (Affymetrix MouseGene2.0ST). Raw data were normalized using the robust Multichip Algorithm (RMA) in Bioconductor R. Quality controls and statistics were performed using Partek GS. A classical ANOVA for each gene was performed and pairwise Tukey's ANOVA was applied to identify differentially expressed genes in THPO-treated and untreated samples. Functional enrichment analysis of differentially expressed genes was performed using ingenuity pathway analysis (Ingenuity Systems). All data have been submitted on GEO Omnibus site under the accession no. GSE84195.

Immunofluorescence

3,000–5,000 HSCs were cytopun on polylysine-coated glass slides, and IF was performed as previously described (de Laval et al., 2013). Monoclonal anti- γ H2AX antibody (clone JBW301) was purchased from Millipore. The presence of the L1 ORF1 protein was assessed in HSCs using the rabbit anti-mouse L1 ORF1p antibody (Martin and Branciforte, 1993; Malki et al., 2014), a gift of A. Bortvin (Carnegie Institution for Science, Baltimore, MA). The antibody was used at a concentration of 3 μ g/ml. To test STAT1 activation, freshly sorted HSCs were stimulated for various times in vitro in the presence of THPO (100 ng/ml), cytopun as above and stained with antiphospho-Tyr(701)STAT1 antibody (clone 58D6 rabbit mAb; Cell Signaling Technologies). Detection was performed using Alexa Fluor 555-coupled anti-rabbit secondary antibody. All slides were visualized using SPE confocal microscope (Leica). Pictures were analyzed using ImageJ software or CellProfiler.

qPCR of genomic LINE-1

Quantification of genomic insertions of L1 was performed as described (Coufal et al., 2009; Muotri et al., 2010). In brief, duplicate samples of 200 HSCs or LSK cells from WT or *Mpl*^{-/-} mice were sorted directly in 10 μ l of prepGEM Tissue DNA extraction buffer (ZyGEM) and lysed according to the manufacturer's instructions. L1 ORF2 was amplified in triplicate using either Taqman- or SYBR-Green-based qRT-PCR. Relative genomic ORF2 content was normalized to the nonmobile 5S ribosomal RNA genomic content as described (Coufal et al., 2009; Muotri et al., 2010).

Analysis of expressed L1 mRNAs

RNA from HSCs and ESCs were extracted and subjected to RNase treatment as above. RNAs were reverse transcribed using SuperScript Reverse Transcriptase IV (Thermo Fisher) for 15 min at 55°C and 10 min at 80°C with 2 μ M mORF2 reverse primer. PCR amplifying a 2,296 bp sequence was then performed using L1-5'-UTR forward and mORF2 reverse primers (Table S1). Actin expression was used as amplification control because unspecific annealing of RT primer produces cDNA from highly abundant RNAs (Parent et al., 2015).

Statistical analysis

Results were statistically evaluated using either one-way ANOVA or *t* test by Prism version 5.0 software (GraphPad Software Inc.).

Online supplemental material

Fig. S1 is related to Figs. 1 and 2. It shows increased L1 expression and retrotransposition in HSCs upon irradiation. Fig. S2, related to Fig. 3, shows that reverse transcription inhibitors rescue irradiation-induced persistent γ H2AX foci HSC loss of function. Fig. S3, related to Fig. 4, shows increased RE expression and retrotransposition in *Thpo*^{-/-} and *Mpl*^{-/-} LSK cells and HSCs. Figs. S4 and S5, related to Figs. 5, 6, and 7 provides supplementary data on the induction of ISGs and Stat1/2 activation by THPO and IFN- α in HSCs, LSK, and human UT7-Mpl cells. Table S1 shows up- and down-regulated genes differentially expressed in THPO stimulated HSCs (fold change > 1.5; P > 0.05). Table S2 describes the primers used in this study.

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Author contributions: D. Barbieri, E. Elvira-Matelot, Y. Pelinski, and L. Genève designed and performed the experiments and analyzed the results. B. de Laval performed microarray experiments. G. Yogarajah and C. Pecquet performed experiments. C. Pecquet and S.N. Constantinescu provided *Stat2*^{-/-} mice. S.N. Constantinescu commented on the manuscript. F. Porteu designed and supervised the study and wrote the manuscript.

