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Alternative splicing by hnRNP L as a new regulator of hematopoietic cell development, survival and migration

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Alternative splicing by hnRNP L as a new regulator of hematopoieitic cell development, survival and migration

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Résumé

Les modifications post-transcriptionnelles de l'ARN messager (ARNm), comme l'épissage alternatif, jouent un rôle important dans la régulation du développement embryonnaire, de la fonction cellulaire et de l'immunité. De nouvelles évidences révèlent que l'épissage alternatif serait également impliqué dans la régulation de la maturation et de l'activation des cellules du système hématopoïétique. Le facteur hnRNP L a été identifié comme étant le principal régulateur de l'épissage alternatif du gène codant pour le récepteur CD45 *in vitro*. Le récepteur CD45 est une tyrosine phosphatase exprimée par toutes les cellules du système hématopoïétique qui contrôle le développement et l'activation des lymphocytes T.

Dans un premier temps, nous avons étudié la fonction du facteur hnRNP L dans le développement des lymphocytes T et dans l'épissage de l'ARNm de CD45 *in vivo* en utilisant des souris dont le gène de hnRNP L a été supprimé spécifiquement dans les cellules T. La délétion de hnRNP L dans les thymocytes résulte en une expression aberrante des différents isoformes de CD45 avec une prédominance de l'isoforme CD45RA qui est généralement absent dans le thymus. Une conséquence de la délétion de hnRNP L est une diminution de la cellularité du thymus causée par un blocage partiel du développement des cellules pré-T au stade DN4. Cette réduction du nombre de cellules dans le thymus n'est pas liée à une hausse de la mort cellulaire. Les thymocytes déficients pour hnRNP L démontrent plutôt une prolifération augmentée comparée aux thymocytes sauvages due à une hyper-activation des kinases Lck, Erk1/2 et Akt. De plus, la délétion de hnRNP L dans le thymus cause une perte des cellules T en périphérie. Les résultats des expériences *in vitro* suggèrent que cette perte est principalement due à un défaut de migration des thymocytes déficients pour hnRNP L du thymus vers la périphérie en réponse aux chimiokines. L'épissage alternatif de CD45 ne peut expliquer ce phénotype mais l'identification de cibles par RNA-Seq a révélé un rôle de hnRNP L dans la régulation de l'épissage alternatif de facteurs impliqués dans la polymérisation de l'actine.

Dans un second temps, nous avons étudié le rôle de hnRNP L dans l'hématopoïèse en utilisant des souris dont la délétion de hnRNP L était spécifique aux cellules hématopoïétiques dans les foies fœtaux et la moelle osseuse. L'ablation de hnRNP L réduit le nombre de cellules progénitrices incluant les cellules progénitrices lymphocytaires (CLPs), myéloïdes (CMPs, GMPs) et mégakaryocytes-érythrocytaires (MEPs) et une perte des cellules hématopoïétiques matures. À l'opposé des cellules progénitrices multipotentes (MPPs) qui sont affectées en absence de hnRNP L, la population de cellules souches hématopoïétiques (HSCs) n'est pas réduite et prolifère plus que les cellules contrôles. Cependant, les HSCs n'exprimant pas hnRNP L sont positives pour l'Annexin V et expriment CD95 ce qui suggère une mort cellulaire prononcée. Comme pour les thymocytes, une analyse par RNA-Seq des foies fœtaux a révélé différents gènes cibles de hnRNP L appartenant aux catégories reliées à la mort cellulaire, la réponse aux dommages à l'ADN et à l'adhésion cellulaire qui peuvent tous expliquer le phénotype des cellules n'exprimant pas le gène hnRNP L.

Ces résultats suggèrent que hnRNP L et l'épissage alternatif sont essentiels pour maintenir le potentiel de différenciation des cellules souches hématopoïétiques et leur intégrité fonctionnelle. HnRNP L est aussi crucial pour le développement des cellules T par la régulation de l'épissage de CD45 ainsi que pour leur migration.

Mots-clés : hnRNP L, cellules T, CD45, cellules souches hématopoïétiques, épissage alternatif

Abstract

Post-transcriptional modifications of pre-mRNA by alternative splicing are important for cellular function, development and immunity. New evidence reveals that alternative splicing is implicated in the regulation of maturation and activation of hematopoietic cells. HnRNP L has been identified as the main regulator of alternative splicing of CD45 in vitro. The receptor tyrosine phosphatase CD45, which is expressed on all hematopoietic cells, is known for its role in the development and activation of T cells.

First, we have investigated the function of hnRNP L in T cell development and CD45 pre-mRNA splicing in vivo using T cell specific deletion of hnRNP L in mice. The hnRNP L deletion results in aberrant expression of CD45 isoforms, predominantly CD45RA, which is usually absent from the thymus. Ablation of hnRNP L results in a partial block in pre-T cell development at the DN4 stage. This reduction in thymic cellularity is not due to an increase in cell death. In fact, hnRNP L deficient thymocytes demonstrate accelerated proliferation compared to wild-type cells due principally to a hyper-activation of the kinases Lck, Erk1/2 and Akt. Moreover, hnRNP L deletion results in a loss of peripheral T cells. In vitro studies suggest that this loss of peripheral cells is caused by a defect in response to chemokine signals. Since CD45 pre-mRNA splicing cannot explain this phenotype, the identification of hnRNP L targets by RNA-Seq has shown that hnRNP L plays a role in the regulation of alternative splicing of factors involved in actin polymerization.

Secondly, we studied the role of hnRNP L in hematopoiesis using knockout mice in which hnRNP L is conditionally deleted specifically in fetal liver and bone marrow hematopoietic cells. Ablation of hnRNP L reduces the number of cell lineage committed progenitors including the common lymphoid progenitors (CLPs), common myeloid and granulocyte progenitors (CMPs, GMPs) and the megakaryocyte-erythrocyte progenitors (MEPs) as well as the mature hematopoietic cells. In contrast to multipotent progenitors (MPPs) that are

affected by the absence of hnRNP L, the hematopoietic stem cell (HSC) population is not reduced. In fact, HSCs from hnRNP L deleted mice demonstrate increased cell cycling. However, hnRNP L deficient HSCs express high levels of Annexin V and CD95, which suggests an increased cell death. As for the thymus, a RNA-Seq analysis of fetal livers revealed different targets of hnRNP L among gene categories related to cell death, DNA damage responses and cell adhesion that may explain the phenotype observed in the hnRNP L deficient HSCs.

These results suggest that hnRNP L and alternative splicing are essential for the survival and maintenance of the differentiation potential of HSCs. HnRNP L is also crucial for the development of T cells by regulating both their migration and the splicing of CD45.

Keywords : hnRNP L, T cells, CD45, hematopoietic stem cells, alternative splicing

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Abbreviations

5-FU : 5-fluorouracil

ADCC : antibody dependent cell mediated cytotoxicity

AGM : aorta-gonad-mesonephros

AIRE : autoimmune regulator

Akt : protein kinase B

APC : antigen presenting cell

ARE:AU-rich element

Arhgap17 : Rho Gtpase activating protein 17

Arhgef2 : Rho/Rac guanine nucleotide exhange factor 2

ARS : activation responsive element

ATP: adénosine-5'-triphosphate

AUF : heterogeneous nuclear ribonucleoprotein protein D0

Bax : Bcl-2 associated X protein

Bcl-2 : B cell CLL/lymphoma 2

Bcl-2A1 : Bcl-2 related protein A1

Bcl-xl: Bcl-2-like 1

Bcl11b : B-cell lymphoma/leukemia 11b

Bim-1 : B lymphoma Mo-MLV insertion region 1 homolog

BM : bone marrow

bp : base pair

Brca2 : breast cancer 2

BrdU : 5-bromo-2'-deoxyuridine

BSA : bovine serum albumin

Card10 : caspase recruitment domain 10

Cbl : Cas-Br-M ectopic retroviral transforming sequence

CCL21 : C-C chemokine ligand 21

CCL25 : C-C chemokine ligand 25

CCR7 : C-C chemokine receptor 7

CCR9 : C-C chemokine receptor 9

cdc42 : cell division cycle 42

CDK : cyclin dependent kinase

CFU : colony forming unit

Ckdn1a : cyclin dependent kinase inhibitor 1a

CKI : cyclin dependent kinase inhibitors

CLIP : cross-linking and immunoprecipitation

CLP : common lymhoid progenitor

CMJ : cortico-medullary junction

CMP : commonmyeloid progenitor

CMV : cytomegalovirus

CRU : competitive repopulating unit

CSK : s-src tyrosine kinase

cSMAC : central supramolecular activation clusters

CTD : carboxy terminal domain

cTEC : cortical thymic epithelial cell

CXCR4 : C-X-C chemokine receptor 4

DAG : diacyl glycerol

DC: dendritic cell

Diap1 : diaphanous homolog 1

DISC : death inducible signaling complex

DL : delta-like

DN : double negative

DNA : deoxyribonucleic acid

DNA-PK : DNA-dependent protein kinase catalytic subunit

DP : double positive

dpc : day post-coitus

DSB : double strand break

EDTA : ethylenediaminetetraacetic acid
EGF: epidermal growth factor
EGFR : epidermal growth factor receptor
Egr1: early growth response protin 1
ELP : early lymphoid progenitor
ENU : N-ethyl-N-nitrosourea
Erk : extracellular signal regulated kinase
ERM : ezrin-radixin-moesin protein
ESE : exonix splicing enhancer
ESS : exonic splicing sequence
EST : expressed sequenced tags
ETP : early T-cell progenitor
FACS : fluorescence activated cell sorting
FADD : Fas associated protein with death domain
Fancm : Fanconi anemia complementation group M
FGFR2 : fibroblast growth factor receptor 2
Fgr: tyrosine-protein kinase Fgr
FITC : fluorescein isothiocyanate
FL : fetal liver
Flt3 : fms-like tyrosine kinase receptor 3
FRET : fluorescence resonance energy transfert
Frz : frizzled receptor
FSC : forward scatter
Fyn : protooncogen tyrosine protein kinase Fyn
G-CSF : granulocyte colony stimulating factor
GAD : glutamic acid decarboxylase
GAIT : IFN-g activated inhibitor of translation complex
Gata-2 : GATA binding protein 2
Gata-3 : GATA binding protein 3

Gfi1 : growth factor independent protein 1

Gfi1b : growth factor independent protein 1b

GFP : green fluorescent protein

Glut1 : glucose transporter 1

GM-CSF : granulocyte-macrophage colony stimulating factor

GMP : granulocyte-monocyte progenitor

GO : gene ontology

Grb2 : growth factor receptor bound protein 2

GSK3 : glycogen synthase kinase 3

Gy : Gray

Hck : hemopoietic cell kinase

HMGB1 : high mobility group protein B1

hnRNP : heterogenous nuclear ribonucleoprotein

hnRNPLL : heterogeneous nuclear ribonucleoprotein L-like

HSC : hematopoeitic stem cell

HSR: hypoxia stability region

ICAM1 : intracellular adhesion molecule 1

ICN : intracellular domainof Notch

Id3 : DNA binding protein inhibitor 3

IEL: intestinal intraepithelial lymphocyte

IFN : interferon

IgD : immunoglobulin D

IgE : immunoglobulin E

IgM : immunoglobulin M

IkB : inhibitor of NF-kB

IL11 : interleukin 11

IL2 : interleukin 2

IL6 : interleukin 6

IL7 : interleukin 7

IL12 : interleukin 12

IL2 : interleukin 2

IL3 : interleukin 3

IL6ST : interleukin 6 signal transducer

IL7R : interleukin 7 receptor

IP3 : inositol triphosphate

ISP : immature single positive

ITAM: immunoreceptor tyrosine based activation motif

Jak : janus kinase

Jnk : c-Jun N-terminal kinases

kDa : kilodalton

KLF2 : krueppel-like factor 2

KO : knockout

LAT : linker of activated T cell

Lck : lymphocyte specific protein tyrosine kinase

LFA-1 : lymphocyte function associated antigen 1

LMPP : lymphoid-primed multipotentp progenitor

LPS : lipopolysaccharide

Lrp5 : low-density lipoprotein receptor related protein 5

Lrp6 : low-density lipoprotein receptor related protein 6

LSK : Lin negative, Sca1 positive, cKit positive

LT-HSC : long-term hematopoieitic stem cell

Lyn : v-yes-1 yamaguchi sarcoma viral related oncogene homolog

MAPK: mitogen activated protein kinase

Mcl1 : myeloid cell leukemia sequence 1

mDC : medullary dendritic cell

MEP :megakaryocyte-erythrocyte progenitor

MFI : mean fluorescence intensity

MHC : major histocompatibility complex

MINK : Misshapen/NIKs (Nck-interacting kinases)-related kinase

MIP-1β : macrophage inflammatory protein 1 beta

MMP9 : matrix metallopeptidase 9

Mpl : myeloproliferative leukemia virus oncogène

MPP : multipostent progenitor

mRNA: messenger ribonucleic acid

mTEC : medulla thymic epithelial cell

mTORC1 : mechanistic target of rapamycin

Myd88 : myeloid differentiation primary response gene 88

Nck : non-catalytic region of tyrosine kinase adaptor protein 1

NF-κB : nuclear factor of kappa light polypeptide gene enhancer in B-cell

NF-κB2 : nuclear factor of kappa light polypeptide gene enhancer in B-cell 2

NFAT : nuclear factor of activated T cell

NHEJ : non homologous end joining

NK : natural killer

NO : nitric oxide

NOS : nitric oxide synthase

NSCLC : non small cell lung carcinoma

Nur77 : nuclear receptor subfamily 4 group A member 1

P-Sp : paraaortic splanchnopleura

p38 : p38 mitogen activated protein kinase

PAF : platelet activating factor

PBS : phosphate buffered saline

PCR : polymerase chain reaction

Pds5b : regulator of cohesin maintenance homolog B

PHD : plant homeodomain

PI3K : phosphoinositide-3 kinase

PIGF : phosphatidylinositol-glycan biosynthesis class F protein

pIpC or poly(I:C) : polyinosinic-polycytidylic

PKCθ : protein kinase C theta

PLCγ : phospholipase C gamma

PSF : polypyrimidine tract binding protein associated splicing factor

PSGL-1 : P-selectin glycoprotein ligand 1

pSMAC : peripheral supramolecular activation clusters

pta : pre-T cell receptor alpha chain

PTB : polypyrimidine tract binding protein

PTPN22 : protein tyroine kinase non-receptor tpe 22

Ptprc : protein tyrosine phosphatase receptor C

Rac : ras-related C3 botulinum toxin substrate

Raf : v-raf-1 murine leukemia viral oncogene homolog 1

Rag : recombination activating genes

RasGRP1 : Ras guanyl releasing protein 1

RBPjk : recombination signal binding protein

RNA: ribonucleic acid

RNAPII : RNA polymerase II

ROS : reactive oxygen species

RPKM : reads per kilobase of exon model per million mapped reads

RRM : RNA recognition motif

RSS : recombination signal sequence

RT-PCR : real time polymerase chain reaction

Runx1 : runt-related transcription factor 1

Runx3 : runt-related transcription factor 3

S1P : sphingosine 1-phosphate

S1P1 : sphingosine 1-phosphate receptor 1

SCF : stem cell factor

SCL : T cell acute lymphocytic leukemia 1

SCZ :subcapsular cortical zone

SDFor CXCL12 : stromal cell-derived factor 1

SEM : standard error of the mean

SF1 : splicing factor 1

SF2/ASF : alternative splicing factor/ splicing factor 2

SFK : Src family kinase

SLP76 : lymphocyte cytosolic protein 2

Smad7: Smad family member 7

snRNP: small nuclear ribonucleoprotein

Sox13 : SRY (sex determining region-Y) box 13

SP : single positive

SR: serine-arginine rich protein

SRPK : serine-arginine protein kinase

ss: splice site

SSC : side scatter

ST-HSC : short-term hematopoeitic stem cell

Stat : signal transducer and activator of transcription

Syk: spleen tyrosine kinase

TCF-1 : T cell factor-1

TCR: T cell receptor

TdT : terminal deoxynucleodityl transferase

tg : transgenic

TGF-β1 :transforming growth factor 1

TGF-βR : transforming growth factor beta receptor

Th-POK : zinc finger and BTB domain containing 7B

Themis : thymocyte expressed molecule involved in selection

TLR : toll-like receptor

TMP : thymic multipotent progenitors

TNF : tumor necrosis factor

TNFR1 : tumor necrosis factor receptor 1

Topbp1 : topoisomerase DNA II binding protein 1

TOR : target of rapamycin

TOX:thymocyte selection associated high mobility group box

TPO : thrombopoietin

TRAILR1 : Tumor necrosis factor related apoptosis inducible ligand receptor 1

TRAP150:thyroid hormaon receptor associated protein 150

Trp53bp1 : transformation related protein 53 binding protein 1

Trp53inp1 : transformation related protein 53 inducible nuclear protein 1

Tyk2 : tyrosine kinase 2

UTR : untranslated region

Vav : vav guanine nucleotide exchange factor

VCAM : vascular cell adhesion molecule

VE : vascular endothelial

VEGF : vascular endothelial growth factor

VLA4 : very late antigen 4

VM : ventral mesoderm

Wasf2 : Wiskott-aldrich syndrom protein family member 2

Wasp: Wiskott-aldrich syndrom protein

Wif1 : wnt inhibitory factor 1

Wnt : wingless-type MMTV integration site family

WT : wild-type

XRCC4 : X-ray repair complementing defective repair inchinese hamster cells 4

XRCC6 : X-ray repair complementing defective repair inchinese hamster cells 6

Zap70 : zeta chain associated protein kinase 70

“La patience est une plante amère mais elle porte de doux fruits”- David Baird

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Introduction

1. Alternative splicing

With genome sequencing data now available, it was unexpected to identify a relatively low number of genes in both human and mouse compared with the complexity of their proteome. One of the explanations is provided by the use of alternative splicing in multiple exon genes, which enables the formation of different mRNA isoforms [1, 2]. It is estimated that as much as 95% of human genes undergo alternative splicing which consists in the removal of introns and the ligation of different combinations of exons from pre-mRNA molecules to form a mature mRNA [1, 2]. While most exons are constitutive, some cassette exons can sometimes be both included or excluded from the mature mRNA. The splicing profile can be tissue specific and/or context dependent meaning that the activation status or maturation stage of a cell type can correlate with the expression of different specific mRNA isoforms [3-5]. Diverse patterns of alternative splicing exist as is shown in Figure 1. It is possible to find mutually exclusive exons, in which only one exon is included at the time, but the use of an alternative 5'splicing site (5'ss) or 3'splice site (3'ss) also exist and can modify the length of a particular exon. Finally, excision of an intron can be omitted and this intron can be retained in the mRNA with the possible consequence of altering reading frames [3, 6].

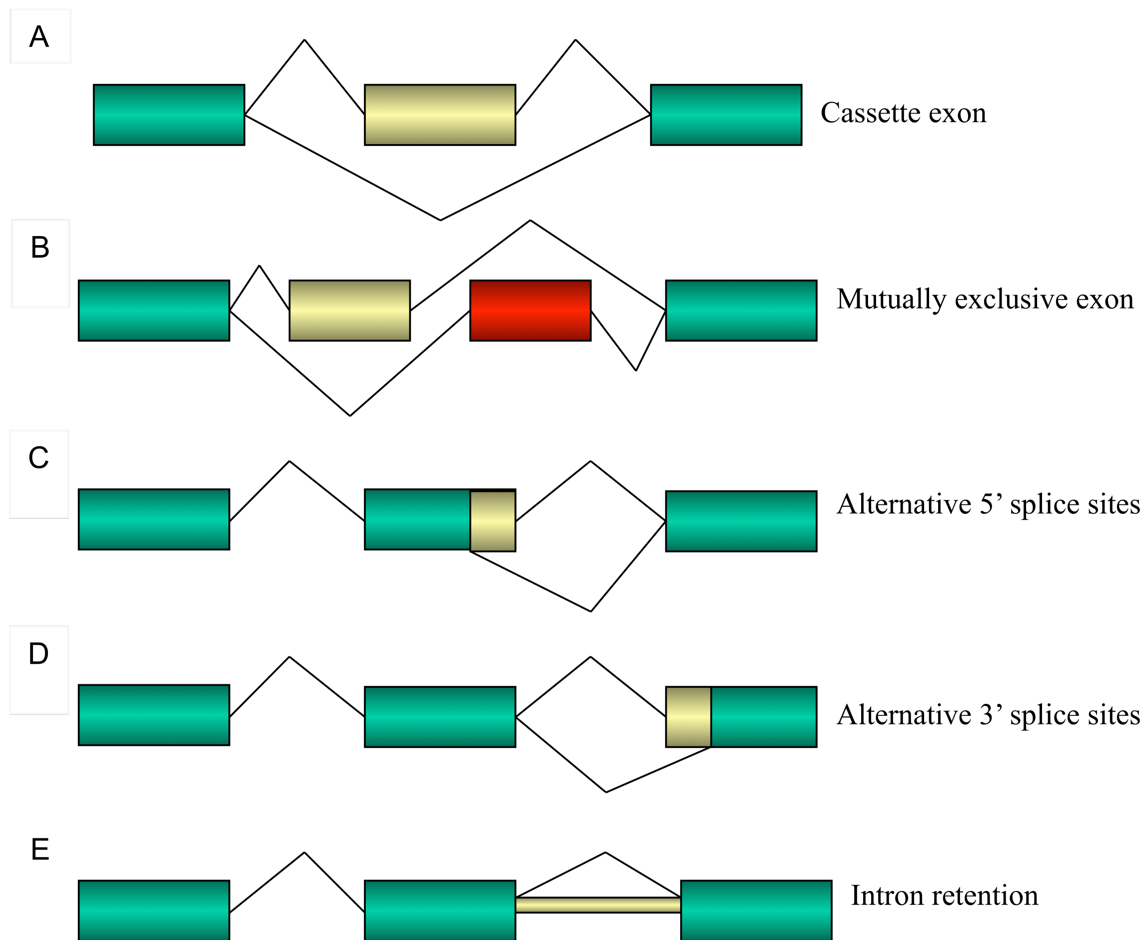


Figure 1. Schematic representation of alternative splicing patterns. Green boxes represent exons that are constitutive and present in all mRNA isoforms. Yellow and red boxes depicte pre-mRNA sequences that are alternatively included in the final mRNA. (A) A cassette exon is an alternatively spliced exon that can either be included or excluded from the final mRNA. (B) Mutually exclusive exons are two or more adjacent cassette exons that are spliced in such a way that only one exon is included in the final mRNA. (C, D) Alternative 5′ and 3′ splice sites allow the modification of the length of a particular exon. (G) An intron can be retained in the final mRNA. Adapted from [3].

1.1. Splicing mechanism

The process of pre-mRNA splicing is carried out by the spliceosome, which is made of a complex of small nuclear ribonucleoproteins (snRNPs) and other protein subunits [3, 7]. Its activity is regulated by cis- and trans-acting elements in both constitutive and alternative splicing processes [8, 9]. The spliceosome mediates the two transesterification steps involved in splicing [3, 6, 10]. At the 5' end of the upstream intron, a pGU rich sequence is found and called the 5'ss. At the 3'splice site, the respective 3'ss contains a branch point, a polypyrimidine tract and a terminal AG sequence [3, 6, 10]. First, the 2' hydroxyl "A" residue from the branch point attacks the phosphate at the 5'ss resulting in cleavage of the 5' end and ligation to the 2' hydroxyl group. Then, the phosphate at the 3' end is attacked by the 3' hydroxyl group of the detached exon and the two exons are ligated [3, 6, 10]. Different complexes have to work together during the splicing process to mediate these transesterification steps (Figure 2). The complex "E" (for "Early") or commitment complex is the primary step. It starts with a U1 snRNP that binds the 5' splice site via base pairing and the association of the SF1 protein to the AG at the intron/exon junction. Just after this step, a U2 snRNP joins the "E" complex by snRNA base pairing at the branch point displacing SF1 followed by the recruitment of U4, U5 and U6 snRNPs to form the complex "B". Finally, a rearrangement is made to produce a complex "C" where the U1 interaction with the 5'ss is replaced by U6 and U1, while U4 snRNPs are lost from the complex. The transesterification steps occur during the complex "C" formation [3, 6, 10].

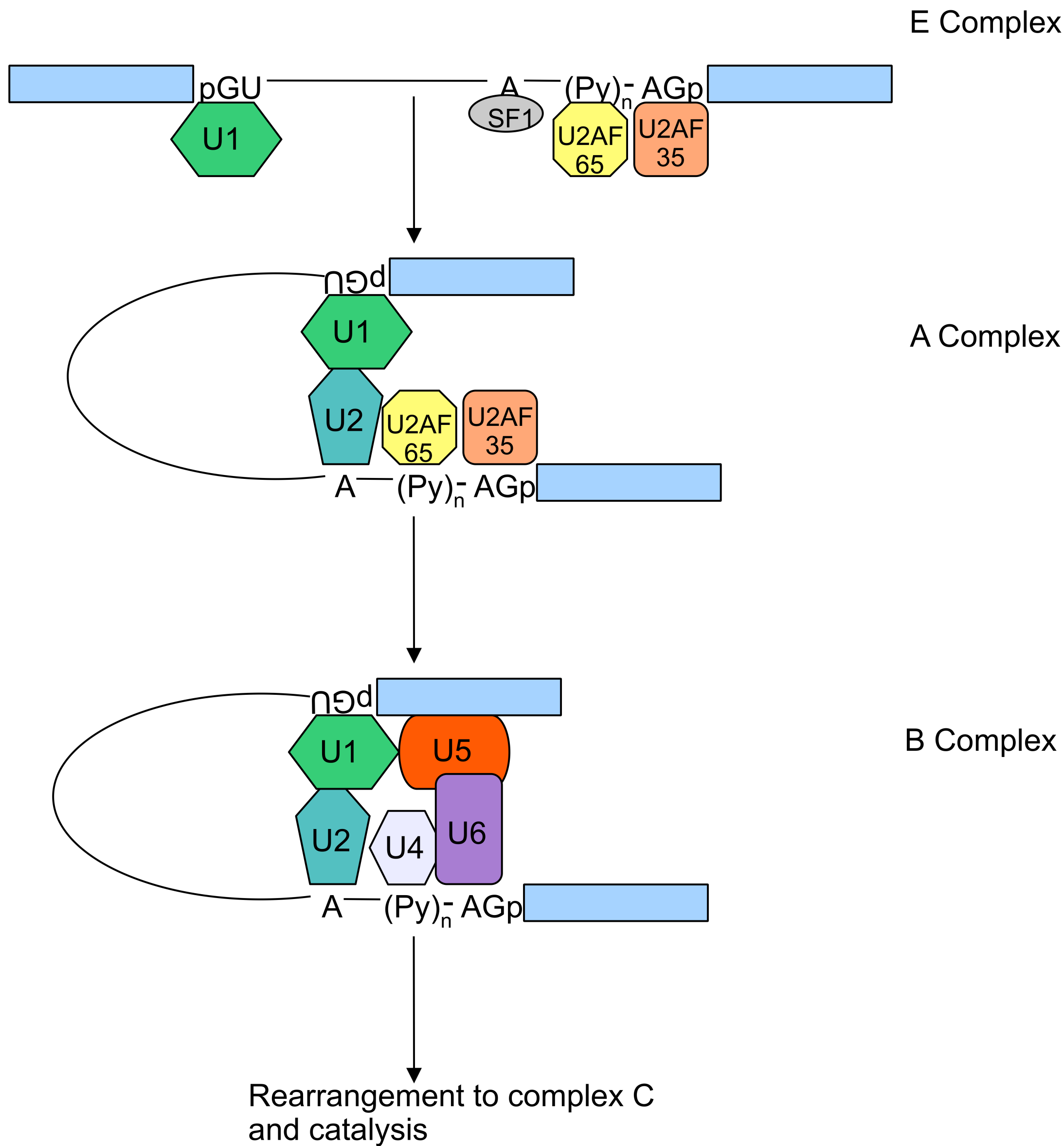


Figure 2. Schematic representation of pre-mRNA splicing. The spliceosome contains five small nuclear ribonucleoproteins (snRNPs). The Early (E) complex is made of the U1 snRNP bound to the 5′ splice site; at the 3′ splice site, the branch point is bound by SF1, the polypyrimidine tract by U2AF65, and the AG dinucleotide by U2AF35. The A complex is formed when the U2 snRNP displaces SF1 at the branch point base-pairing and is looped onto the interacting U1 and U2 snRNPs. This complex is then joined by the U4/5/6 Tri-snRNPs to form the B complex. The B complex is then rearranged to form the catalytic C complex. During this rearrangement the interaction of the U1 and U4 snRNPs are lost and the U6 snRNP is brought into contact with the 5′ splice site. The C complex mediates the two transesterification steps. Adapted from [3].

The decision to include or exclude cassette exons depends on specific signals received by the spliceosome [8, 9] (Figure 3). Part of these regulatory elements are the exonic and intronic splicing sequences. Exonic splicing enhancers (ESE) are diverse in nucleotide sequence but are mostly purine-rich and are embedded within the exon. They are mainly recognized by SR protein family members and participate in the inclusion of an exon [8, 9, 11]. In contrast, hnRNP proteins bind exonic splicing silencers (ESS), which are mostly CA rich elements. The interaction of hnRNP proteins with ESS elements inhibits exon inclusion by different mechanisms - either by blocking the interaction of spliceosomal U1 and U2 snRNPs with the intron splice site or by looping out the exon when the hnRNPs are bound on each side of the exon [3, 8, 9]. Regulatory elements in introns also exist and can be both silencers (ISS), which are highly conserved between species, or enhancers (ISE) [3, 8, 9].

1.1.1. SR proteins

Human cells have 12 SR proteins, designated SRSF 1 to 12, that bind exonic splicing enhancer sequences and are involved in both constitutive and alternative splicing [10, 11]. All canonical SR proteins possess either 1 or 2 RNP type RNA binding domains at the N terminus, which allow the direct interaction with the pre-mRNA [3, 11]. On the other hand, the protein/protein interaction is mediated by the arginine-serine repeat domain (RS) found at the C terminus of SR proteins. The number of RS repeats directly correlates with the splicing sequence recognition potential, such that a higher RS repeat density allows increased binding to the pre-mRNA [3, 10]. In addition, these proteins act by recruiting U1 and U2AF proteins through their RS domains to the pre-mRNA but they can also counteract repressor proteins already bound on the pre-mRNA [3, 10]. The SR domain has the potential to become phosphorylated by SRPK1 and SRPK2 kinases or the Clk/Sty group of kinases and this phosphorylation is required to regulate their activity [12]. Both

hyper- and hypo- phosphorylation has been reported to inhibit their ability to mediate splicing [12].

1.1.2. hnRNP proteins

HnRNPs (heterogeneous nuclear ribonucleoprotein) are a group of abundant nuclear proteins that bind nascent pre-mRNA produced by RNA polymerase II [13-15]. In humans, 13 families of closely related hnRNP members have been identified [11]. These proteins are not only involved in pre-mRNA splicing but also in many other processes including mRNA stability and trafficking as well as RNA editing, translational regulation, polyadenylation, and telomere biogenesis [14, 16]. HnRNPs are similar to SR proteins as they are involved in both the constitutive and alternative splicing processes. They are modular proteins harboring the so-called RRM domain, which enables the direct interaction with the pre-mRNA. An exception to this is represented by the hnRNP E and K proteins, which possess a KH domain instead of a RRM domain [13, 14, 16]. Moreover most of the hnRNP family members contain the so-called RGG boxes (arginine/glycine/glycine tripeptide repeats) and an additional glycine-rich, acidic or proline-rich domain, which enable protein-protein interactions [13, 14, 16]. HnRNP-like proteins have been also characterized including CELF, Fox, Nova and TDP-43 [11].

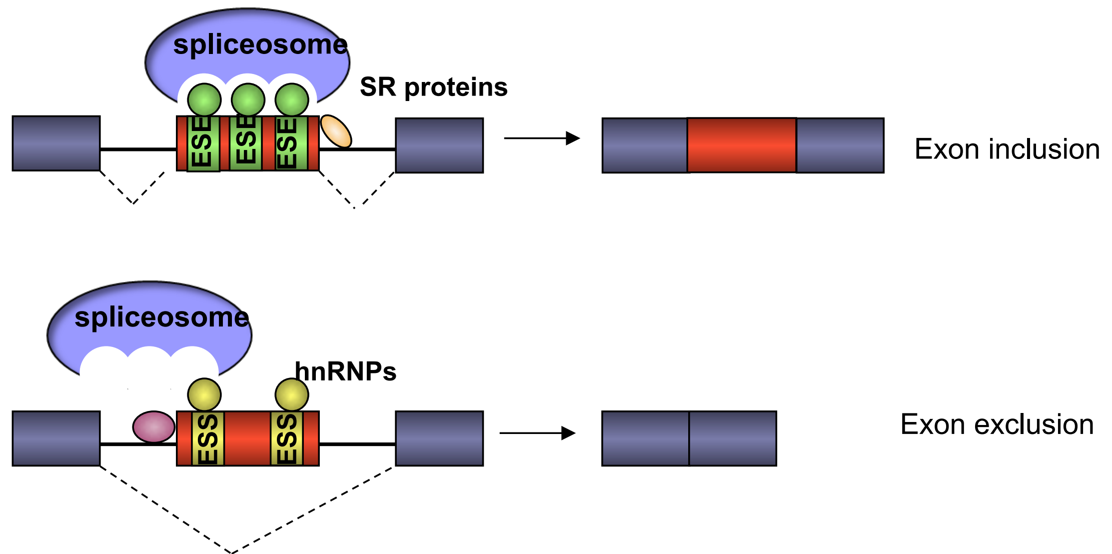


Figure 3. Regulation of alternative splicing by exon definition. The inclusion or exclusion of an exon from a final mRNA depends on which regulatory proteins are bound to mRNA specific sequences. The exonic splicing enhancer (ESEs) sequences are usually bound by members of the SR (Ser-Arg) protein family and allow the spliceosome to recognize the exon and include it in the final mRNA. HnRNP proteins bind the exonic splicing silencers (ESSs), which exclude the spliceosome from the exon and therefore mediate exon exclusion from the final mRNA.

1.2. HnRNP L

Heterogeneous nuclear ribonucleoprotein L (hnRNP L) is one of the 20 members of the hnRNP family [13, 14]. This protein is mostly localized in the nucleoplasm and is distinct from other family members as it associates with the landmark giant loops of lampbrush chromosomes [17]. HnRNP L also possesses the ability to shuttle between the nucleus and the cytoplasm. As with the other members of the hnRNP family, hnRNP L is involved in mRNA stability, mRNA splicing and shuttling [13, 14]. Its predicted molecular weight is 64 kDa and it is expressed in various vertebrates but not in *Drosophilia*

melanogaster nor in *Saccharomyces cerevisiae* [13, 17]. The protein is composed of 558 amino acids and contains a N terminus rich in glycine [17].

HnRNP L was first identified as a regulator of pre-mRNA splicing during the course of the analysis of the human endothelial nitrite oxyde synthase gene (eNOS), which contains a CA rich element [18]. Since then, many studies have confirmed that hnRNP L regulates pre-mRNA splicing by three different mechanisms [19-21]. HnRNP L can act both as an activator or repressor of exon inclusion; it recognizes CA repeats and CA rich elements found either in intronic or exonic sequences [22, 23]. The CA repeats and CA rich elements found in exons act only as repressors of exon inclusion. However, when hnRNP L binds intronic sequences it can activate or repress the inclusion of the upstream exon depending on its binding distance from the 5'ss [19, 20, 22, 23]. For example, the binding of hnRNP L to the intronic sequences of the eNos and Itga pre-mRNA exclusively leads to exonic inclusion. In the case of the TJP1 pre-mRNA, hnRNP L binds to the silencer element adjacent to the 3'ss of the exon 20 and interferes with a stable U2 snRNP association with the pre-mRNA and resulting in exon 20 exclusion. In fact, upon its association with the pre-mRNA hnRNP L impaires the binding of U2AF65 to the TJP1 polyA tract and, as a result, the U2 snRNP is not recruited [23, 24]. In addition, the *SLC2A2* gene contains a CA repeat splicing silencer at the 5'ss of exon 4 where hnRNP L binds as a repressor by interfering with the 5'ss recognition by U1 snRNP leading to exon 4 inclusion [23, 24].

1.2.1. Cross regulation of hnRNP L

Analysis of the *hnRNP L* gene revealed a region of 2 kb inside intron 6, which is the most conserved region between species. The conservation between human and murine sequences is over 87%. The *hnRNP L* gene contains a short exon called exon 6A and the alternative splicing of this sequence, in which exon 6A is included in the final mRNA, generates a premature stop codon initiating a non-sense mediated decay [25]. Further

analysis showed that hnRNP L intron 6 contains a long CA rich cluster, which may be a potential binding site for the hnRNP L protein itself. The CA cluster is divided into two parts: the first part is found at 280 nucleotides from the 5'ss and the second at 330 nucleotides from the 3'ss, each part containing 11 or 14 CA motifs, respectively [25]. Knockdown of hnRNP L results in a reduced exon 6A inclusion and, in contrast, increased concentrations of hnRNP L protein cause exaggerated non-sense mediated decay of hnRNP L mRNA [25]. Therefore, since one CA cluster can bind many hnRNP L proteins, it induces a structural change of the mRNA in a dose dependant manner. It is thus of interest to note that an autoregulatory feedback loop is generated in which hnRNP L apparently functions as an activator of its own "poisonous" exon 6A.

1.2.2. HnRNP L targets

1.2.2.1. Human VEGFA

VEGF (vascular endothelial growth factor) is a protein family that contains five members of which VEGFA is the prototype. The other members are VEGF-B, -C, -D and PIGF [26]. They form homodimers and interact with different ligands including VEGF receptors, heparan sulfate and neurophilins. VEGFA is produced by most parenchymal cells and tumor associated myeloid cells [26]. It is also known to be regulated by alternative splicing with the possibility of generating four different mRNA isoforms [26]. The level of VEGFA expression is regulated by hypoxia-inducible factors with a marked increase in its expression during tissue growth, both in healthy conditions such as wound healing and embryonic development, and health-threatening conditions such as malignant transformation [27]. On the other hand, inflammatory cytokines such as IFN-γ have the potential to decrease VEGF expression in myeloid cells [28]. This increased expression of VEGFA during hypoxic conditions cannot be attributed only to transcriptional regulation,

but it is also related to mRNA stability. The 3'UTR of VEGFA mRNA contains a 126 nucleotide sequence associated with the hypoxia stability region (HSR) made up of a 21 nucleotide AU-rich element (ARE) and 29 nucleotide GAIT element (IFN-γ activated inhibitor of translation complex) [27, 29]. Both elements are involved in regulating the turnover rate of VEGFA mRNA. In normoxia or following IFNγ treatment, the GAIT complex made of glutamyl prolyl tRNA synthetase, the ribosomal protein L13a and the glyceraldehyde-3-phosphate dehydrogenase, which binds the GAIT pre-mRNA element represses VEGFA [27, 28]. In addition, it has been demonstrated that the micro RNAs miR297/299 negatively regulate VEGFA by binding the CA-rich element of HSR and inhibiting its translation [29]. However, hypoxia conditions restore VEGF expression. This change in VEGFA expression under hypoxia conditions is due to hnRNP L binding to AREs within the HSR without forming a complex with other RNA binding proteins [27, 28]. Since this binding of hnRNP L increases VEGFA mRNA stability, the CA repeats can be considered essential for the regulation of VEGFA expression [28]. It has been shown that hypoxia overrides proteasome mediated degradation of hnRNP L to maintain a minimum expression level in the cytoplasm to compete with the activity of the GAIT complex. Hypoxia also influences the subcellular localization of hnRNP L and by maintaining it in the cytoplasm [28, 29]. However, hypoxia or other factors are not necessary for hnRNP L mediated VEGFA mRNA stability since any change in the concentration of hnRNP L in the cytoplasm results in the association of hnRNP L with HRS which counteracts miR297/299 induced mRNA degradation [29].

1.2.2.2. Integrin α2β1

Integrins are heterodimeric glycoproteins formed by the combination of distinct alpha and beta subunits. The α2β1 integrin is ubiquitously expressed on the surface of many cell types of epithelial, endothelial and hematopoietic origin such as platelets, T and B cells, NK and monocyte/macrophages [30, 31]. α2β1 integrins have been identified as

receptors for collagens, laminins, decorin and matrix metalloproteinases. However, when expressed on platelets and megakaryocytes, the binding specificity of α2β1 integrins is restricted to collagen I, II or III [30-32]. The binding of platelet α2β1 to collagen induces platelet adhesion to blood vessel and mediates platelet activation and thrombosis [30-32]. The α2 subunit of the heterodimer is encoded by the *Itga2* gene in the mouse, for which two haplotypes have been identified. Haplotype I is found in mice with the genetic backgrounds 129, C57BL/6, BALB/c, C3H and DBA/2 and is associated with high expression of platelet α2β1 and reduced platelet function. Haplotype-II bearing mice express half the level of α2β1 compared to those bearing haplotype I [19, 33]. This differential gene expression is not due to a change in transcription level of *Itg2a* [19, 33, 34]. In fact, analysis of the *Itga2* gene revealed the presence of a CA-rich region found 250 nucleotides downstream of the 5'splice site. While haplotype I possesses a stretch of 21 CA repeats (CA21), haplotype II contains only 6 CA-repeats (CA6) [19]. After UV-crosslinking, a complex formed by hnRNP L was found to be bound with higher affinity to CA21 compared to CA6. It is known that hnRNP L preferentially binds to longer CA repeats [19]. In a minigene in vivo assay with a *Itg2a* cassette containing exon 1 and 2 separated by a truncated intron 1 with either no CA repeats or with CA21 or CA6, it was demonstrated that in absence of CA repeats only low levels of splicing were detected. However, insertion of CA6 repeats increased splicing and mRNA levels by three times while insertion of CA21 further augmented the levels of the correctly spliced mRNA product [19]. Furthermore, hnRNP L knockdown reduced the levels of α2β1 expression. Therefore, hnRNP L binding to CA repeats within intron 1 of *Itg2a* mediates the correct splicing and therefore increased expression of α2β1 on platelets [19,24].

1.2.2.3. BCL-2

Programmed cell death or apoptosis is a tightly regulated process necessary to remove cells during specific stages of development or during an activation process that has to be terminated. Deregulation of apoptosis is associated with cancer, autoimmune diseases and neurodegeneration. During programmed cell death, chromatin condensation and fragmentation, cellular membrane blebbing and formation of apoptotic bodies have been demonstrated to occur [35, 36]. Apoptosis can be activated through two pathways – the TNF-FADD pathway, or the intrinsic pathway involving cytochrome c release from the mitochondria [35-37]. Bcl-2 is a member of the larger Bcl-2 family of proteins, which are involved in the regulation of apoptosis. Bcl-2 acts as a pro-survival factor and counteracts apoptosis; it is associated with mitochondria and participates in the modulation of calcium homeostasis and proton flux [35, 36].

Bcl-2 mRNA possesses at its 3'UTR an AU-rich element (ARE) known to be involved in mRNA decay and therefore in the activation of an apoptotic program. Two factors have been described to bind the ARE: AUF-1 (hnRNP D) which increases its binding to AREs upon induction of apoptosis, and nucleolin, which stabilizes mRNA [38, 39]. More profound analyses of the Bcl-2 3'UTR revealed a stretch of CA repeats upstream of the ARE. Experiments in vitro and in vivo identified hnRNP L as the major factor regulating Bcl-2 mRNA stability via binding to these CA repeats. HnRNP L knockdown resulted in decreased rates of Bcl-2 mRNA degradation [38]. However, another report from the same group contradicted this conclusion. The involvement of hnRNP L in Bcl-2 mRNA decay has been tested in the context of cell apoptosis or autophagy. Both the reduction and the overexpression of hnRNP L did not affect the Bcl-2 mRNA decay, nor did it affect the binding of nucleolin or AUF-1 to ARE [39]. Thus, they conclude that hnRNP L must interact with other factors to mediate the degradation of Bcl-2 in a pro-apoptotic environment.

1.2.2.4. eNOS

Nitric oxide (NO) is an important mediator in vasodilatation, neurotransmission, macrophage mediated cytotoxicity and gastrointestinal smooth muscle relaxation. NO is synthesized by nitric oxide synthase (NOS). There are three characterized isoforms of NOS; neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS)[40-42]. Both nNOS and eNOS are constitutively expressed and require calcium for activation. On the other hand, iNOS is induced during an immune response by inflammatory mediators and cytokines and is calcium independent [40-43]. NOS produces NO by converting arginine into citrulline. eNOS produces low amounts of NO but its activity is induced by physiological and pathological stimuli such as VEGF, S1P, PAF and shear stress. The cellular localization of eNOS is important for its function. eNOS is mainly found in the plasma membrane and the Golgi complex of endothelial cells, however upon activation it redistributes to the cytosol where its main enzymatic activity takes place [40]. eNOS and NO play a pivotal role in many human pathologies such as diabetes and artherosclerosis [41, 42].

The *eNos* gene is 22 kb long and contains 25 introns of which the largest is intron 13. This intron 13 possesses a polymorphic CA repeat near the 5' splice site and evidence has been gathered to show a link between the number of CA repeats and the risk of coronary artery disease [18, 22]. Studies conducted by Hui demonstrated that this CA repeat acts as an intronic splicing enhancer since the splicing of exon 13 and 14 is possible only in their presence and the number of repeats positively influences the efficiency of splicing. The enhancer factor identified was hnRNP L [18, 22]. Deletion of hnRNP L reduced eNOS mRNA splicing efficiency and exogenous hnRNP L restored the splicing. The binding of hnRNP L is CA specific, depends on the number of repeats and is an ATP-independent process. In addition, binding of hnRNP L does more than just enhance the splicing of eNOS: it also stabilizes its mRNA [18, 22].

1.2.2.5. CD45

The tyrosine phosphatase CD45 is a type I transmembrane protein encoded by the *Ptprc* gene and it is heavily expressed on all nucleated cells of the hematopoietic system with the exception of erythrocytes and platelets [44-49]. The *Ptprc* gene contains 35 exons in mice and one additional exon in humans. Two exons can act as an alternative transcriptional start site and are denoted as exon 1a and exon 1b. Transcription can be initiated at three positions, called P1a, P1b and P2. The *Ptprc* gene lacks a traditional TATA box but contains a strong, non tissue specific promoter within intron 1 [44-48]. Exons 4, 5, 6 and 7 are known to be alternatively spliced. In humans, 1% of the population bears the polymorphism C77G in exon 4, which changes a C to a G and disrupts the ESS1 sequence of exon 4 resulting in aberrant high levels of larger CD45 isoforms in all cell lineages [50]. Evidence linking this polymorphism to an increased incidence of multiple sclerosis and autoimmune hepatitis has been published [51, 52].