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Supplemental material

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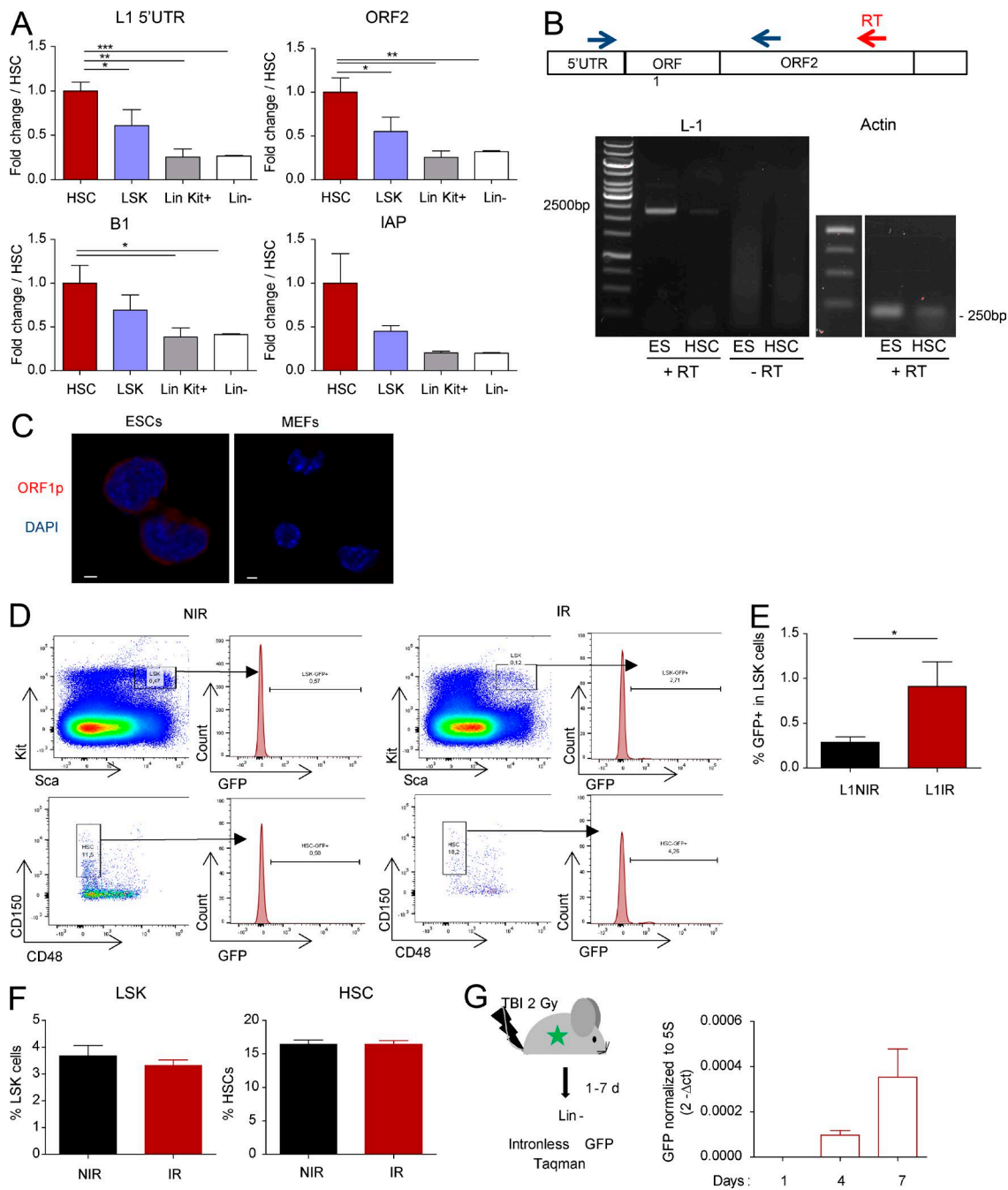


Figure S1. **RE mRNA expression and retrotransposition increase with irradiation.** (A) qRT-PCR analysis of the mRNA expression of LINE, SINE, and IAP elements in HSCs (LSK-CD34⁺Flk2⁻) and various hematopoietic populations, as indicated. Values were normalized to the β -actin and Gapdh levels and to the mean value expression in HSCs. $n = 4$ (B1, ORF2), 5 (5'-UTR), and 8 (IAP) mice from three to four independent experiments. One-way ANOVA with multiple comparison test. (B) PCR amplification of full-length L1 from HSC- and ESC-purified RNA. The schematic positions of the primers used in the RT and PCR reactions are shown. Amplification without RT treatment and of actin were used as negative and loading controls, respectively. (C) Representative images of IF analysis of ORF1p expression in mouse embryonic fibroblasts (MEFs) and ESCs. Bars, 50 μ m. (D) Gating strategy for the detection of GFP⁺ LSK and HSCs by FACS analysis in L1-GFP mice, nontreated (NIR), or one 1 mo after TBI (IR). (E) GFP⁺ LSK cells in L1-GFP mice, nontreated and 1 mo after TBI. Means \pm SEM, $n = 8$ (NIR) and 9 (IR) mice from two independent experiments. Mann-Whitney test. (F) Percentages of LSK and HSCs sorted from L1-GFP mice immediately after TBI ($n = 3$). (G) Kinetics of L1 retrotransposition induced by irradiation in L1-GFP mice. Mice were irradiated (2 Gy) and Lin⁻ progenitor cells were sorted at different times. GFP expression was detected by Taqman assay using exon-exon probe and primers. $n = 2-3$ independent mice/time point. This experiment was performed only one time. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

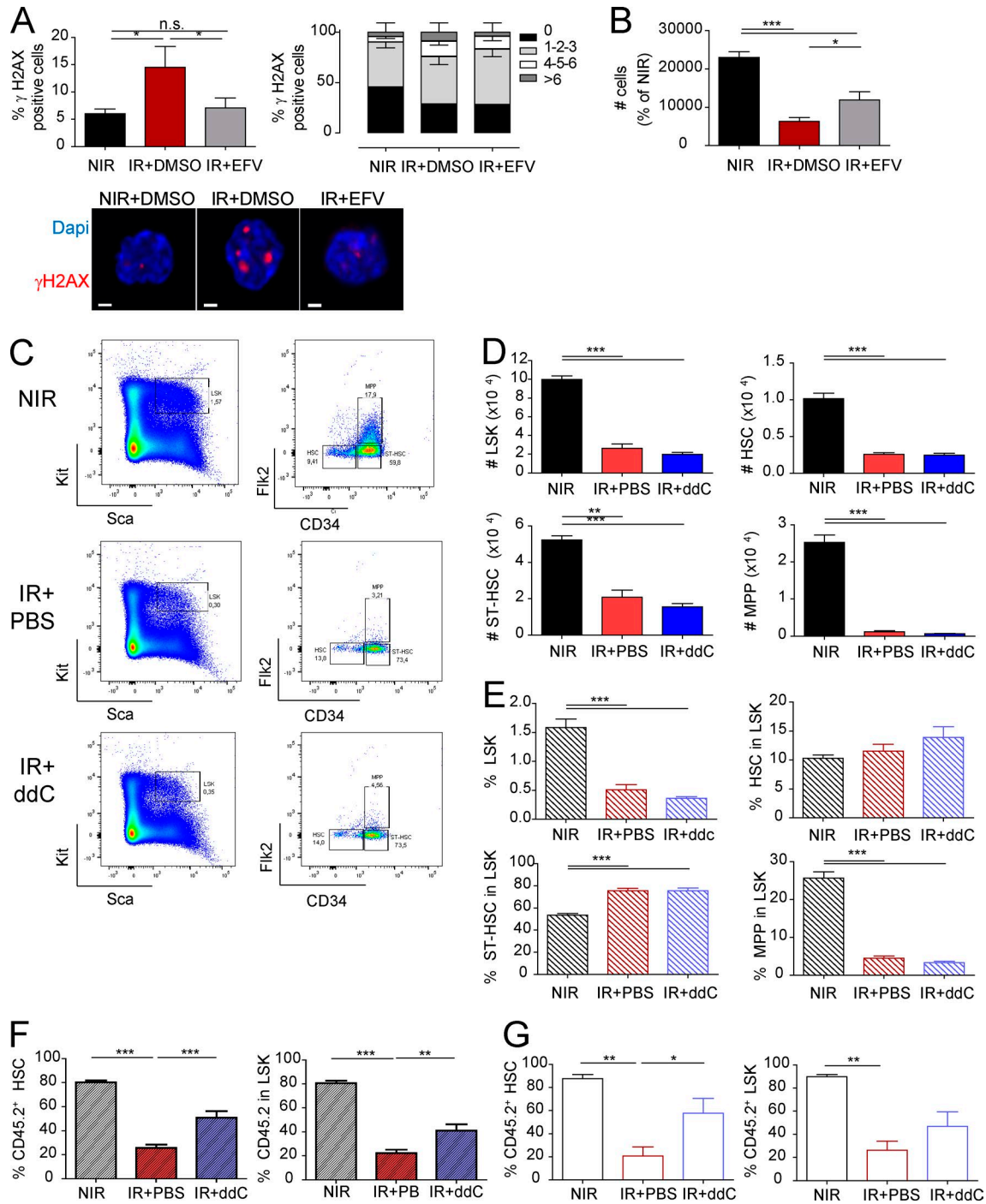


Figure S2. **Reverse transcription inhibitors rescue irradiation-induced HSC loss of function.** (A and B) γ H2AX foci and in vitro proliferation of LSK-CD34⁻Flk2⁻ HSCs isolated from mice subjected to TBI or not and treated daily for 1 mo with efavirenz (EFV) or DMSO as a control. Means \pm SEM, $n = 6$ (NIR), 4 (IR+DMSO), and 5 (IR+EFV) mice. One-way ANOVA with Dunnett's multiple comparison test. This experiment was performed only once. Bars, 30 μ m. (C-E) Quantification of HSCs in the bone marrow 1 mo after irradiation and daily injection of ddC or PBS (stage 1, Fig. 3 A). (C) Representative FACS analysis for each group. (D) Total cell numbers. (E) Relative frequencies. Means \pm SEM, $n = 8$ (NIR) and 12 (IR+PBS and IR+ddC) mice from two independent experiments. One-way ANOVA with Dunnett's multiple comparison test. (F and G) CD45.2⁺ donor contribution 4 and 5 mo after primary (F) and secondary reconstitution (G), respectively, with cells from mice irradiated treated with ddC or not as in Fig. 3 A. Means \pm SEM. One-way ANOVA with Dunnett's multiple comparison test. (F) $n = 7$ (NIR), 9 (IR+PBS), and 10 (IR+ddC) mice from two independent experiments. (G) $n = 4$ (NIR and IR+PBS) and 5 (IR+ddC). Representative independent experiment out of two performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