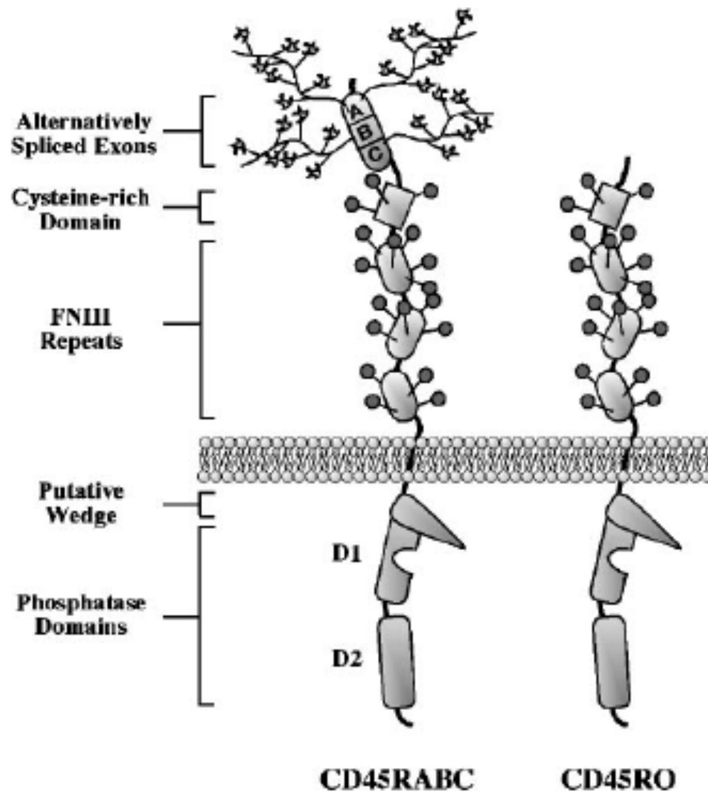
The cytoplasmic tail of CD45 shares 95% homology between all mammals studied while the extracellular domain has only 35% homology [53]. The extracellular portion contains three type III fibronectin domains, which are highly N-glycosylated, a cysteine rich region and a N terminus made of the variable domains A, B, C encoded by the alternatively spliced exons 4, 5 and 6, respectively, which bear O-linked glycan chains (Figure 4A). The level of glycosylation depends on the cell type, developmental stage and cellular activation status [44, 46, 54]. No interacting ligand has been identified until now for CD45 but some non-specific binding, such as interaction with CD22 (sialic acid binding lectin) expressed on B lymphocytes, galectin-1 or serum mannan-binding protein have been characterized [44, 46, 49].

The cytoplasmic tail consists of a wedge-like structure, a phosphatase tandem domain called D1 and D2 and an 80 amino acid stretch at the C terminus [44-46]. Only the D1 domain is enzymatically active but both are required for full phosphatase activity. The D2

domain possesses a 19 amino acid stretch rich in serine and acidic amino acids and is phosphorylated by casein kinase II [55]. Although D2 is not enzymatically active, it associates with the linker protein fodrin, which enables association with the cytoskeleton and modulates the cell membrane mobility of CD45 [44-46].

The size and charge of the extracellular portion of CD45 vary considerably depending on the isoform expressed on the cell surface [44, 54]. Some studies involving FRET analysis and replacement of the extracellular domain of CD45 by EGFR (endothelial growth factor receptor) extracellular domain demonstrated the dimerization potential of CD45 after treatment with EGF (endothelial growth factor) [56] (Figure 4B). The larger isoforms are less likely to dimerize, because they bear O-glycosyl residues and negative charges, which lead to repulsion. The shorter extracellular domain of the CD45RO isoform can dimerize by forming a symmetric interaction between the structural wedge in the juxtamembrane region of one molecule and the catalytic site of the cytoplasmic domain of its partner molecule [57-59]. Other evidences of the importance of dimerization in vivo come from mice bearing the point mutation E613R (glutamate 613 to arginine) in the CD45 wedge domain without affecting its cell surface expression. These mice develop normally but suffer from a lymphoproliferative disease providing evidence of CD45 dimerization in constraining T cell activation [44, 60].

A



B

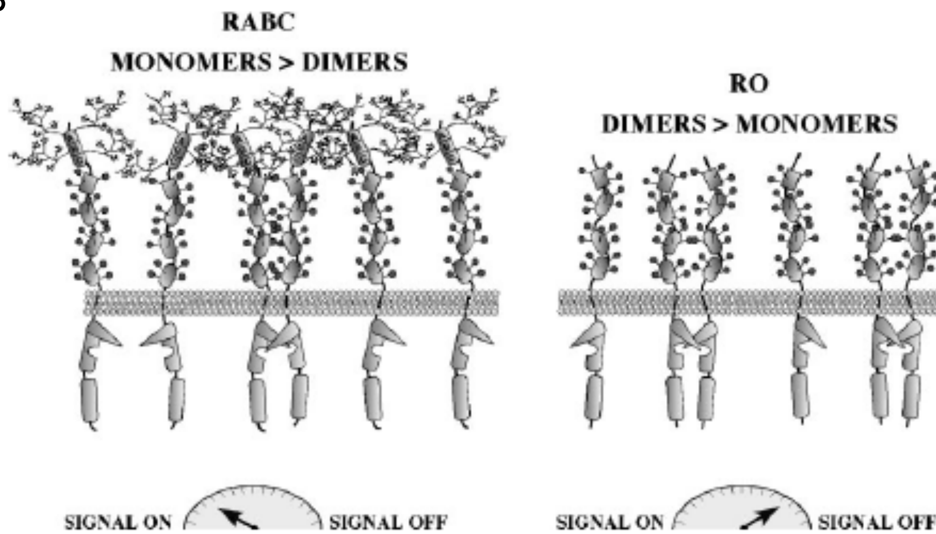


Figure 4. Protein structure of CD45. A) Alternative splicing of CD45 exons 4, 5, and 6, (designated A, B, and C) in the extracellular domain generate CD45 molecules with variable length and molecular weight. The largest isoform is CD45RABC and contains all three variable exons. The smaller isoform that does not include any of the exons A, B or C

is termed CD45RO. The variable exons are O-glycosylated. The rest of the extracellular domain is composed of the cysteine-rich region and the three fibronectin type III repeats. The cytoplasmic tail of CD45 contains two PTPase domains, D1 and D2. B) The largest isoforms of CD45 are heavily glycosylated and negatively charged thus repulsing each other. On the other hand, CD45RO molecules form dimers through the interaction of the juxtamembrane region of one molecule to the inhibitory wedge of a second molecule. This dimerization restrains the PTPase activity of CD45. From [44].

1.2.2.5.1. CD45 alternative splicing

The alternative splicing of the *Ptprc* (CD45) gene is well characterized, in particular those sequences that encompass exons 4, 5 and 6 that encode the three extracellular domains called A, B and C [44]. The exclusion of all three exons from the mature mRNA depends on the binding of hnRNP L to particular sequences, but the mechanisms of exclusion for each exon is slightly different [21, 61]. First, hnRNP L binds to the activation-responsive-sequence (ARS) embedded within the exonic splicing silencer (ESS1) inside exons 4 and 6 [21, 61]. However, the ARS sequence in exon 5 is split in two sections, which are separated by an exonic splicing enhancer (ESE) sequence [61]. It has been described that hnRNP L as well as hnRNP E2, K, D and polypyrimidine tract-binding protein (PTB) bind to the ARS/ESS1 sequences of exon 4 under resting conditions i.e. in which no cell activation occurs. In contrast, only hnRNP L was found to interact with exon 5 sequences under the same conditions and to repress U2 snRNP recruitment and binding to ESE element [62]. On the other hand, SF2/ASF (alternative splicing factor/splicing factor 2) binds the ESE indicating that a competitive balance exists between SF2 and hnRNP L, which controls the exon inclusion or exclusion [62]. In the case of exon 4 and 6, exon skipping under resting conditions is mediated by a stall in spliceosome assembly at step A where U1 and U2 are bound to splice sites flanking the repressed exons which blocks the complex to proceed in “B” complex formation. This mechanism depends on both ESS1 and hnRNP L [21]. Under activating conditions e.g TCR stimulation, there is an increase in exon 4 skipping without affecting the level of expression of hnRNP L and PTB [21, 63].

However, PSF (PTB-associated splicing factor) is now detected in the complex under activating conditions. Also, it has been shown that hnRNP L undergoes post-translational modifications such as phosphorylation during this activating state which may explain how exon skipping is mediated by this protein [63]. In resting T cells, for instance PSF is phosphorylated at threonine T687 and co-precipitates with TRAP150 [64]. T cell receptor signaling induces a decrease in glycogen synthase kinase 3 (GSK3) activity through the phosphorylation of the enzyme at serine 9. The less active GSK3 causes a reduced phosphorylation of PSF, which allows PSF to be released from the TRAP150 complex. The now free PSF can bind CD45 splicing regulatory elements and repress exon inclusion [64].

1.2.2.5.2. CD45 substrates

The main substrates for the CD45 phosphatase are the SFKs (Src family kinase). In T lymphocytes, Lck (lymphocyte specific protein tyrosine kinase) and Fyn (proto-oncogen tyrosine protein kinase Fyn) are the major and the best characterized substrates [45, 65-68]. In B cells it is the Lyn kinase, in macrophages both Lyn (v-yes-1 yamaguchi sarcoma viral related oncogene homolog) and Hck (hemopoietic cell kinase) are substrates and finally in denditric cells CD45 dephosphorylates Lyn, Hck and Fyn. However, not all SFKs are substrates for CD45. For example, Fgr (tyrosine-protein kinase Fgr) in macrophages and dendritic cells and Src in T cells are not affected by the loss of CD45 phosphatase activity [44, 45, 67, 68]. CD45 dephosphorylates two tyrosines in Lck and Lyn: the C terminal inhibitory tyrosines Y505 and Y508 and the activating tyrosines Y394 and Y397, respectively. To explain the complexity of this two phosphatase activities, a model has been proposed in which CD45 first dephosphorylates the C terminal tyrosine of Lck, when it is in a closed conformation, which is upheld by internal SH2 domain interactions. This induces the open or “primed” Lck conformation, which exposes tyrosine 394 for autophosphorylation. This form of Lck is now active and phosphorylates downstream

substrates. Then, another tyrosine phosphatase, PTPN22 (protein tyroine kinase non-receptor type 22), removes the activating phosphate from tyrosine 394 and the intracellular kinase Csk (s-src tyrosine kinase) re-phosphorylates Lck at tyrosine 505 and Lck is again inactive [44-46, 69, 70].

Janus kinases (Jak) have been also proposed to be direct targets of the CD45 phosphatase activity [67, 71]. Some experimental evidence demonstrated a physical interaction between the CD45 D2 domain and the Jak second non-catalytic domain. In vitro experiments also showed a role of CD45 in the dephosphorylation and inactivation of Jak1, -2, and Tyk2 (tyrosine kinase 2) suggesting that CD45 negatively regulates cytokine signaling via interferon receptors [67, 71]. However, there is some controversy around other potential substrates of CD45 including TCRζ, Skapp66 and Dap12 [45, 72, 73]. These proteins have increase phosphorylation in the absence of CD45 but this phosphorylation might be indirectly mediated by other kinases.

1.2.2.5.3. T cell receptor (TCR) signaling

The role of antigen presenting cells (dendritic cells, B cells or macrophages) is to present peptides or antigen bound to MHC (major histocompatibility complex) to T cells [74, 75]. Engagement of the specific TCR with MHC-antigen complex results in the conformational change of the cytoplasmic chain and phosphorylation of the six ITAM (immunoreceptor tyrosine-based activating motif) motifs of the TCR-CD3 complex by Lck, which is associated either to CD4 or CD8. This phosphorylation recruits Zap70 to the TCR and mediates its phosphorylation [74, 75] (Figure 5). In turn, activated Zap70 is responsible for the phosphorylation of the tyrosine residues on the adaptor protein LAT and the assembly of the Grb2-LAT-SPL76 (growth factor receptor bound protein 2- linker of activated T cell - lymphocyte cytosolic protein 2) multimolecular complex signalosome. Activation of the TCR leads also to PKCθ (protein kinase C theta) recruitement, activation

of PLCγ (phospholipase C gamma), production of DAG (diacylglycerol) and IP3 (inositol triphosphate) with finally causes the calcium mobilization [76, 77]. Integration of the signal is mediated by co-receptor molecules including CD4, CD8, CD28, CD2 and LFA-1 that co-engage to amplify the signal, which as a main result leads to cytokine production, cell proliferation, cytolysis, apoptosis or phenotypic differentiation [76, 78]. In a few seconds following TCR ligation, TCR forms microclusters through the action of actin polymerization and the guanine nucleotide exchange factor Vav1 (vav guanine nucleotide exchange factor) [78]. These microclusters are enriched in Zap70, Lck, LAT, CD3, WASP (Wiskott-aldrich syndrom protein), Nck (non-catalytic region of tyrosine kinase adaptor protein 1) and PLCγ. But CD45, Erk1/2 (extracellular signal regulated kinase), Akt (protein kinase B) and Ras are excluded [78, 79]. Within minutes, at the contact interface between the T cell and the APC the accumulation of these microclusters forms the so-called “larger immunological synapse”. This synapse is formed by the central supramolecular activation cluster (cSMAC) whereTCR, CD28 and PKCθ accumulate and by the peripheral pSMAC, which includes LFA-1 (lymphocyte function associated antigen 1), Talin and ERM (ezrin-radixin-moesin protein) proteins [78, 79]. Finally, formation of the cSMAC results in abrogation of the TCR signaling and dephosphorylation of TCR and Zap70, as well as the recycling of TCR through the endosome [78, 79].

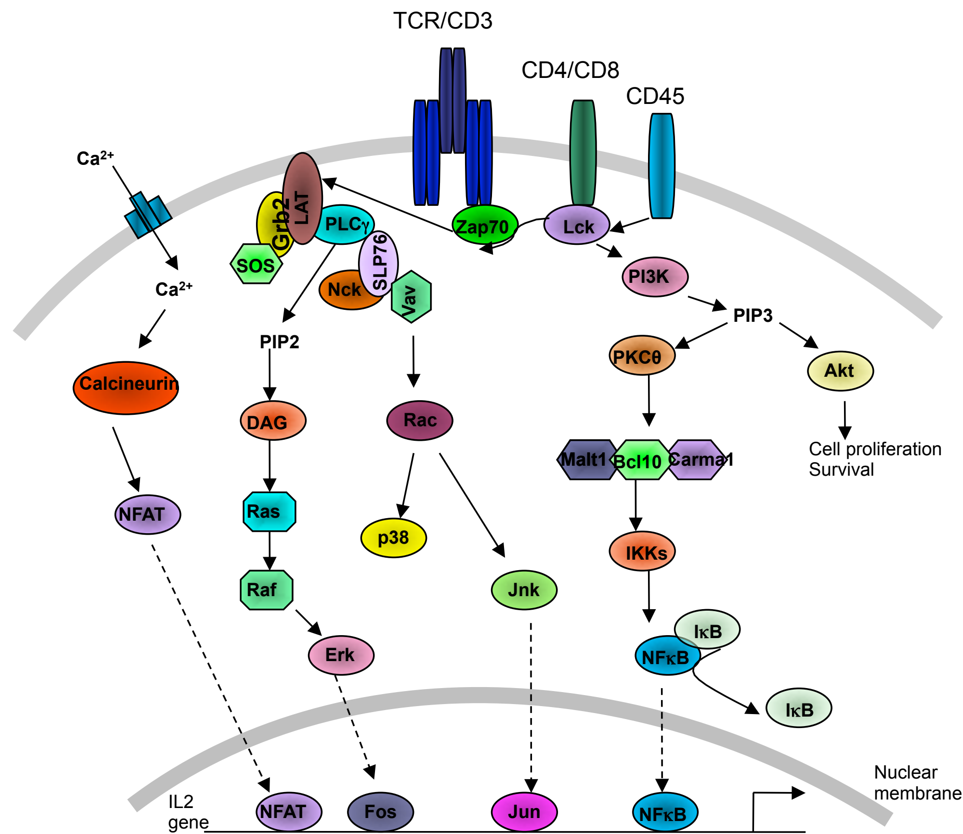


Figure 5. TCR signaling. Upon binding of the TCR to a peptide-MHC complex different signaling cascades are triggered leading ultimately to cell proliferation and IL2 production. The CD45 tyrosine phosphatase first dephosphorylates Lck bound on CD4 or CD8 that causes its activation and phosphorylation of Zap70 and PI3K. The PI3K activates Akt and PKCθ and finally results in NFkB release from IkB and its entry in the nucleus. Zap70 phosphorylates LAT leading to the recruitment of Grb2, SLP76, PLCγ and Vav at the plasma membrane. The Rac/Rho-specific guanine nucleotide exchange factor, Vav activates Rac and therefore mediates p38 and Jnk phosphorylation. After its recruitment, PLCγ cleaves PIP3 to produce DAG and IP3 to activate the downstream molecules Ras and Raf and finally Erk. The production of IP3 causes calcium release from the endoplasmic reticulum that triggers the opening of the calcium channel at the cell surface. The entry of calcium activates calcineurin and the translocation of NFAT (nuclear factor of activated T cell) into the nucleus. Adapted from [74, 78].

1.2.2.5.4. Function in T cells

Expression of the different isoforms of CD45 is stage and cell type specific. In the thymus, the dominant isoform is CD45RO, however cells express more than one isoform on the cell surface and the loss of one isoform is reversible [80, 81]. For example, double negative thymocytes bear in addition to CD45RO, the RB and RBC isoforms. When they develop into the double positive T cells, CD45RO is the predominant form with some RB expression remaining [44, 81]. Single positive thymocytes express only CD45RB and CD45RO while CD4 and CD8 naïve peripheral T cells at resting stage bear CD45RA/RB and CD45RABC/RBC respectively, but CD45RO is expressed after antigen activation and memory phenotype generation for both subsets [44, 81].

Several groups have generated different CD45 knockout mice, which carry deletions of either exon 6, 9 or 12 [82-84]. In all cases, thymocyte development was severely compromised. CD45 null thymocytes contained high levels of Lck phosphorylated at the inhibitory tyrosine Y505 but did not show any changes in the phosphorylation of tyrosine Y394 [69, 85, 86]. As a consequence, these CD45 deficient mice showed a partial block of early (DN to DP) and late (DP to SP) thymocyte differentiation due to a defect in regulating the first step of positive selection [83, 87] [88]. Other experiments investigated the importance of CD45 by generating CD45 heterozygote mice having a reduced expression level of CD45 on cell surface. These CD45 heterozygote mice have increased positive and negative selection [85, 86, 89]. In contrast, CD45 null mice engineered to re-express the CD45RO isoform showed that very low levels of CD45 expression were efficient to restore proper T cell development, but intermediate levels of CD45 re-expression resulted in hyperactive TCR signaling [69]. Finally, it seems that CD45 is more efficient at removing the phosphate of the inhibitory tyrosine Y505 than Y394 therefore a higher level of CD45 expression is required to dephosphorylate Y394 [69]. The complete deletion of CD45 in mice results in a low number of peripheral T cells that are hyper-reactive and represent a danger of autoimmune disease development [83, 84].

1.2.2.5.5. B cells

CD45 is not required for the development of B cells [82, 83]. Although, an augmentation of pro-B cells in the bone marrow and increased numbers and percentages of B cells in the spleen were found in CD45 knockout mice compared to wild-type mice [82]. The increase in B cells in the spleen is characterized by an elevation in the number of immature IgM^{hi} IgD^{lo} (T1), IgM^{hi} IgD^{hi} (T2) and marginal zone B cells [82], but the number of recirculating mature IgD^{hi} is reduced as the B1 population [90]. The B cell kinase Lyn is hyperphosphorylated but there is a reduction in IP3 and calcium mobilization following BCR signaling in absence of CD45 [90]. IgM crosslinking in CD45 deleted cells leads to a reduce proliferation rate but the class switching remains intact as the T cell dependent and independent antigen specific antibodies production [83, 84, 90].

1.2.2.5.6. Mast cells

In mast cells, CD45 activity is involved in the IgE triggered histamine degranulation [44, 91]. CD45 knockout mice are resistant to IgE dependent systemic anaphylaxis and their mast cells are drastically impaired in cytokine production. As expected, Lyn kinase is hyper-phosphorylated at its inhibitory site and calcium mobilization is dampened after IgE crosslinking. Surprisingly, mast cells from CD45 deficient mice are hyper-proliferative in response to IL3 [44, 91].

1.2.2.5.7. NK cells

Deletion of CD45 in NK (natural killer) cells had no effect on their antibody dependent cell mediated cytotoxicity (ADCC) but their number is increased in the spleen of CD45 null mice [92]. ADCC is mediated through the ITAM receptors such as CD16,

Ly49D and NKG2D. However, Ly49d and NKG2D induced cytokines (IFNγ, MIP-1β and GM-CSF) and chemokines secretion is impaired in absence of CD45 [93, 94]. Even if the basal phosphorylation status of CD45 knockout NK cells is upregulated compared to wt cells, the MAPK kinases Erk1/2 and Jnk activation following ITAM receptor ligation is minimally increased [93, 94].

1.2.2.5.6. Dendritic cells

Interest in the role of CD45 in dendritic cells (DC) is rising with the evidence that a positive and negative regulation of different TLRs (toll-like receptor) by CD45 exists [95-97]. Studies of CD45 knockout mice revealed a dispensable role of CD45 in splenic DC and bone marrow DC development. While the maturation of DC is not affected by CD45 absence, the expression of the co-stimulatory molecules CD80, CD40 and CD86 were found increased in CD45 deleted DCs. However, in both wt and CD45 null mice, LPS treated DCs up-regulate the expression of these co-stimulatory molecules [96]. Without affecting the expression of TLR4, absence of CD45 lowered the production of pro-inflammatory cytokines such as IL12 and TNF-α upon LPS induction, i.e. stimulation of TLR4 by LPS. This can be explained by the hyper-phosphorylation status of Lyn, Hck and Fyn at their inhibitory sites without affecting the activation of Erk1/2, Jnk and p38 [95]. In contrast, stimulation of TLR3 by poly I:C or TLR9 by CpG in CD45 null cells increases the amount of pro-inflammatory cytokines compared to wt cells [97]. Both TLR3 and TLR9 pathways are Myd88 independent while TLR4 is dependent and independent of Myd88. Therefore, CD45 negatively regulates Myd88 dependent TLR and positively enhances Myd88 independent TLR [95, 97].

1.3. Transcription and splicing

Experimental evidence demonstrated a role of the transcription machinery in the regulation of alternative splicing [98-100]. Moreover, splicing is largely completed before the release of the transcript but the regulation of this process is not stringent since introns are not necessarily removed in the same exact order that they are transcribed [100-102]. It has been shown that the phosphorylation of the CTD (carboxy terminal domain) of RNA polymerase II (RNAPII) is involved in the regulation of splicing by recruiting elements of the spliceosome and that its deletion disrupts constitutive splicing [98, 99, 103]. Two models have been proposed to explain the role of transcription in splicing. One is the "recruitment model" in which transcription factors and RNAPII interact directly or indirectly with the splicing factors. Evidences for this interaction come from immuno-precipitation experiments that suggested an interaction between SR proteins and RNAPII CTD and other experiments that showed that SR proteins localization and activity depend on CTD [3, 98, 99, 103]. Furthermore, both the structure of a promoter and the set of transcription factors bound to a promoter affect splicing. The best example that supports this view is fibronectin exon 33 for which a promoter dependence of exon inclusion has been directly demonstrated [104-106].

In the second hypothesis, the proposed model touches on the "rate of elongation" [3, 100]. In this model, a reduced speed of pre-mRNA elongation allows more time for the RNA binding proteins and the spliceosome machinery to assemble quickly enough before the 3'ss become available to 5'ss pairing (window time between E to A complex) [3, 107, 108]. In vitro experiments using minigene of tropomyosin in which a G-rich Maz sequence, which promotes intrinsic RNAPII pausing has been inserted resulted in an increased rate of exon 3 inclusion [109]. Moreover, the rate of pre-mRNA synthesis affects the secondary structure of the nascent RNA strand. Therefore, the structural accessibility of spliceosome machinery binding sites may also depend on elongation speed [3, 105].

1.4. Histone modification and splicing

The initial evidence for an involvement of histone modifications in the regulation of splicing came from experiments showing that the splicing of exon 33 of the fibronectin gene is sensitive to the histone deacetylase inhibitor trichostatin-A [110]. Subsequently a number of correlations between chromatin status and splicing have emerged. Nucleosomes are particularly enriched at intron-exon junctions while pseudoexons are exempt of nucleosomes. As well, alternatively spliced exons that are included in pre-mRNA also have more nucleosomes present than the excluded ones and it is known that chromatin complexes facilitate the assembly of the pre-spliceosome [100, 111-113]. More specifically exons that have the epigenetic profile of H3K36me3, H3K4me3 and H3K27me2 and H3K9me3 are absent from mature mRNAs [112-114]. The best example that histone modification is linked to tissue specific expression of an alternatively spliced isoform comes from the FGFR2 (fibroblast growth factor receptor 2) exon IIIb and IIIC. In mesenchymal cells, inclusion of exon IIIc correlates with an accumulation of H3K36me3 and H2K4me1 whereas in the epithelial cells where exon IIIb is predominant, the epigenetic profile is modified towards H3K27me3 and H3K4me3 [113]. These data suggest that histone methylation may act as a platform to recruit splicing factors. Such a platform is evident in the case of splicing factor PTB-dependent genes that are enriched in H3K36me3 and depleted in H3K4me3. In that example, PTB is recruited to its target exons by the adaptor protein MRG15, which interacts directly with modified histone H3 through its chromodomain [100, 113].

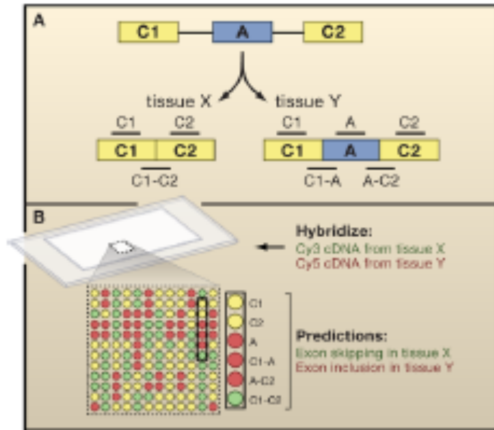
1.5. Methods to study alternative splicing

Bioinformatic analyses take advantage of the availability of sequenced genomes from many organisms including those from mouse and human [1]. The analysis of splicing events relies on the large databases of sequenced transcripts (EST, expressed sequenced tag) and cDNAs. EST sequences are aligned to genomic sequences and spliced candidates are identified from conserved splice site sequences adjacent to the gap created by the introns [1, 115]. One of the limitations of bioinformatic analysis is that ESTs are biased toward 3' and 5' end of the transcript and an insufficient number of ESTs may bias the perceived frequency of splice events in a particular experiment. Another disadvantage is the existence of both false negative and false positive results in the database, with the additional possibility that it contains artefactual ESTs representing non-existing or non-functional isoforms [1, 115].

Genome wide analysis is a fundamental tool to evaluate the overall splicing pattern of a cell or tissue. Initially the first methods employed were hybridization-based microarrays [1, 116]. This technique however, limits the dynamic range of detection. The microarray has evolved into exon-junction arrays that use specific probes for individual exon and splice junctions [1, 116, 117]. However, some difficulties arise with the incomplete nature of gene annotations and the need of predefined targets. To eliminate these restrictions, high throughput sequencing or "RNA-Seq" allows RNA analysis through cDNA sequencing at a massive scale (Figure 6). It is possible to generate billions of nucleotide sequences in a few days and therefore to obtain a complete RNA expression profile, which includes also hitherto unknown transcripts and isoforms [9, 118, 119]. The knowledge thus gained of splice variation is important but is not informative of the role of specific splicing mediators for each splice variant. The combination of CLIP assays with either RNA-Seq or microarray experiment allow this quantification [1, 120, 121]. In this type of procedure a specific immunoprecipitation against a RNA binding protein after crosslinking under

different experimental conditions and in various cell types permits the identification of specific targets for a given of this splicing mediator.

A



B

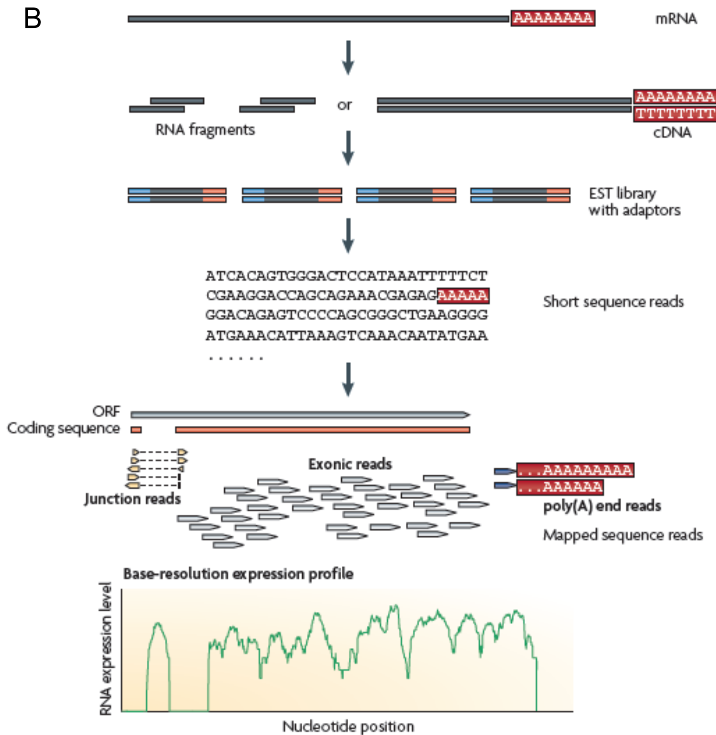


Figure 6. Methods to study the alternative splicing. A) Microarray-based mRNA isoform detection. The microarray probes detect exon-exon junction sequences. Since

different mRNA isoforms will have different exon-exon junctions, this microarray analysis allows the comparison of isoform patterns between diverse tissues. B) Detection of spliced isoforms by RNA-Seq. First, mRNA is extracted from the cells of interest and is converted into a cDNA library. Then sequencing adaptors are added to each library fragment, which are sequenced using high-throughput technologies. Three types of sequence reads are produced: the exonic reads, the junction reads and the poly(A) end reads. Alignment of all reads onto a reference genome allows the identification of alternatively spliced isoforms. From [1, 118].

2. Hematopoieisis

The immune system is composed of more than ten different cell types including T and B lymphocytes, myeloid cells, megakaryocytes and erythrocytes. Each day the hematopoietic system needs to replace millions of non lymphoid peripheral blood cells [122, 123]. During its lifetime, the organism will be subjected to a broad range of stress factors ranging from pathogen infections to cancer or blood deficiencies, which will require the hematological system to compensate and to start the generation of new blood cells from hematopoietic stem cells.

The hematopoietic stem cell (HSC) is at the top of the hierarchy, which spreads further down into several progenitor cells with different lineage potential and renewal capacity until it reaches the more mature differentiated immune effector cells [122, 124] (Figure 7). Several cell surface markers have been identified to specifically distinguish HSCs, progenitors, lineage committed cells and effector cells. In mice, all progenitors and stem cells are enriched within a bone marrow population called LSK (lineage$^-$, c-Kit $^+$, Sca1$^+$) [125, 126]. From these cells, hematopoietic stem cells with long-term repopulating capacity and self-renewal potential are further identified in mice by the expression levels of Flt3, CD48 and CD150 (LSK, Flt3$^-$, CD48^{lo} and CD150$^+$) [124, 127-129]. In addition, the expression of CD34 on these cells is correlated with the cycling status of HSCs [130].

Previously, HSCs with long-term (LT) potential and short-term (ST) potential were separated based on the expression of CD34 with the negative fraction corresponding to LT-HSC and the positive one to ST-HSC on the LSKFlt3^{-} population [131, 132]. Upon commitment HSCs first differentiate further into multipotent precursor cells (MPPs) that can give rise to all cell lineages but loose gradually their self-renewal potential. They are identified through the cell surface marker LSK, CD150^{+} CD48^{+}. As they differentiate, multipotent precursors become lineage- restricted progenitors, biaised either toward the lymphoid branch or the erythrocyte-myeloid branch. Common lymphoid progenitors (CLP) or Lin-IL7R^{+}c-Kit^{+}Sca1^{lo} cells are the precursors of both B and T lymphocytes [133, 134]. In the bone marrow, B cell specific transcription factors will mediate the development of CLPs toward pre-pro B cells [135] [136]. On the other hand, T cell specific gene expression skews the CLPs to develop into early lymphoid precursors (ELPs) that will migrate from bone marrow to the thymus where they become ETPs (early thymic progenitors) [135, 136]. The erythroid-myeloid developmental pathway is distinguished first by the generation of common myeloid progenitors (CMPs) from the MPPs. CMPs are identified through the surface markers Lin^{-}Sca1^{-}c-Kit^{+}CD34^{+}CD16/32i^{nt}. From this point, cells may choose either the erythroid pathway through the expression of Lin^{-}Sca1^{-}c-Kit^{+}CD34^{-}CD16/32^{-}, becoming MEPs (megakaryocyte-erythrocyte progenitors) or the granulocyte-monocyte branch with the expression of CD16/32^{hi} CD34^{+} to become GMPs [137-139].

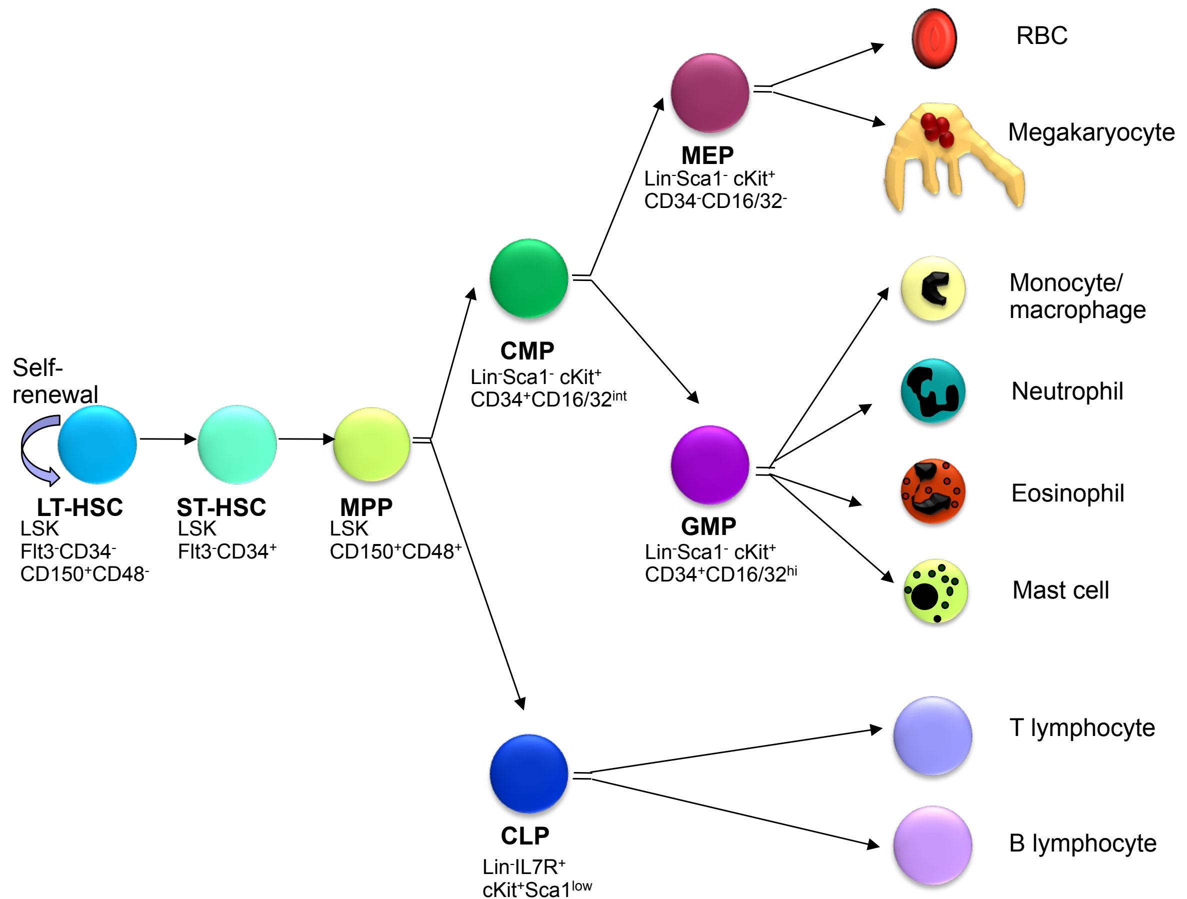


Figure 7. Hierarchical organization of hematopoiesis. The LT-HSCs (long-term) are at the top of the hematopoietic hierarchy and are the only cells that possess both self-renewal capacity and the potential to generate all hematopoietic cell types. As they progress in differentiation, LT-HSCs first loose their self-renewal capacity and then their multipotency. ST-HSCs (short-term) and MPPs (multipotent precursor) still retain multilineage potential. MPPs develop toward the lineage committed progenitors. For example, CLPs (common lymphoid progenitor) give rise to B and T lymphocytes, while CMPs (common myeloid progenitor) differentiate into GMPs (granulocyte/macrophage progenitor) that generate macrophages, neutrophils and granulocytes and MEPs (megakaryocyte/erythrocyte progenitor) that produce erythrocytes and megakaryocytes. Phenotypic markers used to define individual cell types are shown below each cell, LSK stands for lineage negative, cKit positive and Sca-1 positive population. Adapted from [140].

2.1. Ontogeny

Hematopoiesis is established at specific sites in the developing embryo and later in the adult (Figure 8). The concept has been put forward that a particular niche exists, which supports the reproduction of HSCs. This theory stipulates that the microenvironment in which HSCs are located in the developing embryo is the fetal liver while it is the bone marrow in the adult organism [141]. This "stem cell niche" is necessary for the development of HSCs and for the maintainance of their stemness and survival by providing the essential cues for this developmental program [141-143]. Hematopoiesis can be divided into the primitive and definitive stages, which are confined to anatomically distinct sites.

The primitive hematopoiesis is extra-embryonic and it is defined by the production of primitive erythrocytes that are nucleated and produce only embryonic globins. It starts in the yolk sac soon after gastrulation at embryonic day E7.5 [144, 145]. At this stage, the blood islands form from the extraembryonic mesoderm at neural plate. During this phase, there is also the hemangioblastic chord formation where endothelial cells surround the primitive erythrocytes [144-147]. Thus, the primitive hematopoieisis remains confined since the vascularization is not established yet. Around E9 the definitive erythropoiesis starts in the fetal liver, which coincides with a decrease in the numbers of primitive erythrocytes from E10 to E11 [144-147].

The first multipotent hematopotieitic stem cells can be detected in the ventral wall of the dorsal aorta at day E10.5 [144, 146]. However, some experiments demonstrate the presence of a myeloid progenitor in the yolk sac already at the E8.25 stage[145]. Hemogenic sites are found in the mouse as early as E8.5-E10 at the paraaortic splanchnopleura (p-Sp) and then at the aorta-gonad-mesonephros (AGM) at E10-11.5 [144, 146]. It is hypothesized that HSCs are derived from the embryonic endothelium [148]. In vitro assays and transplantation experiments demonstrate that vascular endothelial (VE)-cadherin^{+}CD45^{-}

cells are the ancestors of the definitive HSCs [149]. These cells acquire the expression of CD45 upon differentiation into pre-HSCs and in fact all HSCs found in the AGM are $CD45^+$, $CD34^+$, $CD31^+$, c-Kit^+ and VE-$cadherin^+$ [149-151]. The P-Sp and AGM contain only approximately 500-1000 HSCs. With the onset of blood circulation, HSCs migrate towards the placenta at E11 [152]. At the same time, the colonization of the fetal liver takes place from E11-E12. Until E15, the fetal liver allows the expansion of HSCs where they also acquire their specific surface markers [128]. The fetal liver also supports the differentiation of mature hematopoietic cells such as erythrocytes, myeloid and lymphoid cells. As well, around E13, some HSCs circulate and seed in the fetal spleen. The fetal spleen is only a transitory hematopoietic site where HSCs do not expand but instead differentiate. Finally, the bone marrow is colonized around E18.5 and will remain the definitive site of hematopoiesis after birth. The bone marrow environment permits the maintenance, the expansion and the differentiation of HSCs.

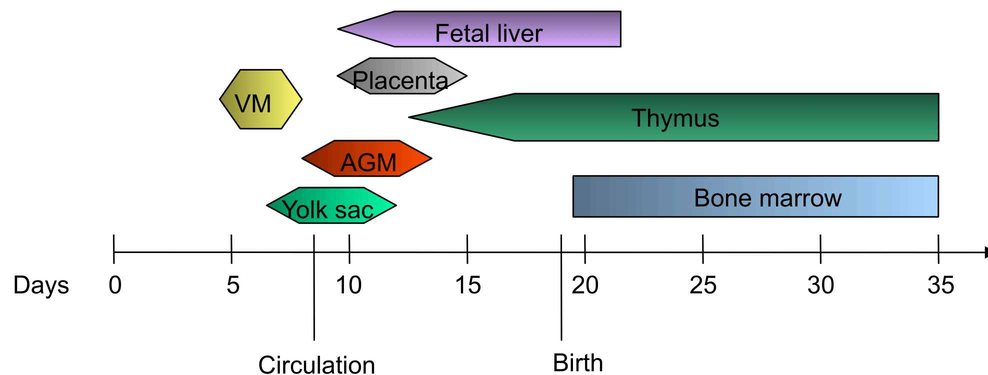


Figure 8. Ontogeny of mouse hematopoiesis. Schematic representation of the kinetic of development and migration of hematopoietic stem cells and progenitors. VM, ventral mesoderm; AGM, aorta-gonad-mesonephros. Adapted from [153].

2.2. HSC Niche

The concept of the HSC niche was postulated over 30 years ago [141, 154]. It claims that a highly organized microenvironment controls HSC homeostasis by providing structural support and physiological signals [123, 143, 155]. With the advance in microscopy, it has been observed that the most primitive HSCs reside closely to the endosteum of the trabecular bone in adult mice henceforth referred to as the "endosteal niche" [154-156]. HSCs interact with the osteoblast cells of the endosteal niche that are producing specific proteins required for their maintenance such as Notch ligands and cytokines including IL6 and TGF-β1 [142, 143, 157] (Figure 9). It has been shown that in the absence of osteoblasts the number of HSCs was considerably reduced and that in vivo activation of the parathyroid receptor, increasing the number of osteoblasts in the bone marrow, also results in an augmentation in HSCs cells [154, 156]. Interestingly, even before the bone formation fetal HSCs self-renewe and differentiate indicating that other niches exist. The bone is a highly vascularized network consisting of arterial vessels, which divide into arterioles and then capillaries and thereafter into sinusoid vessels [143, 158, 159]. This bone marrow vasculature regulates the HSCs function. In fact, 60% of all HSCs reside in the vascular niche, which comprises an alternative microenvironment to the endosteum [143, 158, 159]. The accumulation of HSCs in the vascular niche allows a rapid mobilization of these cells upon G-CSF treatment [143, 158, 159]. This niche mainly consists of endothelial cells but other cell types have been identified recently such as CXCL12-abundant reticular cells around the sinusoids and the perivascular Nestin^{+} mesenchymal stem cells [143, 158, 159].

2.3. Assymetric versus symmetric division

While adult HSCs are quiescent, fetal HSCs divide rapidly to ensure a proper seeding and the establishment of hematopoiesis. BrdU incorporation assays allowed the quantification of cell cycle progression of cells. Using this technique, it has been demonstrated that fetal liver HSC numbers double every day [160]. In contrast, only 25% of adult bone marrow HSCs are cycling while the remaining are at the G0 quiescent stage. It is estimated that only 8% of LT-HSCs will progress into cell cycle each day [130, 160-162].

Most of the cells found in an organism are undergoing symmetric cell division, meaning that one parent cell gives rise to two identical daughter cells. One of the hallmarks of HSCs is that they are capable of self-renewal and differentiation. To accomplish this, the HSCs mostly divide in an asymmetrical pattern in which one daughter cell will be identical to the parent cell with self-renewal capacity and the other one will differentiate [163, 164]. Diverse mechanisms have been proposed to explain these distinct cell fates. For example, an intrinsic factor, such as a transcription factor, in the parent cell may mediate the fate of each daughter cell at the time of the cell division. Another possibility proposed is that a signal from the environment or from cell-cell contact before mitosis instructs the asymmetric division. Finally, two daughter cells, which adopt a different cell fate may have initially divided symmetrically with the cell fate being determined stochasticly by an intrinsic factor [163, 164]. The availability of time-lapse microscopy has allowed the visualization and confirmation of the asymmetrical division of HSCs [165]. Furthermore clone splitting experiments, consisting of the examination of the fate of two daughter cells that have been separated from a single HSC-containing population, have shown that two daughter cells are not functionally equivalent since they possess different cell cycle status and multilineage potential [164, 165]. The combination of time-lapse imaging and Notch reporter mice, in which GFP expression is induced by Notch signaling, can also be used to

track HSC division [166]. Notch is a cell surface receptor and transcription factor that interacts with different ligands including the delta-like (DL) family and the jagged family of proteins. Upon the binding of Notch to its ligand, there is the induction of proteolytic cleavage of Notch by γ-secretase. This releases the intracellular part of Notch (ICN), which will enter the nucleus and act as a transcription factor [167, 168]. Notch is expressed on HSCs and is necessary for their maintenance. Using the notch reporter system three modes of division have been observed in HSCs. The first one is the asymmetric mode where a GFP^+ HSC gives rise to one GFP^+ and one GFP^- cells. On the other hand there are two modes of symmetric division: one producing two HSCs, indicated by a positive GFP signal, and one producing two commited daughter cells which have lost the Notch signal [164, 166].

Another proposed factor in HSC fate is the protein Numb, which is found bound to the membrane and is known to interact with Notch1. As well, Numb plays a role in cell fate determination during development in a variety of other systems [168, 169]. During HSC division, Numb is asymmetrically segregated into the daughter cells [169]. In addition, four asymmetrically distributed proteins in humans have been identified: D53, CD62L/L-selectin, CD63/lamp-3, and CD71/transferrin receptor [170]. Hence, it is most likely that the determinant of asymmetric division is cell intrinsic and does not depend absolutely on the environmental context.

2.4. Regulators of hematopoietic stem cells

2.4.1. Intrinsic factors

2.4.1.1. Runx

Runx1, also known as AML1, is a transcription factor that belongs to the runt domain family [148, 171]. This transcription factor is expressed on almost all bone marrow cell types with the exception of Ter119$^+$ cells and megakaryocytes, including c-Kit$^+$ precursor cells [171]. It has been found to be expressed during early stages of development (E9.5-10.5) in both the endothelial and mesenchymal cells from the dorsal aorta [148, 171]. Runx1 is necessary for primitive and adult hematopoieisis [147, 148]. Furthermore, conditional deletion of Runx1 in the adult bone marrow revealed that Runx1 is a negative regulator of HSC quiescence [172]. Deletion of Runx1 leads to LT-HSC expansion and an accumulation of LSKs in a G0 stage. Moreover, the number of CRU values (competitive repopulation unit), which are calculated by a serial dilution of bone marrow cells that are transplanted into irradiated recipients to determine the lowest number of cells required for reconstitution, show approximately ten times more LT-HSCs in the deleted mice compared to controls [172].

2.4.1.2. Gfi1/GFi1b

Gfi1 and its paralogue Gfi1b are transcription factor proteins that possess at their C terminus 6 zinc fingers that mediate their binding to DNA and at their N terminus a 20 amino acid stretch called the SNAG domain. Gfi1 and Gfi1b differ only in the amino acid sequence that separates both domains [173, 174]. The expression of Gfi1 and GFi1b is complementary with the exception of a few cell types [175]. Gfi1 is known for its

repressive function on many genes such as *PU.1, Cdkn1a* (p21), *Bax* and *IL7R* [174]. Gfi1 is highly expressed in LSK cells as well as in GMPs and CLPs but is absent from MEPs and CMPs. Studies using Gfi1 knockout mice, showed a reduction in the percentage and cell number of LSKs as well as for LT- and ST-HSCs in the bone marrow [176]. In contrast, the LSKFlt3^{-} population was increased in these mice [177]. Functional assays demonstrated a loss of reconstituting potential of HSCs in colony forming assays [176]. Additionally, in bone marrow transplantations cells from Gfi1 knockout mice were still able to repopulate irradiated mice albeit at a much lower efficiency than wt cells. They lost their repopulation ability in secondary transplantations [176, 177]. The defect of Gfi1 deficient HSCs is due to an increased proliferation and cell cycle progression into the G2/M/S phases and also an impaired self-renewal [176]. As well, expression levels of p21, E2F5 and E2F6 were unchanged but protein levels of E2F5 and E2F6 were increased in Gfi1 null bone marrow cells compared to wt controls [176] and in another study, an increase of Gata2 expression and a downregulation of p21 were demonstrated to occur in Gfi1 deficient HSCs [177]. More recently, a role of Gfi1 in protecting HSCs against stress-induced apoptosis through the regulation of pro-apoptotic factors was shown. Transplantation assays of HSCs that are deficient for Gfi1 but overexpress Bcl-2 were shown to have regained their ability to self-renew and to initiate multilineage repopulation in transplanted hosts [178].

It has been described that Gfi1b is mainly expressed in HSCs, CMPs, MEPs, erythrocytes and megakaryocytes but is almost completely absent from the T cell lineage [175]. In the case of Gfi1b, the complete knockout mouse is embryonically lethal due to a failure of primitive erythrocytes to mature [179]. In addition, MEPs found in the fetal liver of Gfi1b deficient embryos were reported to be arrested in their development leading to a lack of megakaryocytes and platelets [179]. Use of a MxCre inducible knockout of Gfi1b in the adult bone marrow, revealed a marked increase in the number of HSCs in the bone marrow and an accumulation of LSK cells and HSCs in blood and spleen [180]. While changes in the number of leukocytes and red blood cells were rather mild, platelets were almost

completely absent from Gfi1b deficient mice [180]. As for the Gfi1 knockout HSCs, the Gfi1b deficient mice show increased proliferation and cell cycle progression of HSCs but in the case of Gfi1b deficiency this did not affect their self-renewal capacity or their ability to initiate multilineage repopulation [180].

2.4.1.3. Bmi-1

Bmi-1 is a transcription factor of the polycomb gene family and is a component of the polycomb repressive complex I, which has a role in stable maintenance of gene repression involved in development, growth and differentiation. This complex containing Bmi-1, Mel18, Mph1/Rae28, M33, Scmh1 and Ring A/B represses genes by antagonizing the chromatin remodeling by the Swi/Snf complex [181, 182]. Using knockin mice where Bmi-1 has been replaced by GFP, it was demonstrated that the expression of Bmi-1 is highest in the long-term HSC compartment and that its expression decreases upon cell differentiation [183]. In this model, only cells harboring high levels of GFP expression were potent to generate multilineage reconstitution in irradiated mice, identifying this gene as a regulator of HSC function [183]. The generation of Bmi-1 knockout mice allowed to demonstrate in more details the role of this transcription factor in HSCs. Deletion of Bmi-1 resulted in death of the animals in early adulthood due to a profound and progressive hematopoietic failure (anemia) [184]. Moreover, there is a ten times decrease in the number of LT-HSCs in the adult bone marrow compared to control mice caused by the replacement of these cells by adipocytes [184]. Furthermore, Bmi-1 deficient HSCs possess a decreased potential in long-term reconstitution capacity. It has also been found that the HSCs from Bmi-1 deleted mice have an impaired long-term maintenance of proliferation potential and a reduction in self-renewal [184-186]. In contrast, forced expression of Bmi-1 promotes symmetrical cell division and self-renewal of HSCs [181].

It has been known for some time that Bmi-1 regulates p16 and p19 expression to inhibit apoptosis and allow cell proliferation [182]. However, a new study using as model a double deletion of Bmi-1 and Arf/inkA locus shows that p16 and p19 do not account for all the phenotypes observed in HSCs in the absence of Bmi-1 [186]. The double deletions allows the rescue of the differentiation capacity of HSCs but does not completely restore the proliferation deficiency seen in the Bmi-1 knockout. This is due to an impaired development of trabecular bone in the metaphyseal area resulting in a reduced or incapacitated osteoblastic niche for HSCs [186].

2.4.1.4. Scl/Tal1

The protein Scl/Tal1 is a member of the basic helix-loop-helix category of transcription factors that bind as heterodimers with E2A gene products to E-box containing DNA sequences. It has been demonstrated that the Scl complex occupies the c-Kit promoter to enhance and transactivates *c-Kit* gene expression [187]. The expression of Scl is first detectable at embryonic day 7.5 and reaches maximum intensity in dormant LT-HSCs [188, 189]. Complete deletion of Scl results in embryonic death around embryonic developmental stage E9 due to a failure in primitive hematopoiesis and the absence of blood island formation [190, 191]. Conditional deletion of Scl in adult mice using the MxCre inducible transgene also causes death due to thrombocytopenia and anemia but has allowed for the analysis of the stem cell compartment [192]. Ablation of Scl in the bone marrow gives rise to an increased number of LSK cells [193]. Bone cells deleted for Scl are able to reconstitute hematopoeisis but their repopulating capacity is significantly diminished [193]. However, serial transplantation assays do not show any difference between knockout and wt mice. Thus Scl is not required for the self-renewal capacity of HSCs [193]. In contrast, mice heterozygous for the Scl allele revealed that under high proliferative demand as during transplantations, $Scl^{+/-}$ cells are impaired in their repopulating ability and are less competitive [188]. Analysis of LT-HSCs from heterozygous bone marrow showed a two

folds increase in cells in the G1 stage over G2/M/S stages. Scl expression in HSCs restricts under steady state the G0 to G1 transition through the transcriptional control of *p21* and *Id1* genes [188].

2.4.1.5. GATA-2

The expression of Gata-2 in HSCs varies according to the cell cycle status and it is highest in S phase and lowest in the G1/S and M phases [194]. Gata-2 is a transcription factor and member of the GATA family that binds to the DNA sequence (T/A) GATA (A/G) [194]. The expression of Gata-2 is essential for embryonic hemangioblast formation and Gata-2 deficient mice die before embryonic stage E11.5 from severe anemia [195, 196]. Chimeric mice generated with Gata-2 knockout ES cells and wt ES cells showed that this transcription factor is important in hematopoiesis since none of the deleted cells contributed to fetal liver development [195, 196]. In addition, dosage of Gata-2 affects the generation and proliferation of immature HSCs from the AGM and yolk sac, both of which containing fewer CFU-s in Gata-2$^{+/-}$ embryos [197]. Once reaching the fetal liver stage, the onset of HSCs development is normal but the bone marrow pool of HSCs is still impaired with an increased quiescence and apoptosis rate [196, 197]. Heterozygous Gata-2 HSCs possess in addition a competitive disadvantage and a reduced serial transplantation potential compared to normal HSCs [196].

2.4.1.6. Cell cycle factors

Progression through the cell cycle is controlled by cyclins and cyclin dependent kinases (CDKs). To avoid unnecessary cycling, the ink4 and the Cip/kip families of cyclin dependent kinase inhibitors (CKIs) negatively regulate cell cycle progression. The ink4 family contains p16, p15, p18 and p19 that act mainly on Cdk4 and Cdk6. On the other

hand, the Cip/Kip family, which includes p21, p27 and p57, inhibits Cdk2 and Cdk4 [198]. Since most HSCs are in a quiescent G0 state, it is conceivable that factors regulating cell cycle progression are important for HSC quiescence and self-renewal. Many studies have been published to dissect the role of CKIs in HSCs. Based on these, p57 seems to be the most important factor in HSCs. p57 is predominantly expressed in LT-HSC and its expression decreases as cells maturate while no p21 and only low levels of p27 can be detected [198, 199]. Constitutive deletion of p57 in mice causes death just after birth and conditional ablation of p57 in adult bone marrow results in a loss of HSC numbers and a reduction of the size of HSC pool in the G0 state without any significant change in levels of p21 and p27 expression [198]. HSCs deficient in p57 loose their self-renewal capacity and their reconstitution potential in serial bone marrow transplantations [198, 199]. A last consequence of the loss of p57 is the exhaustion of HSCs, which occurs by deregulation of HSC quiescence and in part due to apoptosis caused by increased CKI and p53 levels in these cells [198].

The Cip/Kip family of cell cycle inhibitors contains a member, p21, to which many roles have been attributed in HSCs. Surprisingly, deletion of p21 in mice does not affect the number of cells in the peripheral blood [200]. HSCs from these mice are potent to reconstitute even in a secondary transplantation assay [201]. Stress inducible treatment of HSCs by 5-FU or 2Gy leads to the same percentage of cell survival in presence or not of p21 but the number of colony forming units generated is reduced [200, 201]. However, HSCs treated with 2Gy were no longer able to contribute to long-term hematopoeisis [200]. HSCs deleted for p21 were found to cycle at a faster rate than wt HSCs. Therefore p21 impedes the entry of HSCs into the active cell cycle and, in its absence, the self-renewal of HSCs is compromised under stress conditions [201, 202].

The ink4 family member, p18 possesses an opposite effect on hematopoieisis compared to p21. In the absence of p18, HSCs have an increased colony forming capacity compared to their normal counterparts [201-203]. In addition, they are able to sustain hematopoieisis

even after serial transplantations and have a strong competitive advantage over wt cells [202, 203]. Since there is no change in proliferation and apoptosis in p18 knockout HSCs, it seems that ablation of p18 potentiates the self-renewal capacity of HSCs [202, 203].

2.4.2. Extrinsic factors

2.4.2.1. Thrombopoeitin

Thrombopoietin (TPO) and its receptor Mpl are known to be essential for megakaryocyte development and platelet production but it is becoming obvious that they are key components of the stem cell niche and exert functions as HSC regulators [204, 205]. Osteoblast cells found in the endosteal compartment of the bone secrete TPO in adult mice [206]. Surface analysis of HSCs reveals that the quiescent pool of HSCs expresses Mpl, which allows the cell to adhere to the endosteal surface [206]. Reconstitution experiments demonstrated that only the fraction of HSCs expressing Mpl was capable of long-term repopulation [206]. Thus it is not surprising that deletion of either TPO or Mpl in mice affects the HSC compartment [207, 208]. It is believed that the interaction between TPO and Mpl maintains the immature HSC phenotype by suppressing p57. Furthermore, stimulation of Mpl by TPO transiently increases the proportion of quiescent HSCs [206].

2.4.2.2. TGF-β

TGF-β is a cytokine produced by bone marrow stromal cells (including osteoblasts, adipocytes and mesenchymal cells) and it is known for its potential to arrest hematopoiesis in culture [209, 210]. In vitro studies show that TGF-β is able to delay the time of the first cell division and inhibits colony formation and differentiation. TGF-β modulates also the ability of the cells to respond to other cytokines [209, 211]. This latter effect was confirmed

by demonstrating that TGF-β represses expression of SCL by stromal cells [210]. Moreover, cell cycling of HSCs is inhibited by TGF-β without affecting their cell survival [211]. Also it is known that expression of CD34 on HSCs decreases with proliferation, while treatment of HSCs with TGF-β maintains and/or up-regulates the level of CD34 on these cells [211]. However, conflicting results were obtained by using the TGF-β or TGF-β receptor knockout mice. The complete deletion of TGF-β resulted in 50% embryonic death and the survival mice developed a massive T cell-dependent inflammatory syndrom [212, 213]. While in the double knockout mice for TGF-β and MHC-II the number of quiescent LT-HSCs were almost absent, the conditional deletion of TGF-β receptor I using Mx-Cre induced deletion had no effect on LT-HSC population nor on their capacity to reconstitute lethally irradiated mice [212, 213]. This discrepancy might come from the microenvironment itself or the redundant use of TGF-β receptor II and III.

2.4.2.3. Stem cell factor (SCF)

c-Kit is highly expressed on HSCs serving to phenotypically identify the HSC compartment and is the receptor for the cytokine stem cell factor (SCF) [125, 126, 214]. Bone marrow stromal cells and endothelial cells are the main producers of this cytokine, which can be both soluble or membrane bound based on the alternative splicing of its pre-mRNA [215]. SCF has different roles in the hematopoietic niche: one of these being the modulation of the avidity of the α4 and α5 integrins to their respective ligands [215]. Mice deleted for either the receptor c-Kit or its ligand SCF have been generated and both die at early embryonic stages from severe anemia [216, 217]. Non-lethal point mutations in c-Kit generate mice with a reduction in the number of functional HSCs resulting in a loss of both CFU-S (colony forming unit-spleen) production and the ability of multilineage reconstitution [218, 219]. Cell culture of HSCs requires SCF for survival and proliferation but the cytokine is unable to potentiate self-renewal capacity [214].

2.4.2.4. Wnt/Notch

Wnt proteins are a group of secreted signaling proteins that are lipid-modified (palmitoylation). They are able to induce cell signaling through the binding to their respective Frizzled (FRZ) receptors together with their transmembrane partners Lrp5 and Lrp6 [220]. They have been recognized for their roles in embryonic development, cell differentiation and cell polarity. Wnt signaling can be either canonical, which involves β-catenin mediated Lef/TCF transcription factor activation or non-canonical [220]. Stromal cells of the endosteal lining of the bone marrow accumulate high amounts of β-catenin, which enhances the expression of jagged-1 and thus activates Notch signaling in HSCs [221]. While stromal cells express the receptors FRZ1, 2, 7 and 8 the HSCs secrete Wnt1, 2b, 4 and 10b [221]. β-catenin possesses a pivotal role in the maintenance of HSCs self-renewal and the undifferentiated status of HSCs throughout the bone marrow microenvironment [221, 222]. In fact, deletion of β-catenin causes a loss of LT-HSC numbers due principally to increase apoptosis without any changes in cell cycle [222]. Transplantation of wt bone marrow into β-catenin deficient mice results in the same HSC reduction in number and loss of self-renewal [222]. This was due to the decrease in osteoblast cells in the bone marrow niche. Other indications aside from this exclude a bone marrow architecture defect link Wnt signaling to HSC function. Mice constitutively expressing Wif1 (wnt inhibitory factor 1) that blocks both the canonical and non-canonical pathways have been generated [220]. Analysis of these mice revealed no change in bone marrow cellularity or white blood cell count in the blood. However, LT-HSC population was found to be increase while ST-HSCs, LSKs and progenitor cells remain unchanged. Transgenic Wfi1 HSCs were less quiescient and more of these cells were found in the G1 stage than in G0 [220].

2.4.2.5. Integrins and chemokines

The chemokine SDF or CXCL12 is constitutively secreted by the bone marrow stroma and interacts with its receptor CXCR4, which is expressed on hematopoietic cells to mediate its effects [223]. One of the suggested roles of SDF is its action on the survival of peripheral blood CD34 positive cells through induction of Bcl-2 and Bcl_{xL} [223]. As well, other effects of SDF/CXCR4 signaling in hematopoiesis have been characterized using conditional knockout mice for CXCR4. As for many important HSC regulators, CXCR4 is mainly found on the surface of long-term HSCs. Bone marrow from CXCR4-deleted mice contains less total HSCs but an increased number of ST-HSCs [224]. The long-term reconstitution potential of HSCs deficient for CXCR4 was altered as well as their survival after 5-FU treatment. Since in the absence of CXCR4, HSCs have a reduced interaction with their environment, there is an accompanying increase rate of exit from the G0 phase [223, 224].

Integrins are potent mediators of cell adhesion and HSCs are not exempt of their expression. These surface proteins form functional heterodimers by combining different alpha and beta chains [225]. Alpha4 integrin or (VLA4) is found on HSCs in association with beta1 integrin, which allows the cell to bind both fibronectin and VCAM on bone marrow stromal cells [225, 226]. Two different studies using alpha4 deficient mice unveil the role of this adhesion molecule on HSCs. Absence of alpha4 integrin did not affect the phenotype nor the number of HSCs in mice. Strikingly, though alpha4 integrin deficient HSCs had reduced competition potential in part due to altered engraftment and overtime all deficient cells were replaced by non-deleted cells and the expansion of HSCs after 5-FU treatment was impaired suggesting a potential loss of self-renewal [225, 226].

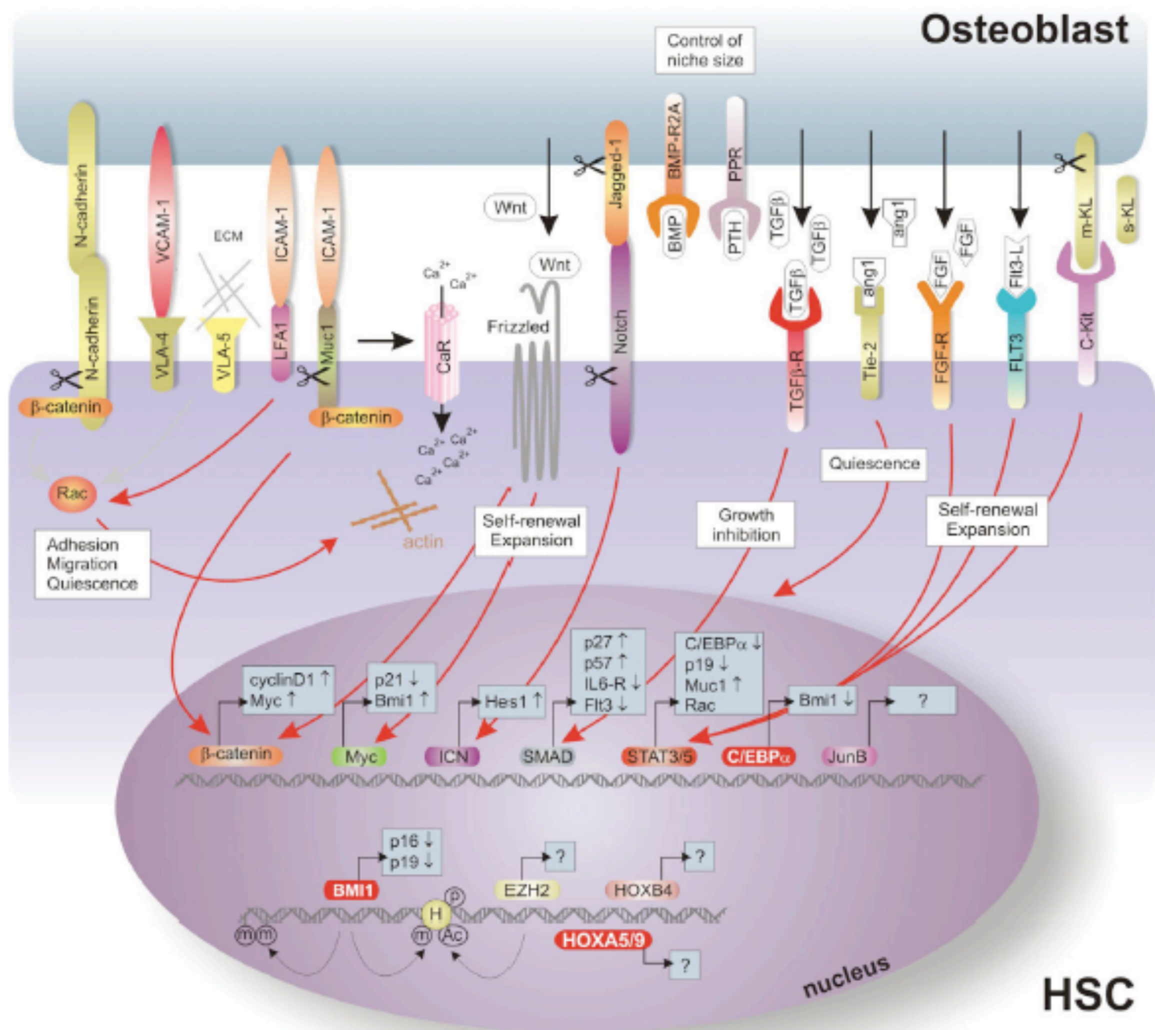


Figure 9. Interactions of HSC with their niche. Schematic representation of the interactions of HSCs with the niche that regulate their cell fate. Direct cell contact between HSCs and the osteoblast is mediated by N-cadherin, VLA4 binding to VCAM-1 and LFA1 interaction with ICAM. This direct cell-cell contact allows access to regulators that mediate self-renewal capacity or expansion. These regulators include cytokines such as TGF-β or Wnt proteins as well as Notch and Kit ligands. All these signals induce transcription of specific genes that are necessary for HSC maintenance. From [227].

3. T cell development

T lymphocytes are one of the main components of the acquired immune response. Antigenic specificity is obtained through the α/β T cell receptor (TCR) expressed on the cell surface interacting with MHC class I and II expressed on antigen presenting cells (APC). γ/δ T cells, which express a γ/δ TCR, recognize non-MHC restricted molecules and are mainly required for innate and mucosal immunity [228, 229]. T cell differentiation occurs in the thymus and is very tightly regulated stepwise process through which different subpopulations of pre-T cells are generated based on the signal they receive [229, 230] (Figure 10). This developmental process starts from the cells double negative for CD4 and CD8, which are divided into four subsets (DN1-DN4) based on their expression of the CD44 and CD25 surface markers. DN4 cells differentiate into CD4/CD8 double positive cells (DP) to be selected and to become mature CD4 or CD8 single thymocyte (SP), which will leave the thymus to settle in the peripheral lymphoid organs [229, 230]. From this, TCRα/β bearing thymocytes can develop into cytotoxic CD8 T cells and CD4 T cells with different effector functions that can be subdivided in either Th1, Th2, Th17 or $CD4^+$ T regulatory cells [229, 231]. Unconventional TCRα/β lymphocytes are also generated and reside mainly in the intestinal mucosa and include CD8αα+ IEL and CD8αβ+ IEL (intestinal intraepithelial lymphocytes). On the other hand, thymocytes that harbor the TCRγ/δ are generated at earlier stages of differentiation usually at the DN3 stage and remain double negative for CD4 and CD8 co-receptors [229, 230].

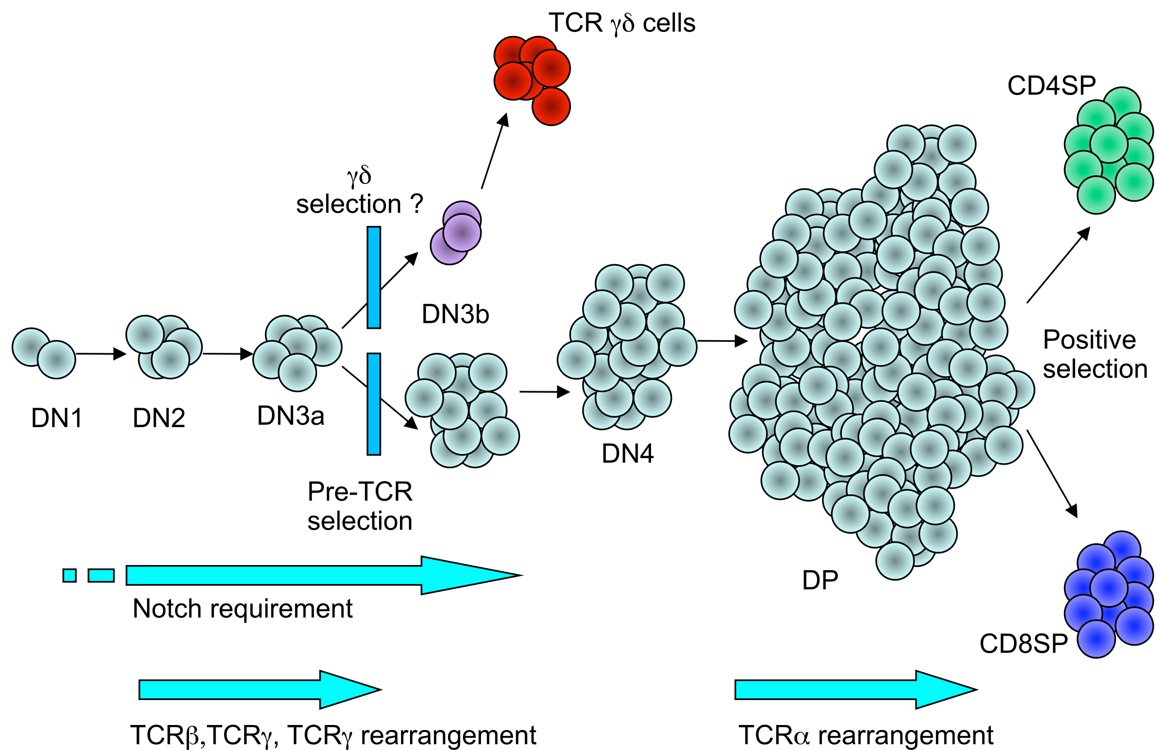


Figure 10. Thymocyte development. Early steps of T cell differentiation (from DN1, DN2 to DN3a pre-T cells) take place in the thymus. Notch signaling is required from the late DN1 stage until DN3b. TCRβ, γ, δ rearrangement starts at DN2 and terminates at β-selection. TCRγδ cells develop from DN3b cells that successfully rearrangement the TCRγδ. DN3a cells that have productively rearranged a TCR beta chain express a pre-T cell receptor made from a TCR beta chain and the pTalpha chain on the cell surface and receive proliferation and survival stimuli. After β-selection is completed, DN4 pre-T cells proliferate extensively to generate the DP cell subset where TCRα rearrangement occurs. Positive selection of DPs allows the development of non-self reactive CD4SP or CD8SP cells. DN, double-negative; DP, double positive; SP, single positive. Adapted from [229].

3.2. T lymphocyte progenitors

The thymus is the organ where the early steps of pre-T cell differentiation take place. Since the thymus is devoid of progenitors with self-renewal capacity, its long-term repopulation is achieved by the recruitment of progenitor cells from the bone marrow through the bloodstream [135, 229, 232]. In the mouse, these progenitors start homing from the fetal liver to the fetal thymus at embryonic day 11.5 [233, 234]. Several progenitors have been postulated to exist, each discovered through different experimental approaches. The first candidate cells, the CLP-2 cells detected in the blood and the bone marrow, were discovered using pre-TCR::hnCD25 transgenic mice the CLP-2 cells detected in the blood and the bone marrow and defined as Lin^{-}CD19^{-}B220^{-}c-Kit^{-}hnCD25^{+} [235, 236]. The TMPs or thymic multipotent progenitors characterized phenotypically as Lin^{-}CD44^{+}CD25^{+}c-Kit^{+} and CCR9^{+} are found in the bone marrow, blood and thymus and were also candidates for the earliest T cell progenitors [235, 237]. Also found in the BM, were the L-selectin^{+} progenitors that express Lin^{-}Sca^{+}c-Kit^{+}Thy1.1^{-}CD62L^{+} and possess T cell potential [235, 238]. There are also the Flt3^{+}LMPPs, which are biased toward lymphoid and myeloid lineages and finally, the Ly6D^{-} CLPs that generate both B and T cells but also NK cells and DCs [239]. From all these candidates, the only cell that contributes to T cell development is the ELP (early lymphoid progenitor). ELPs are found in the bone marrow and in the blood and are defined as LSK Flt3^{lo}CCR9^{+} cells. There are only 30-60 ELPs per mouse, which corresponds to the limited thymic niche that is available [235, 239, 240].

3.3. Early T cell development

As the ELPs settle in the thymus, they become ETPs (early T cell progenitor) that are Lin^{-}Sca^{+}CD4^{lo}CD44^{+}CD25^{-}c-Kit^{hi}. At this stage, the expression of IL7R is almost absent, however fate mapping experiments following the expression of GFP under IL7R

promoter activity revealed that ETPs must have passed at some point in their development through a step in which IL7R signaling was necessary [241]. In addition, as ETPs differentiate to DN1 and then DN2 cells, IL7R expression is upregulated on the cell surface to allow cell survival through Jak1/3-Stat5 mediated transcription of Bcl-2 and Mcl1 [242-245]. The other role of IL7 at this stage is to promote the proliferation of pre-T cells [244, 245]. Two subsets of ETPs are distinguished by the expression of Flt3. The Flt3 positive ETPs are 10-20 times more efficient at generating DP thymocytes than are their Flt3 negative counterparts [239]. As ETPs commit to the T cell lineage, they progress toward the DN1c to DN1e sub-populations which are defined based on the expression of CD24 and c-Kit to obtain $CD24^{hi}$c-Kit^{hi} DN1c, $CD24^{+}$c-Kit^{-} DN1d and $CD24^{-}$c-Kit^{-} DN1e [229]. The DN1 population is heterogeneous and the T cell fate of pre-T cell is not exclusive. It has been shown that the DN1 still have the potential to become NK cells, DCs or myeloid cells but not B cells [246, 247].

A particular regulator of pre-T cell differentiation at this commitment step is Notch signaling that is crucial to repress genes that may mediate other lineage choices [248, 249]. As mentioned earlier, mammals express four different Notch receptors referred to Notch1 to Notch4, and the thymus harbors Notch1, 2 and 3. Two ligand families are available for interaction with Notch; the jagged family including jagged-1 and 2 and the delta-like (DL) family, which includes DL-1, 3 and 4 where DL4 is the main interacting protein in the thymus for Notch1. DL1 is also an interaction partner for Notch1, albeit to a lesser extent. Upon binding of Notch to its ligand, the proteolytic cleavage of Notch by the γ-secretase releases the intracellular part of Notch (ICN) that will enter the nucleus. Once in the nucleus, ICN forms a heterodimer with RPB-Jk, converting this transcription factor from a repressor to an activator [135, 167, 248]. This heterodimer binds the CSL element located in Notch target gene promoters, which allows their transcriptional activation. Notch signaling in pre-T cells promotes the expression of T lineage specific genes such as *Gata-3*, *Runx1*, *Hes1* and *Ptcra* [157, 181, 248-251].

Transcription factors Gata-3 and Runx1 as well as several E-box family proteins are necessary to initiate T cell differentiation [135, 232]. Gata-3 is a protein from the zinc finger category of transcription factors, detected in cells from the ETP to the DN3 stages of development [252-254]. Its ablation in mice results in a complete loss of fetal T cells. However, few ETPs and normal LMPPs and CLPs in the fetal liver of these mice remain [252]. In contrast, LckCre recombinase mediated deletion of Gata-3 starting in DN3 does not affect the thymus [253]. It seems that Gata-3 is indispensable for ETP development without affecting survival and cell cycle [253, 254]. Hes1, a known direct Notch target gene, is also a transcription factor but from the basic helix loop helix family [255, 256]. High levels of Hes1 are present in ETPs and DN1 subsets. Hes1 knockout mice show no DP cells and low DN numbers [257]. Runx1 cooperates with CBFβ, which is the non DNA binding partner of Runx1 in early T cell. Deficiency in CBFβ impairs the differentiation of ETPs to DN2 and abrogates the formation of more mature T cells in the thymus [239, 258]. Strikingly, Notch expression was unable to overcome this defect. E-box transcription factors such as E2A and HEB that form heterodimers cooperate with Notch and directly bind Notch target genes at E-box sites [255]. Loss of either E2A or HEB causes a partial block at the DN or ISP (immature single positive) stage [259]. E2A also seems to play a role in the bone marrow, as its absence induces Flt3 down-regulation and loss of CCR9 expression. This may affect the ability of ELPs to seed the thymus and the suppression of their myeloid potential [239, 260].

Other factors are crucial for T cell commitment; the transcription factor Bcl11b is one of these. Its expression is elevated in ETPs and DN2 cells. Mice deficient for Bcl11b show a block at the DN2 stage and instead of pursuing a T cell commitment process, they switch towards an NK cell fate [261, 262]. Even though PU.1 is considered to be a transcription factor specific for the myeloid lineage, restriction or control of its expression in the early T cells is necessary for their development, as PU.1 ablation results in a complete loss of T cells [263, 264]. PU.1 regulates Flt3 and IL7R, which are crucial for the survival and commitment of early T cells [264, 265]. However, higher levels of PU.1 convert cells with

a T cell lineage fate towards macrophage and DC cell development [263, 266]. Recently, TCF-1 (T cell factor 1) has been identified as the main downstream Notch signaling effector in pre-T cells with a function of imprinting a T cell fate [267, 268]. In the absence of TCF-1, ETPs were reduced and DN2 cells were almost completely lost [267, 268]. In addition, these TCF-1 deficient ETPs were able to sense Notch signaling by up-regulating the Notch targets Deltex and Hes1, but T cell lineage specific gene expression was impaired [267, 268]. In contrast, over expression of TCF-1 enhances T cell specific gene expression even in the absence of Notch signaling, however both transcription factors need to collaborate to ensure optimal expression of T cell genes [267, 268]. Since Notch binds directly to the CSL domain of the TCF-1 promoter, it is likely that Notch induces TCF-1 in ETPs to drive T cell commitment [267, 268].

3.3.1. TCR recombination

As ETPs progress to the DN2 stage, the cells become committed to the T cell lineage and TCR gene recombination is initiated and completed at the DN3 stage. The lymphoid specific recombinases Rag1 and Rag2 are upregulated in DN2 and DN3 cells by IL7 but are already present in HSCs and lymphoid precursors [239]. The T cell receptor (TCR) comes in two variations, either as an α/β or a γ/δ heterodimer. Recombination of β, γ and δ TCR gene loci is initiated at the DN2 stage [269-271]. The TCR chain is composed of four parts for the TCR β and δ; the V (variable), D (diversity), J (joining) and C (constant) regions and of three parts for the TCR α and γ; the V (variable), J (joining) and C (constant) regions. To produce a functional TCR, the multiple exons that are dispersed throughout the loci must be joined by a process called V(D)J recombination, which is catalyzed by the recombinase enzymes Rag1 and Rag2 [269-271]. There are two copies of each TCR locus (one on each allele) but only one fully rearranged locus will be produced, a phenomenon called allelic exclusion even if there are some exceptions that have been found including dual bearing $TCRV_{\alpha}$ and $TCRV_{\beta}$ T cells [272]. However, if

errors in the V(D)J recombination process occur, recombination can continue on the same chromosome until all available exons are used, and at that point, rearrangement is pursued on the second allele [269-271]. Many gene segments are located in each locus. For the β locus, 20Vβ, 13Jβ and 2Dβ elements are present and for the α locus, 50 Vα and 50Jα exist. In the case of the γ and δ genes the number of segments is much smaller. However, the possibility to re-arrange two consecutive D segments considerably increase the probability to re-arrange correctly the TCR δ chain [269-271]. The first step to occur in V(D)J recombination is the pairing of D to J segments, following which a V region will join the DJ segments. A recombination signal sequence (RSS) made of a conserved heptamer and nonamer separated by spacer of either 12 or 23 bp flanks each gene segment. The 12-23 rule consists in the recombination of a 12 bp spacer RSS only with a 23 bp spacer. The Rag1 and Rag2 enzymes recognize the RSS and cleave DNA [269-271]. Rag cleavage is a two steps process: Rag1/2 and HMGB1 (high-mobility group protein B1) bind one RSS forming a signal complex to capture a second RSS, which lacks bound protein forming a paired complex. In the first endonuclease step, Rag1/2 create a single strand nick between the heptamer and the gene. The 3' hydroxyl group then attacks the other strand to make a double strand break. This process generates a blunt 5' end at the heptamer sequence and a hairpin at the coding end [269-271]. The double strand break created is repaired through the non-homologous end joining pathway (NHEJ) [273, 274]. Rag1/2 recruit Ku70/80 at the DNA ends, working principally as a docking site for other NHEJ proteins. Ku proteins form a complex with DNA-PKs and Artemis, which has the nuclease activity to open the hairpin. The TdT (terminal deoxynucleotidyl transferase) processes the DNA ends by randomly adding nucleotides to the single strand. Finally, the DNA ends are joined by the DNA ligase IV in cooperation with XRCC4 [273, 274] (Figure 10).

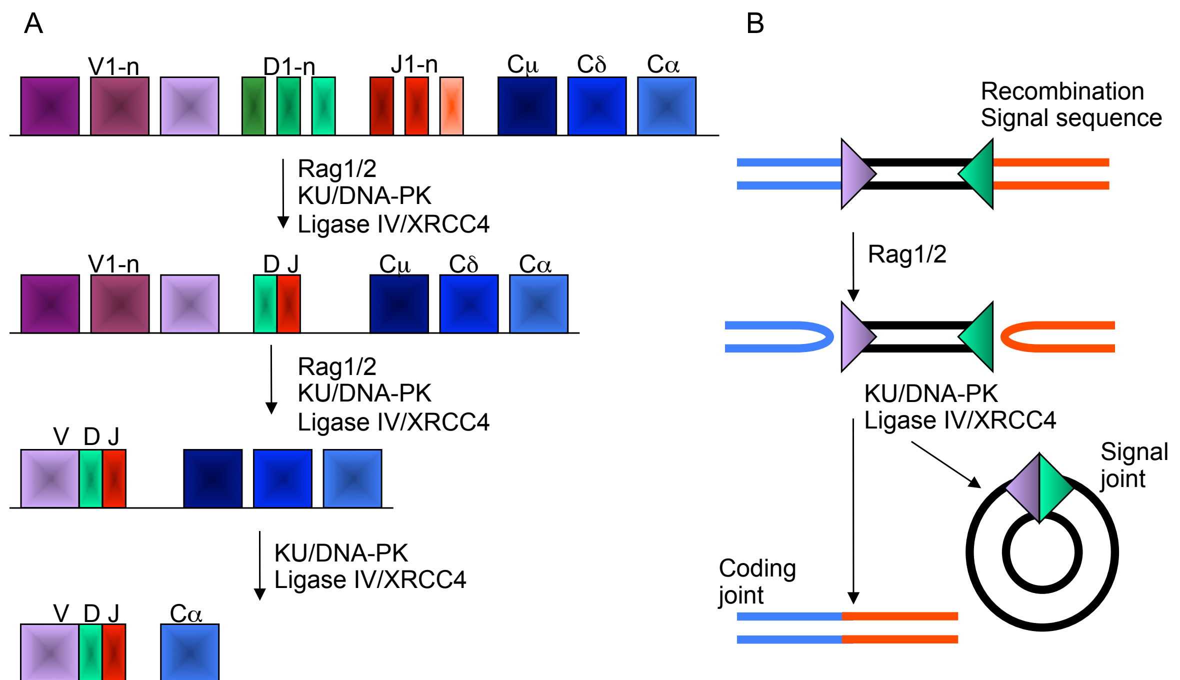


Figure 11. TCR recombination. A) The TCR locus is composed of V, D, J and C segments that need to be brought together during TCR rearrangement. By the action of Rag1/2, KU/DNA-PK, ligase IV and XRCC4, the D and J segments are recombined first. This is followed by the joining of the V segment to the DJ segment and the joining of the VDJ segment to the constant region (C). B) Rag1 and Rag2 recognize the recombination signal sequences found in the vicinity of the V, D and J segments. Rag1/2 cleave the DNA, which produces a hairpin structure. The non-homologous end joining pathway (NHEJ) proteins are recruited and form a coding joint and a signal joint that will be eliminated by the cell. Adapted from [273].

During recombination, both IL7R and Notch are important mediators, which induce a different outcome for the β and the γ/δ loci [229, 261]. In the absence of IL7R, thymocytes are blocked at DN2 stage and TCRγ/δ cells fail to develop [275]. IL7R signaling is known to promote cell survival with the expression of Bcl-2, however, crossing of IL7R$^{-/-}$ with a transgenic mice expressing Bcl-2 fails to rescue TCRγ/δ cells [242, 243, 276]. IL7 sustains the expression of Rag1/2, however in absence of IL7R, TCRγ/δ cells are more affected than TCRα/β cells, so the Rag1/2 expression level cannot explain the entire phenotype observed

[275, 277]. To be rearranged, the TCR loci should be accessible. IL7R is composed of the IL7Rα chain and the common γ chain that is associated with Jak3 kinase [243]. Deletion of either of these molecules, results in the same impaired TCRγ/δ rearrangement, therefore signaling through Jak3 is essential [277]. Surprisingly, when the enhancer of the TCRγ locus was analyzed there was no change in the binding complex in the presence or absence of IL7 signaling. However, the sterile transcript from both the constant and variable genes were decreased and careful analysis of the methylation status of the TCRγ gene revealed a hyper-methylation in $IL7R^{-/-}$ mice, restricting the accessibility of recombinases to the gene. Hence, IL7 regulates TCRγ/δ rearrangement by modifying the epigenetic landscape of the genes [277, 278].

Some experimental procedures revealed a Notch requirement for both TCRβ and TCRγ/δ rearrangement [279-281]. First, complete deletion of Notch results in a partial block at DN3 with an impaired V to DJ recombination [281]. Also, fetal liver HSCs cultured on OP9-DL1 give rise to both TCRα/β DP and TCRγ/δ that are fully functional. Treatment with the γ-secretase inhibitor that inhibits Notch signaling affects the generation of both subsets in a dose dependent fashion but to a greater extent the TCRα/β cells. In contrast, sorted DN3 in OP9 culture generated TCRγ/δ cells [282]. Thus only uncommitted TCRγ/δ cells require Notch signaling.

3.3.2. TCR commitment

Because all three chains of the TCR are recombined at the same time, the question remains open as to how a cell chooses to become $TCR\beta^{+}$ or $TCR\gamma/\delta^{+}$. Two hypotheses have been proposed to explain this conundrum; TCR signaling strength and TCR commitment [229, 261, 283]. One piece of evidence for pre-committed T cells is the identification of a specific regulator of the TCRγ/δ lineage: the transcription factor Sox13 [283, 284]. It has

been demonstrated that a DN2 IL7R^{hi} population, which is prone to γ/δ commitment, expresses higher levels of Sox13 than the IL7R^{lo} population that gives rise to αβ cells. The deletion of Sox13 blocks the generation of TCRγ/δ cells. In contrast the over-expression of this transcription factor impairs transition from DN to DP transition, which requires a TCRβ chain [283, 284].

However, the TCR signal strength hypothesis is supported by strong evidence. DN1 cells are bipotent for αβ and γδ TCR while DN2 cells express at 55% TCRβ alone, at 39% TCRγδ and TCRβ, and only 3 % of DN2 cells have on their surface TCRγδ alone . Definitive commitment arises only in DN3 where cells are either TCRβ or TCRγδ [282]. The hypothesis claims that strong TCR signaling will promote TCRγδ cell development, whereas weak signaling will result in TCRαβ commitment [283, 285, 286]. TCRγδ is expressed at higher levels on the cell surface than TCRαβ. This expression level might potentiate the TCRγδ signal observed in these cells instead of a "ligand-TCR" interaction control of the TCR intensity signal. TCRγδ positive DN3b cells exclusively produce TCRγδ cells when subjected to strong TCR stimulation. On the other hand, TCRαβ DP cells could become TCRγδ positive when a strong signal was induced [285, 286]. Signaling through TCRγδ is associated with strong Erk1/2 activation leading to increased expression of Id3 (DNA binding protein inhibitor 3) a negative regulator of the E-box transcription factor E2A, which is known to promote TCRαβ cells development [287, 288]. The use of a knockin strategy to produce a GFP tagged TCRδ provided visual evidence that TCRδ expression in DN3 cells potentiates the induction of more TCRγδ rearrangement [289]. Furthermore, stronger TCR signaling induces CD5 expression on the cell surface and in the IL2-GFP reporter mice, the CD5^{hi}DN3b population becomes almost exclusively TCRγδ cells [290, 291]. Therefore, even if the TCR pre-commitment hypothesis cannot be ruled out, the TCR signaling strength model remains the more accepted model to date.

3.3.3. β-selection

Upon functional rearrangement, TCRγδ or TCRβ chains are expressed on the surface of DN3 cells. The TCRγδ is expressed directly at the cell surface without a need for a pre-TCR stage. However, there is a checkpoint at the DN3 stage mediated by an unknown signal, as DN3 cells up-regulate the expression of CD5 and CD27 and increase in size [283]. With maturation, the level of CD24 on cell surface is down-regulated. However, it has been suggested that Vδ is able to mediate oligomerization of TCRγδ, an observation based on the results of an in vitro study which showed that Vδ forms homodimers [283]. In the case of TCRβ, it associates with the surrogate pTα chain and CD3 to form the pre-TCR, which mediates β-selection [292]. Cells undergoing β-selection (DN3a cells) reduce expression of CD25 and up-regulate CD27 to become DN3b cells [293]. Failure of DN3a cells to successfully recombine the TCR results in cell death. The signaling cascade produced by the pre-TCR is ligand independent and is either mediated by the dimerization of the pre-TCR on the cell surface or as it has been proposed, by a constitutively activated pTα that is palmitoylated, which targets the pre-TCR to lipid microdomains [294, 295]. The β-selection process promotes cell survival, expansion and differentiation of selected pre-TCR positive cells (DN3b cells) towards DN4 cells. Then, expression of both CD4 and CD8 are induced and DN4 cells become DP cells [296]. Like the TCR, pre-TCR signaling involves phosphorylation of ITAM domain of CD3 and the kinase activity of Lck, Zap70 and Syk, as well as exchange factors and GTPases [296-298].

At this transition step, p53 plays a role by inducing cell death in DN3 cells that failed to generate successful TCR rearrangement [299]. Rag knockout mice are known to be unable to generate DP cells, however deletion of p53 or γ-irradiated induced mutation of p53 in these mice results in the differentiation of DN3 cells into DP bypassing the β-selection checkpoint [299-301]. In addition, constitutively active Lck also inactivates p53 and therefore prevents cell death [298]. A new study identified a particular role of Miz-1 (Myc-

interacting zinc finger protein 1) as a potential regulator of p53 during β-selection. Inactivation of Miz-1 causes a block in DN3 and increased apoptosis due to up-regulation of p53-dependent target genes, which induce cell death [302].