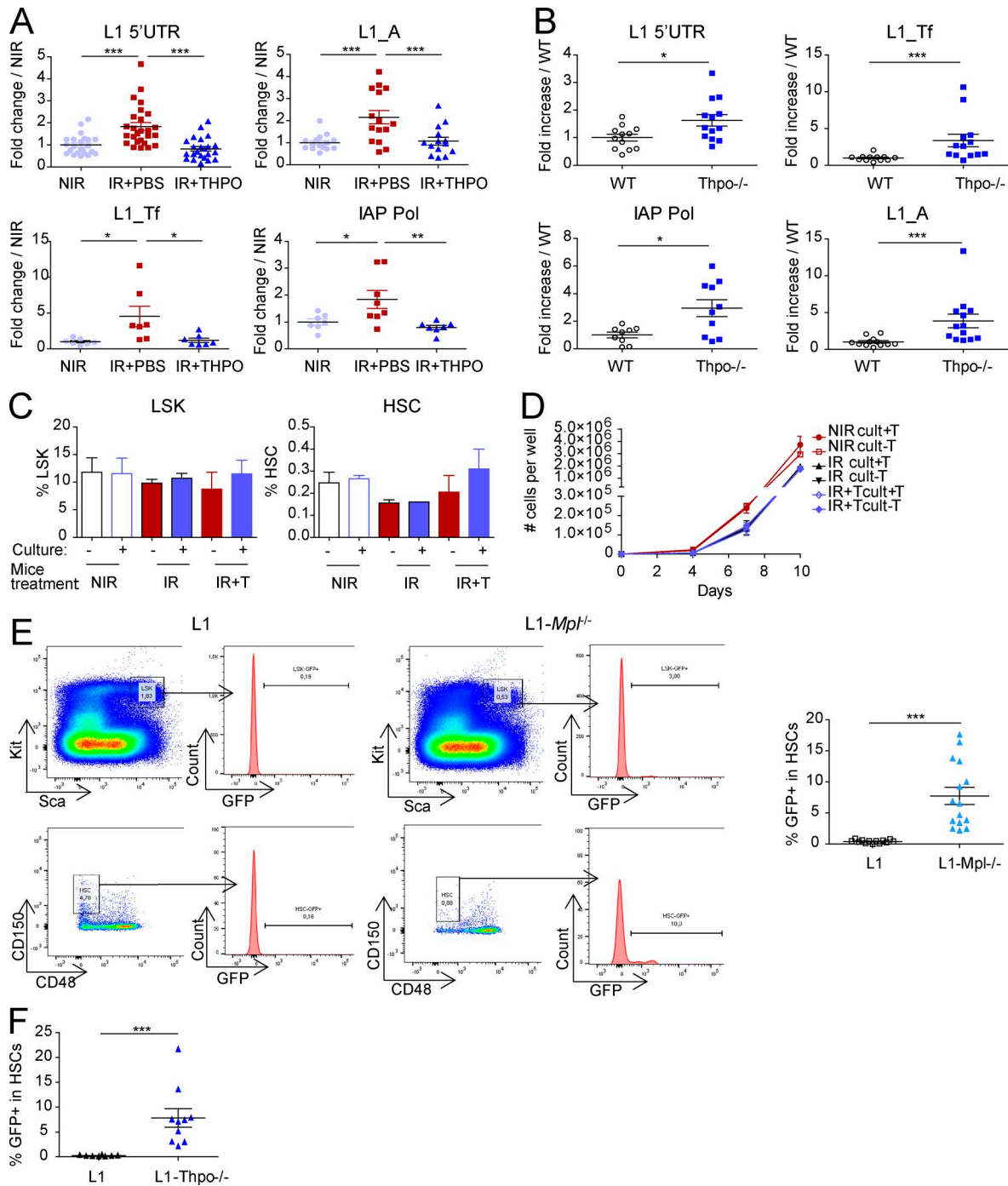


Figure S3. THPO signaling restrains L1 expression and retrotransposition in HSCs in the absence of irradiation. (A) RE mRNA expression in LSK cells isolated 1 mo after 2 Gy TBI with (IR+THPO) or without (IR+PBS) THPO injection or nontreated (NIR). Results represent means \pm SEM and are expressed as fold change from the NIR mean value after normalization. Each dot represents an individual mouse. Data are pooled from three to four independent experiments. One-way ANOVA with Dunnett's multiple comparison test. **(B)** qRT-PCR analysis of RE expression in LSK cells from WT and *Thpo*^{-/-} mice. Results are normalized to the mean values obtained with WT cells. Each dot represents an individual mouse. Mean \pm SEM from two to four independent experiments. Mann-Whitney test. **(C and D)** Percent of HSCs and LSK cells measured by FACS analysis at day 10 (C) and total cell counts (D) in vitro cultures of L1-GFP HSCs isolated immediately after TBI with or without THPO injection and cultured in the presence (+T) or absence of THPO (-T). Means \pm SEM of two to three independent cultures. **(E)** Representative gating strategy and quantification of retrotransposition detected by FACS analysis in HSCs (LSK-CD48⁺-CD150⁺) from L1-GFP and L1-*Mpl*^{-/-} mice. Means \pm SEM from four independent experiments. Mann-Whitney test. **(F)** Retrotransposition detected by FACS analysis in HSCs (LSK-CD48⁺-CD150⁺) from L1-GFP and L1-*Thpo*^{-/-} mice. Each dot represents an individual mouse. Means \pm SEM from two independent experiments. Mann-Whitney test. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