Notch is required during β-selection as it is for pre-T cell commitment, but its effector role differs from previous developmental steps [303-306]. In vitro experiments show that DN3 cells from $Rag2^{-/-}$ mice transduced with a transgenic TCRβ cultured on OP9/DL1 cells were able to differentiate into DP, but on OP9 alone they were blocked at the DN3 stage [306]. Notch withdrawal induced apoptosis, which was independent of TCRβ expression and the anti-apoptotic protein Bcl-2 [303, 305]. In fact, during β-selection, Notch regulates glucose metabolism through Glut1 (glucose transporter 1). This activity of Notch on glucose uptake is mediated by Akt-PI3K, which has been confirmed by constitutive activation of Akt, which can substitute for Notch signaling [303]. Recently, new evidences have demonstrated a pivotal role for the chemokine receptor CXCR4 as a co-stimulator of the pre-TCR during β-selection [307-309]. CXCR4 is found mostly on DN2 and DN3 subsets and it interacts with CXCL12 produced by the cortical stroma of the thymus [310]. Deletion of CXCR4 or its inhibition by the chemical compound AMD3100 results in the accumulation of DN3 cells and a loss of DN4 cells principally due to increased cell death. These deleted cells were unable to up-regulate Bcl-2A1 (Bcl-2-related protein A1), which is known to be a pre-TCR inducible pro-survival molecule [309]. In contrast to Notch, CXCR4 associated pre-TCR signaling activates principally Erk1/2 kinase, but cooperates with Notch to modulate Akt [307, 308]. E-box transcription factors such as HEB and E2A are crucial for early T cell development but also for β-selection [311]. Over-expression of E2A results in growth arrest and apoptosis of DN3 cells while its deletion in $Rag2^{-/-}$ background induces DP formation [296]. It therefore seems that E2A is required to inhibit the differentiation in the absence of a pre-TCR. It is known that both E2A and HEB are negatively regulated by Id3 by forming a complex which blocks their DNA binding capacity [296, 312]. Both pre-TCR and CXCR4 co-stimulation induces Erk1/2 activation. Therefore, Id3 expression is induced

by Erk1/2 through Egr1 (early growth response protein 1) during β-selection allowing the cell to develop into ISP and then DP [293].

3.3.4. Positive Selection

After the β-selection step that promotes differentiation to DN4 and finally induces the expression of both CD4 and CD8, the DP subset reduces the levels of the pre-TCR on the cell surface. Rag1/2 recombinases are re-expressed but this time in an IL7 independent manner, to allow the TCRα locus rearrangement [313]. The lifespan of these cells is short, unless they are able to engage their fully rearranged TCRαβ with the MHC class I and II. The DPs move throughout the cortex to find MHC molecules presenting self-antigen, to form a complex expressed on the cell surface of cTEC (cortical thymic epithelial cells) [314, 315]. TCR rearrangement generates a diverse repertoire of TCRs with different specificities. In the case of a thymocyte bearing a certain TCR that is unable to recognize the MHC-self-peptide complex, the cell dies by neglect [315, 316]. The majority of the DP cells, however, will interact with low avidity with the MHC-self-peptide complex, resulting in a weak signal and be therefore positively selected [316-318]. These selected DPs will then increase TCRαβ and CD69 expression. In contrast, CD4 and CD8 co-receptors will be downregulated ($CD4^{lo}CD8^{lo}$). Thereafter, DPs finally commit to becoming CD4 or CD8 SP cells and harbor on their cell surface only one of the co-receptors and a TCRα/β having only one specificity [229, 313].

The TCR signal induced during positive selection is similar to the signal generated at the β-selection stage. This includes the phosphorylation of CD3ζ and the tyrosine kinases Lck, Zap70 and Erk1/2, and finally calcium mobilization leading to calcineurin mediated NFAT relocalization and signaling [313, 319]. To be positively selected, the signal received should result in low but sustained Erk1/2 phosphorylation. In cells missing CD3ζ chains

there is a block in positive selection due to a lack of Erk1/2 activation [320-322]. In fact, a positive selecting ligand needs to be able to lead to an incremental increase in activation of CD3ζ and Zap70, which in turn mediates the translocation of RasGRP1 to the Golgi apparatus. This translocation of RasGRP1 is correlated with sustained Erk1/2 phosphorylation [313, 323].

The transcription factor Bcl11b (B-cell lymphoma/leukemia 11b) has also been identified as a factor controlling positive selection [262, 324]. In the absence of Bcl11b, TCRα is rearranged and assembled with TCRβ chains on cell surface. However, the proximal TCR signals and calcium mobilization are impaired [262, 324]. Bcl11b deficient DPs are more susceptible to spontaneous apoptosis and there is an altered balance between the pro-apoptotic and the pro-survival factors. The cell survival action of Bcl11b is thought to be mainly Bcl-2 independent [262, 324].

Recent studies also found a potential role for Themis (thymocyte expressed molecule involved in selection) in positive selection [325-327]. Themis is part of a gene family of unknown function but its expression in thymcocytes correlates with late DN and DP subsets and decreases after the positive selection stage [326, 327]. Furthermore, following TCR activation Themis is tyrosine phosphorylated. In a knockout mouse model of Themis, a defect in positive selection was detected and was related to a slight change in calcium mobilization and Erk1/2 phosphorylation [325-328]. Since the threshold of activation is crucial during positive selection, a small difference in Erk1/2 and calcium through the action of Themis might be the key feature.

The co-receptor CD4 recognizes self-peptides in the context of MHC class II. This antigen presentation through MHC class II depends upon the cleavage of the invariant chain leading to CLIP (class II-associated invariant chain peptide) formation by the cathepsin protease family in the endosomes [329-331]. While in almost all cell types the proteolytic cleavage is mediated by Cathepsin S, the cTECs only express Cathepsin L [329-331]. This

substitution may select different antigens to be presented on cTECs surface for positive selection. The principle is slightly different in CD8 cells, since they recognize peptides through MHC class I presentation. In this case, the antigen presentation depends on the activity of the proteasome [332-334]. In cTEC the proteasome composition differs from the mTECs (medullary TEC) and the mDCs (medullary DC). They express exclusively the subunit β5T, which is associated with an increase in the subunits β1i and β2i [332-334]. This complex has been identified as the thymoproteasome and it possesses a decrease chymotrypsin-like activity compared to other proteasome complexes. Even though that deletion of β5T results in an altered positive selection of CD8, not all cells depend on antigen presentation mediated by this thymoproteasome complex [332-334].

3.3.5. CD4 versus CD8 fate

Once the pre-selected DP cells have successfully passed through positive selection they become the post-selected DPs that express CCR7 and move toward the medulla [335]. These cells down regulate *Cd8* gene transcription to generate the intermediate subset $CD4^{+}CD8^{lo}$. They then need to choose their lineage fate by becoming either CD4SP or CD8SP [336, 337]. Diverse models have been proposed to explain the mechanism of CD4/CD8 lineage fate. The first, which has been almost eliminated was the "stochastic model". It suggested that the lineage choice was random: the correlation between lineage choice and MHC class restriction was achieved by the elimination of the cells whose co-receptors did not match their MHC-TCR specificity. Experimental evidence suggests that the second model better explains the CD4/CD8 fate better. The "instructive model" suggests that the strength and/or duration of the TCR signal after engagement with the MHC dictates the divergence between CD4 and CD8. In this model, a strong TCR signal will generate CD4SPs while a weak signal will give rise to CD8SPs [336, 337]. It is known

that the co-receptor CD4 possesses a higher affinity for Lck than CD8, therefore CD4 is more potent to recruit Lck and augments TCR signaling intensity [69].

Both *Cd4* and *Cd8* gene transcription are regulated by cis-elements as opposed to only promoter action; with regards to CD4, it is a silencer element in the first intron while the CD8 locus contains 4 enhancer elements termed E8I-IV [336, 337]. Lineage specific factors have been identified to regulate the expression of CD4 and CD8. Among them, there is ThPOK a transcription factor of the BTB/POZ domain family containing a kruppel-like zinc finger and a regulator BTB/POZ domain [336-338]. The expression of ThPOK is highly specific; it is absent from DN and DP populations and starts at a very low level in the $CD4^{lo}CD8^{lo}$ DP subset. However, there is a marked increase and sustained expression in the intermediate DP population, while CD8SPs contain only background levels [336-338]. ThPOK is necessary and sufficient for CD4SP commitment. Constitutive expression of ThPOK leads to a complete developmental bias toward CD4 while $b2m^{-/-}ThPOK^{-/-}$ mice (b2m is the β2 microglobulin component of MHC class I molecule i.e. MHC class I knockout) generate CD8 cells that are both MHC I and MHC II restricted [336, 337].

Another transcription factor regulating CD4 transcription is Gata-3. It is thought to be upstream of ThPOK and to bind the Gata consensus sequence found in the *Thpok* locus [337, 339]. Its expression starts at early positively selected DP stage cells and is controlled by the TCR signaling strength. Gata-3 deficient mice have a block in CD4 development without affecting the CD8 population [254, 337, 339]. Alone, Gata-3 is insufficient to direct CD4 lineage fate but it is essential to support their commitment. Recently, the discovery of Tox has increased the complexity of CD4 lineage choice [340, 341]. Even if its expression is not CD4 biased, it is required for CD4SP development. In mice deficient for the *Tox* gene, there is no $CD4^{+}CD8^{lo}$ subset present in the thymus. The CD4SP population is absent but the CD8SP is only moderately affected without a re-direction of MHC class II cells toward CD8 fate. The over expression of ThPOK in these cells gives rise to some CD4SP cells. However, the expression of *Cd4* genes was still affected by the

lack of TOX, including *ThPok, Cd4* and *Id2*, suggesting a role for Tox in CD4 commitment independent of ThPOK [340, 341].

The CD8 lineage specific transcription factor is Runx3 whose expression is absent in CD4 cells [342]. The CD4 silencer contains a binding site for Runx3 [343]. Constitutive expression of Runx3 causes a redirection of MHC class II specific cells into CD8. In contrast, deletion of Runx3 does not produce the reciprocal result, but leads to a decrease in CD8 cells without abrogating them completely [337, 342]. Two hypotheses are proposed to explain the role of Runx3; either Runx3 constitutively represses ThPOK unless it is counteracted by a CD4 specific factor, or Runx3 functions upstream of a factor that blocks ThPOK during lineage commitment [336, 338, 343-345].

TCR signal interruption in the $CD4^{+}CD8^{lo}$ population makes these cells responsive to IL7 signaling. In fact, the IL7R signaling pathway is required to generate CD8SPs. IL7R signals through Stat5a/b and Stat6. E8III-Cre induced deletion of Stat5 and Stat6 at the DP stage leads to a loss of CD8 cells. These results have been confirmed using a Socs1 transgenic mouse. Socs1 is a negative regulator of cytokine signaling through binding to Jak and inhibiting its kinase activity. Signaling through the IL7R induces Runx3 expression even in DPs that have not received a TCR signal, demonstrating a requirement for IL7 to generate CD8SP in the absence of a strong TCR signal [346].

Taken together TCR signaling generated during positive selection might end the transcription of *Cd8* gene and increase Tox expression to enable the maintenance of CD4 expression on the cell surface. There are two possible outcomes at this intermediate stage; either the TCR signal obtained is strong and persists, increasing the levels of Gata-3, which will induce ThPOK to prevent CD4 silencing or, the TCR signal ceases or weakens in comparison to the IL7R mediated signal, which provokes the transcription of Runx3. This will, in return, inhibit ThPOK to block *Cd4* gene expression to generate CD8SP cells (Figure 12).

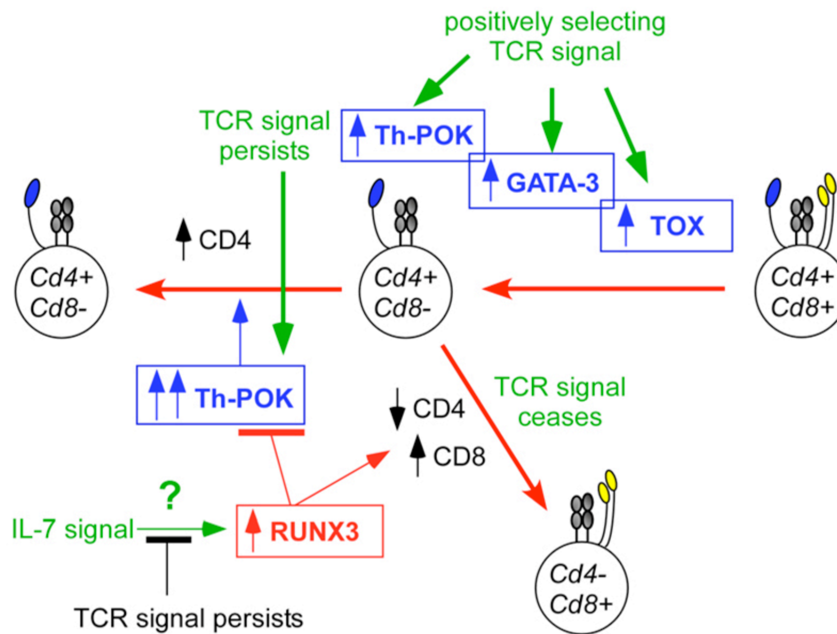


Figure 12. Environmental signals that regulate CD4/CD8 lineage choice. TCR signals generated during positive selection upregulate the expression of the transcription factors TOX, Gata-3 and ThPOK. At the $CD4^{+}CD8^{low}$ intermediate stage and if the TCR signal is persistent, Th-POK expression is significantly upregulated, which prevents *Cd4* gene silencing. However if the TCR signal ceases, IL7 is thought to induce Runx3 expression, which silences *Cd4* gene expression either by directly binding the silencer element of the *Cd4* gene or by inhibiting ThPOK and therefore promoting CD8SP differentiation. From[337].

3.3.6. Negative selection

With such a wide diversity in TCR specificity, SP cells bearing TCRs specific for self-antigen must be deleted to avoid autoimmunity once they are in the periphery. Five pathways of antigen presentation to SP cells have been postulated. First, there is the constitutive production of ubiquitously expressed or thymic-specific proteins presented through MHC class I and II. Second, there is the non-classical sampling of intracellular proteins by autophagy. Third, circulating DCs can import extrathymic antigen. Medullary DCs may also present antigen delivered by the bloodstream. Finally, the one that has been

the most considered hypothesis is the promiscuous gene expression of tissue-restricted antigens by mTEC [347, 348].

The latter involves the discovery of AIRE (AutoImmune Regulator) a transcription factor expressed by a subset of mature mTEC that are CD80^{hi} and MHC class II^{hi}. AIRE knockout mice develop multiple organ autoimmunity [349-351]. The expression of AIRE is regulated by NF-kB2 (p52) and promotes the stochastic expression of genes strictly expressed in peripheral organs. The main mechanism of action of AIRE is not yet resolved. It has been suggested that since it contains several zinc finger domains, AIRE may act as a general transcription factor in mTEC. It is also possible that AIRE regulates differentiation of thymic stroma cells since not all thymic epithelia are equal. However, chromatin immuno-precipitation assays showed that AIRE directly binds to its targets genes, even though no consensus sequence has been found. At its N terminus, there is a CARD/HSR domain, which mediates homodimerization [349, 352, 353]. AIRE also possesses 2 PHD domains (plant homeodomain), the first of these having been described as a histone reader that binds to H3K4me0 [354]. Futhermore, many AIRE interacting proteins have been identified including DNA-PK. However, even if Aire regulates expression of thousands of tissue specific genes in the mTEC, such as insulin 2 and fatty acid binding protein not all genes are AIRE dependent including, for example, GAD (glutamic acid decarboxylase) [347, 351].

How SP cells interpret the signal obtained through the interacting of TCR with MHC during positive and negative selections is obscure, since the downstream events are different (Figure 13). It may involve a conformational change of the TCR by the ligand avidity. While Erk1/2 is important in positive selection, it is rather Erk5 that mediates the downstream signal during negative selection [355]. Negative selection also results in MINK (Misshapen/NIKs (Nck-interacting kinases)-related kinase) activation leading to p38 and Jnk phosphorylation [356]. The two main downstream regulators of negative selection are Bim and Nur77. Constitutively active Erk5 has been shown to promote Nur77 activity [355,

357]. Nur77 is an orphan steroid nuclear receptor which translocates to the nucleus and upregulates the expression of TRAIL (TNF-related apoptosis-inducing ligand) and FasL, both part of the death receptor family. Nur77 is also able to translocate to the mitochondria to associate with Bcl-2. Nur77 interacts with Bcl-2 through the linker between the BH3 and BH4 domains. This interaction leads to the exposure of the BH3 domain converting Bcl-2 from an anti-apoptotic mediator to a pro-apoptotic molecule [358, 359]. This can explain why over expression of Bcl-2 cannot rescue cells from negative selection. Jnk-induced activation by MINK results in an increase in Bim levels, which can inhibit the anti-apoptotic Bcl-2 and Bax leading to apoptosis. The direct role of Cbl in negative selection is unclear. However, deletion of cCbl and Cbl-b potentiates negative selection and induces constitutive phosphorylation of NFkB. Cbl is an ubiquitin ligase known to negatively regulate TCR signaling; it may influence the pathway through Bim [347, 351].

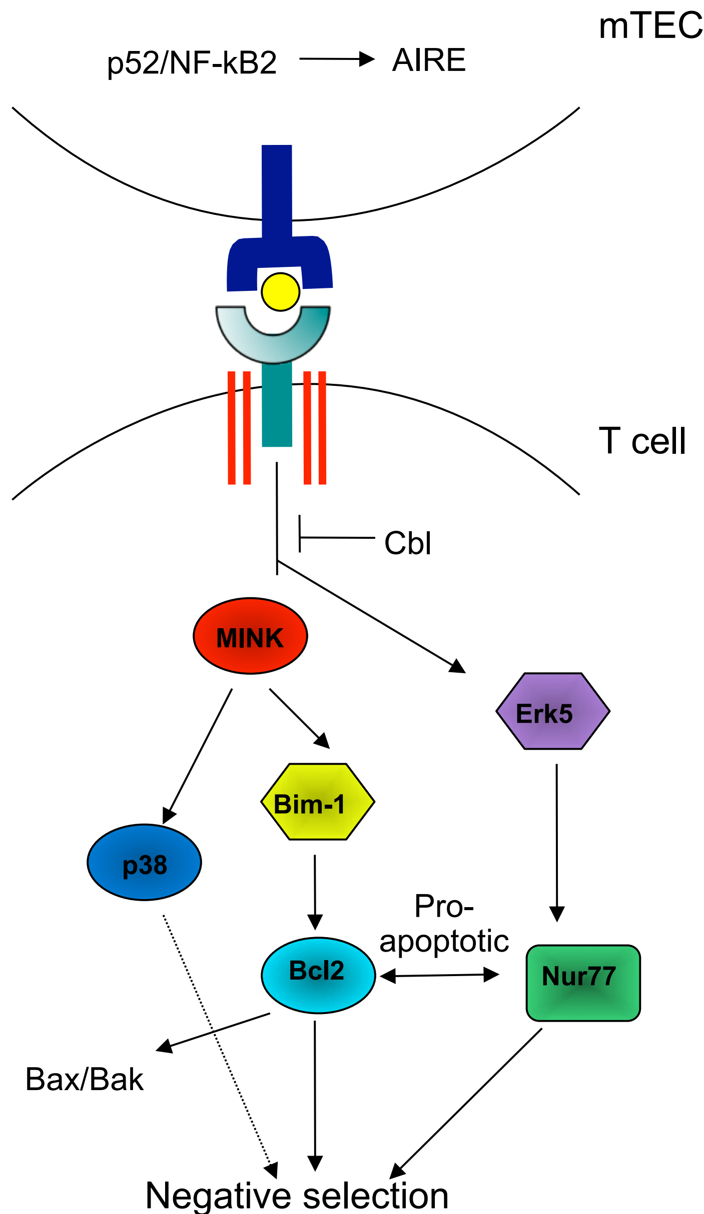


Figure 13. Signal transduction during negative selection. AIRE expression is regulated by NF-κB2 in TEC cells (thymic epithelial cells). AIRE induces the expression of tissue specific antigen in a MHC restricted context. Thymocytes bearing a TCR specific to these AIRE induced self-antigens receive a negative signal. The negative TCR signal induces a cascade that involves Erk5 phosphorylation and MINK (Misshapen/NIKs (Nck-interacting kinases)-related kinase) activation as well as its downstream targets p38 and Bim-1. Nur77 is activated by Erk5 and interacts with Bcl-2 to reverse its anti-apoptotic activity to one that induces cell death. Adapted from [351].

3.4. Migration

Thymocytes do not remain static, but show a dynamic relocalization within different compartments of the thymus to obtain the right signal for development and maturation [335, 360] (Figure 14). The thymus is composed of a peripheral cortex enclosed by an outer capsule that contains the densely packed immature thymocytes, and the central medulla with fewer mature cells. The control of thymocyte migration is mainly coordinated by chemokines, which are secreted by or anchored on stromal and endothelial cells and their respective receptors expressed by the thymocytes. Chemokines are basic low molecular weight proteins (8-10 kDa) that interact with 7 transmembrane protein G-protein coupled chemokine receptors. They are divided into four groups depending on how many residues separate their two cysteines [335, 360].

During fetal thymic development starting at E11.5, prior to vascularization, the chemokine receptors CCR9 and CCR7 and, to a lesser extent CXCR4 become important for T cell progenitors to be able to colonize the thymic primordium [234]. CCL25, CCL21 and CXCL12, the ligands for CCR9, CCR7 and CXCR4 respectively are synthesized by the neighboring parathyroid primordium. A recent study demonstrated through deletion of all three chemokine receptors, that even if the progenitors were able to differentiate efficiently into T cells, they were impaired, as they were unable to reach the fetal thymus. Absolutely no $CD45^{+}$ were detected near the parathyroid anlage [234].

In adult mice, both chemokines and adhesion molecules coordinate the recruitment of ELPs. Expression of P-selectin and CCL25 on endothelial cells at the cortico-medullary junction (CMJ) is correlated with intrathymic occupancy. ELPs from blood are positive for PSGL-1 (P-Selectin Glycoprotein Ligand 1) and CCR9. As for the $CCR7^{-/-}$ $CCR9^{-/-}$ double knockout mice, deletion of PSGL-1 decreases the number of ETPs without changing the total thymic cellularity [310, 335, 360, 361]. The ETPs stay in the CMJ for 10 days to

expand up to 1000-fold and become DN1 cells. As they up-regulate CD25 and differentiate into DN2 and then DN3 cells, they migrate from the inner cortex outwards, toward the subcapsular cortical zone (SCZ) [310, 335, 360]. This migration step inside the cortex is modulated by CXCR4. In its absence, thymocytes are arrested at the DN1 stage and accumulate at the CMJ. At the DN3 stage the cells up-regulate CCR9 following a pre-TCR signal. However, CCR9 seems to be dispensable since CCR9 negative DN3 cells fail to accumulate at the SCZ but thymic development is normal [310, 335, 360]. Developing DP cells travel back to the inner cortex by sensing the level of CXCL12 expressed by the cTEC to obtain the right signal for positive selection. After positive selection, DPs up-regulate CCR7 and move towards the medulla. CCR9 is also expressed on almost all DPs while only a small percentage of them express CCR4 on their surface. The medulla is the critical environment to induce tolerance with mTEC and mDC presenting tissue-restricted antigens. In fact, mice in which CCR7 has been deleted develop autoimmune diseases [362-364]. The level of CCL19 and CCL21 is much higher in the medulla than in the cortex. CD4SP and CD8SP cells remain in the medulla for approximately 4-5 days [335, 363, 364].

Each day, around one percent of all thymocytes are exported to the periphery, which represents one million cells per mouse. It is only recently that the mediator involved in this thymocyte egress was identified. Using pertussis toxin which blocks G-protein coupled proteins and the specific chemical FTY720, S1P and its receptor S1P1 have been shown to be implicated in the thymic egress of T cells [365-367]. S1P is produced by stromal cells in the thymic medulla and by hematopoietic cells in the bloodstream. A S1P gradient is maintained by the balance between its activation by S1P kinase and its degradation by S1P lyase [368]. However, it seems that the only critical source of S1P for the egress of thymocytes is the blood vessel-ensheathing pericytes. The direct regulator of S1P1 expression is the transcription factor KLF2 (Krueppel-like factor 2), which also controls CD62L levels [369, 370]. As for the complete knockout of S1P1, KLF2 deficient cells are absent from the peripheral lymphoid organs and they accumulate in the thymus. Newly committed thymic T cells are $CD69^{hi}$ while mature T cells are $CD69^{lo}$, which coincides

with the expression of S1P1 on their surface. CD69 and S1P1 mutually antagonize the expression of each other on the cell surface [371, 372]. However, the exact mechanism remains unknown.

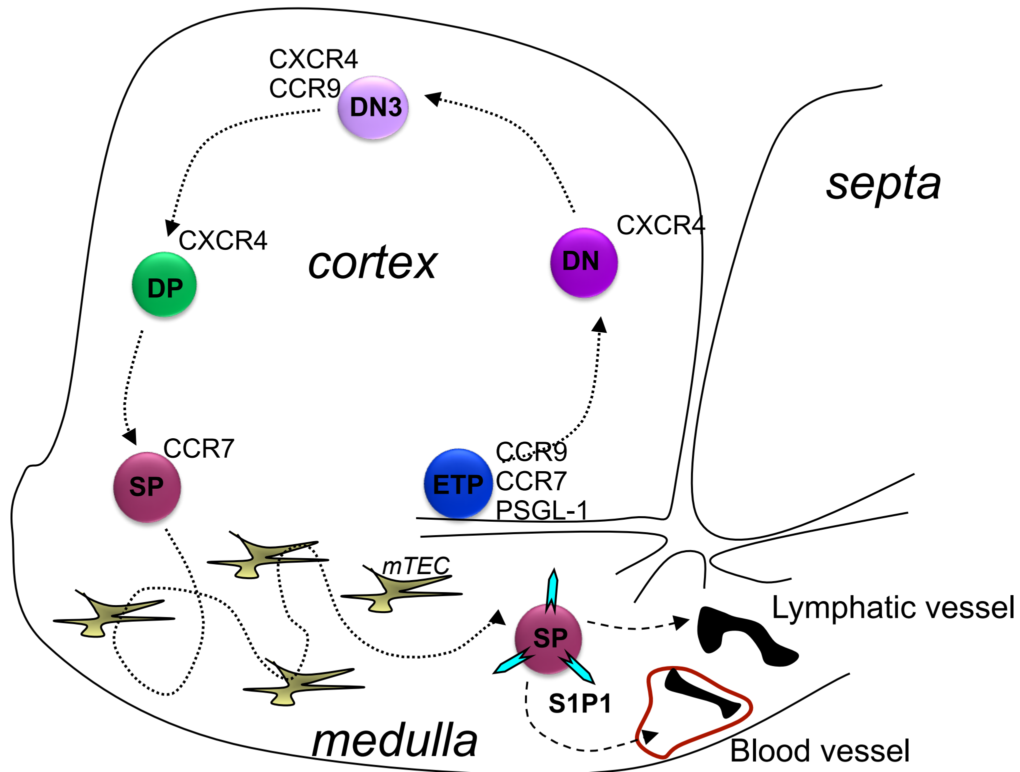


Figure 14. The thymic journey of progenitor cells. Early T cell precursors (ETP) are recruited from the bloodstream through CCR9, CCR7 and PSGL-1 and begin to seed the thymus at the cortico-medullary junction (CMJ). Early DN cells that express CXCR4 migrate towards the outer cortex by sensing a CXCL12 gradient. At the DN3 stage, cells express CCR9 and CXCR4 and are found in the subcapsular cortical zone. After beta-selection, DN3 cells move back inside the cortex and at the DP stage cells are found at the CMJ. Post-selection DPs start to express CCR7 and SP cells migrate into the medulla to sense the antigen presented by the mTEC. Egress of SP cells is achieved through a S1P gradient in the blood. Adapted from [373].

4. Hypothesis and Objectives

As mentioned above, hnRNP L is an important regulator of alternative splicing but it is also involved in mRNA stability and shuttling. HnRNP L binds to exon splicing silencers excluding targeted exons from mature mRNA. Its best known target is CD45, the major tyrosine phosphatase expressed on all hematopoietic cells with the exception of erythrocytes and platelets. The differential isoforms of CD45 generated by alternative splicing control the intensity of TCR signaling in T lymphocytes. The pattern of CD45 isoform expression varies with hematopoietic cell types and differentiation stages. Some of the targets of hnRNP L are known, including CD45, VEGF-A, eNos and integrin alpha2 but most of them still remain unidentified.

The hypothesis of this thesis is that alternative splicing mediated by hnRNP L is crucial for hematopoiesis. To verify this hypothesis, three objectives have been defined. The first objective was to investigate the function of hnRNP L in T cell differentiation and activation and characterize the effect of hnRNP L ablation on the alternative splicing of CD45 in vivo in T cells. The second objective was to determine the consequences of hnRNP L ablation with regards to HSCs and early hematopoietic differentiation. The third objective was to identify the specific targets of hnRNP L in T cells and hematopoietic stem cells by assessing the global effect of hnRNP L on all transcripts.

5. Experimental model to study hnRNP L function-generation of a loss of function mouse mutant

A very powerful way to characterize the function of a protein is to generate a loss of function mutant, a knockout mouse for instance, in which the gene encoding the protein is deleted. For this thesis, a mouse model deficient for hnRNP L was generated and is the first one to be developed for this protein. The gene targeting of hnRNP L resulted in an arrest of embryonic development at a very early stage (E3). Therefore a conditional knockout mouse using the Cre-Lox recombination system was used to generate animals where the gene of interest is flanked by LoxP recognition sequences. These floxed alleles were generated by homologous recombination of a targeting vector in embryonic stem cells that allowed the integration of the LoxP sites into the germline at the hnRNP L locus. The expression of the targeted hnRNP L gene is not affected since the LoxP sites are situated within introns. Upon expression of a Cre recombinase, the two LoxP sites are brought together, DNA is cleaved and re-ligated resulting in the deletion of several exons of the hnRNP L gene.

To mediate cell specific deletion of hnRNP L, mice containing two alleles of the floxed hnRNP L gene were crossed with either mice that carry the Lck-Cre transgene that is expressed in T cells from the DN3 stage onwards for a T cell specific deletion or with animals that bear the Vav-Cre transgene which is active in all hematopoietic cells including HSCs. Since Vav-Cre mediated deletion is also embryonic lethal, we also used mice that carried the IFN-α inducible Mx-Cre transgene. To counteract the lethality of Vav-Cre hnRNP $L^{fl/fl}$ mice, we crossed them with transgenic mice carrying a Bcl-2 transgene under the H2K promoter. And finally, to be able to track hnRNP L deleted cells, we used the $Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}$ animals. These mice carry a beta actin promoter, which drives the expression of a dtTomato transgene flanked by loxP sites. A GFP transgene is located downstream of the floxed dtTomato gene and is silent as long as the dtTomato gene is present. These mice constitutively express the tdTomato fluorescent

protein in all cells. Upon Cre recombinase activity, the tdTomato transgene is excised which leads to the expression of the GFP protein since the GFP gene is now under the direct control of the beta actin promoter. This allows for detection of cells that have activated the Cre recombinase by simply following green fluorescence.

Results

Chapter II: Article 1

Alternative splicing controlled by heterogeneous nuclear ribonucleoprotein L (hnRNP L) regulates development, proliferation and migration of thymic pre-T cells

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Running title: hnRNP L in T cell development and migration

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Contribution: M.-C.G. performed research, analyzed data, and wrote the manuscript; F.H. designed and generated the described mouse strain and reviewed the manuscript; R.B. performed actin polymerization experiments; B.W. performed and analyzed the RNA-Seq data and T.M. designed the research, analyzed data, wrote the manuscript, and provided funding.

Abstract

The regulation of post-transcriptional modifications of pre-mRNA by alternative splicing is important for cellular function, development and immunity. The receptor tyrosine phosphatase CD45, which is expressed on all hematopoietic cells, is known for its role in the development and activation of T cells. CD45 is known to be alternatively spliced, a process that is partially regulated by hnRNP L. To investigate the role of hnRNP L further, we have generated conditional hnRNP L knockout mice and found that LckCre mediated deletion of hnRNP L results in a decreased thymic cellularity caused by a partial block at the transition stage between DN4 and DP cells. In addition, hnRNP $L^{-/-}$ thymocytes express aberrant levels of the CD45RA splice isoforms and show high levels of phosphorylated Lck at the activator tyrosine Y394 but lack phosphorylation of the inhibitory tyrosine Y505. This indicated an increased basal Lck activity and correlated with higher proliferation rates of DN4 cells in hnRNP $L^{-/-}$ mice. Deletion of hnRNP L also blocked the migration and egress of SP thymocytes to peripheral lymphoid organs in response to S1P and the chemokines CCL21 and CXCL12 very likely as a result of aberrant splicing of genes encoding GTPase regulators and proteins affecting cytoskeletal organisation. Our results indicate that hnRNP L regulates T cell differentiation and migration by regulating pre-TCR - and chemokine receptor signaling.

Introduction

Thymocytes must pass through a tightly regulated developmental process to mature into effector T cells. First, early lymphocyte precursors migrate from bone marrow to the peripheral blood and enter the thymus where they undergo differentiation (1, 2). The most immature thymocytes termed “double-negative (DN)” are characterized by the absence of both CD4 and CD8 co-receptor surface markers and are subdivided based on the expression of CD44 and CD25 into four fractions called “DN1 to DN4” (3). In DN3 cells, β-selection takes place, which assures the selection of cells with a productive rearrangement of the T cell receptor beta (TCRβ) gene and the correct assembly of a surface pre-TCR complex. From this, DN4 cells emerge and develop further by up-regulating both CD4 and CD8 marker to become “double positive or DP” cells (4-9). These cells then undergo a positive and negative selection to eliminate autoreactive T-cells and to produce the final single positive (SP) CD4 and CD8 expressing T effector cell populations. During these two selection processes, thymocytes receive different signals from the pre-TCR, the TCR, the co-receptors and probably other receptors that promote cell survival and proliferation (4, 10-16).

One of the critical factors that regulate the strength of TCR or pre-TCR signaling is the transmembrane tyrosine phosphatase CD45, which is not only expressed on T cells, but is found on a wide variety of hematopoietic cells, except platelets and erythrocytes (17, 18). This protein exerts its regulatory function by modulating the activity of the src kinases Lck and Fyn (18-22). Multiple isoforms of CD45 can be generated by alternative splicing of the variable exons 4-6, also called A, B and C, which code for different extracellular domains of the protein (23). The expression of a specific isoform of CD45 is cell-type specific and changes during thymocyte development. Immature DPs predominantly express CD45RO, which lacks the domains encoded by exons 4-6, whereas CD4SP or CD8SP cells express the high molecular weight isoform CD45RB, which contains the domain encoded by exon 5 (24-26). The smaller isoforms of CD45 form predominantly homodimers, whereas the

high molecular weight isoform lose this potential, resulting in a less efficient signal transduction (27). A number of studies using CD45-deficient mice have demonstrated a crucial role for this protein, since its absence results in a severely impaired TCR signal transduction and in a differentiation block during positive and negative selection that occurs during the differentiation of DP thymocytes to mature SP cells (28-30).

Several lines of evidence provided by *in vitro* studies of alternative splicing of CD45 reveal the implication of heterogeneous nuclear ribonucleoprotein L (hnRNP L) in mediating this process. The hnRNP proteins belong to a family of RNA-binding factors that regulate alternative splicing by binding exonic splicing silencer elements (ESS) resulting in exon exclusion from the mature mRNA (31). In the case of hnRNP L, its binding to the ESS1 sequence in the alternatively spliced CD45 exons results in their exclusion from the mature, spliced RNA (23, 31).

We show here that deletion of hnRNP L in the mouse results in a very early block of embryonic development emphasizing its crucial role in morphogenesis. In order to study a role of hnRNP L in T cell development and function we have restricted hnRNP L deletion to the T cell compartment by using a T cell specific Cre recombinase transgene (LckCre). This strategy allowed us to reveal new and important roles of alternative splicing mediated by hnRNP L in T cells maturation and migration.

Material and Methods

Mice

C57BL/6, LckCre transgenic, Gt(ROSA)26Sor$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$ and CD45.1 mice were purchased from Jackson Laboratories or were maintained at the Institut de recherches cliniques de Montreal. For the hnRNP L targeting construct, a phage screen was performed that yielded a clone containing around 18 kb of the genomic hnRNP L locus (kindly performed and provided by Dr. R Waldschütz, University of Duisburg-Essen). An 11 kb EcoRV-NdeI fragment was cloned into pcDNA3 for further manipulation. A single loxP site was inserted into the EcoRI site and a neo-cassette flanked by 2 loxP sites was introduced into the Sfi1 site (thereby destroying this site). This construct was found to efficiently recombine in bacteria stably expressing Cre-recombinase. For homologous recombination in ES cells the targeting construct was freed from vector sequences by EcoRV-NdeI digestion. After transfection into ES cells, homologous recombination was confirmed by verifying presence of the neo cassette (StuI digestion) as well as the third loxP site (XmnI digestion) by Southern blot. A positive clone was transiently transfected with a plasmid encoding Cre-recombinase and partially recombined clones with the neo-cassette removed leaving the endogenous locus with only 2 loxP sites inserted were identified by PCR and confirmed by Southern blot (Bcl1/Sfi1 digestion). One clone was used to generate hnRNP L floxed mice using standard procedures. hnRNP L floxed mice were backcrossed to C57BL/6 for more than 8 generations. All animals were housed under specific pathogen-free conditions at the Institut de recherches cliniques de Montreal animal facilities, and all experiments conformed to ethical principles and guidelines approved by the institutional animal care committee.

Antibodies

Anti-mouse CD4 (RM4-5), CD8a (53-6.7), CD25 (PC61), CD44 (1M7), TCRγ/δ (GL3), TCRα/β (H57-597), CD3 (145-2C11), B220 (RA3-6B2), CD45RA (14.8), CD45RB (16A)

antibodies and streptavidin fluorescent conjugated form were purchased from BD biosciences. The "Lineage cocktail" was made from B220, Ter119, CD3, Mac1, Gr1, NK1.1, CD49b, TCRγ/δ and CD8, all biotinylated, and were from either BD biosciences or eBioscience. Phospho-specific antibodies were for LckY505 from BD biosciences, Lck Y394 from Santa Cruz and ERK and Akt from Cell Signal. Goat anti-hamster Ig and HRP and FITC-conjugated anti-rabbit and anti-goat were purchased from Jackson ImmunoResearch laboratories and anti-actin from Santa Cruz.

Immunoblotting

Cell lysates from equal number of cells were prepared by lysis in buffer (20 mM Tris (pH 7.5), 150 mM NaCl and 5mM EDTA), supplemented with complete mini protease inhibitors (Roche Applied Science), 1mM Na_3VO_4 and 1% Nonidet P-40. Western blot analysis were done following SDS polyacrylamide electrophoresis and transferred onto nitrocellulose membranes (GE healthcare). General and specific tyrosine phosphorylated proteins were detected with antibodies followed by goat anti-rabbit or donkey anti-goat coupled HRP. All immunoblots were visualized with ECL chemiluminescent (Thermo Scientific) detection system and images were taken on film

Cell stimulation

Thymocytes were pre-incubated with 10µg/ml of anti-CD3 (2C11) for 10 min on ice. Bound antibodies were cross-linked with goat anti-hamster Ig at 20µg/ml and immediately incubated at 37°C for the indicated time. Cells were processed for surface and intracellular staining according to standard procedure.

Proliferation assays

For *in vivo* proliferation assays, mice were injected intraperitoneally with 200 µl of 10mg/ml solution 5-bromo-2-deoxyuridine (Sigma Aldrich) 16h before sacrifice. Thymocytes were stained with specific fluorescent antibodies, fixed and treated with

Perm/Wash (BD biosciences). DNase I (Sigma Aldrich) treatment at 30μg/ml was applied for 1h at 37°C. Anti-BrdU FITC conjugated (BD biosciences) was added for 30 min at room temperature and events were acquired on a LSR (BD biosciences).

RT-PCR

Total RNA was extracted from thymocytes using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For RT-PCR analysis, RNA was used to prepare cDNA using SuperScript II reverse transcriptase (Invitrogen). PCR for CD45 and Gapdh were done according to previous report (32). To analyze CD45 alternative splicing, an established radioactive RT-PCR protocol was used (33). Briefly, 0.5 μg purified RNA of sorted thymic subpopulations was used for reverse transcription with a gene specific primer and PCR was performed with a 32P-labeled forward primer. Products were separated on a denaturing polyacrylamide gel electrophoresis and quantified by Phosphoimager analysis (Typhoon 9400, ImageQuant both GE Healthcare).

Quantitative PCR

DN3 and DN4 thymocyte subsets were sorted using a MoFlo (Cytomation) and RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA were prepared from RNA using SuperScript II reverse transcriptase (Invitrogen) and detected using Taqman probes (Applied biosystems) for *Ptcra* and *Gapdh* as an internal control. A Mx-3005 system (Stratagene) was used and relative expression was calculated via the 2-ΔΔCT method.

Migration Assay

Total thymocytes were harvested and re-suspended in migration media (RPMI containing 0.5% BSA) at $2.5x10^{6}$ cells/ml. Migration assays were performed in transwell plates (Corning, 5μM pore). Chemokines CXCL12 (Peprotech), CCL21 (Peprotech) and S1P (Avanti Polar Lipids) were diluted in migration media at different concentrations and added

to the lower chamber. After resting for 2h at 37°C, 100μl of cell suspension was added to the upper chamber and cells were allowed to migrate for 3h at 37°C. Cells were collected and stained for CD4 and CD8, re-suspended in 200μl PBS supplemented with 2% FCS and a fixed number of countbright beads (Beckman Coulter) was added. The percentage of migrating cells was calculated by dividing the number of cells that migrated into lower chamber by the input cell number.

Actin polymerization

Thymocytes were collected and re-suspended in RPMI supplemented with 0.5% BSA and rest at 37°C for 2h. Cells were then activated with different concentration of S1P, CXCL12 or CCL21 for 30s at 37°C. Cells were fixed with Cytofix/Cytoperm (BD bioscience) for 10 min on ice and Perm/Wash (BD bioscience). Cells were then incubated for 30 min at room temperature with 1U of phalloidin-Alexa488 (Invitrogen) followed by surface staining for CD4 and CD8. Events were acquired on a LSR (BD bioscience). Relative F-actin polymerization was calculated as the ratio of mean fluorescence of phalloidin-Alexa488 of activated cells over non-stimulated cells.

Recent thymic emigrants

Four to five week old mice were anesthetized with isofluorane. The shaved skin at the thoracic inlet was cut open by a 1 cm incision, one-third of the sternum was longitudinally bisected and the thymus exposed. Each thymic lobe was injected with 10μl of FITC (Sigma) dissolved in PBS at 10mg/ml and the wound was closed by suture. Mice were sacrificed 4 days later.

RNA-Seq

RNA was extracted from total thymocytes using Tri Reagent (Molecular Research Center) followed by a purification using RNeasy Mini kit and RNase-free DNase on column (Qiagen) for 15 min at room temperature both following manufacturer’s instructions. RNA

integrity and quality have been confirmed using a bioanalyzer (Agilent). rRNA from each biological sample was depleted from total RNA using a RiboMinus kit (Invitrogen) and the treated RNA was then fragmented using RNase III. Ligation of the adaptor mix A and reverse transcription were performed following the manufacturer's protocol. Libraries were size selected for fragments between 150 and 300 bp, amplified for 12 cycles of PCR, and purified using PureLink PCR micro kit (Invitrogen). Barcoded library concentrations were determined by quantitative PCR using a standard curve of template at known concentrations (DH10B), and approximately 0.25 ng of each library was used for each full emulsion PCR (emPCR) reaction (4 emPCR/sample). Approximately 200 millions of beads from each two samples were deposited on single full slides (2 slides for 4 samples in total) and sequenced using the Opti Fragment Library Sequencing kit-Master Mix 50 on a SOLiD machine (Version 3+). Mapping of DNA sequence reads was performed using Bioscope (Ver 1.3) against both the mouse genome (mm9) and all annotated splice junctions to calculate gene expression and splice site usage counts.

Statistical Analysis

Two-tailed Student *t* test was used to calculate *p* values where indicated. A p value ≤ 0.05 was considered statistically significant: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Two-way ANOVA was used to calculated *p* value for the migration assay and the actin polymerization.

Results

Constitutive deletion of hnRNP L impedes early embryonic development

To be able to analyze the consequences of a constitutive or inducible ablation of hnRNP L for the immune system and hematopoietic differentiation we used a previously published strategy (34) to generate mice carrying loxP (fl) sites flanking coding exons 2-6 of the hnRNP L gene (Suppl Fig. 1A, B). Animals carrying two hnRNP L floxed alleles (hnRNP $L^{fl/fl}$) were bred with germline deleter strains (CMV Cre) to generate constitutively hnRNP L deficient mice. However, live pups or embryos beyond stage 3.5 dpc could not be generated with this strategy (data not shown) suggesting that the ablation of hnRNP L is lethal at a very early stage of murine development.

To overcome this problem and since RT-PCR analyses revealed expression of hnRNP L in a wide range of hematopoietic cells including lymphoid progenitors and thymocyte subsets (suppl. Fig. 1C), we chose to restrict our analysis to the T cell compartment. To determine the function of hnRNP L in T lymphocytes, we deleted the hnRNP L floxed alleles using mice expressing Cre recombinase driven by the Lck proximal promoter. In these mice, Cre expression starts at the pro-T cell DN2/DN3 stages and is most efficient at and after the DN4 stage. As expected, the floxed alleles in LckCre, hnRNP $L^{fl/fl}$ mice efficiently deleted in pro-T cells and hnRNP L protein expression is almost eliminated in the thymus of these animals (Suppl. Figure 1D,E).

Loss of hnRNP L expression affects thymic cellularity in a cell autonomous manner

Flow cytometric analysis revealed a normal frequency of the different thymocyte subsets in $LckCre^{+}$hnRNP $L^{fl/fl}$ mice with a slight increase of DN cells compared to wild-type thymus (Fig. 1A,B). However, loss of hnRNP L protein led to a decrease in overall thymic cellularity by 75% compared to wt mice (Fig. 1C). Without affecting the absolute DN cell number, deletion of hnRNP L resulted in the loss of DP thymocytes and the more mature

CD4 and CD8 SP cells in about the same proportions (Fig. 1C). In addition, the loss of thymic cellularity in LckCre^{+}hnRNP L$^{fl/fl}$ mice was specific to TCRα/β cells and did not affect TCRγ/δ cells since their absolute number remained unchanged (Fig. 1D,E). Consistent with this, the DN4 population of LckCre^{+}hnRNP L$^{fl/fl}$ mice had a reduced frequency of cells expressing surface TCRβ, but all hnRNP L deficient DN4s expressed the cytoplasmic form of TCRβ. In addition, the expression of *Ptcra* was not disturbed by the ablation of hnRNP L suggesting that a pre-TCR can be made in hnRNP L deficient mice (Fig. 1 F,G).

To test whether the reduced thymic cellularity of LckCre^{+}hnRNP L$^{fl/fl}$ mice could be due to an effect of hnRNP L deletion in thymic epithelial cells, we generated mixed bone marrow chimeras. Congenic CD45.1^{+} recipient mice that were reconstituted with bone marrow cells from LckCre^{+}hnRNP L$^{fl/fl}$ mice (CD45.2^{+}) showed the same defect in thymocyte development as was observed in the original hnRNP L deficient mice (data not shown), suggesting that the phenotype observed upon hnRNP L deletion is cell autonomous.