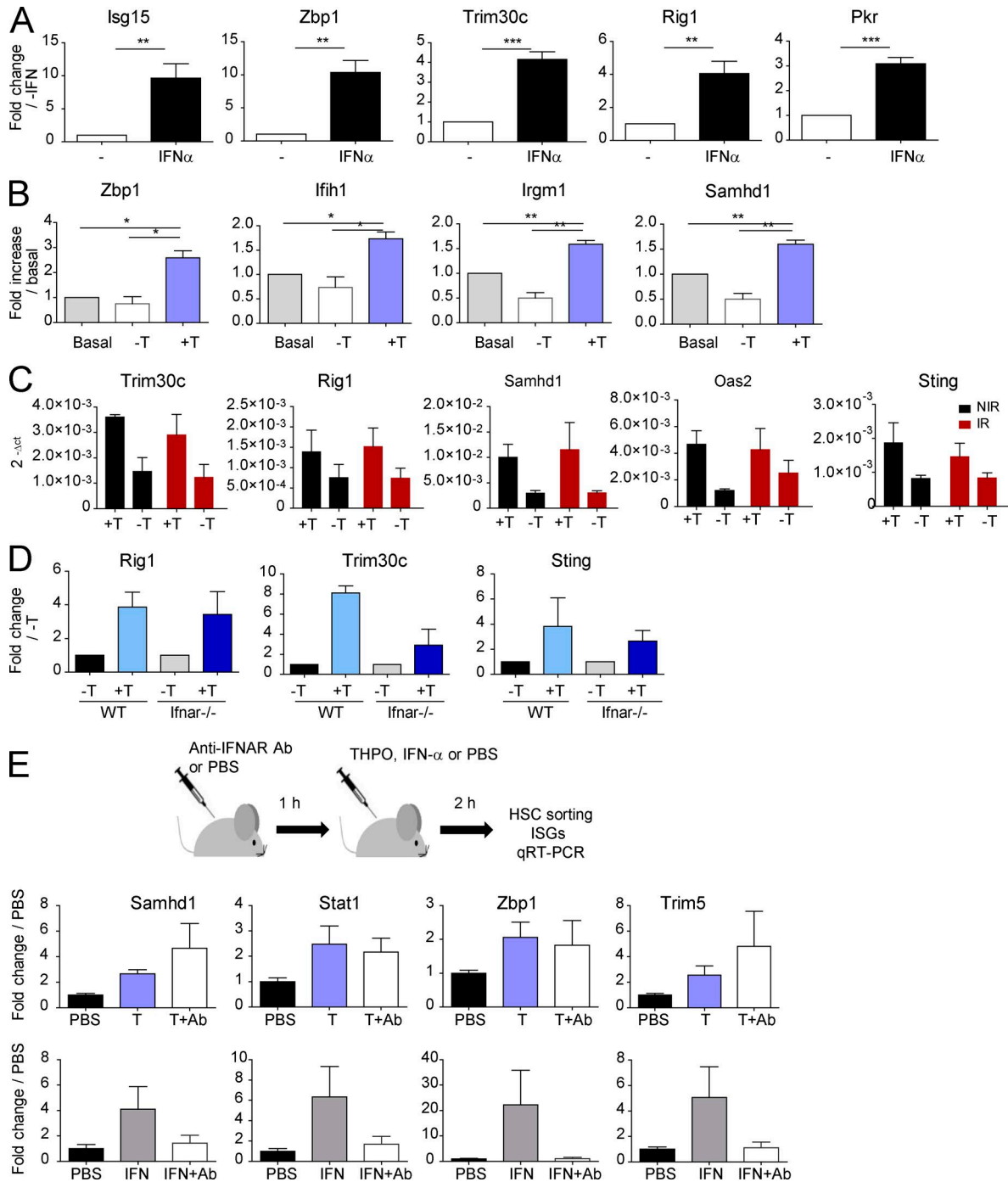


Figure S4. THPO induces IFN-regulated genes in HSCs. (A) qRT-PCR analysis of ISG expression in LSK cells cultured for 90 min in a medium containing or not containing 100 ng/ml IFN- α . Ct values were normalized to β -actin and/or Gapdh. Results are expressed as fold change from cells isolated from the same mice and cultured without IFN- α . Means \pm SEM. $n = 5$ (Rig1 and Pkr), 7 (Trim30c, Zbp1, and IRF7), 10 (Isg15, Oas2, and Ifi44) mice from two to three independent experiments; paired t test. **(B)** qRT-PCR analysis for THPO-up-regulated genes in HSCs just after sorting (Basal) or stimulated for 90 min in vitro in medium containing cytokines with or without THPO as in Fig. 6 A. Data normalized to the mean values of expression in unstimulated cells. Means \pm SEM; $n = 3$ pools of 5 mice in two independent experiments. Repeated measure ANOVA with multiple comparison test. **(C)** ISG analysis in HSCs treated as in Fig. 5 A. Means \pm SEM of Ct values obtained from three pools of six to eight mice, representative experiment out of two performed. **(D)** THPO-induced ISGs in *Ifnar*^{-/-} HSCs. HSCs from WT and *Ifnar1*-deficient mice were incubated for 90 min in vitro in medium supplemented with (+T) or without (-T) before qRT-PCR analysis. Means \pm SEM. $n = 4$ –5 pools of five mice in two independent experiments. **(E)** Experimental design for testing the effect of anti-IFNAR blocking antibodies on ISG induction by THPO or IFN- α in vivo. Top: $n = 7$ –10 mice from two to three experiments. Bottom: $n = 3$ mice from a representative experiment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

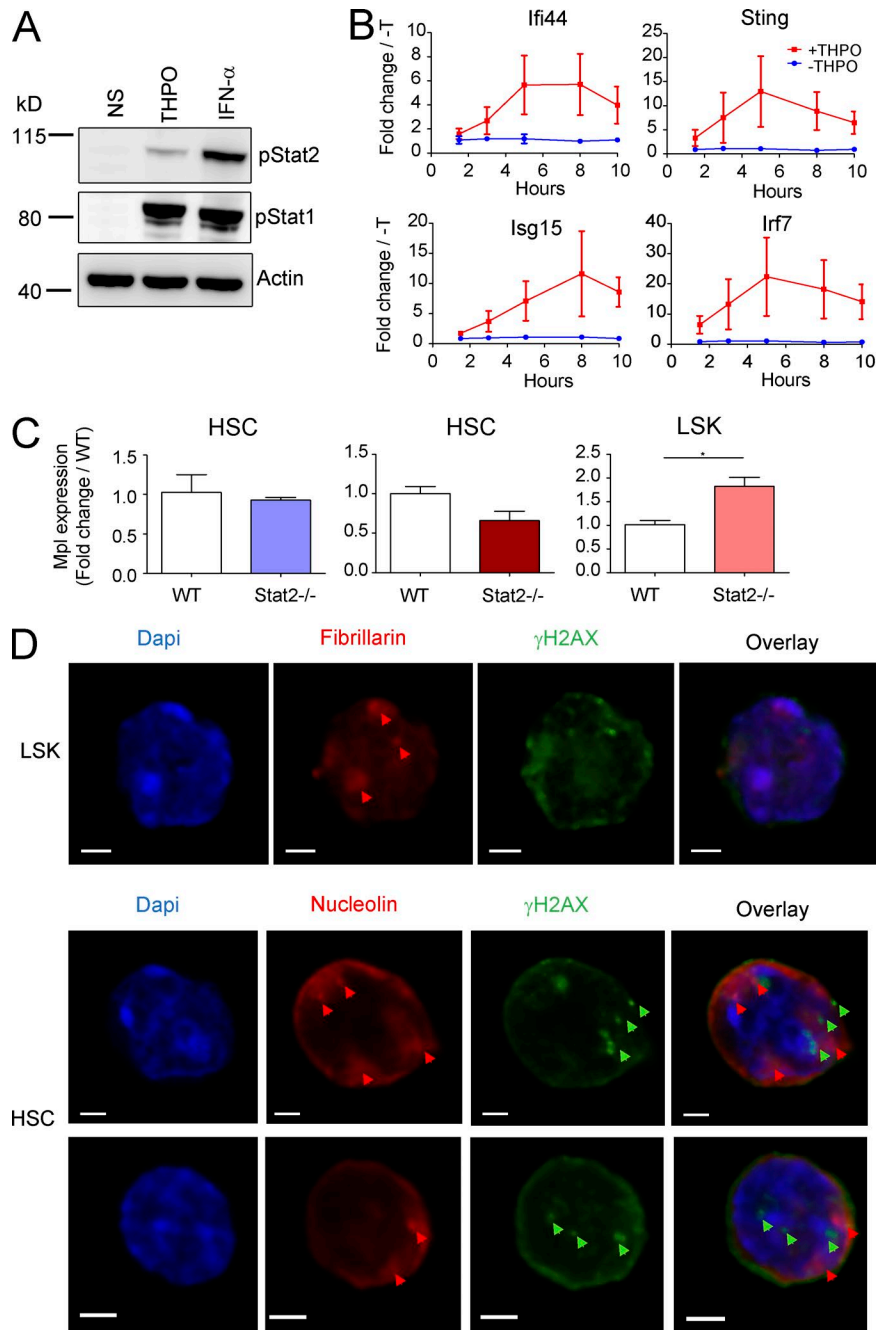


Figure S5. **THPO induces IFN type-I signaling in UT7-Mpl cells. (A)** Human UT7-Mpl cells were stimulated for 10 min with THPO peptide (10 nM) or IFN- α (50 ng/ml) before lysis and Western blot analysis. Representative experiment out of two performed. **(B)** qRT-PCR analysis of ISG induction in UT7-Mpl cells stimulated with 3 nM THPO peptide or not. Means \pm SEM from two independent experiments. **(C)** qRT-PCR analysis for Mpl mRNA expression in HSCs (LSK-CD34⁺Flk2⁻) or LSK cells isolated from WT or *Stat1*- or *Stat2*-deficient mice. Results are expressed as fold change from the mean $2^{-\Delta\Delta CT}$ values of WT mice. $n = 2$ (*Stat1*^{-/-} HSC) and 4 (*Stat2*^{-/-} HSC and LSK) pools of six mice from two to three independent experiments. Unpaired *t* test. *, $P < 0.05$. **(D)** Long-lasting TBI-induced γ H2AX foci do not colocalize with the nucleolus. LSK cells and HSCs were sorted 1 mo after TBI and stained with mouse anti- γ H2AX and rabbit anti-nucleolin (Ab22758; Abcam) or fibrillarin (2639; Cell Signaling Technology), as indicated. The slides were counterstained with Dapi analyzed using a confocal microscope. Arrowheads indicate the position of the nucleolus (red) and γ H2AX foci (green). Bars, 30 μ m.

Tables S1 and S2 are provided online as Excel files. Table S1 shows up- and down-regulated genes differentially expressed in THPO stimulated HSCs (fold change > 1.5; $P > 0.05$). Table S2 describes the primers used in this study.

Titre : La désorganisation de la chromatine régule l'expression des L1Md induite par l'irradiation et la fonction des cellules souches hématopoïétiques

Mots clés : Cellule souche hématopoïétique, retroélément, irradiation, thrombopoïétine