To discriminate between cells before and after positive and negative selection we stained thymocytes from both wt and hnRNP L deficient mice for CD4, CD8, CD69, and TCR β expression which enables the definition of four maturation stages (35). This analysis showed that the relative percentages of the mature subsets as defined by CD69 and TCRβ expression were not different with regard to the relative frequency of cells between wt and hnRNP L deficient mice. In this analysis, CD4 cells appear before CD8 cells and this sequential appearance is also unaffected by hnRNP L deletion (Suppl. Figure 2A,B). This suggests that there are no major defects in either positive or negative selection or in CD4/CD8 lineage choice due to the loss of hnRNP L.

hnRNP L regulates the splicing of CD45 in vivo

Since it has been suggested that hnRNP L regulates the alternative splicing of CD45 *in vitro* (23, 31) we tested whether the expression of this transmembrane phosphatase was affected by hnRNP L ablation *in vivo*. It has been described that CD45RO and CD45RB isoforms are expressed in thymocytes, whereas the CD45RABC isoform is absent in these cells (26). We detected an increased expression of CD45RB on hnRNP $L^{-/-}$ DP and CD8SP cells compared to the respective wt controls and found the expression of CD45RA upregulated 4-fold over the wt in all hnRNP $L^{-/-}$ thymocytes subsets except DN3 (Fig. 2A,B). An assessment of the mRNA expression level of all possible isoforms of CD45 by RT-PCR indicated that the mRNAs for the CD45RABC isoform that is absent from wt thymus is now present in hnRNP L deficient DN4 and CD8SP cells and to a lesser extent also in DP and CD4SP cells compared to their respective controls (Fig. 2C). In addition, the same analysis also suggested that the mRNA encoding CD45RB and CD45RBC are enriched in hnRNP $L^{-/-}$ DP and DN4 cells over wt controls (Fig. 2C). A more quantitative RT-PCR method using the incorporation of radioactive nucleotides confirmed an enrichment of the CD45RB mRNA over the CD45ROΔ7 mRNA isoforms in DN4 and DP cells (Fig. 2D). These data indicate that absence of hnRNP L leads to an enrichment of mRNA isoforms that encode the larger isoforms of CD45, which is consistent with the role of hnRNP L as one of the proteins that regulate the exclusion of CD45 exons.

hnRNP L modulates the TCR signal in thymocytes

It is known that CD45 is a tyrosine phosphatase that controls the duration of pre-TCR or TCR signaling by removing the inhibitory phosphate group at tyrosine (Y) 505 in the Lck kinase that is associated with the cytoplasmic domains of the TCR complex (22, 36). The removal of the Y505 phosphate enables Lck to phosphorylate its substrate, the ZAP70 kinase (Fig. 3A). It has been shown that larger CD45 isoforms are more efficient phosphatases than the smaller variants such as CD45RO probably owing to their different potential to form homodimers (27). Since the $LckCre^{+}hnRNP\ L^{fl/fl}$ thymocytes, in particular

DN4 cells, expressed aberrant level of the larger CD45 isoforms, we tested whether this affected the phosphorylation status of LckY505. We found a lower level of Lck phosphorylation at the inhibitory Y505 in hnRNP L deficient DN4 cells both at steady state and after anti-CD3 activation (Fig. 3B,C). Interestingly, the phosphorylation of the activating tyrosine Y394 of Lck as well as the downstream tyrosine kinases ERK and Akt was increased in these DN4 cells over wt controls at steady state levels (Fig. 3 C,D). These results suggest that hnRNP L controls TCR signaling intensity through the alternative splicing of CD45.

Deletion of hnRNP L in thymocytes results in aberrant cell proliferation

Next, we assessed whether the aberrant expression of CD45 isoforms and the ensuing deregulation of Lck phosphorylation had any affect on cell proliferation or cell death, which could explain the lower thymic cellularity in hnRNP $L^{-/-}$ mice. Staining with Annexin V for apoptotic cells did not show any differences between hnRNP L pre-T cell subsets compared to wt controls (Fig. 3E). However, *in vivo* BrdU pulse labeling clearly indicated that DN4 and CD4SP cells from hnRNP L mice progressed faster through S phase than their wt counterparts (Fig. 3F). This result indicated that thymocytes from $LckCre^{+}$hnRNP $L^{fl/fl}$ mice are not apoptotic, but have shortened cell cycle phases resulting in higher proliferation in stages following the β-selection checkpoint.

Deletion of hnRNP L results in loss of peripheral T cells

We next investigated whether hnRNP L deletion and the differential expression of CD45 isoforms affected CD4 or CD8 SP cells. We noted that the frequency of peripheral CD3 positive cells was considerably reduced in blood and spleen of $LckCre^{+}$hnRNP $L^{fl/fl}$ mice compared to wt animals and that this reduced frequency corresponded to a significantly reduced absolute number of peripheral $CD3^{+}$ T cells (Fig. 4 A, B). Importantly, the loss of peripheral T cells affected both CD4 and CD8 cells equally (Fig. 4B). A genotyping PCR analysis showed the presence of both floxed and excised hnRNP L alleles in sorted

peripheral $CD3^+$ T cells of $LckCre^+hnRNP\ L^{fl/fl}$ mice, although only fully excised alleles were found in CD4SP and CD8SP subsets in the thymus (Suppl. Fig 1D). This suggests that peripheral T cells are counterselected for complete excision, or that CD4SP or CD8SP thymocytes that have completely excised hnRNP L are not migrating to the periphery. To investigate this hypothesis further, we quantified recent thymic emigrants by injecting FITC into the thymus of wt or $LckCre^+hnRNP\ L^{fl/fl}$ mice. Four days after injection the mice were sacrificed and blood and spleen were analyzed for the presence of FITC labeled CD4 or CD8 T cells by FACS. A quantification showed clearly that FITC labeled cells were significantly reduced in the periphery of hnRNP L deficient mice over wt controls (Fig 4D), suggesting a reduction of thymic egress caused by the deletion of hnRNP L.

Next, $LckCre^+hnRNP\ L^{fl/fl}$ and $LckCre^+hnRNP\ L^{wt/wt}$ were crossed with $Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}$ mice (hereafter $ROSA^{fltomatofl-GFP}$). These animals allow the tracking of cells by measuring red fluorescence (tomato) and Cre activity by measuring green fluorescence (GFP). Both $ROSA^{fltomatofl-GFP}$ $LckCre^+hnRNP\ L^{fl/fl}$ and $ROSA^{fltomatofl-GFP}$ $LckCre^+hnRNP\ L^{wt/wt}$ mice had 95-98% GFP positive thymocytes (data not shown). We found 73-85% GFP positive T cells ($CD8^+$ or $CD4^+$) in blood or spleen in $ROSA^{fltomatofl-GFP}$ $LckCre^+hnRNP\ L^{wt/wt}$ mice indicating efficient deletion by the Lck promoter driven Cre recombinase (Fig. 4E). However, T cells from blood or spleen of $ROSA^{fltomatofl-GFP}$ $LckCre^+hnRNP\ L^{fl/fl}$ mice were mostly tomato positive (81-93%) and only very few (0.2-2.5%) were GFP positive (Fig. 4E), indicating that cells which have deleted the tomato marker in the thymus, and by inference also the floxed hnRNP L alleles and are thus GFP^+, do not leave the thymus or fail to migrate into the blood and settle in the peripheral lymphoid organs.

hnRNP L regulates the migration of thymocytes

It is known that the motility of pre-T cells within the thymus and their egress into the bloodstream is tightly regulated by different chemokines such as CCL21, CXCL12 or

sphingosine-1-phosphate (S1P) (37, 38). Since the previous experiments suggested an impaired motility or egress of hnRNP L deficient thymic T cells, we tested the chemotactic response of hnRNP $L^{-/-}$ thymocytes towards these chemokines. The expression of CXCR4, CCR7 and S1P1, which are the receptors for CXCL12, CCL21, and S1P, respectively, was not altered in hnRNP L deficient thymocytes excluding a role of hnRNP L in the regulation of chemokine receptor expression (Fig. 5A). However, a transwell assay, which allows a quantification of thymocytes migration towards chemokines enriched media, demonstrated that hnRNP $L^{-/-}$ CD4SP and CD8SP thymocytes migrated poorly toward the chemokines compared to wt cells (Fig. 5B). In addition, hnRNP L deleted cells were deficient compared to wt controls to polymerize actin upon chemokine treatment, suggesting a defect in the relay of signals from the chemokine receptor to actin polymerization, which is required for cell migration (Fig 5C).

hnRNP L regulates alternative splicing of other gene targets

The aberrant splicing of CD45 in hnRNP L deficient thymocytes may not account for all phenotypes observed in this mouse mutant. To gain more insight into the global effects of hnRNP L deletion, we undertook a genome-wide analysis of all splicing events through next-generation RNA-Sequencing of thymus from wt and $LckCre^{+}hnRNP\ L^{fl/fl}$ mice. To survey differences in splicing between control and hnRNP L deficient thymocytes, sequence reads were mapped against all possible annotated splice junction combinations within a gene locus, regardless of prior evidence of their usage *in vivo*. The total number of spliced reads in the sum of the 2 biological replicates was scaled using all mapped sequence reads to account for differences in sequencing depth. Scatterplots comparing exon junction usage between wt and hnRNP $L^{-/-}$ cells were generated with evidence of junction usage in at least one cell type as a threshold for inclusion. Green diagonal lines indicate a +/- 2 fold difference between wt and hnRNP $L^{-/-}$ and blue dotted lines on either axis represents a minimum of 32 (2^5) reads spanning junction for either wt or hnRNP L deleted cells (Fig. 6A). In order to avoid evidence from low confidence junctions, genes lists were generated

from data over the blue dotted lines (e.g. >=32 junction spanning reads) for GO/Annotation pathway analysis. Alternative methodologies of filtering splice junctions yielded similar results (Fig. 6B) but were not used.

The gene lists were analysed for enrichment of genes of specific functions using the DAVID tool (http://david.abcc.ncifcrf.gov/summary.jsp). The most significant annotation cluster generated for hnRNP L deficient cells was for genes involved in cytoskeleton/microtubule organization, and no elements of this annotation cluster were contained in analysis of the wt cells (Fig. 6C). This cytoskeleton/microtubule category includes genes such as *Wasf2, Diap1 and Arhgef2* (Table I). Another GO category that is significantly enriched in hnRNP $L^{-/-}$ thymocytes concerns gene involved in "G protein mediated signaling" (Fig. 6C). This suggests that hnRNP L may affect thymocyte egress by regulating chemokine receptors that are implicated in cell migration and bind GTPases and GTPase regulators like Dock9, Arhgef2 and Arhgap17 (Fig. 6C, Table I). Most importantly, the analysis also identified the gene for CD45, *Ptprc*, as alternatively spliced with high significance. This confirms and validates our results from RT-PCR and semi-quantitative radioactive PCR analysis of sorted DN4 cells (Fig. 2 and Table I).

Discussion

In this study, we provide evidence for an important role for the splicing factor hnRNP L in the development, intrathymic migration and thymic egress of pre-T cells. In particular, our study shows that hnRNP L regulates the alternative splicing of CD45 *in vivo* as was suspected from previous *in vitro* studies, but also indicates that hnRNP L controls the alternative splicing of hundreds of other genes implicated in early T cell differentiation and migration.

CD45 expression is crucial for T cell development as well as in controlling the threshold of antigen-mediated activation in peripheral T lymphocytes (22, 29, 39, 40). Larger CD45 isoforms (e.g. CD45RABC, -RAB, -RBC or –RB) facilitate the activation through the TCR whereas T cells expressing CD45RO need a stronger signal to obtain the same level of activation (27, 41). Our observation that both the basal and TCR-induced inhibitory phosphorylation of Lck at tyrosine Y505 is reduced in LckCre^{+}hnRNP L$^{fl/fl}$ DN4 pre-T cells is in agreement with this, since we show that hnRNP L deficient cells express higher CD45RB and RA levels than wt cells. This is also consistent with the increased basal activating phosphorylation of Lck at Y394 in hnRNP L deficient cells compared to wt controls. It is known that Lck mediates activation of T cells through recruitment and phosphorylation of its substrates, such as ZAP70, but also activates PI3K (42-45). Hence, the higher basal Akt and ERK phosphorylation and related increased proliferation rate observed in hnRNP L deficient DN4 cells is likely to be a consequence of the aberrant Lck signaling in these cells. We conclude that alternative splicing of CD45 is regulated by hnRNP L and represents a mechanism to control pre-TCR signaling in DN4 cells and thus the early steps of pre-T cell differentiation at the DN to DP transition.

Recently, new studies suggested that instead of hnRNP L, the related protein hnRNP L-like (hnRNP LL) plays a major role in controlling the alternative splicing of CD45 (46, 47).

Both ENU induced mutations in the *hnRNP LL* gene or shRNA knockdown showed the same shift in CD45 isoform expression than our hnRNP L deficient model. It is possible that hnRNP L and hnRNP LL may have overlapping functions but with different mRNA binding requirements. Alternatively, hnRNP L may be mediating the basal CD45 alternative splicing while hnRNP LL may be necessary in cells that receive a TCR signal to induce exon skipping (46-48). Further investigation has to clarify the shared or specific role of both proteins in TCR signaling and CD45 alternative splicing.

Our observation that hnRNP L deficiency correlates with a loss of peripheral T cells was unexpected and suggested a role of alternative splicing in thymic egress or the intrathymic migration of pre-T cells. Indeed, a number of experiments support this view. First, our transwell assays showed that hnRNP L deficient CD4SP and CD8SP cells failed to adequately respond to chemotactic signals provided by CCL21, CXCL12, or S1P, although they express normal levels of the respective CCR7, CXCR4 or S1P1 receptors. Second, hnRNP L deficient mice show very low yields of labeled T cells in the periphery after intrathymic injection of FITC. Third, almost no peripheral T cells in LckCre^{+}hnRNP L$^{fl/fl}$ mice show complete excision, which have be verified by both genotyping PCR and by GFP expression in the ROSA$^{fltomatofl\text{-}GFP}$ LckCre^{+}hnRNP L$^{fl/fl}$ mice. This suggests, that thymic egress or intrathymic migration selects against cells that lack hnRNP L.

Stage dependent migration of thymocytes is important to obtain the right signal at the right time (37). It has been shown in Jurkat cell lines that CD45 and CXCR4 co-localize to mediate signal transduction following CXCL12 treatment (49). Furthermore, a recent study linked CD45 expression in DN1 cells with the migration towards CXCL12 (50). Absence of CD45 results in a deficiency in CXCL12-induced migration of DN1 cells in the cortex which is in opposite of our model since CD45 is present on cell surface. Finally, it has been shown that ablation of SC35, which also regulates alternative splicing of CD45, correlates with a reduced number of peripheral CD4 and CD8 T cells in the spleen.

However, in contrast to our findings, the CD45RO isoform was predominant and CD45RB was undetectable in SC35 deficient mice (51). Although our hnRNP $L^{-/-}$ deficient SP cells show a similar migration defect as SC35 deficient animals, higher molecular weight isoforms of CD45 are upregulated in the absence of hnRNP L, which differs from the situation seen in SC35 deficient mice. These observations suggest that aberrant CD45 alternative splicing may not account for the migratory deficiency seen in hnRNP L deficient mice.

One of the first steps in the chemotactic response after binding of the chemokine to its receptor is the activation of a GTPase dependent events and the polymerization of F-actin. Rho-GTPases such as cdc42 and Rac are converted upon receptor activation from the inactive form to the active GTP bound form to control F-actin polymerization through interaction with Arp2/3, WASP and the Wave complex (52, 53). Our evidence suggests that hnRNP L controls the steps between chemokine receptor engagement and F-actin polymerization and thereby affects cell motility leading to the phenotype observed in hnRNP L deficient mice. Two sets of data support this notion: first, we show that F-actin polymerization is indeed disturbed by the ablation of hnRNP L in CD4SP and CD8SP thymic cells. Second our next-generation RNA sequencing data identified different alternatively spliced RNAs belonging to GO categories such as GTPase binding and regulation and cytoskeletal proteins among them the genes *Arhgap17*, *Arhgef1*, *GEF-H1*, *Wave2* and *Diap1*.

Previous studies have already shown the importance of these molecules in immune cell chemotaxis (54-56). For instance, macrophage migration toward CSF-1 requires the Wave2 complex and its phosphorylation by Map kinases since the reduction of its expression through iRNA abrogated F-actin rich membrane protrusions (54). In addition, GEF-H1 plays a pivotal role in uropod formation during transendothelial migration of T cells (57). Among these potential hnRNP L targets, the *Diap1* gene, which is involved in actin

nucleation and polymerization, might be the key regulator of lymphocyte migration in the thymus (56, 58). Similarly to the results presented here, it has been reported that mDia1$^{-/-}$ mice show reduced numbers of peripheral T cells and an impaired chemotactic response to CXCL12 and CCL21. Moreover, the block in migration in mDia1$^{-/-}$ mice was due to a suppressed production of F-actin (58), which is very similar to the results we obtained with hnRNP L deficient cells. It remains to be shown how the ablation of hnRNP L leads to a loss or alteration of mDia1 function, but it can be speculated that hnRNP L does not necessarily need to affect the protein expression of the target gene candidates identified here. Altered mRNA isoforms generated by hnRNP L controlled alternative splicing might either encode proteins that undergo differential post-translational modification, may loose their binding capacity to specific interaction partners or produce truncated proteins with altered functions.

Our study not only reveals a new, critical role of the splicing factor hnRNP L in the differentiation and migration or thymic egress of pre-T cell, it also illustrates to which extent alternative splicing serves as a regulatory mechanism of biological processes at a post-transcriptional level. The fine-tuning of CD45 isoform expression at differential stages of thymocytes development is one example how alternative splicing regulates the generation of a functional T cell mediated immune response. The regulation of alternative splicing of genes involved in relaying signals from chemokine receptors to F-actin polymerization is another example. Our findings link alternative splicing controlled by hnRNP L to defined cellular processes in the acquired immune system and thus ascribe this factor important new biological roles.

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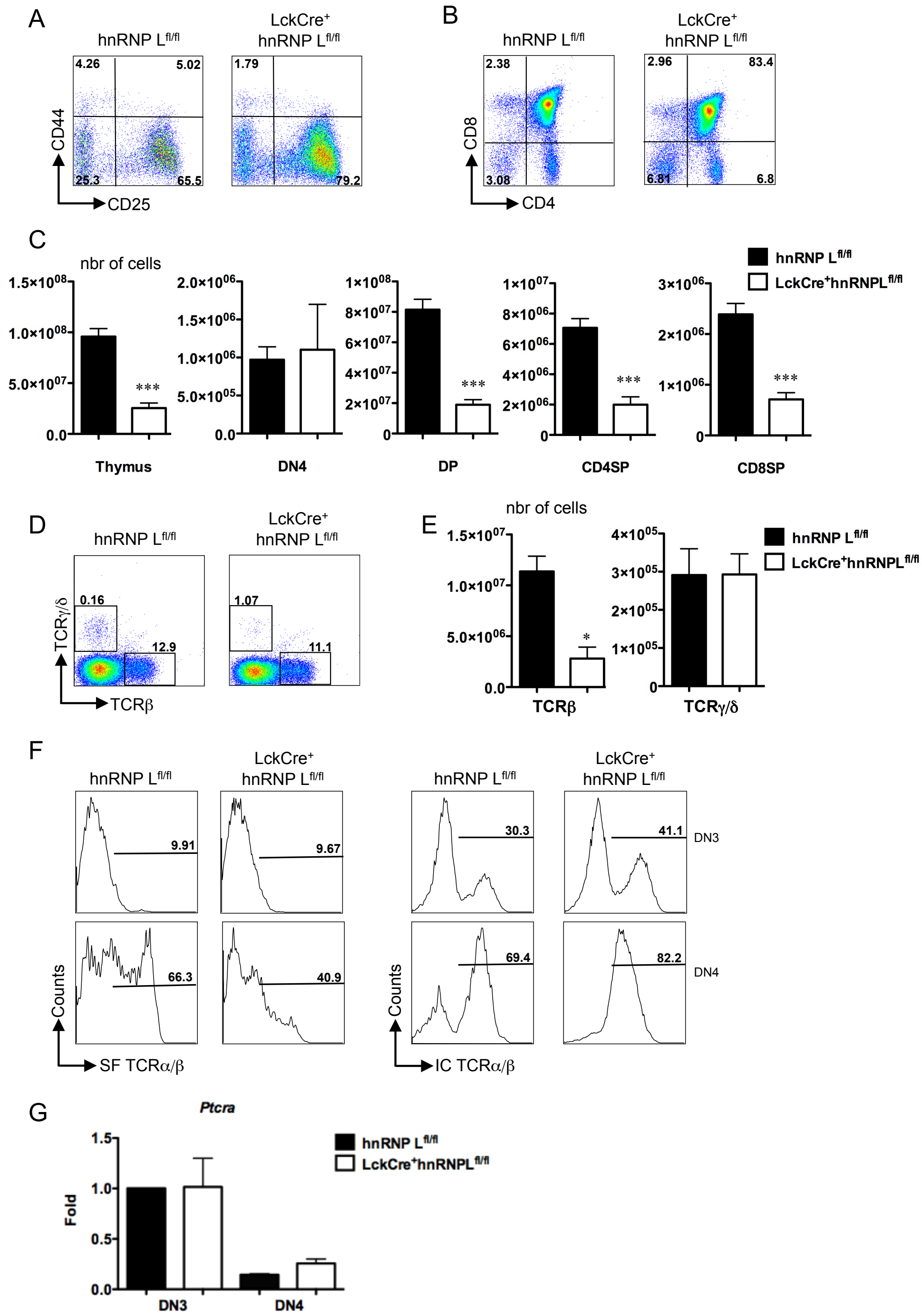


Figure 1

FIGURE 1. **Deletion of hnRNP L and its effect on thymocyte populations**. Flow cytometric analysis of wt and hnRNP L deficient thymocyte populations assessed by Lin-/CD44/CD25 (A) and CD4/CD8 staining (B). C) Histograms representing the absolute cell number of total thymus and different thymocyte subsets from LckCre^{+}hnRNP L$^{fl/fl}$ and wt mice at 6 weeks of age. A minimum of 6 mice per group was analyzed. D) Flow cytometry and E) absolute cell number of TCRα/β and TCRγ/δ thymocyte populations. F) Intracellular and surface expression of TCRβ by flow cytometry performed on DN3 and DN4 gated cells. The plot is representative for three independent analyses. G) Expression of *Ptcra* measured by qRT-PCR on DN3 and DN4 cells sorted by flow cytometry. Expression was measured and normalized to the expression of the *Gapdh* gene and is presented as the fold increase relative to wt cells (set as 1-fold). Data represent three independent experiments each done in triplicate. All error bars are means ± SEM (* $p<0.01$, ** $p<0.001$, *** $p<0.0001$).

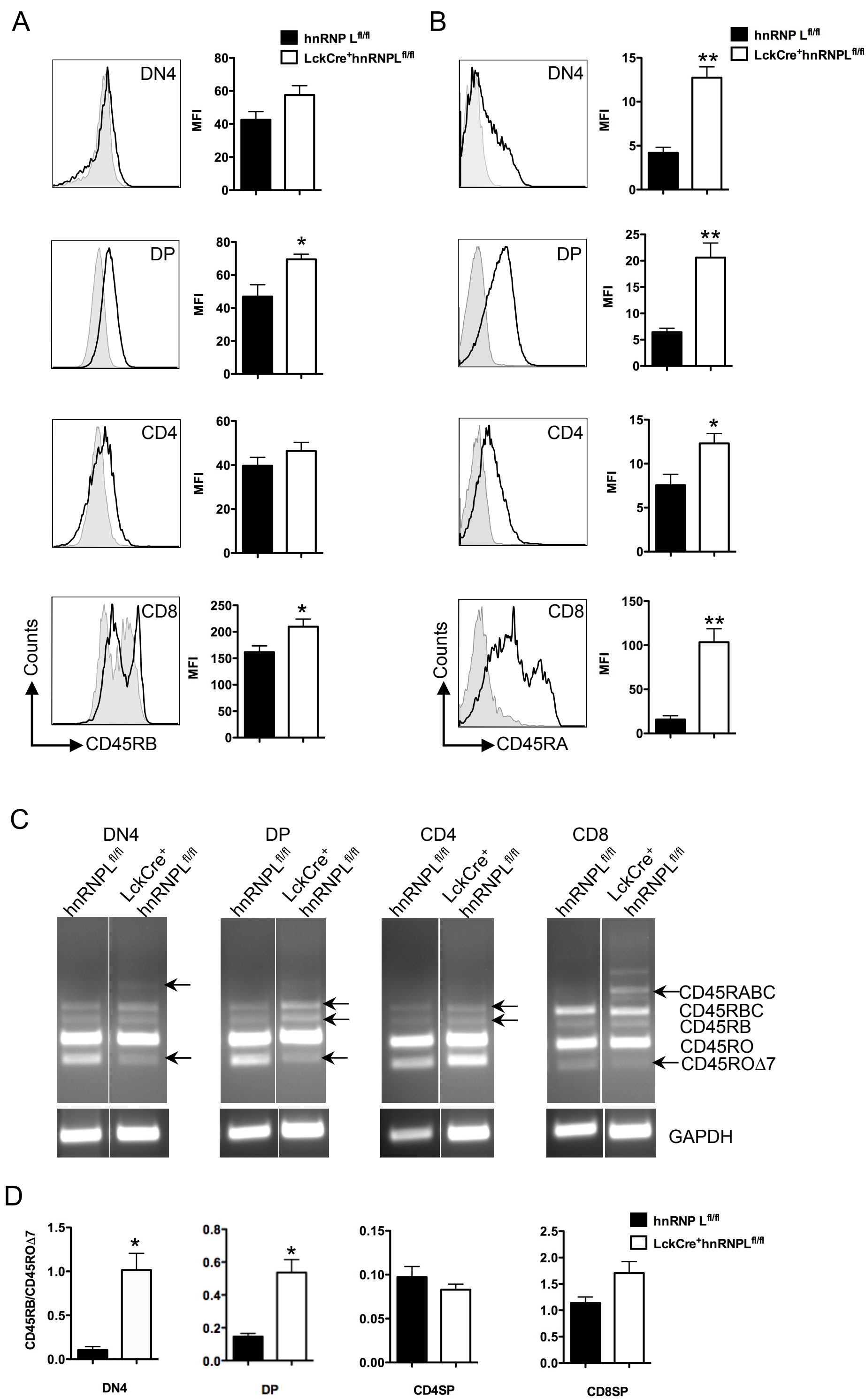


Figure 2

FIGURE 2. hnRNP L regulates the alternative splicing of CD45. Flow cytometric histograms and bar graphs represent the mean fluorescence of CD45RB (A) and CD45RA (B) on different population of thymocytes from hnRNP L deficient (black) or control mice (gray). RNA was extracted from sorted DN4, DP, CD4SP and CD8SP from wt and LckCre^{+}hnRNP L$^{fl/fl}$ and RT-PCR detection of the different isoforms of CD45 mRNA and Gapdh control on an agarose gel (C) or D) Radioactive RT-PCR was performed with a genespecific primer and a 32P-labeled forward primer. Products were separated on a denaturing PAGE and quantified by Phosphoimager analysis. Ratio was calculated between the isoform containing both exons (CD45RB) and the isoform containing neither (CD45R0DE7) and compared between the different thymic subpopulations (n=3). All error bars are means ± SEM (* $p<0.01$, ** $p<0.001$, *** $p<0.0001$) from at least three independent experiments.

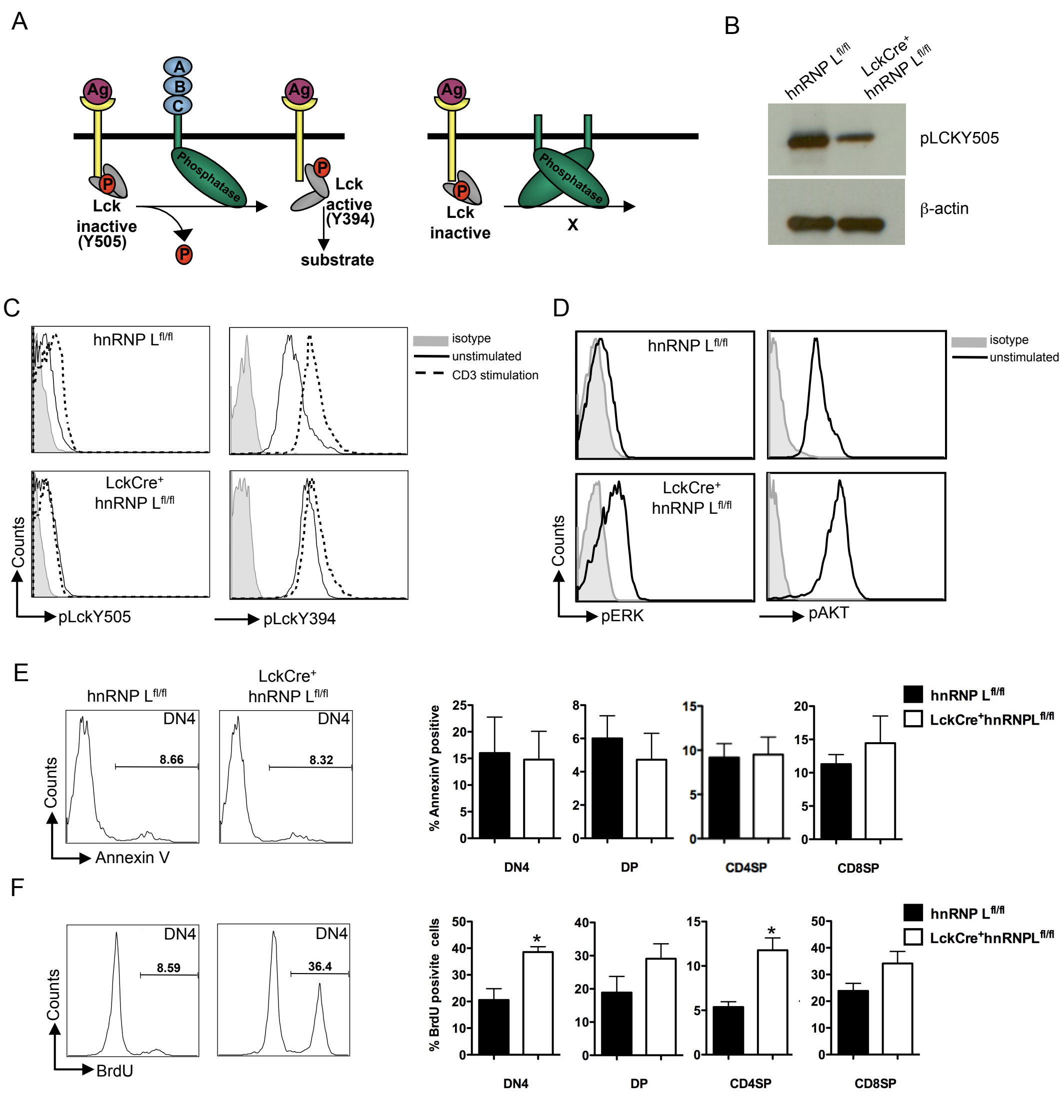


Figure 3

FIGURE 3. **Loss of hnRNP L in thymus does not affect cell death but causes aberrant TCR activation**. A) Schematic representation of CD45 phosphatase activity on Lck following TCR activation. B) Phosphorylation levels of Lck in total thymus from the indicated mice were assessed by Western blot or C) by flow cytometry on gated DN4 cells using antibodies specifically recognizing LckY505 or LckY394 at basal level and following 2 min α-CD3 stimulation (gray = isotype control, black = non-stimulated, dotted line = 2 min CD3 activation). D) Basal phosphorylation of ERK and Akt in wt and hnRNP L deficient DN4 gated cells by flow cytometry analysis. Data are representative of three independent experiments. E) Flow cytometric analysis of cell death by Annexin V staining on different thymocyte subpopulations and bar graph representing the percentage of Annexin V positive cells. F) Proliferation was assessed by incorporation of BrdU in all thymocytes subsets after 16 hr post-administration and quantified by flow cytometry. All graph represent the mean ± SEM from n= 6 mice (* $p<0.01$).

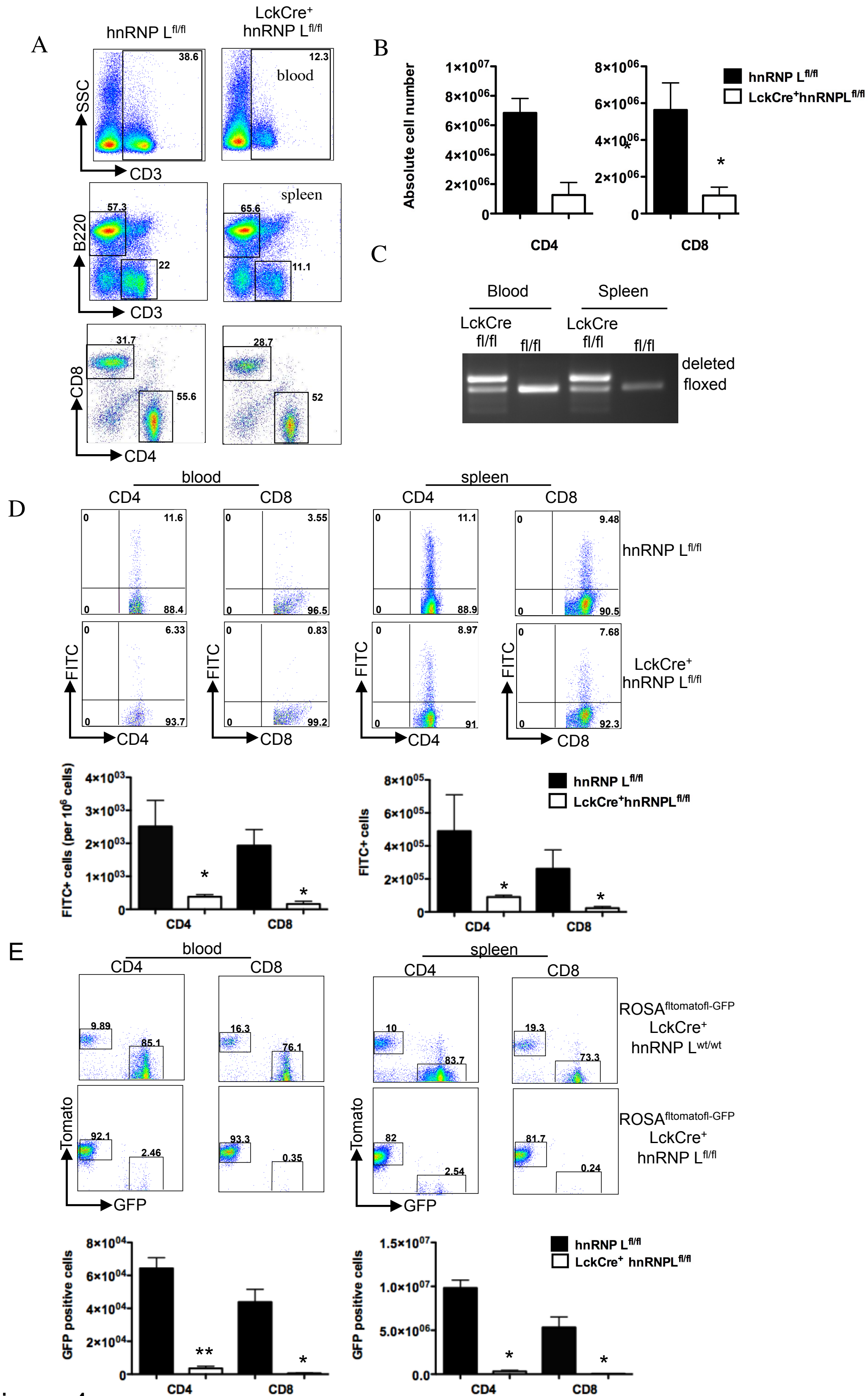


Figure 4

FIGURE 4. **HnRNP L deficient mice show a loss of peripheral T cells**. A) Flow cytometric analysis of blood and splenic T cells assessed by CD3 and CD4/CD8 staining from both control and hnRNP L deficient mice. B) Absolute cell numbers of splenic CD4 and CD8 T cell subsets. C) Excision of floxed hnRNP L alleles visualized by PCR on sorted CD3 positive cells from blood and spleen from $LckCre^{+}hnRNP\ L^{fl/fl}$ and control mice. D) Recent thymic emigrant assay. Briefly, 10 μg of FITC were injected intra-thymically and mice were sacrificed four days later. Blood and spleen were harvested and analyzed by flow cytometry for SSC/FSC and FITC expression on gated CD4 and CD8 cells. Graphs show the numbers of CD4 and CD8 FITC positive cells in blood and spleen. E) $ROSA^{fltomatofl\text{-}GFP}$ mice were crossed with $LckCre^{+}hnRNP\ L^{wt/wt}$ or $LckCre^{+}hnRNP\ L^{fl/fl}$. Cells expressing the LckCre transgene delete the floxed tomato allele and switch the expression from tomato (red) to GFP (green) allowing to track cells with active Cre deletion. The numbers of GFP positive cells were assessed in blood and spleen by flow cytometry after staining for CD4 and CD8 on CD3 gated cells. Absolute cell numbers are shown in the bar graphs. All error bars are means ± SEM (* $p<0.01$, ** $p<0.001$, *** $p<0.0001$) and a minimum of six mice were used in all experiments.

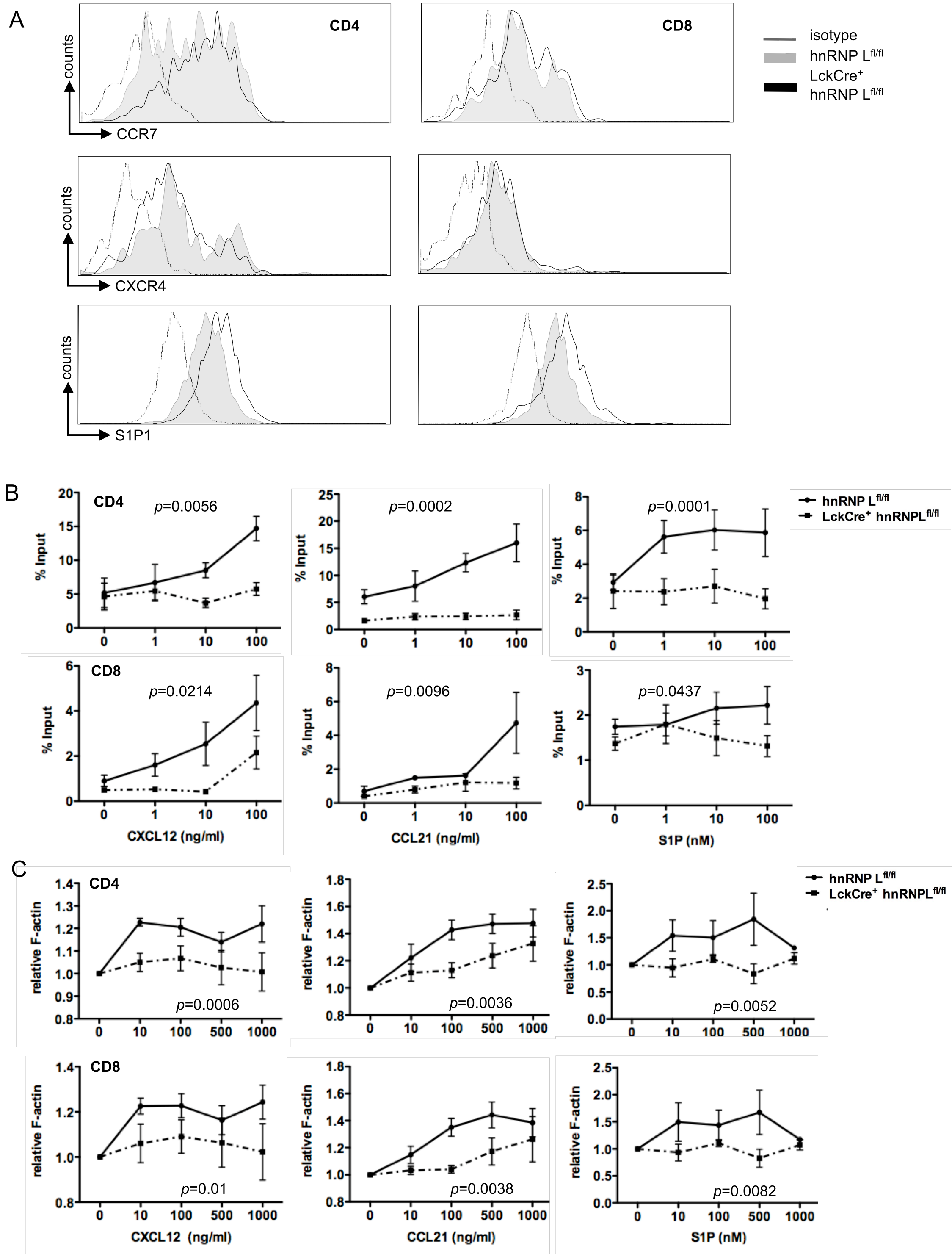
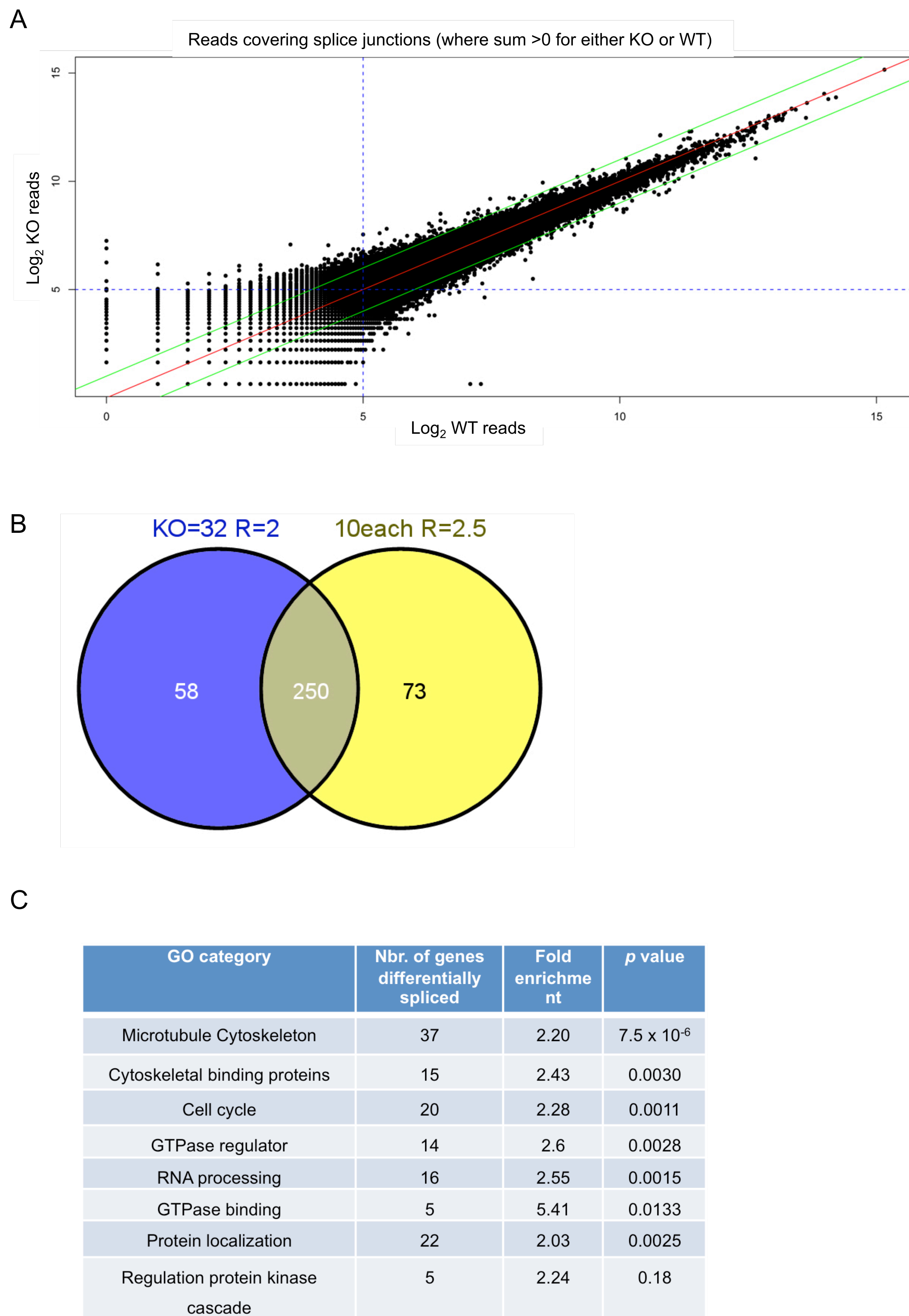


Figure 5

FIGURE 5. **HnRNP L deleted thymocytes show defects in chemotaxis**. A) Cell surface expression of chemokine receptors CXCR4, CCR7 and S1P1 on CD4 and CD8 gated thymocytes by flow cytometry. Histograms show cells from wt (gray) or $LckCre^{+}$hnRNP $L^{fl/fl}$ (black) mice and dashed line the isotype control. B) Transwell migration assay: Chemotactic response of CD4SP and CD8SP to CXCL12, CCL21 and S1P after 3h incubation depicted as the percentage ± SEM of cells that migrated into the lower chamber normalized to the corresponding input cells. At least three different mice were used for each measurement. C) hnRNP L deficient CD4SP and CD8SP cells have reduced actin polymerization compared to wt control cells. To detect F-actin, cells were stained with phalloidin-Alexa488 and with antibodies for CD4 or CD8 after 30s stimulation with different concentrations of CXCL12, CCL21 or S1P. Cells were analysed by flow cytometry. Relative F-actin content was calculated as the mean fluorescence intensity (MFI) of phalloidin-Alexa488 after stimulation over non-stimulated cells. Graphs show means ± SEM of at least three different mice.



GO category	Nbr. of genes differentially spliced	Fold enrichment	*p* value
Microtubule Cytoskeleton	37	2.20	7.5×10^{-6}
Cytoskeletal binding proteins	15	2.43	0.0030
Cell cycle	20	2.28	0.0011
GTPase regulator	14	2.6	0.0028
RNA processing	16	2.55	0.0015
GTPase binding	5	5.41	0.0133
Protein localization	22	2.03	0.0025
Regulation protein kinase cascade	5	2.24	0.18

Figure 6

FIGURE 6. RNA-Seq analysis of splicing events affected by hnRNP L ablation. A) Scatterplot of all junction spanning sequence reads from either normal control thymocytes (WT) or from hnRNP L deficient thymocytes (KO) where the sum of one or the other condition (or both) is at least 1. Green lines indicate a +/- 2 fold difference between WT and KO samples and blue dotted lines at 5 represent a minimum of 32 (2^5) reads spanning either WT or KO junctions. Differentially spliced junctions with low absolute counts (below the blue lines) may be valid, but were not considered in the analysis to avoid inclusion of junctions resulting from sampling noise. B) For differential splice junction usage, two different approaches were assessed. First, splice junctions were filtered based on those which had at least 10 reads in both WT and KO samples and were then sorted based on the ratio of reads in KO versus WT. Second, the threshold of reads in either KO or WT samples was set to 32 and genes were then sorted based on a KO versus WT ratio. The Venn diagram shows the ~80% overlap between both approaches indicating robust detection of differentially spliced junctions. For GO pathway analysis, genes lists were generated from data using former threshold (32 reads). C) GO categories enriched in a set of 250 genes with differential exon junction usage between wt and hnRNP L deficient thymocytes.

Table I. Selected genes which contain exon(s) showing preferential splicing in hnRNP L deleted thymocytes

Genes	Gene Description
Ptprc	Protein tyrosine phosphatase, receptor type C (CD45)
Diap1	diaphanous homolog 1, related formin
Wasf2	WAS protein family member 2
Arhgef2	rho/rac guanine nucleotide exchange factor (GEF) 2
Dock9	Dedicator of cytokinesis 9
Cd8b1	CD8 antigen beta chain 1
Akt3	Thymoma viral proto-oncogene 3
Tln1	Talin 1
Arhgap17	Rho GTPase activating protein 17
Bcl10	B cell leukemia/lymphoma 10
Hdac4	Histone deacetylase 4
Tbk1	TANK-binding kinase 1
Fn1	Fibronectin 1

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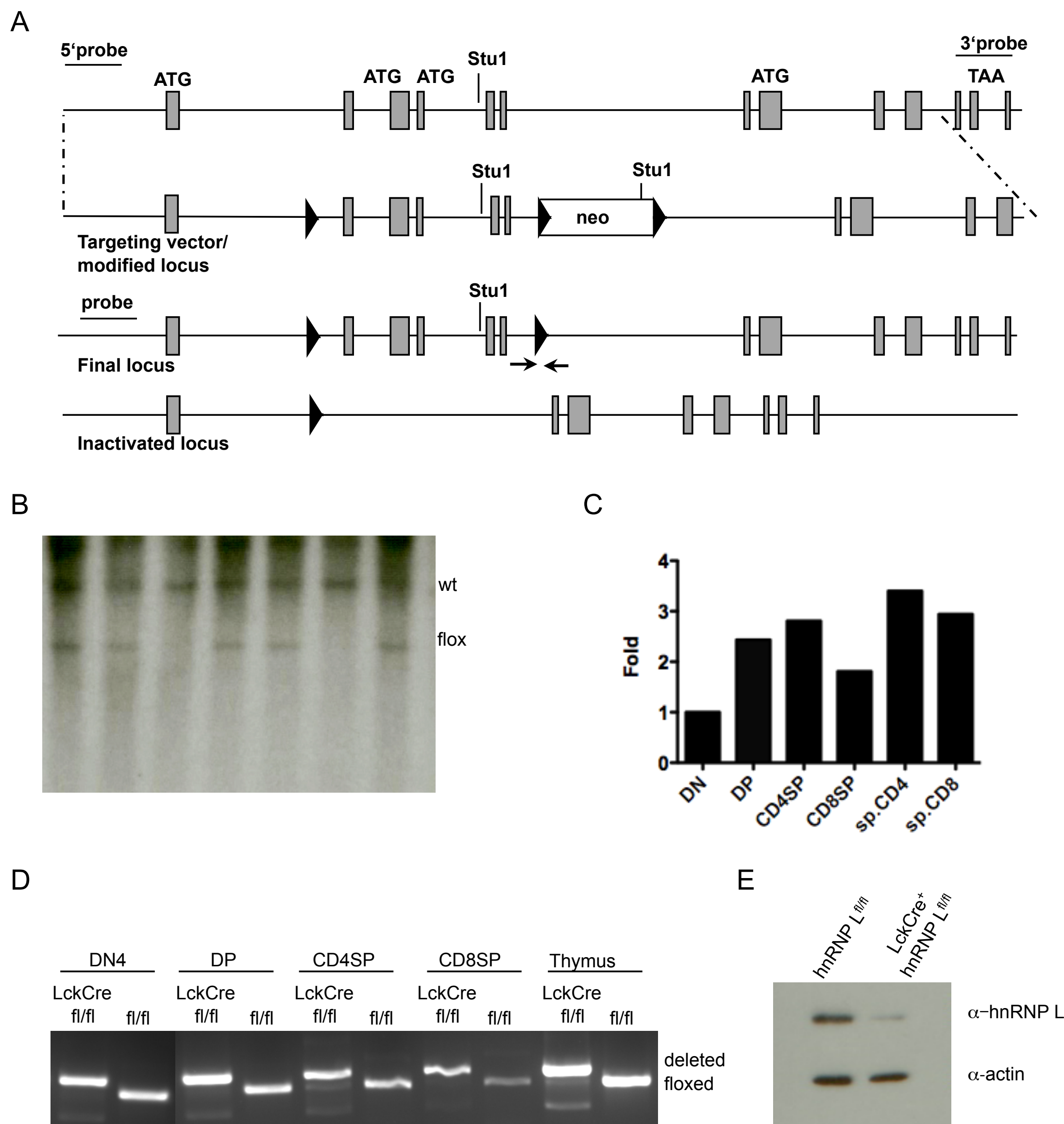
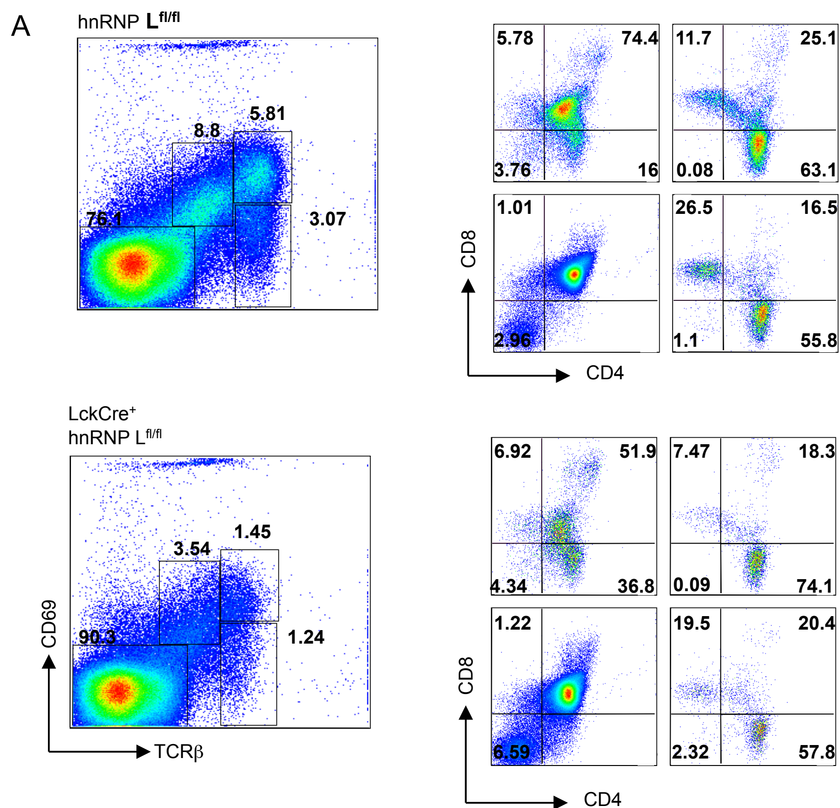
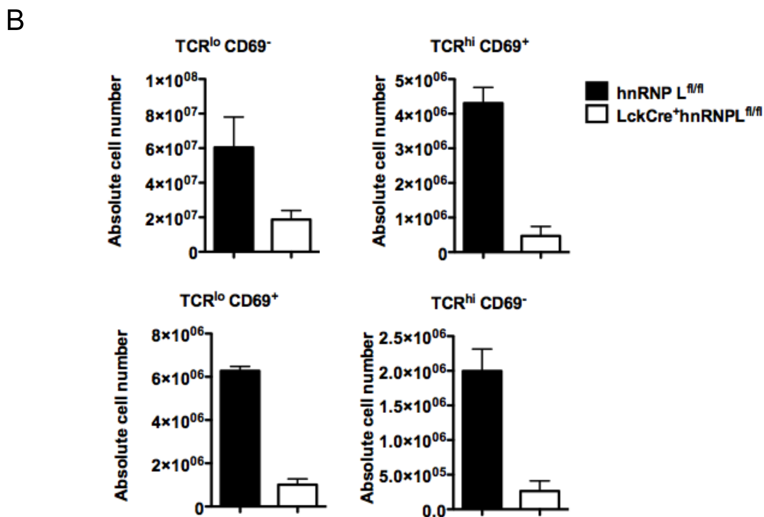


Supplemental Figure 1

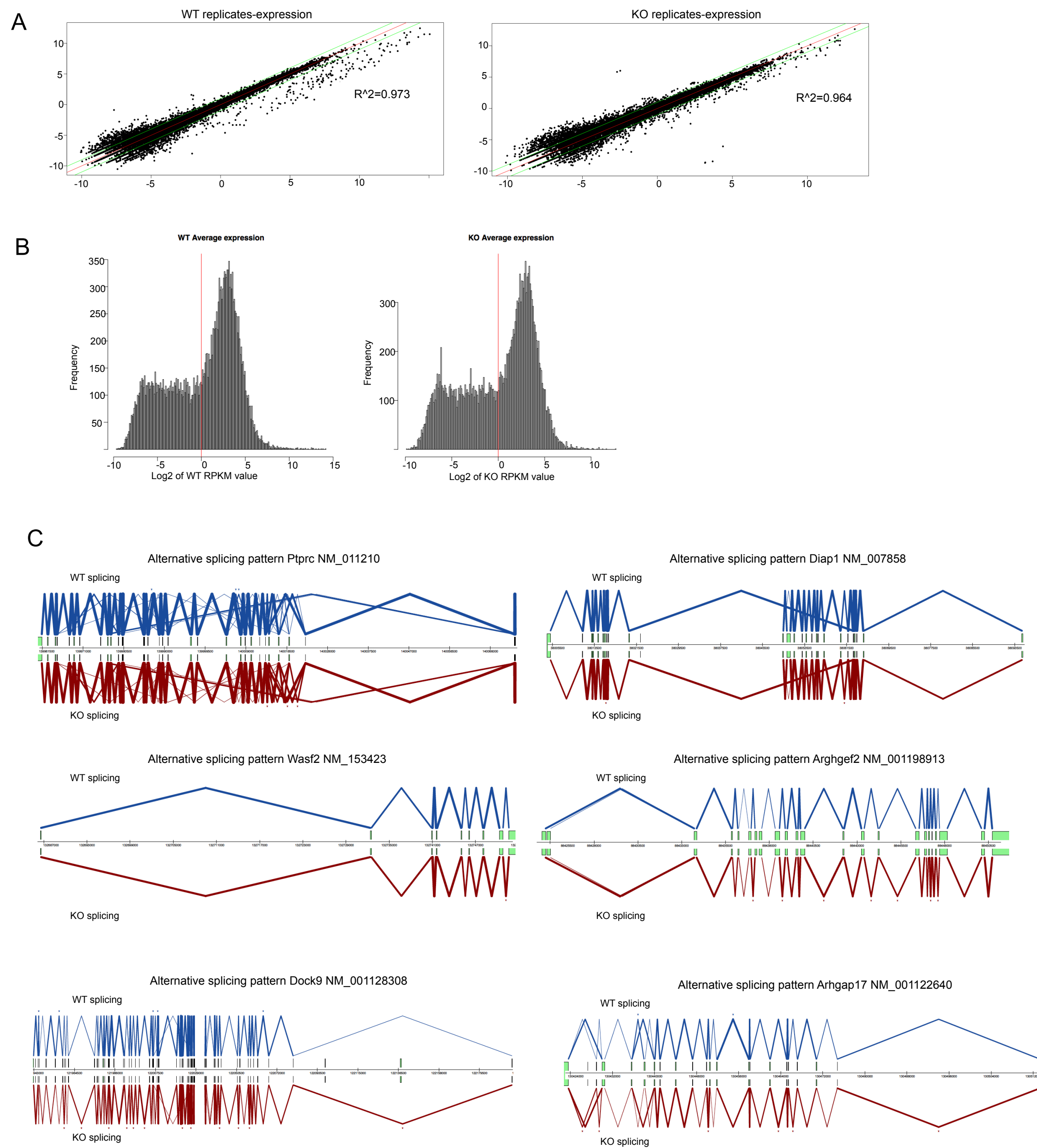
Supplemental Figure 1. A) Schematic representation of the murine hnRNP L locus and the targeting strategy to generate the conditional deletion of *hnRNP L* allele. Exons 2 and 6 are flanked by loxP sites. Upon Cre recombinase activity, the exons 2 to 6 will be excised, inactivating the locus and therefore the expression of the hnRNP L protein. B) Southern blot of Southern blot of ES cells clones partially recombined with the neo cassette removed leaving the endogenous locus with only 2 loxP sites inserted were identified. C) Relative expression of hnRNP L on different sorted T cell subset by qRT-PCR. D) Genotyping PCR to detect floxed and deleted hnRNP L alleles performed on sorted thymocyte subpopulations or complete thymus from control or $LckCre^{+}hnRNPL^{fl/fl}$ mice. E) Western blot analysis of expression of hnRNP L protein in thymus from wt or $LckCre^{+}hnRNPL^{fl/fl}$ mice and the loading control Gapdh.





Supplemental Figure 2

Supplemental Figure 2. A) Positive selection was assessed by flow cytometry on thymus from control wt or hnRNP L deleted based on surface expression of CD4 and CD8 on gated population $TCR^{lo}CD69^{-}$, $TCR^{lo}CD69^{+}$, $TCR^{hi}CD69^{+}$ and $TCR^{hi}CD69^{-}$. B) Absolute cell number of each positively selected population. Graphs show mean ± SEM (n=3).



Supplemental Figure 3

Supplemental Figure 3. A) Plot of the gene expression profile of each duplicate from wt and mutant thymus. The data indicate that both biological replicates are highly similar with regard to the global gene expression levels. B) Gene expression profiles (histograms) from wt and hnRNP L deleted thymocytes. Shown is the log_2 RPKM (Reads per kilobase of exon model per million mapped reads) values against the number of genes

Chapter III: Article 2

Heterogeneous Nuclear Ribonucleoprotein L (hnRNP L) is required for the functional integrity of hematopoietic stem cells

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Contribution: M.-C.G. performed research, analyzed data, and wrote the manuscript; F.H. designed and generated the described mouse strain and reviewed the manuscript; B.W. performed and analyzed the RNA-Seq data and T.M. designed the research, analyzed data, wrote the manuscript, and provided funding.

Abstract

The function of hematopoietic stem cells (HSCs) has to be tightly regulated since uncontrolled growth and development of blood cells may lead to leukemia or autoimmune diseases. Here we report that hnRNP L, a factor that regulates alternative splicing, is necessary for the functional integrity of HSCs. Using conditional hnRNP L knockout mice we found that hnRNP L deficiency is incompatible with hematopoietic differentiation and leads to premature death. HSCs deficient for hnRNP L are unable to elicit colonies in semisolid medium or to differentiate into lymphoid or myeloid lineages. In addition, hnRNP L deficient HSCs cannot generate lineage-committed progenitor cells or foster multilineage differentiation in a transplanted host. HSCs that lack hnRNP L are no longer quiescent, show a high level of reactive oxygen species and a prone to undergo apoptosis. An RNA-Seq analysis showed that hnRNP L ablation affects the alternative splicing of genes that regulate cell cycle progression, apoptosis and DNA damage response pathways. Our data show that alternative splicing mediated by hnRNP L is a mechanism that regulates HSC quiescence and survival at a post-transcriptional level.

Introduction

In mice, hematopoiesis originates from hematopoietic stem cells (HSC) that migrate from the aorta-gonad-mesonephros region (AGM) towards the fetal liver at embryonal stage 10.5 day post-coitus (dpc) and later on takes place in the bone marrow of adult mice[1,2]. In both fetal liver and adult bone marrow, hematopoietic stem cells (HSCs) possess a unique self-renewal capacity and the potential to generate all mature blood and immune cells of an organism throughout its lifetime[3-5]. The commitment of HSCs to differentiate into specific blood cell lineages is tightly regulated and starts with the formation of multipotent progenitors (MPPs) that have a reduced self-renewal capacity and are already restricted in their multilineage potential [6,7]. The earliest precursors that emerge from MPPs still have both myeloid and lymphoid potential and are called LMPPs (lymphoid-myeloid multipotent progenitors) [8,9]. These cells lack lineage markers but express the Flt3 receptor tyrosine kinase. They give rise to common lymphoid progenitors (CLPs)[10,11], which already bear the IL-7 receptor at their surface, and to common myeloid progenitors (CMPs), that express Fcgamma receptor. CMPs in turn differentiate into the granulo-monocyte progenitors (GMPs) on one hand and into the megakaryocyte-erythrocyte progenitors (MEPs) on the other and assure the differentiation of myeloid and erythroid lineage cells[12,13].

HSCs reside in the bone marrow or the fetal liver and are part of the $Lin^-Sca^+cKit^+$ (LSK) subset. They can be further defined by the expression of the so-called "SLAM" markers CD150 and CD48 (i.e. HSCs are $Lin^-Sca^+cKit^+CD150^+CD48^-$)[14-17]. While most HSCs in the bone marrow of adult mice are in a quiescent stage, embryonic HSCs are proliferating to generate the adult pool of stem cells [5,18,19]. Many transcription factors including Runx1, Gfi1, Gfi1b, GATA2, SCL and Notch1 have been identified as important regulators of lineage commitment as well as HSCs quiescence and survival[20-24]. However, with the exception of Runx1 isoform expression in HSCs [25] and some studies about low

mRNA transcript levels[26-28], the role that RNA metabolism and in particular pre-mRNA splicing may have for HSCs has remained unexplored. In particular, very little is known about splicing factors and their potential role as regulators of HSC function, although it is known that the majority of genes undergo alternative splicing [29].

Heterogeneous nuclear ribonucleoprotein L (hnRNP L) is a RNA-binding protein that has been identified to regulate alternative splicing by binding negative exonic splicing silencers elements (ESS) resulting in exon exclusion from the mature mRNA [30,31]. One of the major hnRNP L targets is the membrane tyrosine phosphatase CD45, which is expressed on all hematopoietic cells with the exception of erythrocytes and platelets [32]. In addition, it has been reported that hnRNP L acts as the mediator for the alternative splicing of the human endothelial isoform of the nitric oxide synthase (eNOS)[33] and mouse integrin $\alpha2$ [34]. Futhermore, hnRNP L is also involved in the stabilization of the VEGF mRNA under hypoxic conditions [35].

To investigate the role of hnRNP L in HSC function and hematopoietic differentiation, we have generated conditional hnRNP L knockout mice carrying floxed alleles that can be deleted either by the pIpC inducible MxCre transgene or the VavCre transgene, which is expressed in all hematopoietic cells starting at embryonic stage E14. Here, we present evidence that ablation of hnRNP L is incompatible with a functional hematopoiesis and blood cell formation. In particular, HSCs that are deficient for hnRNP L are prone to undergo programmed cell death and thus are unable to exert their typical stem cell functions. This particular role of hnRNP L to assure cell survival in HSCs appeared to be specific for these cells since other cell types such as B cells remained unaffected by the ablation of hnRNP L.

Materials and Methods

Mice

The generation of hnRNP L floxed mice was described previously (Gaudreau et al., submitted). MxCre, VavCre and H2kBcl2 transgenic mice were obtained from Jackson laboratories or from a colony maintained at the Institut de recherches cliniques de Montreal. All mice were housed under specific pathogen-free (SPF) conditions at the animal facilities of the Institut de recherches cliniques de Montréal (IRCM). All animal experiments were approved by the animal ethics committee of the IRCM. $MxCre^{+}hnRNP\ L^{fl/fl}$ or $hnRNP\ L^{fl/fl}$ mice were injected intraperitoneally with 500 μg of polyinosinic-polycytidylic acid (pIpC; Sigma-Aldrich) every other day for a total of 5 injections to initiate the excision of floxed alleles.

Flow cytometric analysis and cell sorting

Single cell suspensions were generated from bone marrow or fetal liver as previously described. A mouse lineage depletion kit (Miltenyi) was used to enrich HSC and progenitor cells from either bone marrow or fetal liver cells using an AutoMacs (Miltenyi). Cell surface staining of HSCs and progenitor cells was done using a lineage cocktail containing antibodies against B220, Ter119, CD3, Mac1, Gr1, NK1.1, CD49b and CD8 that were all biotinylated and streptavidin labeled with a fluorescent dye (BD biosciences). Antibodies against CD150, CD48, cKit, Sca1, CD34, Flt3, CD16/32, AA4.1, IL7R, CD95 and Annexin V were either from BD biosciences, ebiosciences or biolegends. Hoechst staining and the analysis of reactive oxygen species (ROS) was performed as previously described [21]. Briefly, cells were first surface stained with specific markers and then incubated at 37°C for 30 min with 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA; Invitrogen) and analyzed by fluorescence activated cell sorting. All analyses were done using a LSR FACS (BD biosciences). LSK cells were sorted on a MoFlo cell sorter (Cytomation). Peripheral cells were stained with fluorescent conjugated antibodies against CD3, B220, CD19, Mac1, Gr1, Ter119, CD71 that were all from BD biosciences.

In vitro differentiation

OP9 or OP9DL1 cells were plated in AMEM with either IL-7 and SCF or IL-7, SCF, GM-CSF, IL-3 and IL-6 at a density of $2x10^4$ cells in 24-well plates. Two thousand LSK cells from fetal liver of E14.5 embryos were sorted into each well. Cells were harvested 7 or 14 days later and were stained for CD4, CD8, B220, CD19, Gr1 and Mac1.

Transplantation Assays

Non-competitive repopulation assays were performed using total fetal liver cells from embryos E14.5 ($2X10^5$) or total bone marrow cells ($1x10^6$). Competitive transplantation assays were done by pooling total bone marrow cells from $MxCre^+$hnRNP $L^{fl/fl}$ or $MxCre^+$hnRNP $L^{wt/fl}$ ($CD45.2^+$) or fetal liver cells from $VavCre^+$hnRNP $L^{fl/fl}$ or hnRNP $L^{wt/fl}$ ($CD45.2^+$) with bone marrow cells from wt $CD45.1^+$ mice at a 1:1 ratio for a total of 2×10^6 cells and 4×10^5 respectively. Cells were injected intravenously into lethally irradiated syngenic $CD45.1^+$ mice.

Methylcellulose assay

Five hundred LSK cells sorted from E14.5 fetal liver or adult bone marrow were seeded on methylcellulose (StemCell Technologies) supplemented with erythropoetin, IL-3, IL-6, SCF, transferrin and insulin. After 10 days, the number of colonies was determined.

Real-time quantitative PCR

Total RNA was isolated from lineage depleted fetal liver or bone marrow cells using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared from RNA using SuperScript II reverse transcriptase (Invitrogen) and was analyzed using TaqMan probes (Applied biosystems) for hnrnpl, Bcl-2 and Gapdh as an internal control. A Mx-3005 system (Stratagene) was used and relative expression was calculated via the 2-ΔΔCT method.

Western blotting

Extracts of lineage depleted bone marrow or fetal liver cells were prepared with lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl and 5mM EDTA), supplemented with complete protease inhibitors (Roche Applied Science), 1mM Na_3VO_4 and 1% Nonidet P-40. Western blot analyses were done following SDS polyacrylamide electrophoresis and transfer of separated proteins onto nitrocellulose membrane (GE healthcare). Caspase-8 (Cell Signaling) was detected with a primary anti caspase-8 antibody, which was detected by a goat anti-rabbit coupled to HRP. All immunoblots were visualized with ECL chemiluminescent (Thermo Scientific) detection system and images were taken on film.

RNA-Seq

RNA was extracted from total fetal liver of E14.5 embryos using Tri Reagent (Molecular Research Center) followed by a purification using RNeasy Mini kit and RNase-free DNase on column (Qiagen) for 15 min at room temperature both following manufacturer's instructions. RNA integrity and quality have been confirmed using a bioanalyzer (Agilent). rRNA from each biological sample was depleted from total RNA using a RiboMinus kit (Invitrogen) and the treated RNA was then fragmented using RNase III. Ligation of the adaptor mix A and reverse transcription were performed following the manufacturer's protocol. Libraries were size selected for fragments between 150 and 300 bp, amplified for 12 cycles of polymerase chain reaction (PCR), and purified using PureLink PCR micro kit (Invitrogen). Bar-coded library concentrations were determined by quantitative PCR using a standard curve of template at known concentrations (DH10B), and approximately 0.25 ng of each library was used for each full emulsion PCR (emPCR) reaction (4 emPCR/sample). Approximately 200 millions of beads from each two samples were deposited on single full slides (2 slides for 4 samples in total) and sequenced using the Opti Fragment Library Sequencing kit-Master Mix 50 on a SOLiD machine (Version 3+).

Statistical analysis

Two-tailed Student *t* test was used to calculate *p* values where indicated. A *p* value ≤ 0.05 was considered statistically significant: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. Statistical analysis of survival curves was performed using the Log-rank test.

Results

Absence of hnRNP L results in altered hematopoeisis and premature death

RT-PCR analyses showed that hnRNP L is expressed in all hematopoietic cell subsets including HSCs and early hematopoietic progenitors (Figure 1A). To gain more insight into the function of hnRNP L in hematopoiesis, we generated conditional knockout mice carrying floxed hnRNP L alleles, since a constitutive deletion of hnRNP L resulted in early embryonic lethality (Gaudreau et al., submitted). To delete hnRNP L alleles in hematopoietic stem cells and thereby in all hematopoietic cells we used Vav-Cre transgenic mice, which express the Cre recombinase under the pan-hematopoietic Vav promoter that is also active in HSCs ([36] and references therein). VavCre^{+}hnRNP L$^{fl/fl}$ mice were not viable and did not progress in their development further than embryonic stage E17.5. To be able to investigate the role of hnRNP L in adult hematopoiesis, hnRNP L$^{fl/fl}$ mice were crossed with animals carrying the interferon alpha inducible Mx-Cre transgene. However, the ablation of hnRNP L by pIpC injection that activates the interferon alpha pathway caused a high rate of mortality in adult MxCre^{+}hnRNP L$^{fl/fl}$ mice compared to control animals (Figure 1B).

To better understand how and why hnRNP L ablation causes premature death during embryonic development and in adult mice, we analyzed both the fetal liver of VavCre^{+}hnRNP L$^{fl/fl}$ mice and the bone marrow of adult MxCre^{+}hnRNP L$^{fl/fl}$ mice. We observed that the ablation of hnRNP L significantly reduced the cellularity of both hematopoietic tissues (Figure 1C). The loss of cellularity in the fetal liver correlated with a loss of differentiated cells notably within the T- and B-cell lineage and to a lesser extent within the erythroid lineage in the fetal thymus and fetal liver of VavCre^{+}hnRNP L$^{fl/fl}$ mice (Figure 1D). Moreover, analysis of peripheral blood of adult pIpC injected MxCre^{+}hnRNP L$^{fl/fl}$ mice showed a loss of differentiated cells but revealed in addition a strong thrombocytopenia and neutropenia (Figure 1E). This was observed although the deletion of the hnRNP L alleles in total bone marrow of MxCre^{+}hnRNP L$^{fl/fl}$ mice was less efficient

than the deletion of floxed hnRNP L alleles in fetal livers of VavCre$^+$hnRNP L$^{fl/fl}$ mice (Figure 1F). These findings indicate that even an inefficient ablation of hnRNP L causes a severe a dysruption of hematopoiesis at very early stages possibly at the level of hematopoietic stem cells.

Differential requirement of hnRNP L for fetal and adult HSCs and progenitors

Mature blood and immune cells arise from progenitor cells, which are generated from HSCs and are programmed to differentiate toward either the myeloid-erythroid or the lymphoid lineage. Consistent with the previous results, flow cytometric analysis of the Lin$^-$ cKit$^+$Sca1$^-$ population revealed a significant reduction in the frequency and absolute numbers of CMPs and GMPs and at a lesser extend MEPs in both fetal liver and bone marrow of hnRNP L deficient mice compared to normal controls (Figure 2A, B). Similarly, the progenitors for both T and B cells, the CLPs, were almost completely absent from fetal liver or bone marrow when hnRNP L was deleted either during embryonic development or at adult stages (Figure 2C, D), which corroborates with the difficulty to detect mature lymphocytes, white blood cells or neutrophils in the peripheral blood of adult hnRNP L deficient mice (Figure 1E).

The low cellularity of both myeloid and lymphoid lineage precursors and mature blood cells in hnRNP L deficient mice prompted us to investigate in more detail the integrity of HSCs in absence of hnRNP L. Fetal liver cells from control and hnRNP L deficient embryos (E14.5) were collected and analyzed for number and frequencies of LSK Flt3$^-$, CD150$^+$CD48$^-$ HSCs as well as the multipotent progenitors (MPP1 and MPP2) in hnRNP L deficient fetal livers. While the LSK fraction and the more differentiated MPP1 and MPP2 subsets were affected by the abrogation of hnRNP L expression, HSCs were increased in frequency but their absolute numbers remained unchanged compared to controls (Figure 3A-B). However, in the bone marrow of adult MxCre$^+$hnRNP L$^{fl/fl}$ mice, absolute numbers of both LSK Flt3$^-$, CD150$^+$CD48$^-$ HSCs and the MPP1/MPP2 progenitor subsets were significantly reduced after ablation of hnRNP L compared to the respective control subsets

(Figure 3 C, D). This suggests that hnRNP L may is required to maintain the cellularity of both adult HSCs and MPPs and fetal MPPs, but also indicates that hnRNP L may play different roles in fetal or adult HSCs or may have a specific role in the transition from fetal to adult hematopoietic stem cells.

Loss of HSCs and multipotent progenitors in the absence of hnRNP L is a cell autonomous effect

It is known that the MxCre transgene is active in HSCs but also in bone marrow stroma and liver cells. To test whether the phenotype observed in the hematopoietic system of hnRNP L deficient mice is cell autonomous, we used bone marrow cells from $MxCre^+hnRNP\ L^{wt/fl}$ or $MxCre^+hnRNP\ L^{fl/fl}$ mice and induced the deletion of hnRNP L in these cells after a competitive or a non-competitive transplantation into syngenic $CD45.1^+$ mice. The non-competitive transplantation recapitulated the loss of HSCs and multipotent progenitors that was originally observed in $MxCre^+hnRNP\ L^{fl/fl}$ mice (Figure 4A). The competitive transplantation assays allowed to maintain the hematopoietic system in absence of hnRNP L since $CD45.1^+$ wild-type cells were present, however the transplanted mice also showed a strongly reduced percentage of $CD45.2^+$ progenitors and HSCs after the deletion of hnRNP L alleles (Figure 4B). This indicates that the loss of HSCs that is observed after the ablation of hnRNP L is indeed cell autonomous and is not due to an aberrant bone marrow microenvironment in $MxCre^+hnRNP\ L^{fl/fl}$ mice.

Both fetal and adult hnRNP L deficient HSCs have impaired repopulating capacity

Even if differentiated blood cells are reduced in hnRNP L deficient embryos and adult mice, there are still some HSCs remaining, which may have conserved their function. To test this, we sorted LSK cells and co-cultured them in vitro on OP9 and OP9DL1 stroma cells or in methylcellulose to assess their potential of differentiation and colony formation. Clearly, hnRNP L deficient LSKs were unable to generate B- or T- lymphoid or myeloid cells or to generate colonies, whereas wild type LSKs efficiently gave rise to all three lineages and produced the expected number of colonies under these conditions (Figure 5A,

B). Next, we transplanted competitively or non-competitively fetal liver cells from either wt or hnRNP L deficient embryos ($CD45.2^+$) into lethally irradiated syngenic recipient mice ($CD45.1^+$). Analysis of peripheral blood of the recipients showed that hnRNP L deficient cells were unable to generate detectable numbers of $CD45.2^+$ cells upon transplantation, whereas transplanted wt control cells demonstrated a very efficient reconstitution of all lineages (Figure 5C-F). These results suggest that both fetal and adult HSCs that lack hnRNP L are no longer functional since they are unable to reconstitute transplanted hosts and to foster multilineage differentiation either alone or against wt competitors.

hnRNP L is required to restrict cell cycle progression of adult HSCs and to ensure their survival

HnRNP L deficient HSCs are non functional and are inefficient in generating committed hematopoietic progenitors or more mature hematopoietic cells. To investigate the underlying causes of this defect, we determined the cell cycle status of HSCs. Hoechst staining revealed that the frequency of HSCs that are in S/G2/M phases of the cell cycle is higher in hnRNP L deficient mice than in wild-type control animals. In addition, the level of reactive oxygen species (ROS) was elevated in hnRNP L deficient HSCs over wt controls (Figure 6A, B), suggesting that hnRNP L deficient HSCs are no longer quiescent and undergo cell division at a higher rate than expected. Since high level of ROS may cause cell death, we analyzed fetal liver cells from hnRNP L deficient embryos and found that the percentage of HSCs and MPPs positive for AnnexinV was higher compared to wt controls. The same situation was found for the equivalent subsets from hnRNP L deficient adult bone marrow cells (Figure 6C), suggesting that hnRNP L is required for the survival of HSCs and MPP subsets.

Since previous studies had reported a role of hnRNP L in preventing the decay of the mRNA coding for Bcl-2, a pro-survival protein[37], we tested by RT-PCR whether Bcl-2 mRNA stability was involved in the survival of hnRNP L deficient HSCs, but did not find evidence that the deletion of hnRNP L affected the steady state expression level of Bcl-2

mRNA (Figure 6D). In addition, fetal livers from VavCre^{+}hnRNP L$^{fl/fl}$ mice that also carry a H2k-Bcl-2 transgene showed similar frequencies of AnnexinV positive cells than fetal livers from VavCre^{+}hnRNP L$^{fl/fl}$ embryos (Figure 6E). This excludes a role of Bcl-2 mRNA stability or expression level as the cause of the increased apoptosis rates seen in hnRNP L deficient HSCs.

A Bcl-2 independent apoptosis inducing pathway involves the expression of the CD95 cell surface receptor (Fas), which upon stimulation by its cognate ligand (FasL) is activated and mediates the cleavage of pro-caspase 8 [38]. Although HSCs usually do not express CD95[39] staining of hnRNP L deficient HSCs from fetal liver or bone marrow with antibodies recognizing CD95 revealed an abnormally high expression levels of this death receptor compared to control cells from wild type animals (Figure 6F). Consistent with a high CD95 expression, we also found different cleaved forms of pro-caspase-8 in lineage-depleted cells from hnRNP L deficient embryos or adult mice, which are absent in wild type cells (Figure 6G). This suggests that hnRNP L deficient cells activate CD95 signaling, which initiates a Bcl-2 independent cell death pathway.

hnRNP L regulates alternative splicing of other gene targets

To gain more insight into the underlying mechanisms causing the activation of a CD95 death pathway and accelerated apoptosis and cell cycle progression in hnRNP L deficient HSCs, we undertook a genome-wide analysis of mRNA expression through next-generation RNA-Sequencing using fetal liver cells from wt or VavCre^{+}hnRNP L$^{fl/fl}$ E14.5 embryos. To survey differences in splicing, sequence reads were mapped against all possible annotated splice junction combinations within a gene locus, regardless of prior evidence of their usage *in vivo*. The total number of spliced reads in the sum of two biological replicates was scaled using all mapped sequence reads to account for differences in sequencing depth. Scatterplots comparing exon junction usage between wt and hnRNP L$^{-/-}$ cells were generated with evidence of junction usage in at least one cell type as a threshold for inclusion (Figure 7A). In order to avoid evidence from low confidence junctions,

GO/Annotation analysis and genes lists used were generated from data with >=32 junction spanning reads. Alternative methodologies of filtering splice junctions based on those which had at least 10 reads in both wt and hnRNP L deficient cells yielded similar results but were not used. To look at enrichment in gene lists for genes of specific function, the DAVID site was used (http://david.abcc.ncifcrf.gov/summary.jsp). Interestingly, the most significant differences observed in splicing between wild-type and VavCre^{+}hnRNP L$^{fl/fl}$ fetal liver cells appeared in the GO pathways “response to wounding” and “coagulation” that correlate with the thrombocytopenia observed in absence of hnRNP L (Table 1). However, a number of other genes that were differentially spliced in wt or hnRNPL deficient cells were enriched in the GO categories “cell death” and “apoptosis” containing genes such as *Cdkn1a, Card10, Prkdc* (DNA-PK) and *Trp53inp1* (Figure 7B, Table 1 and Table 2). In contrast, the genes with splice junctions preferentially used in wt cells compared to hnRNP L deleted cells fell into the GO pathways “cell cycle”, “DNA repair” and “cell division” (Table 1), which is consistent with the aberrant cell cycle progression in hnRNP L deficient HSCs.

Discussion

Hematopoiesis is a well-orchestrated hierarchical system that generates mature blood and immune cells from a multipotent stem cell that has the capacity for indefinite self-renewal. Each step during hematopoietic differentiation is tightly regulated by different factors and at several levels. For this, the proper function, survival and proliferation of HSCs and their controlled differentiation into lineage committed progenitors is essential. Several studies have revealed that transcription factors play an important role in these steps. They control the maintenance of HSC quiescence, their self-renewal and survival and their differentiation into progenitors [20-22,24]. Although this is well established, other post-transcriptional mechanisms must be at play to ensure the functional integrity of HSCs and with it hematopoietic differentiation. For instance, it is well known that during or after transcription, almost all murine and human genes are alternatively spliced [29], to enable the generation of a proteome, i.e. a repertoire of proteins that surpasses largely the limited number of genes. Given the significance of this process for the function of many differentiated cells, it is likely that alternative splicing is also a critical regulatory mechanism for hematopoiesis in general and for HSCs in particular as much as transcription itself or the post transcriptional modification of proteins.

We have therefore investigated the importance of alternative splicing in the maintenance of HSC function and integrity and in their ability to foster the development of mature hematopoietic cells. To address this question, we have chosen to study the splicing factor hnRNP L, which is expressed in all hematopoietic cells, in particular in HSCs and in hematopoietic progenitors. Using gene targeting, we found that mice lacking hnRNP L in all hematopoietic cells including HSCs show signs of hematopoietic failure characterized by neutropenia, thrombocytopenia, mild anemia and low number of lymphocytes. We also found that all progenitors including those for the specification of lymphocytes (CLPs), and for erythrocyte-myeloid development (MEPs, CMPs, GMPs) were affected in hnRNP L deficient embryos and adult mice. From this it can be inferred that hnRNP L is indeed

required for the proper function and integrity of HSCs, since these cells are at the top of the hierarchy and are at the origin of all lineage committed progenitor cells. This is supported by our findings that a cell type specific abrogation of hnRNP L for instance in mature B cells (suppl Figure 1) had no consequences indicating that the deletion of hnRNP L is not generally incompatible with the normal function of a cell. Hence our findings suggest that hnRNP L exerts a biological role that is specific for a certain cell type and differentiation stage, in our study here HSCs, as opposed to a more general housekeeping function.

HSCs are the most primitive cells of the hematopoietic hierarchy and are multipotent and fully able to self-renew [3-5]. HSCs seem to have differential requirements for hnRNP L depending on their developmental stage, since their numbers are clearly reduced in adult bone marrow of hnRNP L deficient mice, but are found at wt numbers in hnRNP L deficient fetal livers. One possible explanation for this may be the fact that fetal liver HSCs have a higher proliferative capacity than adult HSCs, which are almost all quiescent [18,19]. However, both fetal and adult hnRNP L deficient HSCs have lost critical stem cell functions. This is evident from a series of experiments such as colony formation in semi-solid medium, differentiation of OP9 feeder layers and both regular and competitive transplantation assays. The results of these experiments clearly establish a critical role of hnRNP L in maintaining the functional integrity of HSCs regardless whether they are of fetal or adult origin.

One of the characteristic features of adult HSCs is their quiescence, which is only given up in situation such as an injury that requires the emergency generation of new blood cells [5]. Our data suggest that hnRNP L exerts regulatory functions that restrict adult HSC proliferation and thus contribute to keeping them a quiescent state. Hoechst staining to determine the frequency of cells in cycle and the detection of increased levels of reactive oxygen species in HSCs that lack hnRNP L support this notion. Our finding that adult hnRNP L deficient HSCs from bone marrow divide faster but have a reduced absolute cell number compared to their wild type counterparts can only be explained by a higher rate of

cell death. This was indeed confirmed by AnnexinV staining of HSCs, which showed a higher percentage of AnnexinV positive cells in hnRNP L deficient mice.

As expected, these experiments also showed that not only HSCs but also both MPP progenitor populations MPP1 and MPP2 showed accelerated rates of cell death. It is known that accelerated progression through the cell cycle requires energy and higher metabolic rates and thus generates increased level of reactive oxygen species (ROS), which in turn increase the risk of single and double strand DNA breaks [40,41]. It is thus possible that a persistent DNA damage response exists in hnRNP L deficient HSCs and MPPs and that this DNA damage response initiates apoptosis. This is supported by the finding that a number of genes that are affected in their alternative splicing according to our RNA-Seq analysis are enriched in GO categories such as “DNA repair”, “Cell death” and “Apoptosis”. In addition, our data show that a CD95-caspase-8 cell death pathway is activated in hnRNP L deficient cells, since both CD95 expression and caspase-8 cleavage products are detectable in hnRNP L deficient cells. This is also consistent with our observation that altered Bcl-2 expression or decreased Bcl-2 mRNA stability is not implicated in the accelerated cell death of hnRNP L deficient HSCs. It is unlikely that hnRNP L regulates the splicing or expression of CD95 itself since no evidence for this was found in our RNA Seq experiment. It is thus possible that the activation of the CD95-caspase-8 pathway is a result of a persistent DNA damage response in hnRNP L deficient cells.

Our RNA Seq experiment also showed that significant differences in expression levels exist between wild-type and hnRNP L deficient fetal liver cells, many concerning mainly liver specific genes. Since the relative percentages of hematopoietic cells were lower in hnRNP L deficient fetal liver sample than in the wt sample, we excluded these genes from further analysis, since it is likely that their different expression levels reflect this disparity. However, we also found that many genes were differentially spliced in hnRNP L deficient fetal liver cells when compared to their wild-type counterparts. The fact that these differentially spliced genes genes were enriched in the GO categories “DNA repair”, “cell

cycle" and "apoptosis" correlated well with the phenotype of hnRNP L deficient HSCs. It is thus possible that hnRNP L is required to maintain the quiescent state of HSCs by mediating the alternative splicing of genes involved in cell cycle progression such as *Clsp, Pds5b, Cdt1* and *Topbp1*. Moreover, genes such as *Xrcc6, Brca2* and *Rad50* were also found differentially spliced further supporting the hypothesis that a DNA damage signal persist in hnRNP L deficient HSCs that triggers p53 activation, which then induces the CD95/caspase-8 death pathway [42].

It is likely that other targets of hnRNP L that are not directly related to cell cycle progression or apoptosis are required for HSCs maintenance. The interaction of HSCs with their niche plays a primordial role in their homeostasis and in particular is required for maintaining quiescence[5]. Our RNA-Seq analysis revealed also genes that are members of the "cell adhesion" GO category. The list includes the genes encoding integrins beta2 and alphaL, which are already known for their importance for the microenvironment and homing of HSCs because their gene products ensure interacting with adhesion molecules such as ICAM-1 [43,44]. Other factors may also contribute to balance the survival and proliferation of hnRNP L deficient HSCs. For instance Lrp5 and Lrp6 are highly expressed in early stages of hematopoiesis and associate with Frizzled receptors that initiate the Wnt signaling pathway [45]. Both Lrp5 and Lrp6 have been found to be potential hnRNP L targets. In addition, TGF-β1 restricts the proliferation of HSCs to keep them in a quiescent state [46]. Our analysis also identified Smad7 as a potential target of hnRNP L. Smad7 is a negative regulator of TGF-β1 signaling [47], and ablation of hnRNP L may lead to a Smad7 isoform that is more potent in interfering with TGF-β1 signaling causing the HSCs to enter the cell cycle. It is thus possible that a dysregulation in the signal between HSCs and the microenvironment might skew hnRNP L deficient HSCs towards proliferation and cell death instead of quiescence and differentiation.

In this study we have identified the splicing factor hnRNP L as being necessary for the functional integrity of hematopoietic stem cells and thereby for hematopoietic

differentiation and blood formation in general. Our findings bear a particular significance by the fact that hnRNP L regulates alternative splicing, a process that is recognized to be of critical importance both as a post-transcriptional regulatory mechanism and as a mechanism to ensure a vast and diverse repertoire of proteins. Our findings link the process of alternative splicing to the function of hematopoietic stem cells and suggest that HSC quiescence and survival are regulated also at a post-transcriptional level by alternative splicing of genes that control cell cycle progression and apoptosis.

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Authorship contribution

Contribution: M.-C.G. performed research, analyzed data, and wrote the manuscript; F.H. designed and generated the described mouse strain and reviewed the manuscript; B.W. performed and analyzed the RNA-Seq data and T.M. designed the research, analyzed data, wrote the manuscript, and provided funding.