Résumé : L'exposition à l'irradiation, comme lors des radiothérapies, affecte l'intégrité et la fonction des cellules souches hématopoïétiques (CSH). L'IR est donc associée au développement de maladies myéloïdes ainsi que aux syndromes myélodysplasiques (SMD) et les leucémies myéloïdes aiguës secondaires. Par conséquent, l'étude des mécanismes moléculaires qui contribuent à la perte de fonction des CSH induite par le stress, pourrait aider à identifier les patients à risque et à trouver éventuellement de nouvelles stratégies pour prévenir ces maladies.

Nous avons récemment découvert un nouveau mécanisme responsable de la perte de fonction des CSH murines suite à l'IR qui implique les L1Md, les sous-familles jeunes et actives des éléments LINE-1. Nous avons montré que l'expression des L1Md est augmentée suite à l'IR et que cela entraîne une accumulation de dommages à l'ADN et de défauts des CSH. Nous avons également montré que la thrombopoïétine (TPO), une cytokine de niche des CSH, prévient la perte de fonction des CSH induite par l'IR, l'accumulation de dommages à l'ADN et la dérégulation des L1Md. Au début de ma thèse, j'ai participé à une étude qui a montré que la TPO contrôlait l'expression des L1Md par cette voie de signalisation.

Les L1Md sont reconnues comme des contributeurs majeurs des réseaux de régulation des gènes. Leur expression est étroitement régulée par des mécanismes épigénétiques, tels que la marque répressive de l'histone H3K9me3.

Les principaux objectifs de mon projet de thèse sont donc de comprendre les mécanismes par lesquels l'IR affecte l'épigénétique des CSH, et en particulier l'hétérochromatine; déterminer comment, l'expression des L1Md peut affecter l'expression génique des CSH; déterminer si la TPO, via sa signalisation de type IFN, peut réguler la répression des L1Md par des mécanismes épigénétiques.

Des expériences de ChIP-qPCR sur des CSH un mois après IR, montrent que la dérégulation des L1Md est liée à une perte de H3K9me3. Ces résultats ont été confirmés par des expériences de ChIPseq montrant qu'une grande majorité des loci L1Md présentaient une perte de H3K9me3 suite à l'IR par rapport à la condition non irradiée. Ce n'était pas le cas pour les sous-familles de rétroéléments plus anciens, comme le Lx5, ou pour les rétrovirus endogènes. Les données RNAseq ont montré que l'IR dérégule fortement le transcriptome des CSH. Nous montrons également que les gènes réprimés lors de l'IR sont significativement plus susceptibles de contenir un L1Md dans leurs introns que par hasard ($p < 0,05$). Ceci est spécifique aux L1Md et aux gènes qui sont réprimés par l'IR. Certains de ces gènes sont impliqués dans l'oncogenèse ou la fonction des CSH. L'IR induit une perte de la signature CSH. Il est intéressant de noter que 55% des gènes appartenant à la signature CSH et qui sont réprimés lors de l'IR contiennent un L1Md dans leurs introns. L'orthologue humain de 75% des gènes réprimés lors de l'IR et hébergeant une L1Md, contient également un L1 jeune chez l'homme, suggérant une fonction conservée dans la régulation de l'expression des gènes hématopoïétiques. Nous avons analysé plusieurs cibles et validé une diminution de l'expression en IR accompagné d'une perte de H3K9me3 au niveau de leur L1Md intronique respective.

Ces résultats montrent un lien entre l'IR et l'épigénétique des CSH, et suggèrent un rôle pour les L1Md dans la régulation de l'expression des gènes hématopoïétiques.

Title : Chromatin disorganization as a regulator of irradiation-induced L1Md expression and hematopoietic stem cell function

Keywords : Hematopoietic stem cell, retroelements, irradiation, thrombopoietin

Abstract : Exposure to ionizing radiations (IR), like in radiotherapy, affects hematopoietic stem cell (HSC) integrity and function. As a consequence, IR is associated with the development of therapy-related myeloid malignancies such as myelodysplastic syndromes (MDS) and secondary acute myeloid leukemias. Therefore, studying the molecular mechanisms that contribute towards stress-induced HSC loss of function, could help identify patients at risk and eventually find new strategies to prevent these diseases.

Our team has recently uncovered a new mechanism responsible for murine HSC loss of function upon IR that involves L1Md, the young and active subfamilies of Long Interspersed Elements LINE-1. We showed that L1Md expression is increased following IR and that this leads to an accumulation of DNA damage and HSC defects. We have also shown that thrombopoietin (TPO), a niche HSC cytokine involved in self-renewal, prevents IR-induced HSC loss of function, accumulation of DNA damage and L1Md derepression.

Microarray analysis had shown that TPO induced an enrichment of IFN-I signaling genes in HSCs, many of which are viral restriction factors. At the beginning of my PhD I was involved in a study that showed that TPO controlled L1Md expression via this signaling pathway. These results were published in J Exp Med in 2018, in an article in which I am co-first author.

L1Md are recognized as major contributors of gene regulatory networks. Their expression is tightly regulated by epigenetic mechanisms, such as the repressive histone mark H3K9me3.

The main objectives of my PhD project are thus to understand the mechanisms by which IR affects HSC epigenetics, and in particular heterochromatin; determine if TPO, via its IFN-like signaling, may regulate L1Md repression through epigenetic mechanisms; determine if, and how, L1Md expression may impact HSC gene expression.

We performed ChIP-qPCR experiments on HSCs one month post IR, and found that L1Md derepression is linked to a decreased H3K9me3 enrichment at their promoters, which is prevented by TPO. These results were further confirmed by ChIPseq experiments that showed that a vast majority of L1Md loci showed a reduced H3K9me3 enrichment upon IR compared to the non-irradiated condition, and that this was prevented by TPO. This was not the case for older retroelement subfamilies, such as the Lx5, or for endogenous retroviruses (ERV). RNA-seq data showed that IR strongly deregulates the HSC transcriptome. These effects are prevented by TPO injection 1h prior to IR. We also show that genes repressed upon IR, and not in the IR+TPO condition, are significantly more prone to contain an L1Md in their introns than by chance ($p < 0.05$). This is specific for the L1Md family and for genes that are downregulated upon IR. Some of these genes are involved in oncogenesis or HSC function. IR induces a loss of the HSC signature. Interestingly, 55% of the genes belonging to the HSC signature and that are repressed upon IR contain an L1Md in their introns. The human orthologous of 75% of the genes repressed upon IR and hosting an L1Md, also host young human and primate L1, suggesting a conserved functional role of young L1 in regulating hematopoietic gene expression.

We have analyzed in more details several target genes, and validated a decreased expression upon IR that is accompanied by a loss of H3K9me3 at their respective intronic L1Md.

These results show for the first time a link between IR and HSC epigenetics, and suggest a role for L1Md in regulating hematopoietic gene expression.