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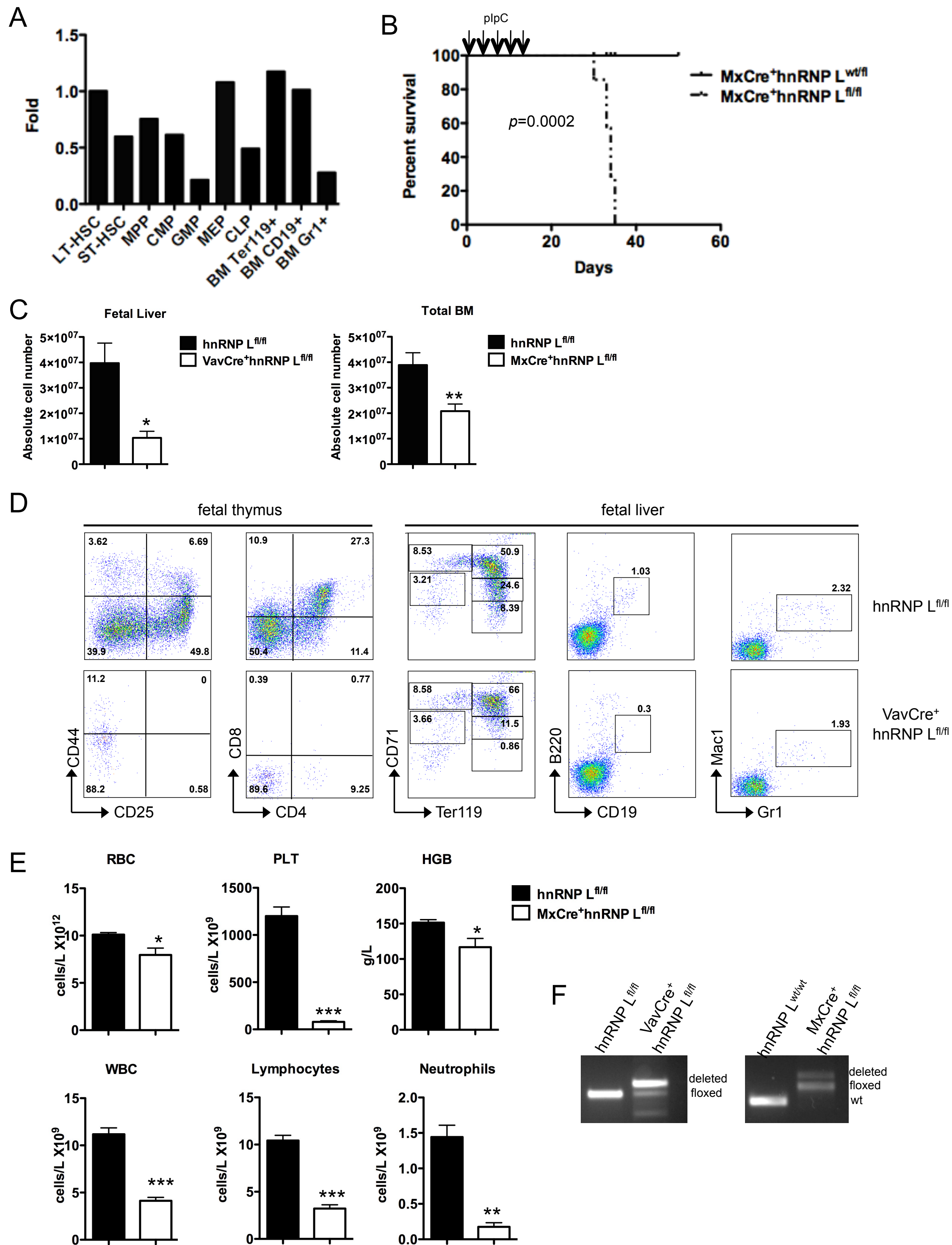


Figure 1

Figure 1. Impaired hematopoiesis in the absence of hnRNP L.

A) hnRNP L expression level in sorted hematopoietic cell populations determined by real-time PCR done in triplicate. B) Survival curve of control or $MxCre^{+}hnRNP\ L^{fl/fl}$ mice injected five times with 500 μg of pIpC. C) Histogram showing absolute cell numbers in fetal liver of wt or $VavCre^{+}hnRNP\ L^{fl/fl}$ embryos (at E14.5) or in total bone marrow from wt or $MxCre^{+}hnRNP\ L^{fl/fl}$ mice. A minimum of 6 mice per group was analyzed. D) Flow cytometric analysis of lymphoid, erythroid and myeloid subsets different in fetal thymus or fetal liver from $hnRNP\ L^{fl/fl}$ or $VavCre^{+}hnRNP\ L^{fl/fl}$ deleted embryos (stage E14.5). FACS plots are representative of three independent experiments. E) Analysis of blood parameters from control ($hnRNP\ L^{fl/fl}$) or adult $MxCre^{+}hnRNP\ L^{fl/fl}$ mice performed using an Advia system. (RBC: red blood cell, PLT: platelets, HGB: hemoglobin, WBC: white blood cells). A minimum of 6 mice per group was analyzed. F) PCR analysis to detect the deletion of hnRNP L floxed alleles in fetal liver cells of embryos at the E14.5 stage ($VavCre^{+}hnRNP\ L^{fl/fl}$) or in total bone marrow ($MxCre^{+}hnRNP\ L^{fl/fl}$). Bands representing the deleted wt, or floxed alleles are indicated. The excision by the Mx promoter driven Cre is less efficient (around 50%) than the excision in cells expressing Cre recombinase from a Vav promoter (>90%). All error bars are means ± SEM (* $p<0.01$, ** $p<0.001$, *** $p<0.0001$).

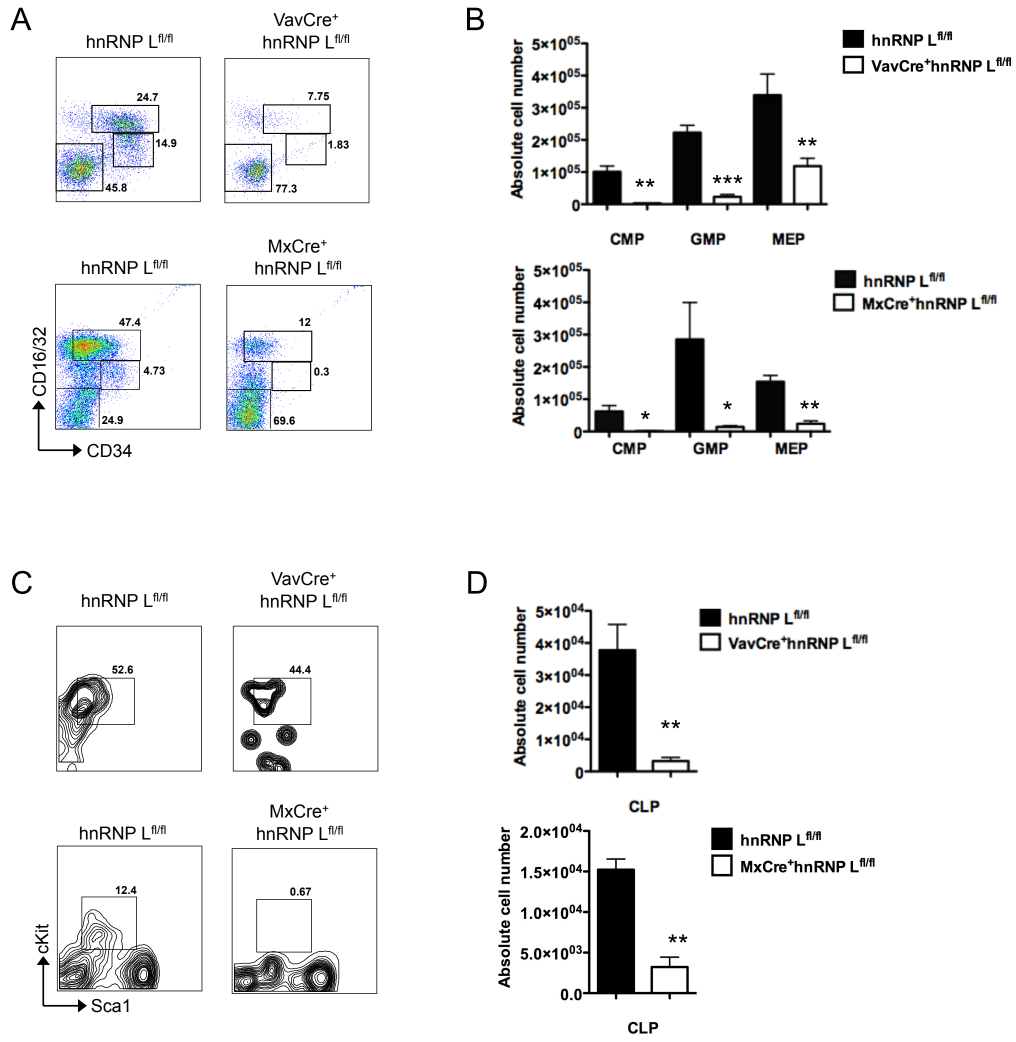


Figure 2

Figure 2. Loss of lineage committed progenitors in hnRNP L deficient mice.

(A) Flow cytometric analysis and (B) absolute cell numbers of granulo-myeloid-erythroid progenitors GMP, CMP and MEP. Shown are data from fetal liver or bone marrow from control mice (hnRNP $L^{fl/fl}$) or animals where hnRNP L was deleted in fetal over ($VavCre^{+}$hnRNP $L^{fl/fl}$) or in adult bone marrow ($MxCre^{+}$hnRNP $L^{fl/fl}$). A minimum of 3 mice per group was analyzed. (C)(D) as in (A) and (B), flow cytometric analysis (C) or absolute cell numbers (D) of common lymphoid progenitor (CLP) in both fetal liver and bone marrow of control or hnRNP L deficient mice (n=5). All error bars are means ± SEM (* $p<0.01$, ** $p<0.001$, *** $p<0.0001$).

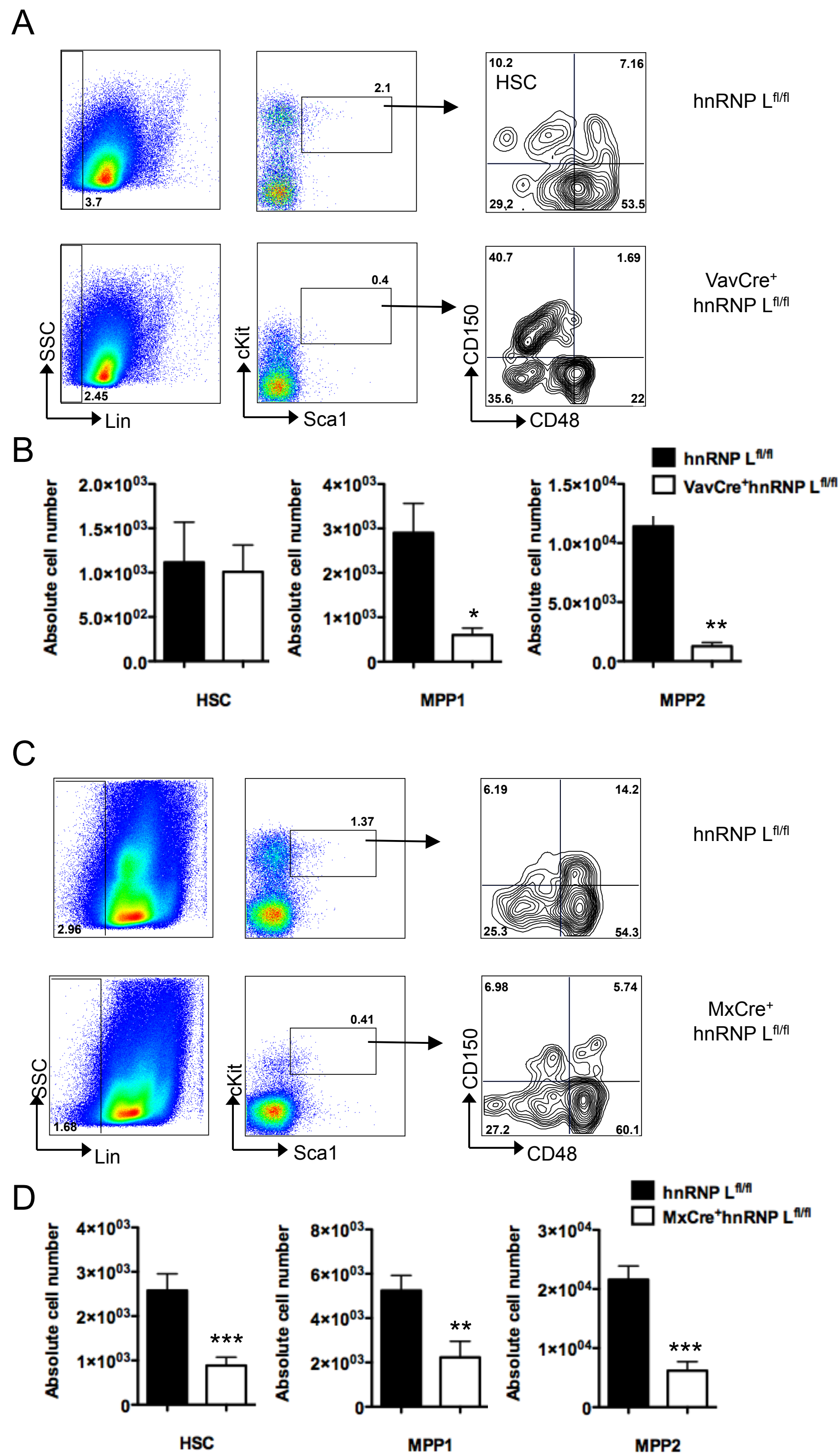


Figure 3

Figure 3. Differential role of hnRNP L in embryonic and adult HSCs.

Flow cytometric analysis and absolute cell numbers of HSCs defined as LSK $Flt3^-$ $CD150^+CD48^-$ and MPP1 and MPP2 defined as LSK $Flt3^-$ $CD150^+CD48^+$ or LSK $Flt3^-$ $CD150^-CD48^+$, respectively, from fetal liver (stage E14.5, A, B) or bone marrow (C, D) compared to control mice (hnRNP $L^{fl/fl}$). In fetal liver of hnRNP L deficient embryos only precursor cells (MPP1 or MPP2) are lost in contrast to bone marrow where both HSCs as well as the MPP1 and MPP2 subste are affected by the ablation of hnRNP L (n=6). All error bars are means ± SEM (* $p<0.01$, ** $p<0.001$, *** $p<0.0001$).

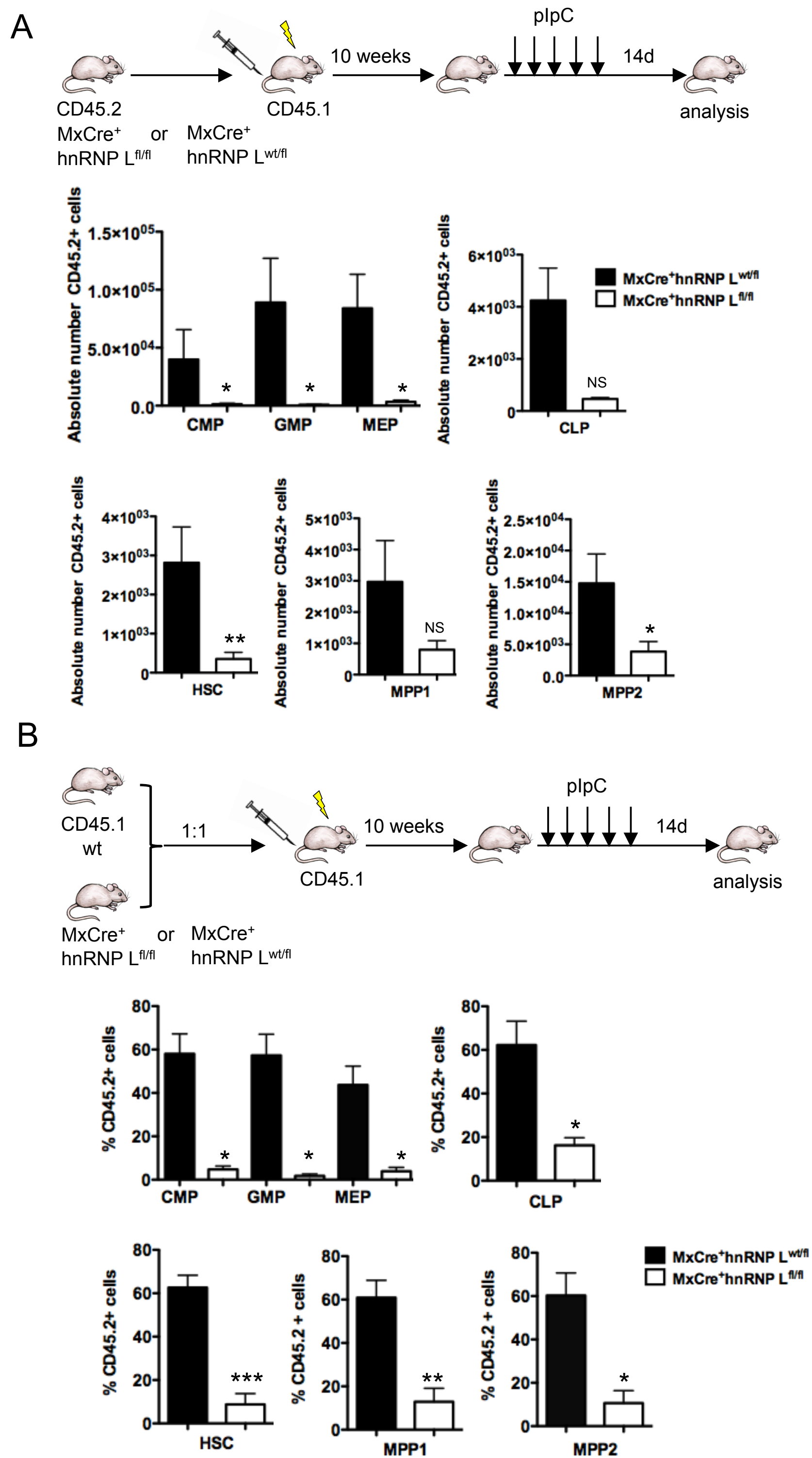


Figure 4

Figure 4. Loss of HSCs and progenitor cells in absence of hnRNP L is cell autonomous.

A) Non-competitive and (B) competitive transplantation of total bone marrow cells from $MxCre^{+}hnRNPL^{wt/fl}$ or $MxCre^{+}hnRNPL^{fl/fl}$ mice either alone (A) or mixed with wt $CD45.1^{+}$ bone marrow cells at a ratio of 1:1 for a total of 2×10^{6} cells (B) into lethally irradiated syngenic $CD45.1^{+}$ recipient mice. Ten weeks post-transplantation reconstitution was tested and mice were injected 5 times every other day with 500μg of pIpC and analyzed 14 days later. The frequency of $CD45.2^{+}$ cells and absolute cells numbers were quantified for progenitors and HSCs in the bone marrow (n=4). All error bars are means ± SEM (* $p<0.01$, ** $p<0.001$, *** $p<0.0001$).

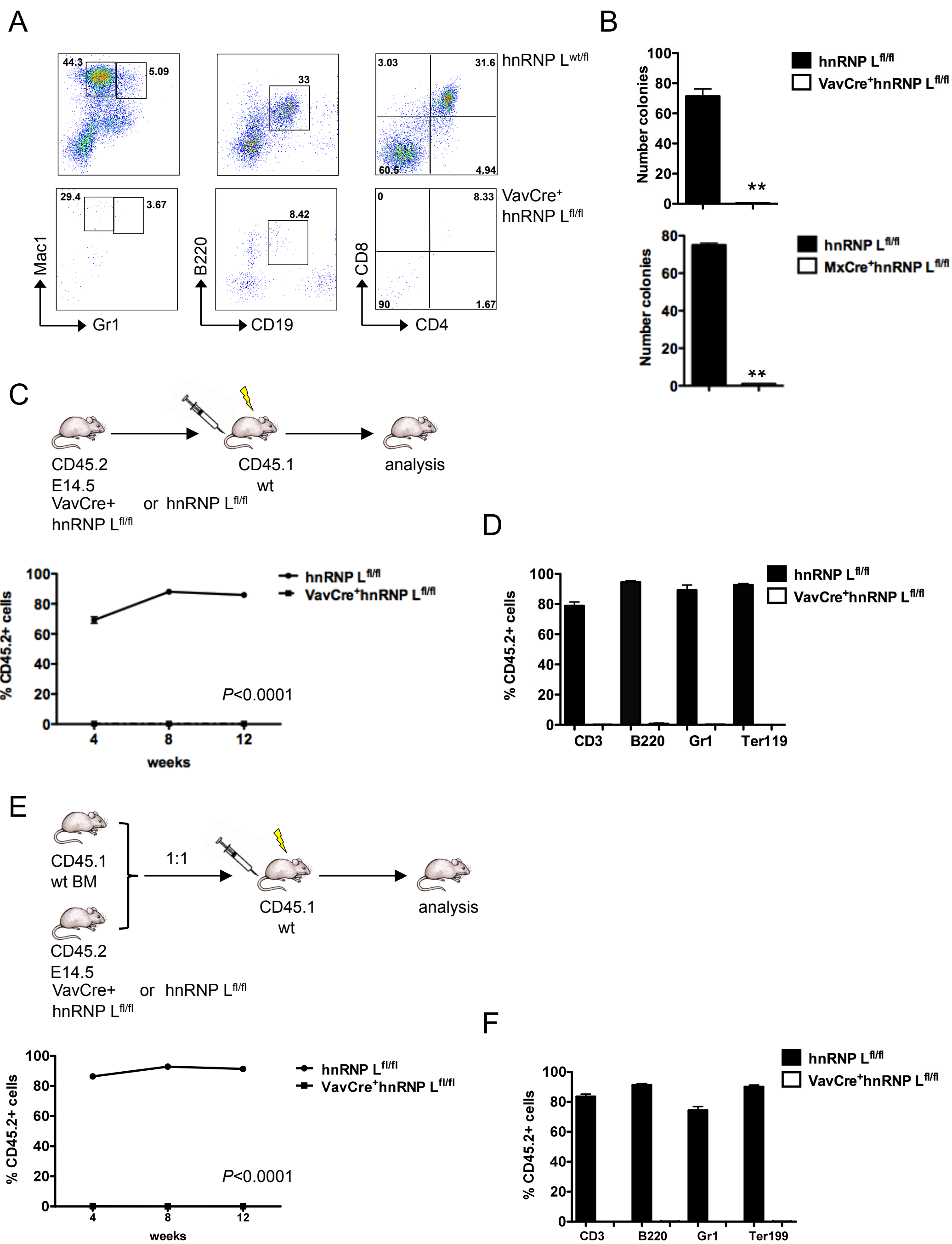


Figure 5

Figure 5. HSCs require hnRNP L to be functional.

(A) 2000 fetal liver LSK cells from the indicated animals were sorted onto OP9DL1 cells in T cell medium or onto OP9 cells in complete medium and were cultured for 7 to 21 days. Cells were then harvested and stained for the presence of the indicated T, B or myeloid specific cell markers. Data are representative of three independent experiments. (B) Colony assay on methylcellulose; 500 LSK cells from fetal liver or bone marrow from the indicates mice were sorted directly onto methylcellulose. Colonies were counted after 10 days of culture (n=3). (C) 2×10^5 fetal liver cells of either VavCre$^+$hnRNP L$^{fl/fl}$ or control (hnRNP L$^{fl/fl}$) stage E14.5 embryos (both CD45.2$^+$) were transplanted into lethally irradiated syngenic CD45.1$^+$ mice and the percentage of total CD45.2$^+$ cells in the blood were measured at 4, 8 and 12 weeks post-transplantation. (D) Frequency of CD45.2$^+$ cells within the T-, B-, myeloid or erythroid subpopulations (i.e. within CD3, B220, Gr1, Ter119 positive cells) in the blood of the recipient mice 12 weeks post-transplantation (n=6). E) Competitive transplantation: 2×10^5 fetal liver cells from VavCre$^+$hnRNP L$^{fl/fl}$ or VavCre$^+$hnRNP L$^{wt/fl}$ were mixed with 2×10^5 normal CD45.1$^+$ bone marrow cells at a ratio of 1:1 for a total of 4×10^5 cells. The mixtures were transplanted into lethally irradiated CD45.1$^+$ recipient mice and the percentages of CD45.2$^+$ cells in the blood were measured at 4, 8 and 12 weeks post-transplantation. F) Frequency of CD45.2$^+$ cells within the T-, B-, myeloid or erythroid subpopulations (i.e. within CD3, B220, Gr1, Ter119 positive cells) in the blood of the recipient mice 12 weeks post-transplantation (n=3). All error bars are means ± SEM (* $p<0.01$, ** $p<0.001$, *** $p<0.0001$).

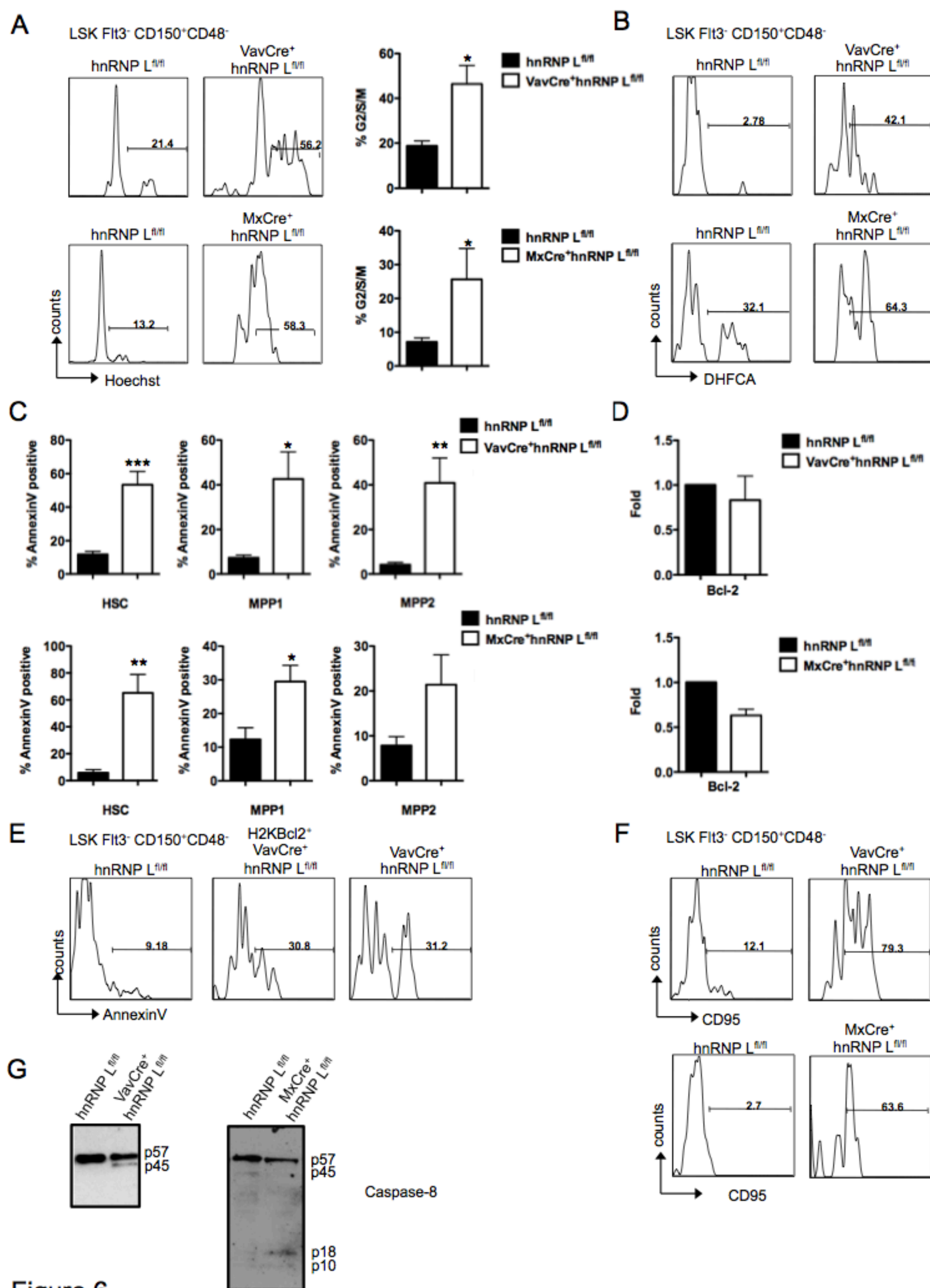

A
LSK Flt3- CD150+CD48-
hnRNP L fl/fl
VavCre+ hnRNP L fl/fl
MxCre+ hnRNP L fl/fl
21.4
56.2
13.2
58.3
counts
Hoechst
% G2/S/M
VavCre+hnRNP L fl/fl
MxCre+hnRNP L fl/fl
B
DHFCA
2.78
42.1
32.1
64.3
C
% AnnexinV positive
HSC
MPP1
MPP2
D
Fold
Bcl-2
E
H2KBcl2+ VavCre+ hnRNP L fl/fl
AnnexinV
9.18
30.8
31.2
F
CD95
12.1
79.3
2.7
63.6
G
p57
p45
p18
p10
Caspase-8
Figure 6

Figure 6. HnRNP L restricts cell cycle progression of HSCs and is required for their survival.

A) HSCs (defined as LSK $Flt3^-$, $CD150^+CD48^-$) from fetal liver or bone marrow of animals with the indicated genotype were analyzed by Hoechst staining. The percentage of cells in the G2/S/M phases of the cell cycle as indicated by the gates in the histograms was quantified. A minimum of 6 mice per group was analyzed. B) Level of reactive oxygen species (ROS) was assessed by flow cytometry using DHF-CA staining on gated HSCs (LSK $Flt3^-$, $CD150^+CD48^-$) after incubation for 30 min at 37°C. Data are representative a three independent experiments. C) Cell death was assessed by Annexin V staining on HSCs (LSK $Flt3^-$, $CD150^+CD48^-$) and MPP1 and MPP2 precursor cells (defined as LSK $Flt3^-$, $CD150^+CD48^+$ and LSK $Flt3^-$, $CD150^-CD48^+$, respectively) from fetal liver and bone marrow (n=6). D) Quantification of Bcl-2 mRNA expression by real-time PCR on lineage depleted fetal liver or bone marrow cells from embryos (upper part) or adult mice (lower part) with the indicated genotype. Data are shown as fold induction relative to values obtained for wt control. Average of triplicate values ± SEM are shown. E) Annexin V staining of fetal liver HSCs (defined as LSK $Flt3^-$, $CD150^+CD48^-$) from embryos with the indicated genotype. Data are representative of 6 embryos per group. F) CD95 expression was tested by flow cytometry on HSCs (defined as LSK $Flt3^-$, $CD150^+CD48^-$) from fetal livers of hnRNP $L^{fl/fl}$ or $VavCre^+$hnRNP $L^{fl/fl}$ embryos (upper part) and from bone marrow of hnRNP $L^{fl/fl}$ or $MxCre^+$hnRNP $L^{fl/fl}$ mice (lower part). FACS plots are representative of a minimum of three mice per group. G) Western blot analysis of caspase-8 cleavage in lineage depleted fetal liver (left) or bone marrow cells from embryos and animals with the indicated genotype. All error bars are means ± SEM (* $p<0.01$, ** $p<0.001$, *** $p<0.0001$).

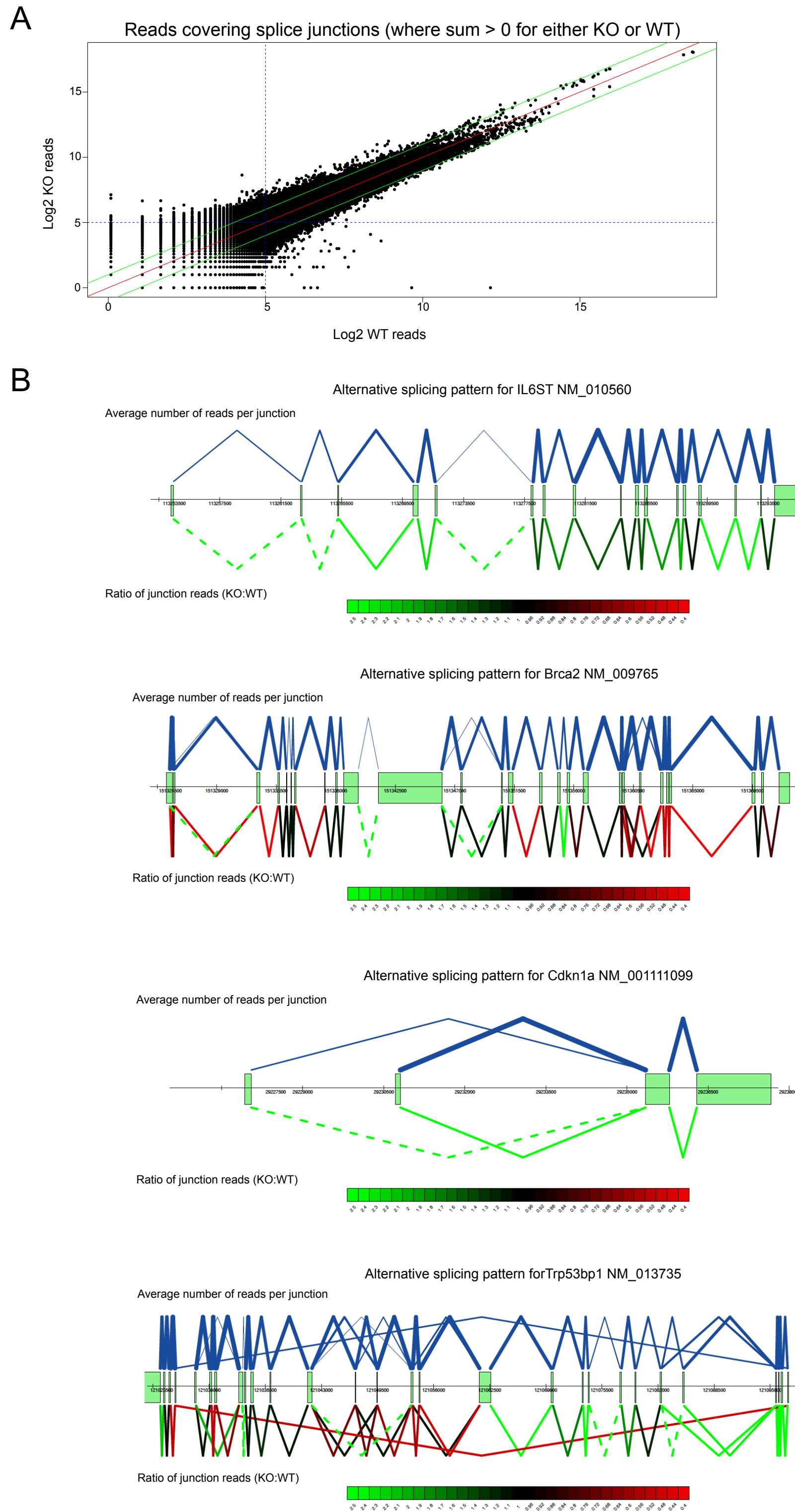


Figure 7

Figure 7. RNA-Seq analysis of fetal liver deficient for hnRNP L.

A) Scatterplot of all junction spanning sequence reads from either normal control fetal liver cells (WT) or from hnRNP L fetal liver cells (KO) where the sum of one or the other condition (or both) is at least 1. Green lines indicate a +/- 2 fold difference between WT and KO samples and blue dotted lines at 5 represent a minimum of 32 (2^5) reads spanning either WT or KO junctions. Differentially spliced junctions with low absolute counts (below the blue lines) may be valid, but were not considered in the analysis to avoid inclusion of junctions resulting from sampling noise. B) Graphical depiction of differential splice junction usage of some genes shown in Table I in normal control (WT) or hnRNP Ldeficient (KO) fetal liver cells. The exons of the genes are shown in the middle (green filled boxes). The average number of reads spanning each junction is shown on the top (in blue) where thicker lines indicate more transcripts, which have an isoform with these exons. The ratio between WT and KO is represented below in red to green where lines are all the same width. Green lines mean there are more reads for this junction in hnRNP L deficient cells (KO) than in normal cells (WT), black means equal (ratio of 1) and red means there are more reads for this junction in normal control cells than in hnRNP L deficient cells. A dotted green line indicates that this junction is only used in hnRNP L deficient cells.

Table I. *GO categories of spliced genes in hnRNP L deficient fetal liver cells*

GO categories wild-type	Nbr of genes differentially spliced	Fold enrichment	*p* value
GO:0007049, Cell cycle	69	4.01	3.10E-23
GO:0051301, Cell division	39	4.93	5.81E-16
GO:0006281, DNA repair	25	4.00	1.54E-8
GO:0006974, Response to DNA damage	27	3.34	1.45E-7
GO:0030218, Erythrocyte maturation	7	5.18	0.00212
GO categories hnRNP L deficient	Nbr of genes differentially spliced	Fold enrichment	*p* value
GO:0009611, Response to wounding	100	2.03	1.46E-12
GO:0007596, Coagulation	34	3.43	3.48E-11
GO:0007155, Cell adhesion	122	1.15	7.48E-7
GO:0008219, Cell death	105	1.46	4.21E-5
GO:0006915, Apoptosis	95	1.41	4.21E-4

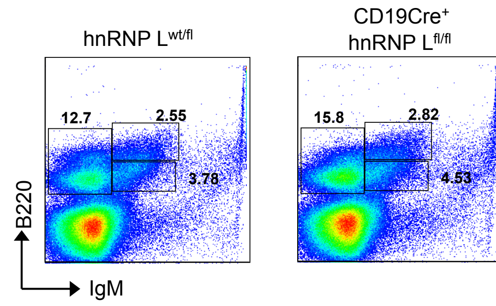
Table II. *List of genes differentially spliced in hnRNP L deleted fetal liver*

Genes with splice junction preferentially used in hnRNP L deficient cells	**Gene Description**
Cdkn1a	Cyclin-dependent kibase inhibitor 1A (p21Waf)
Vwf	von Willebrand factor
Card10	Caspase recruiting domain family member 10
Prkdc	Protein kinase DNA activated catalytic polypeptide (DNA-PKcs)
Itgb2	Integrin beta 2
Smad7	SMAD family member 7
Trp53bp1	Tumor protein p53 binding protein 1
Trp53inp1	Tumor protein p53 inducible nuclear protein 1
IL6ST	Interleukin-6 signal transducer
ItgaL	Integrin alpha L
Lrp5	Low density lipoprotein receptor related protein 5
Lrp6	Low density lipoprotein receptor related protein 6

Table III. *List of genes differentially spliced in wild-type fetal liver*

Genes with splice junction preferentially used in wild-type cells	**Gene Description**
XRCC6	X-ray repair complementing defective repair in chinese hamster cells 6
Brca2	Breast cancer 2, early onset
Rad50	DNA repair protein Rad50
CDT1	chromatin licensing and DNA replication factor 1
Pds5B	PDS5, regulator of cohesion maintenance, homolog B
TopBP1	topoisomerase (DNA) II binding protein 1
hnRNP L	Heterogeneous nuclear ribonucleoprotein L
Sox6	SRY (sex determining region Y)-box 6
Tal1	T-cell acute lymphocytic leukemia 1
Clspn	clapsin

A



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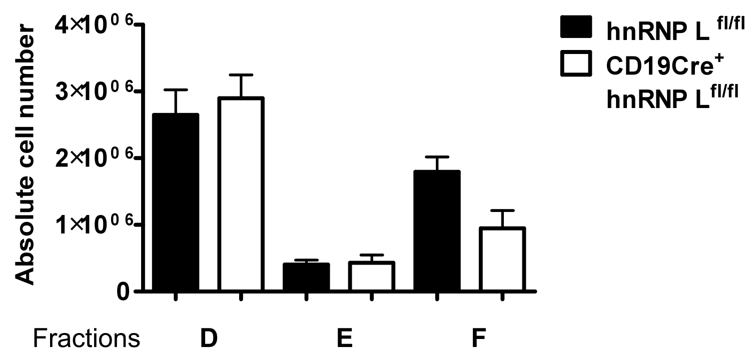

Supplemental Figure 1

Supplementary Figure 1

Deletion of hnRNP L in B cells does not affect their development.

A) Flow cytometric analysis of B cell development in the bone marrow of hnRNP L $^{wt/fl}$ or CD19Cre^{+}hnRNP L$^{fl/fl}$ mice based on IgM and B220 staining. Data are representative of 6 mice per group. B) Histogram showing the absolute cell number of fractions D, E, F based on IgM and B220 staining of bone marrow from hnRNP l $^{wt/fl}$ or CD19Cre^{+}hnRNP L$^{fl/fl}$ mice (n=4). All error bars are means ± SEM.

Discussion

In this thesis, it has been shown for the first time using an in vivo model that alternative splicing mediated by hnRNP L is required at different hematopoietic developmental stages. As mentioned in the introduction, the majority of pre-mRNAs are alternatively spliced in mammals and is widely spread in all tissues of the organism [380]. In the mouse, the hnRNP L protein is ubiquitously expressed with relatively higher expression level in neurons and primary oocytes compared to other tissues [381]. Since the constitutive ablation of hnRNP L expression led to an arrest in embryonic development at very early stages before gastrulation, a conditional Cre-lox strategy to generate loss of function mutants was chosen. This allowed to abrogate hnRNP L expression in specific cell lineages and tissues and enabled to investigate the roles of hnRNP L in cell lineage determination, proliferation and cell survival in a cell type specific manner. The results of this study indicate that hnRNP L has specific functions in different cell types and suggested that the process of alternative splicing is used to control a variety of processes such as stem cell integrity and T cell proliferation and migration. In the following chapter the findings obtained with a conditional and cell type specific hnRNP L deficient mouse mutants will be discussed.

1. The role of hnRNP L mediated alternative splicing in pre-T cell development

1.1. Role of the alternative splicing of the pre-mRNA encoding CD45 in early T cell development

1.1.1. CD45 pre-mRNA alternative splicing by hnRNP L

Previously, many *in vitro* studies implied hnRNP L as the main factor regulating the alternative splicing of the CD45 encoding pre-mRNA [21, 61, 62]. However, recent *in vivo* ENU-induced mutagenesis experiments suggested that it is rather the related factor hnRNP L-like (hnRNP LL) protein, which is the main regulator of CD45 pre-mRNA splicing [382, 383]. These studies principally demonstrated that hnRNP LL mediates CD45 pre-mRNA splicing after T cell activation but not in resting T cells [382, 384]. Most results of hnRNP LL were obtained by studying a mice mutant (thunder mice) that carried a mutation and a partial loss of function of the hnRNP LL allele. With the conditional hnRNP L knockout mouse model that has been generated for this thesis, it is now possible to test whether the role of hnRNP L overlaps or is different from the function of the related hnRNP LL protein in T cells.

The tyrosine phosphatase CD45 is known to regulate TCR signaling intensity through the Src kinase Lck [45]. Thymic pre-T cells start to express a pre-TCR at the DN3 stage and it is only at the DP stage that the fully functional TCR$\alpha\beta$ is found on the cell surface. During these steps of pre-T cell differentiation, the range of CD45 isoforms expressed differ from one cellular subset to another suggesting a differential requirement in CD45 phosphatase

activity. While DN4 cells bear mostly CD45RB and CD45RBC isoforms on their surface, CD45RO is the predominant isoform found on DP and both SP subsets [81]. The CD45RO isoform on DP and SP cells is probably present to restrict the level of TCR signaling when it engages to self-peptide-MHC complexes during positive and negative selection. The data obtained with conditionally deficient mice indicate that hnRNP L is regulating CD45 alternative splicing in pre-T cells. In absence of hnRNP L, the level of CD45RB already high in normal cells was further upregulated over CD45RO. In addition, CD45RA containing isoforms appeared in hnRNP L deficient pre-T cells. This is in striking difference to wild-type pre-T cells in which these isoforms are completely absent. Considering that hnRNP L binds to ESS sequences in the CD45 pre-mRNA and represses the inclusion of exons 4, 5 and 6 of CD45, the predominance of CD45RA isoforms in cells that lack hnRNP L was expected. Since it is known that the binding of hnRNP L to CD45 ESS sequences takes place in association with PTB under resting conditions, it is thus likely that hnRNP L is a bona fide regulator of CD45 alternative splicing under resting conditions during pre-T cell development [61, 63, 64]. Although TCR signaling induces post-translational modifications in hnRNP L and modulates the association of PSF on CD45 pre-mRNA, hnRNP L is still present in that context [61, 63, 64]. Therefore, it is also likely that hnRNP L is also implicated in the regulation of CD45 pre-mRNA splicing during TCR signaling.

1.1.2. Role of hnRNP L at the pre-TCR checkpoint

As they progress in their development, DN3 cells rearrange their TCRβ locus. Upon production of a functional rearrangement, the β-chain assembles with the surrogate pTα to generate the pre-TCR on the cell surface [385]. Signaling through the pre-TCR is required for the cells to proliferate and to give rise to the DN4 subset. These cells upregulate the CD4 and CD8 co-receptors and form the DP subset. The observation that the abrogation of

hnRNP L expression from the DN3 stage onwards affects DN4 cell proliferation and the subsequent differentiation to DP cells indicated defects at the pre-TCR selection step. In addition, the low thymic cellularity in hnRNP L deficient mice would be consistent with this interpretation. It is known that the pre-TCR selection step is a major checkpoint at the DN3 stage where only those cells survive that have productively undergone V(D)J recombination and express a TCRβ chain on their cell surface. Those cells that lack a β chain owing to a non-productive rearrangement are eliminated by apoptosis [229]. Also, since V(D)J recombination generates double strand break, a DNA damage response has to be contained at this stage to avoid premature cell death due to p53 mediated apoptosis [299, 386]. Since no increase in the level of Annexin V has been detected in DN3 or DN4 cells from hnRNP L deficient mice, the loss of DPs that is also observed in the absence of hnRNP L is very likely not a consequence of accelerated apoptosis. In fact, both the DN3 and the DN4 subsets in hnRNP L deficient mice do express the TCRβ chain as for the pTα. Therefore, the blockage between DN4 and DP cells cannot be due to a deficient pre-TCR.

However, hnRNP L deficient DN cells express aberrant levels of CD45RA isoforms. It is known that pre-TCR signaling involves the activation of Lck and downstream substrates including Zap70, Erk1/2 and Akt; the best characterized substrate of CD45, Lck, setting the threshold for TCR signaling [44, 69, 88]. The tyrosine phosphatase activity of CD45 acts principally by removing the inhibitory phosphate on tyrosine 505 (Y505) of Lck which allows a conformational change of Lck resulting in the auto-phosphorylation of the activator tyrosine 394 (Y394). It has been demonstrated that larger CD45 isoforms were less potent to dimerize than smaller isoforms such as CD45RO because of their level of O-glycosylation, which repulses CD45 molecules. Dimerization is a way to restrict the intensity of the TCR signal [57, 58, 60, 387]. The data presented here shown that with the overrepresentation of aberrant CD45 isoforms such as CD45RA on hnRNP L deficient DN4 cells, Lck is hyperactive showing a lower level of Y505 phosphorylation and increased auto-phosphorylation of Y394. This result was in concordance with the hypothesis that deletion of hnRNP L affects expression of CD45 isoforms and as a

consequence TCR signal intensity. This is in agreement with the finding that the downstream effectors of Lck; Erk1/2 and Akt were also activated in resting pre-T cells from hnRNP L deficient mice (Figure 15).

The activation of the kinase Akt is required for the transition of DN to DP and pre-T cells from Akt deficient mice are blocked at DN3 stage [388]. Moreover, it is known that the role of Akt at the DN3 stage is to induce cell proliferation [303, 388]. Given that Akt is activated in pre-T cells that lack hnRNP L, it is likely that the partial DN4 to DP block of differentiation observed in hnRNP L deficient mice is the consequence of accelerated proliferation. This is supported by data that show enhanced BrdU incorporation in several pre-T cell subsets but in particular in DN4 cells from hnRNP L deficient mice.

Since hnRNP L knockout DN cells proliferate extensively but do not accumulate compared to the respective wild-type control subpopulations, nor differentiate into DPs the question remains what the fate of these cells is? Annexin V staining data excluded that they die by accelerated programmed cell death and propidium iodide staining excluded that necrosis occurs in the thymus of hnRNP L knockout mice. Given that both apoptosis and necrosis have been tested and were eliminated it is still possible that these cells are removed from the thymus by autophagy. The autophagy is a different cell death pathway that involves the degradation of cytoplasmic components in lysosomes. The autophagic process is activated by physiological stress, the accumulation of unfolded proteins or nutrient starvation [389-391]. The main signaling molecules regulating the autophagy are PI3K and TOR (target of rapamycin). It is likely that the aberrant phosphorylation of Akt in hnRNP L deficient pre-T cells leads to the autophagic process through the activation of TOR1\mTORC1 which correlates possibly with the nutrient starvation of these pre-T cells induced by their proliferative status [389-391]. It will be therefore interesting to test the role of hnRNP L depletion in autophagy or to characterize to cell death pathway involved in DN4 cells deleted for hnRNP L.

The model that allows to conciliate the findings and provides a plausible explanation for the obtained data would be that those splicing steps of the CD45 pre-mRNA that depend on hnRNP L during the β-selection are necessary to restrict the intensity of the pre-TCR signal and to allow normal development from DN4 to the DP stage including the up-regulation of CD4 and CD8. In the absence of hnRNP L, the more potent tyrosine phosphatase CD45RA isoform is expressed which results in uncontrolled cell proliferation of DN4 cells and a block from DN4 to DP development.

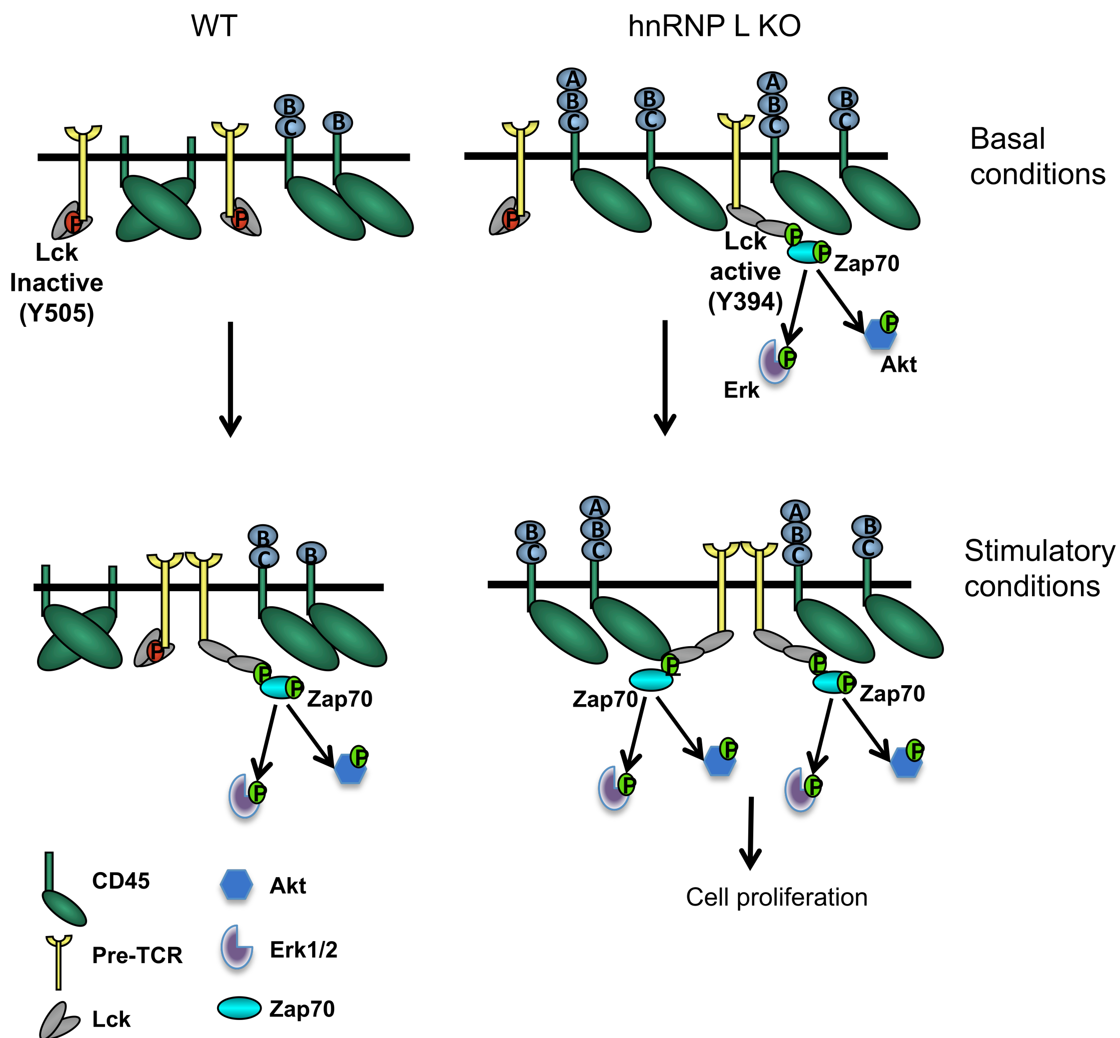

Figure 15. Schematic representation how CD45 isoforms mediate T cell activation. CD45RO is the main isoform expressed on wild-type T cells and as a homodimer it restricts TCR signaling. In contrast, in absence of hnRNP L, the larger isoforms CD45RA and CD45RB, which do not form dimers, are present on the cell surface. The phosphatase domain of CD45 is now exposed in these larger isoforms, and as a consequence, a basal TCR signaling occurs. Upon TCR stimulation, Lck is dephosphorylated and phosphorylates its downstream effectors such as Zap70, Akt and Erk1/2 kinases. In wild-type cells, the CD45 dimers restrict the intensity of the TCR signal while in hnRNP L deficient cells, the signal intensity is increased and leading to an uncontrolled proliferation and activation since the higher molecular weight CD45 isoforms prevail.

1.1.3. Is hnRNP L important in positive and negative selection?

Previous studies using CD45 knockout mice revealed an important role for this transmembrane phosphatase in mediating positive and at some extent negative selection [82, 88, 89]. CD45 null DP cells were defective in positive selection in particular in generating a selected CD4 subset mainly because CD45 affects Lck activity and Lck binds preferentially and more efficiently to the CD4 than to the CD8 co-receptor [69]. Reconstitution of these mice with different levels of CD45RO isoform demonstrated that CD45 phosphatase activity is dose dependent in promoting positive selection. In fact, a restoration of only 3% of the wild-type CD45 expression level was sufficient to re-establish positive selection in CD45 knockout mice [69, 86, 89]. Since both positive and negative selection are induced through TCR signaling and since the intensity of the signal is crucial and depends on the level of CD45 surface it was plausible to consider hnRNP L as a mediator of positive or negative selection. In hnRNP L deficient mice pre-T cell development is partially blocked at the DN4 stage, but differentiation into DP and later on in SP cells still occurs at appreciable levels. The percentage of DP cells showing CD69 and TCRβ expression is similar in wild-type and hnRNP L deficient mice and the generation of CD4 and CD8 remains unaffected in the absence of hnRNP L. Even though, the absolute numbers of CD4SP and CD8SP cells are reduced, this decrease is proportional to the loss

of the DP population in hnRNP L deficient animals compared to controls. It is thus likely that hnRNP L does not affect the processes of positive or negative selection.

The findings presented in this thesis obtained with hnRNP L deficient mice that also carry the OT-I and OT-II transgenes support this conclusion (Annexe I). OT-I and OT-II transgenes allow the expression of a specific TCR receptor against ovalbumin in the context of MHC class I (OT-I) and MHC class II (OT-II) restriction enabling to test the requirement of hnRNP L for positive selection. No obvious changes have been observed in CD69 and TCRβ expression in OT-I or OT-II transgenic mice regardless whether hnRNP L was present or not. Nevertheless, in absence of ovalbulmin expression, the role of hnRNP L in negative selection cannot be definitively determined. To clearly demonstrate a role of hnRNP L in negative selection, either a crossing of OTI and OTII transgenic mice with mOVA transgenic mice or H-Y TCR transgenic mice that are deficient for hnRNP L have to be used. The mOVA/OTI or OTII transgenic mice will express both the OVA antigen and the OVA specific TCR, which will result in the deletion of the T cells bearing this TCR. The H-Y mice would express a specific TCR for the H-Y antigen that is a male specific protein. In this system, positive selection occurs in female H-Y TCR transgenic mice, but in male the H-Y TCR bearing SP cells are eliminated by negative selection [392]. Future experiments with male and female hnRNP L deficient mice that carry the H-Y TCR will unequivocally demonstrate whether or not hnRNP L is involved in negative selection.

1.2. Role of hnRNP L in pre-T cell migration and thymic egress

The observation that only T cells with only one non-excised hnRNP L can be found in the periphery even if the hnRNP L floxed alleles were almost completely excised in pre-T cells was unexpected. During their development, pre-T cells need to migrate within the thymus towards a chemokine gradient, which is specific for each thymic sub-compartment [335,

360]. Migration assays revealed that the hnRNP L knockout SP cells were unable to respond to the chemokines CXCL12 and CCR7 and the sphingolipid S1P. Further analysis showed that this migration defect correlated with inefficient actin polymerization upon chemokine exposure. It is thus conceivable that the deletion of hnRNP L interferes with the migration of SP cells inside the thymus so that they cannot reach the right thymic sub-compartment to access the S1P gradient and respond to it. As a consequence, SP cells that have excised both floxed hnRNP L alleles cannot egress the thymus, and only the small fraction of non-excised cells can reach the periphery.

As discussed in the article of chapter II, the altered CD45 isoform pattern expressed in the absence of hnRNP L cannot fully explain the observed migration defect or the lack of thymic egress. However, a direct interaction between CD45 and CXCR4 has been demonstrated before and CD45 deficient T cells were shown to be unable to migrate towards a CXCL12 gradient [393]. It is also known that CD45 associates with CXCR4 and probably mediates CXCL12 mediated signaling [393]. A similar association of CD45 with CCR7 and S1P1 has not been reported. In hnRNP L deficient mice, CD45 is present and the signaling cascade involving p38 is intact in contrast to the process of actin polymerization, which is mediated by G proteins including Rho GTPases and calcium mobilization [394, 395] suggesting that hnRNP L deficiency does not affect chemokine signaling through the control of CD45 isoform expression. It remains to be shown however, whether a dimerization of CD45 with CXCR4 or CCR7 and S1P can occur in hnRNP L deficient thymocytes.

To identify new targets of hnRNP L in T cells that may potentially explain the defect in migration, a high throughput sequencing strategy (RNA-Seq) has been used. Interestingly, a high proportion of splicing events affected by the lack of hnRNP L concerned genes encoding proteins of the microtubule cytoskeleton, cytoskeletal binding proteins and GTPase binding proteins (Figure 16). Together with the finding that actin polymerization is altered in hnRNP L deficient cells, this suggests that hnRNP L controls the alternative

splicing of pre-mRNAs encoding genes that are involved in the organization of the cytoskeleton after chemokine stimulation. The list of alternatively spliced genes notably contained those implicated in thymocyte mobility for instance mDiap and Wasf2 [396-398]. Reports that mice lacking mDiap present the same T cell migration defect than hnRNP L deficient mice support this conclusion [398]. In addition, mDiap null cells are unable to respond to CXCR4 and CCR7 and mDiap knockout mice lack T cells in their peripheral lymphoid organs.

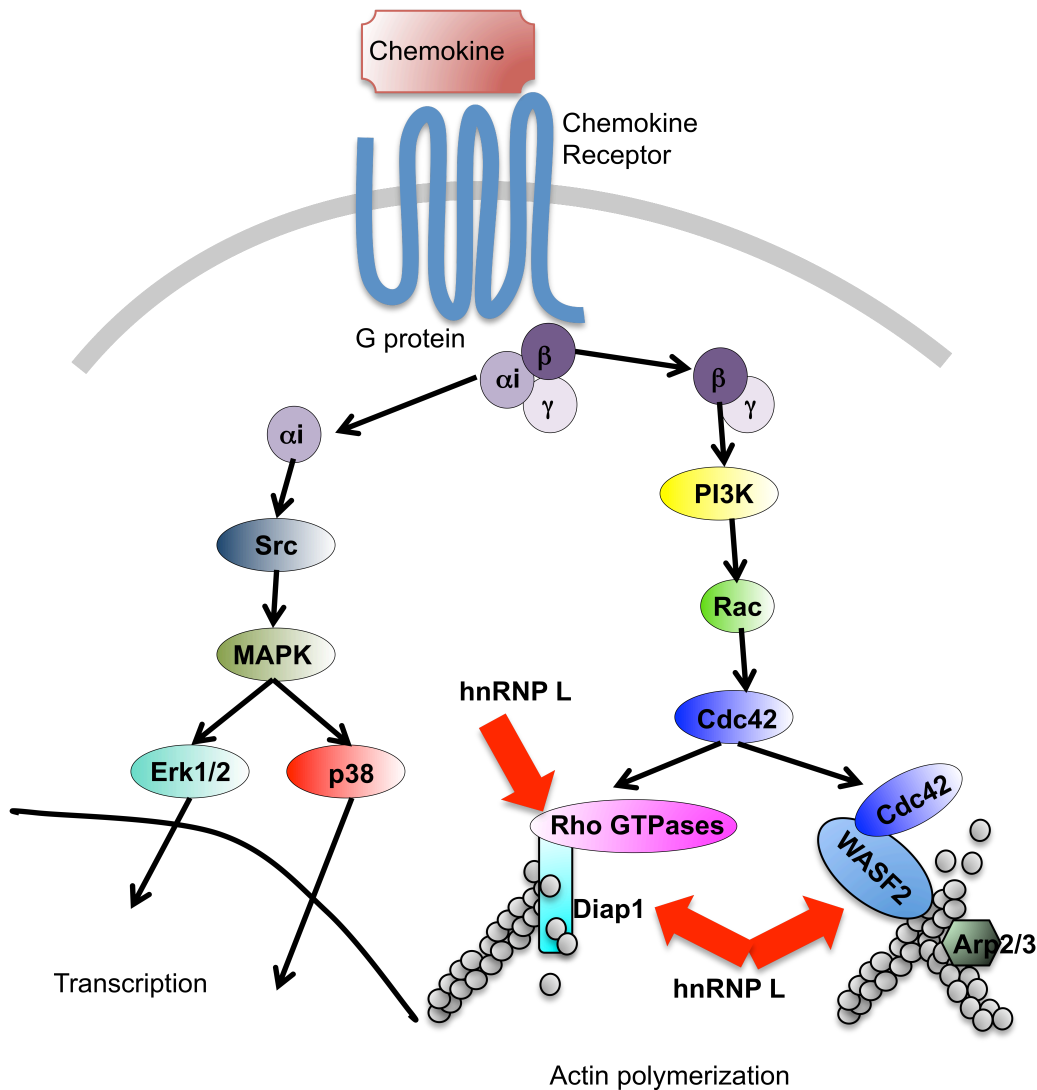

Figure 16. Role of hnRNP L in chemokine receptor signaling. Ligation of chemokines with their cognate receptors results in the initiation of a signal cascade involving - among others – G proteins. Through the α1 G protein subunit, the p38 and Erk1/2 kinases are activated leading ultimately to the activation of gene transcription. In the other side, the G protein β/γ subunits mediate the activation of PI3K and Rac, which induces actin polymerization enabling cell migration. Two possible complexes can modulate actin polymerization: the Formin-driven complex and the WASP-Arp2/3 complex. RNA-Seq analysis revealed that hnRNP L is an important regulator of the alternative splicing of components of this two complexes. In regard to the Formin complex, hnRNP L is involved in the splicing of both Diap1 and Rho GTPases such as Arhgef2 and Arhgap17. With regard to the WASP-Arp2/3 complex, hnRNP L regulates splicing of Wasf2. Therefore, the inability to polymerize actin in absence of hnRNP L might be due to aberrant isoforms of components of the Formin or Wasp complexes produced in these cells. Adapted from [395, 399]

Whether these genes are directly responsible for the phenotype seen in hnRNP L deficient T cells remains to be clarified. Further investigation is needed to conclude which of these genes is the main regulator and how their splicing affects its activity. Different pre-mRNA isoforms of a specific gene encode proteins that have diverse functions in a cell. They may be non functional either as a result of a truncation, or because of mRNA degradation. The new isoforms produced may also have a post-translational modification that may change their localization or function and or may affect the interaction with a binding partner. The list of differentially spliced genes obtained from the comparison between wt and hnRNP L deficient cells and the precise mapping of the preferentially used splice sites will enable the identification of hnRNP L targets. To confirm a target an alternatively spliced isoform would have to be reintroduced into hnRNP L deficient cells to rescue the mutant phenotype.

1.3. Role of hnRNP L in peripheral T cells

Since hnRNP L deficient thymocytes do not reach the peripheral organs it is difficult to characterize the role of hnRNP L in the acquired immune response. It is possible that the TCR signal threshold upon MHC-antigen encounter will be modified due to the possibly aberrant CD45 isoforms present on the surface of naive and memory T cells. Considering that a point mutation in the inhibitory wedge of CD45 abrogates its dimerization and as a consequence induces polyorgan autoimmunity [60], it is likely that a similar phenomenon will develop in absence of hnRNP L in peripheral T cells which express more active CD45 isoforms. Furthermore, given the defect in migration of thymocyte, it is possible that T cells deficient for hnRNP L will bear the same phenotype and be inefficient in re-circulating and in getting access to antigen presenting cells in the lymph nodes or to mediate the immune response at specific site. The interaction between APCs and T cells depends also on chemokine and chemokine receptor interaction. One possibility to study peripheral T cells in hnRNP L deficient mice will be to directly inject into the thymus pre-T cells that carry floxed hnRNP L alleles, let these cells seed and circulate and finally induce deletion by either poly(I:C) injections in the case of MxCre transgenic T cells or tamoxifen treatment for CreER positive T cells. Alternatively, a Cre transgene has to be expressed in hnRNP L deficient mice that allows expression solely in peripheral T cells.

1.4. Role of hnRNP L in human T cells

HnRNP L is known to be expressed and to be involved in CD45 splicing in vitro in both human and mice [62, 384] It is then conceivable to transpose our results obtained with our mouse model into human. However, some differences remain between thymocyte development in mouse and human. As mentionned above, murine thymocytes do not express on their cell surface the CD45RA isoform [81]. In the case of human, the CD45RA

isoform has been detected in almost all sub-populations [80]. This difference might reflect a need for a greater TCR signal in human thymocytes to develop compared to murine cells. The same discrepancy arise for peripheral naive T cells where the mouse compartment expressed only CD45RB isoform while human naive T cells beared CD45RA [80, 81]. HnRNP L still can play a role in T cell development and T cell activation in human since as for murine thymocytes, the CD45 isoforms expression vary from one sub-type to another and CD45 splicing by hnRNP L is needed to restrict the TCR signal. Even if we considere that the genome differs between the two organisms, we can assume that hnRNP L might splice the human actin polymerization regulators Diap1 and Wasf2 and therefore regulates the migration of human thymocytes.

2. Role of hnRNP L in hematopoiesis

As observed for the constitutive deletion of hnRNP L, a specific deletion of hnRNP L in all hematopoietic cells either by VavCre or MxCre transgenes is lethal. Fetuses and adult mice lacking hnRNP L in hematopoietic cells have low number of lymphocytes, neutropenia, thrombocytopenia and incomplete erythrocyte development showing a major requirement of hnRNP L in hematopoiesis. The hematopoietic system is a complex hierarchy in which mature cells developed from progenitors that are pre-committed to a certain lineage mainly by specific transcription factors [122, 149, 400]. These progenitors possess a restricted multipotent capacity and do not have the self-renewal capacity of hematopoietic stem cells (HSCs) [122, 149, 400]. In absence of hnRNP L all progenitors were reduced in numbers including those for lymphocyte differentiation (CLPs) and erythrocyte and myeloid specification (MEPs, CMPs, GMPs). Since all progenitors were affected to almost the same extent than mature cells it is likely that hnRNP L deficiency affects hematopoietic stem cells.

HSCs are self-renewing, multipotent and are defined as LSKFlt3$^-$ CD150$^+$CD48$^-$. HSCs possess a long-term potential of repopulation in irradiated mice since they are the most primitive cells of the hierarchy with the highest self-renewal capacity and complete multipotency [130, 140]. In contrast, the multipotent progenitors MPP1 and MPP2 identified as LSKFlt3$^-$CD48$^+$; CD150$^+$ and CD150$^-$ respectively, are still able to generate all cells of the hematopoietic system, but their self-renewing ability is reduced and they can only reconstitute irradiated mice for a restricted period of time [130, 140]. Both the fetal liver and the bone marrow deficient for hnRNP L showed a drastic reduction in the number of MPPs. Interestingly, the effect of hnRNP L ablation was different for HSCs between the embryos and adult mice. In adult mice, the percentage of HSCs was similar to control mice, but their absolute cell numbers were reduced compared to wild-type. In contrast, in the fetal liver, the HSCs were increased in percentage but there was no significant change in the total number of cells. This suggests that hnRNP L is more important in adult HSCs than the embryonic one maybe due to their difference in quiescence. However, in both case hnRNP L plays a pivotal role in the transition from HSC to MPP.

Different in vitro and in vivo methods are available to test the functionality of HSCs and progenitors. Some of these were used to evaluate the potential of the remaining HSCs found in the fetal liver and the bone marrow of hnRNP L deficient mice. The hypothesis was that even if a blockage at the transition from HSC to MPP existed and developmental progress was inhibited, HSCs that lack hnRNP L might keep their long-term potential of reconstitution and development. However, the results of all tests presented in this thesis indicate that hnRNP L ablated HSCs are non functional. A number of independent lines of experiments support this conclusion. First, when hnRNP L knockout LSK cells were seeded on stromal layer cells to potentiate their development into B and T lymphocytes or were put into semisolid medium to generate granulocyte-monocyte or erythrocyte colonies, no mature cells or colonies could be generated. Reconstitution assays using transplantation of fetal liver cells into irradiated mice are the main methods to evaluate the long-term potential and the multipotency of HSCs. With hnRNP L deficient cells, it was impossible to

observe any reconstitution of the hematopoietic system in recipient animals. Still, very few HSCs were found in bone marrow of animals reconstituted with hnRNP L deficient fetal liver cells 12 weeks post-transplantation. However the variability of their numbers was too wide to make any conclusion on the long-term survival of these cells. An additional experiment to further support the conclusion that hnRNP L is required for HSC functionality would be the use of cells from hnRNP L deficient mice carrying Gt(ROSA)26Sor$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$ alleles, which will allow to track GFP positive and hnRNP L deleted HSCs in the bone marrow of reconstituted mice.

Fetal HSCs are dividing to constitute the stem cell pool for the lifetime of the organism while adult HSCs are mainly in a quiescent state [376, 401]. However, in both systems, HSCs after division can either self-renew, differentiate or die [164]. To dissect which one of these possibilities is mis-regulated in the absence of hnRNP L, the cell cycle status of the HSCs has been verified. First, it is likely that hnRNP L deficient HSCs do not have a higher capacity of self-renewal than wild-type HSCs since their absolute number is unchanged or even decreased compared to wt controls. In fact, we observed a marked increase in the percentage of HSCs that were progressing through the cell cycle in both the fetal liver and adult bone marrow. Hence, the deletion of hnRNP L resulted in a loss of the quiescent state. In correlation to this cycling status, the HSCs from the deficient animals were producing high levels of reactive oxygen species (ROS), which are known to be generated during increased metabolic rates [377, 378]. ROS predominantly contribute to oxidative DNA damage by making single strand breaks but they can also induce double-strand breaks during replication or when they encounter the transcriptional machinery [377, 378]. Cells including HSCs possess a DNA damage response machinery to repair DNA breaks. In the case of an inefficient repair, cells undergo apoptosis as was observed in hnRNP L deficient HSCs and MPPs. It is thus possible that HSCs require hnRNP L to restrict their cell cycle progression and cycling. If hnRNP L is missing, cells start to cycle more often and generate ROS as a consequence of a higher metabolic rate and subsequently induce a DNA damage response that leads to apoptosis. This hypothesis is in concordance with the data presented

here; to further support it more experiments are required that show that a DNA damage response indeed is initiated in the absence of hnRNP L (Figure 17).

2.1. Proliferation and apoptosis in hnRNP L deficient cells

2.1.1. Alternative splicing of CD45 pre-mRNA

The list of known hnRNP L targets does not include genes that would point to the role of hnRNP L in HSC function. It is unlikely that the alternative splicing of CD45 pre-mRNA and the expression of altered CD45 isoforms in absence of hnRNP L could account for the HSC phenotype seen in hnRNP L deficient mice. HSCs as other hematopoietic cells express CD45 mainly as the CD45RB and CD45RO isoforms [44]. Studies using CD45 null mice revealed no change in bone marrow cellularity nor the number of white blood cells, supporting this view [82]. However, two recent publications showed a possible involvement of CD45 in HSC mobilization. CD45 deficient HSCs had an impairment to engraft and mobilization of HSCs by GM-CSF treatment was inefficient in the absence of CD45. This mobilization defect was due to the effect of CD45 on CXCR4 adhesion and migration potential [402, 403]. Another indirect consequence was the bone architecture that was altered in CD45 null mice, because osteoclasts that are expressing CD45 were secreting less MMP9 [402]. In the model favoured here, the deletion of hnRNP L is cell type specific and therefore should not affect osteoclasts nor bone or fetal liver architecture. However, even if we cannot exclude completely an indirect role of Poly(I:C) induced IFN-α on osteoclast differentiation, both wild-type and hnRNP L deficient mice received the same treatment [404]. Moreover, even a complete deletion of CD45 did not result in a major change in bone marrow cellularity in contrast of the observations made with hnRNP L deficient animals. In addition, CD45 null mice did not show an increased rate of cell death and, finally, all HSC and progenitor populations are present in CD45 knockout mice but show a reduced mobilization after a stress induced challenge. Therefore, it can be

concluded that the apparent requirement of hnRNP L for HSC function and integrity is not based on its function to regulated CD45 alternative splicing and isoform expression.

2.1.2. Role of Bcl-2

A number of previous and contradictory studies were linking hnRNP L to Bcl-2 mRNA stability. One study claimed that hnRNP L binds directly to the ARE element of Bcl-2 mRNA to regulate its degradation [38]. A second study showed the requirement of another protein to mediate the binding of hnRNP L to the Bcl-2 mRNA [39]. Bcl-2 is an anti-apoptotic molecule blocking the release of cytochrome c from the mitochondrion and the subsequent caspase-9 cleavage and activation [405, 406]. Since Bcl-2 is being implicated in apoptosis and was shown to be required for the maintenance of HSCs [407], it was necessary to determine whether its expression was affected by the absence of hnRNP L. However, the data presented here do not show any significant differences in Bcl-2 mRNA level between hnRNP L deleted cells and control cells. It is thus unlikely that hnRNP L is a regulator of Bcl-2 in HSCs. Moreover, as rescue experiment in which VavCre^{+}hnRNP L $^{fl/fl}$ mice had been crossed with H2K-Bcl2 transgenic mice which overexpress constitutively Bcl-2 in all hematopoietic cells was done, but did not reveal any information of a regulatory link between hnRNP L and Bcl-2 in HSCs. In fact, the analysis of fetal liver from H2K-Bcl2, VavCre^{+}hnRNP L$^{fl/fl}$ combinatorial mutant mice showed a similar level of apoptosis in the HSC compartment compared to the VavCre^{+}hnRNP L$^{fl/fl}$ embryos. Therefore, it can be concluded that accelerated cell death in absence of hnRNP L is independent of the Bcl-2 pathway and cytochome c release from the mitochondria.

2.1.3. Role of other unknown hnRNP L targets

One other possible mechanism to induce apoptosis is through the expression of death receptors such as TRAILR1, FAS and TNFR1 at the cell surface [37, 408]. The FAS receptor or CD95 is usually absent from HSCs [409]. Upon interaction with its ligand, FAS trimerizes to form the Death inducing signaling complex (DISC) with the FADD adaptor molecules and pro-caspase-8. Two types of CD95 signals have been characterized [37, 408]. Type I signals rely on a high level DISC formation leading to increased cleavage of pro-caspase-8 into its active form which mediates apoptosis through its downstream caspase effectors. On the other hand, CD95 type II signalling results from a less dense DISC formation with low level of active caspase-8. This pathway can be inhibited by Bcl-2 and needs an amplification loop involving Bid and cytochrome c to generate active caspase-9 [37, 408]. Analysis of HSCs revealed that in absence of hnRNP L, CD95 is present on the cell surface while control HSCs did not express this death receptor. As a consequence, the accumulation of the cleaved form of caspase-8 was observed in the lineage depleted fraction of bone marrow and fetal liver deficient for hnRNP L. Surprisingly, it has been described that in HSCs, CD95 might possess a different role than the typically observed induction of cell death. It is possible that CD95 is also able to induce cell proliferation using another signaling pathway than the one employed in cell death induction [409, 410]. However, the data presented here show that in hnRNP L deficient HSCs caspase-8 is cleaved and Bcl-2 over expression is unable to block cell death. It is thus possible that a type I, Bcl-2 independent CD95 signaling takes place and induces cell death in hnRNP L deficient HSCs. A more detailed analysis of the DISC formation would be one possible experimental way to acquire more support for his hypothesis.

It has been shown before that hnRNP L can affect the alternative splicing of caspase-9 pre-mRNA. This role of hnRNP L had been discovered in non small cell lung cancer (NSCLC), where hnRNP L is phosphorylated and binds to the exon 3 of caspase-9 [411, 412]. Upon this binding, exon 3 is excluded from the final mRNA, which generates the isoform b of

caspase-9 that is anti-apoptotic instead of pro-apoptotic like the regular caspase-9a isoform [411, 412]. This phenomenon cannot be analyzed in hnRNP L deficient cells since the *Caspase-9* gene in mouse considerably differs from the human gene.

Although death receptor signaling through CD95 may be the cause of apoptosis in hnRNP L deficient cells, a direct action of hnRNP L on the CD95 pre-mRNA seems implausible. It is more likely that the upregulation of CD95 seen on hnRNP L deficient HSCs is caused by another event, for which the ablation of hnRNP L could be responsible. High throughput sequencing of fetal liver cells from wt and hnRNP L deficient embryos was chosen to find novel hnRNP L targets. Most of the cells found in the fetal liver are erythrocytes mainly enucleated, immature hematopoietic cells and precursors with little or no mature hematopoietic cells. The high throughput sequencing generated a wide list of targets genes showing differential alternative spliced patterns in absence of hnRNP L.

One limitation of our RNA-Seq analysis was the use of the total fetal liver instead of being restricted to HSCs only. The availability of HSCs in wild-type fetal liver is already restraint, but it is almost impossible to obtain a sufficient number of HSCs from hnRNP L deficient embryos to generate RNA-Seq analysis. The problem arose that the main genes with upregulated expression in hnRNP L deficient cells were those found in GO pathways related to liver and liver development. As explain above, the fetal liver is constituted of hematopoieitic cells but also liver cells. We have demonstrated that deficient hnRNP L fetal liver have reduced number of erythrocytes, progenitors cells and HSCs. This reduction alters the ratio between liver and hematopoietic cells compared to control mice. Therefore, we excluded from our analysis the liver genes that were over represented in absence of hnRNP L. To overcome this problem, one possible method will be the validation of our candidates using a quantitative PCR specific to each isoforms identified in sorted HSCs.

Lists of genes with spliced junctions preferentially used in either wild-type or knockout cells which are all potential targets of hnRNP L, could be identified with this method. The

gene ontology (GO) pathways that contained genes from these lists at a frequency that is statistically significant were identified. Among those, many corresponded to "cell cycle", "DNA repair" and "apoptosis", which reflects well the phenotype of hnRNP L deficient HSCs. Genes such as *Cdkn1a* (p21Waf), *Card10*, *Trp53bp1* and *Trp53inp1* are mediators of apoptosis and are alternatively spliced in hnRNP L knockout cells. DNA repair genes including *Xrcc6*, *Brca2*, *Fancm* and *Rad5*0 were also found alternatively spliced primarily in wild-type fetal liver. Thus, one possible mechanism by which hnRNP L deficient HSCs are dying consists of a two steps process: HSCs are induced to divide by one of the aberrant isoforms of genes involved in cell cycle progression such as *Cdt1, Topbp1* and *Pds5b.* This augmented cell cycling produces increased metabolites such as ROS that provoke DNA double-strand breaks. Once the DNA is broken, the DNA repair machinery is in action. However, inefficient damage repair leads to apoptosis through the activation of p53 [413]. The hypothesis laid out here involves that one or more of the target genes of hnRNP L in the DNA "damage response" GO pathway may be non functional as result of their alternative splicing for instance by post-translational modification or a modified binding or association to a partner protein. The gene expression of CD95 is known to be mediated by p53, which in turn is induced by a DNA damage response [414]. In this case, an enhanced expression of CD95 could trigger a cell death pathway in hnRNP L deficient HSCs. However, the high level of expression and the alternative splicing of p21 (Ckdn1a) is still difficult to reconcile with this hypothesis, since this is a negative regulator of cyclins and therefore should stop cell cycle progression in particular when DNA damage occurs. On the other hand, overexpression has been implicated in a number of studies in apoptosis and p21 knockout mice do only show mild alteration in cell cycle progression [200, 415]. It is thus still possible that a high level expression and potential aberrant splicing of Ckdn1a contributes to the apoptotic phenotype seen in hnRNP L deficient HSCs.

It is tempting to simplify the induction of apoptosis in hnRNP L deficient HSCs by the mechanisms proposed. But it should be taken in account that hnRNP L is a splicing regulator of other important target genes required for HSCs maintenance and survival. As

mentioned in the introduction, the interaction of HSCs with its environment is crucial [142, 143]. Some targets of hnRNP L detected by the RNA-Seq analysis are also members of the GO category “cell adhesion”. Both integrin alphaL and integrin beta2 were found to be alternatively spliced by hnRNP L in this analysis. Together they form a heterodimer called LFA-1, which interacts with ICAM-1 expressed on osteoblasts [227]. Even if the integrin alpha 4 can compensate for the loss of LFA-1 in the homing of HSCs, LFA-1 needs to be present in absence of integrin alpha4 [379, 416]. Hence, a defect in adhesion might cause HSCs to detach and/or migrate from the niche and lose their quiescence. It was also found that the FRZ co-receptors LRP5 and LRP6, which are constituents of the Wnt signaling pathway are potential targets of hnRNP L in the fetal liver [417]. Both canonical and non-canonical Wnt signaling pathways are important in maintaining the self-renewing ability of HSCs and to keep them in a quiescent state [418, 419]. Also among potential hnRNP L targets is the negative regulator of TGF-β signaling Smad7 [420]. As already described, TGF-β is required to maintain the HSCs in a G0 state [211, 421]. Therefore, if Smad7 action is potentially modified by hnRNP L mediated alternative splicing it is possible that it becomes more potent in inhibiting the TGF-β signaling and causing HSCs to enter the cell cycle. Finally, IL6ST (gp130) receptor show a differential spliced pattern in hnRNP L knockout fetal liver cells compared to the respective control cells. IL6ST is the receptor for both IL6 and IL11. It has been described that IL6ST null mice had a reduced number of primitive HSCs [422]. In addition, IL6ST activation by both IL6 and IL11 promotes the HSC self-renewal in a dose dependent manner. In fact, in vitro stimulation with one of the two ligands induces maximal amplification of HSCs as shown in a CRU assay [423, 424]. As a target of hnRNP L, alternative spliced variant of IL6ST might be responsible for the cycling of HSCs and consequently their cell death.

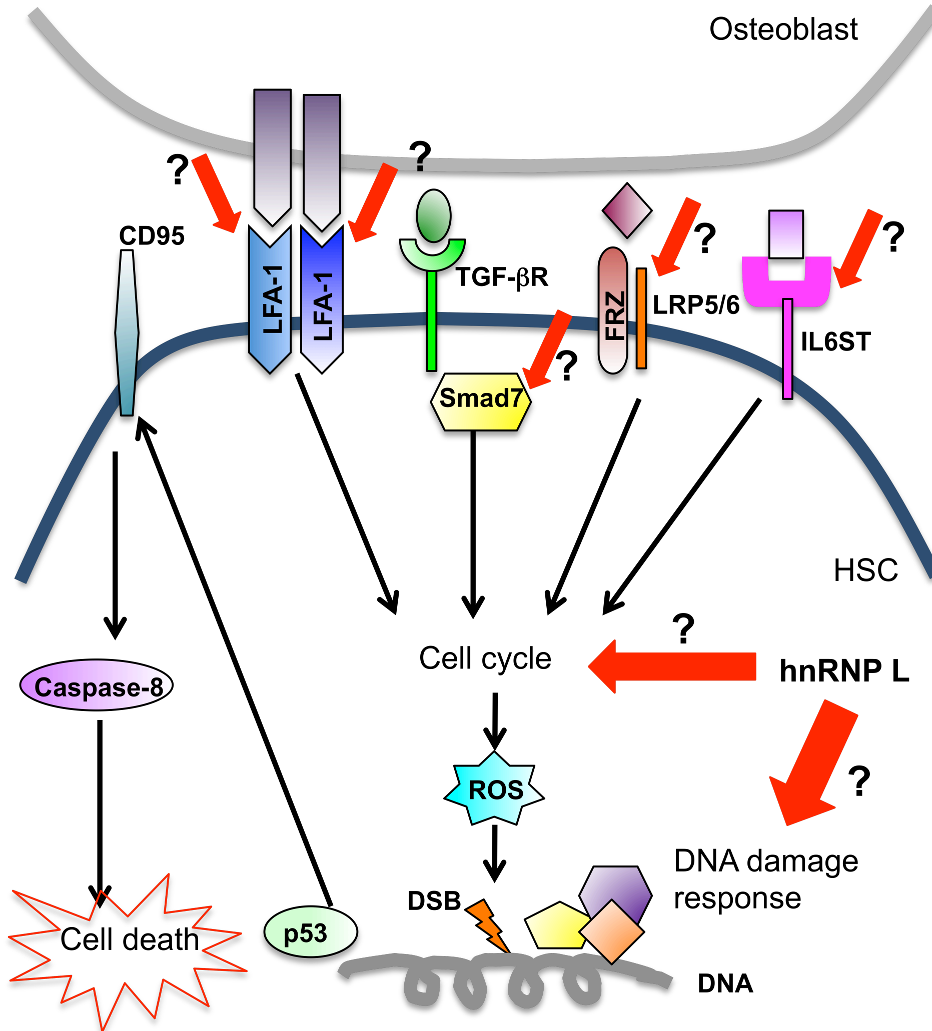

Figure 17. Possible roles of hnRNP L in HSC integrity. RNA-Seq analysis of fetal liver cells revealed several important candidates for hnRNP L targets. Diverse mechanisms may explain the increased cell death observed in HSCs lacking hnRNP L. First, an enhanced cell cycle progession might be caused directly by aberrantly expressed isoforms of some of these new hnRNP L targets or indirectly by mediators of quiescence such as the integrin LFA-1 (integrins β2 and αL), Smad7, Lrp5/6 and IL6ST whose splicing are also affected by hnRNP L deficiency. Enhanced cell cycle progression can lead to increased ROS (reactive oxygen species) production that might cause double-strand breaks (DSB) inducing a DNA damage response. Since some hnRNP L targets that were identified to be alternatively spliced comprise genes that regulate DNA repair and the response to DNA damage, it is thus possible that inefficient DNA repair occurs in hnRNP L deficient cells. This impaired repair can cause the CD95 upregulation and caspase-8 cleavage that is observed in hnRNP L deficient HSCs through p53. Adapted from [227].

2.2. Role of hnRNP L in human hematopoieisis

Both human and murine HSCs can be found in the fetal liver of embryos or in the bone marrow of adults. Even if there are some phenotypic differences in the HSC compartment between the two organisms, both are maintained in a quiescent stage in adult but proliferate in the fetal liver [155, 227, 425, 426]. The quiescent stage of adult human HSCs requires the same bone microenvironment than the murine ones [155, 227, 425, 426]. Therefore, we can assume that the role of hnRNP L in human hematopoiesis will be similar to mouse. However, we cannot exclude that there will be some differences since the gene targets may differ between species as for example the caspase 9 splicing found only in human [412].

3. Role of post-transcriptional mechanisms regulating hnRNP L splicing

Splice variants generated in the absence of hnRNP L in the fetal liver or in the thymus do not necessarily generate functionally defective proteins. Therefore, it will be important to check the functionality of each of the spliced isoforms of the potential mediators of HSC proliferation and cell death. In addition, the list generated might not contain all direct targets of hnRNP L. Since hnRNP L is a ubiquitous splicing factor and autoregulates its own splicing [25], it is possible that it is involved in the splicing of another regulator which is implicated in the alternative splicing of some of the target genes discovered through the RNA-Seq analysis. The only way to determine the direct targets of hnRNP L is by combining a CLIP assay with the RNA-Seq in which only the immuno-

precipitated RNA bound by hnRNP L will be sequenced. In addition, such an analysis performed on sorted, pure populations of pre-T cells or HSCs will be more accurate. It has also to be taken into account that complete deletion of hnRNP L does not reflect exactly what happens usually in the cells. As other splicing factors, hnRNP L is regulated by post-translational modifications that might change its binding partner and the sequence recognition. For example the phosphorylation at Ser43 of hnRNP L in NSCLC that allows the binding of hnRNP L on caspase-9 exon 3 that does not contain CA rich elements [412]. Moreover, exclusion or inclusion of CD45 exon 5 depends on the phosphorylation of PSF by GSK3 that becomes a partner of hnRNP L in exon skipping [64, 412]. Nevertheless, probably all these genes identified are related to hnRNP L splicing but it will depend on the cell status and the signals received, whether they might be spliced or not by hnRNP L.

Conclusion

Alternative splicing of genes is cell type specific and depends on the developmental stage and activation status of cells. Splicing factors such as hnRNP L are ubiquitously expressed however their function differs for each cell type; this difference is in part mediated by post-translational modification of splicing factors. In this study with conditional hnRNP L deficient mice this cell type specificity and the dependency on cellular context could be well demonstrated using the example of pre-T cells and hematopoietic stem cells. In the pre-T cells, hnRNP L regulates proliferation of one subtype of cells through the control of CD45 activity and migration and thymic egress of another cell subtype by regulating the splicing of another set of pre-mRNAs. In hematopoietic stem cells, hnRNP L is required for restricting cell cycle progression and cell survival by regulating the alternative splicing of yet another set of target genes. The list of target genes in the two cell types differ considerably in accordance to their phenotype and thus provide a proof of the specificity of alternative splicing by hnRNP L.

By demonstrating that a factor regulating alternative splicing controls such different processes as T cell differentiation and migration on one hand and the functional integrity of hematopoietic stem cells on the other is intriguing. It demonstrates that another layer of regulation exists that is different from but interwoven with the action of transcription factors, epigenetic regulators, receptors and cytokine signaling and posttranscriptional processes. Alternative splicing significantly contributes to the regulation of hematopoietic differentiation and has to be recognized as a key process that can also be exploited to interfere with aberrant hematopoietic processes such as the emergence of leukemia, lymphoma or myeloma or other type of blood cancers. Alternatively spliced variants of many important genes thought to be cancer related may serve as biomarkers in the future. In addition, specific isoforms of proteins generated by alternative splicing as well as the

splicing factors themselves might turn out to be useful clinical targets once their function has been fully elucidated. This study shows the implication of one of those splicing factors, hnRNP L in hematopoietic development. Together with other studies, a clear picture of the role of alternative splicing in normal and malignant hematopoiesis will appear and pave the ground for new therapeutic strategies.

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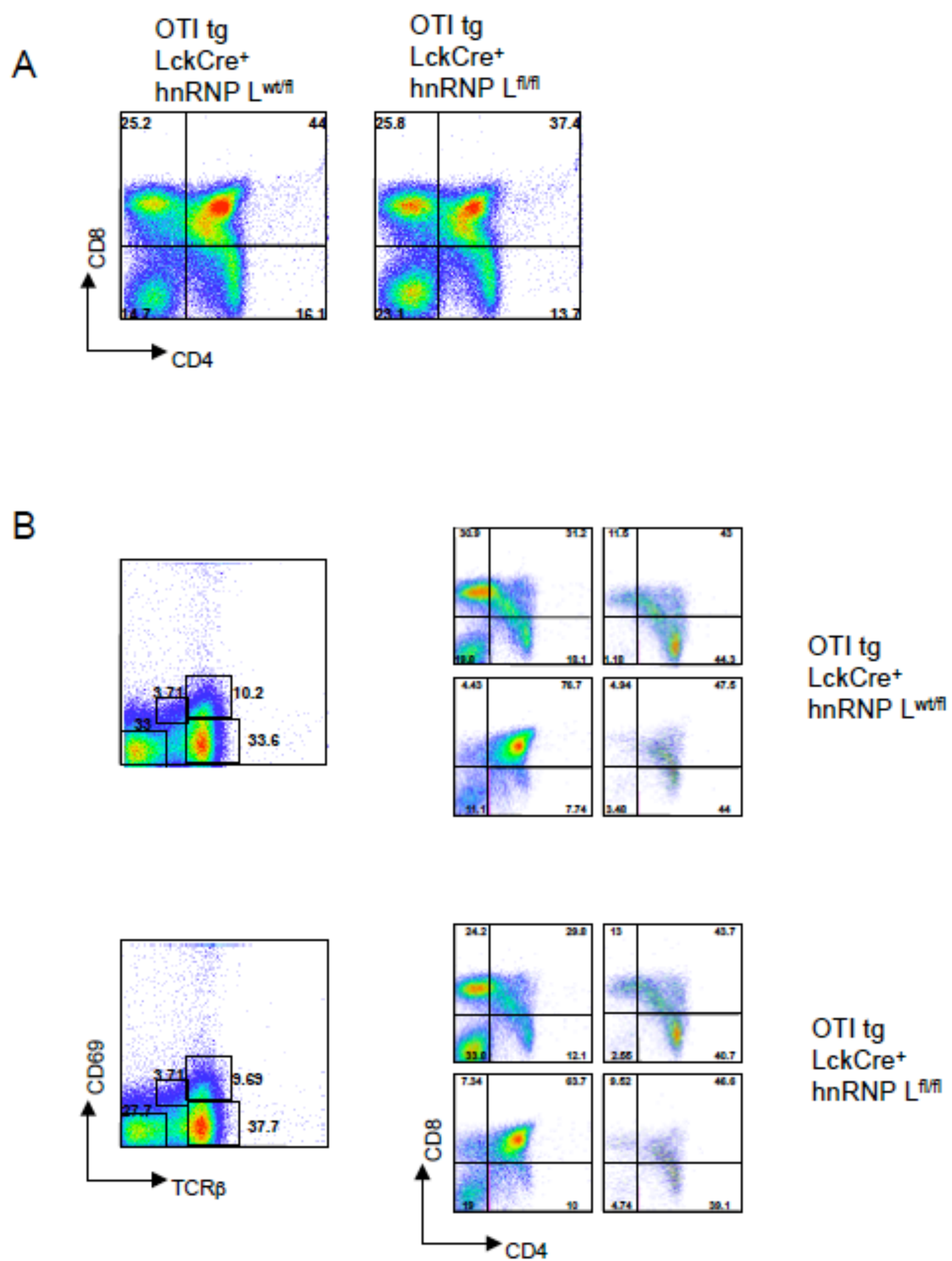
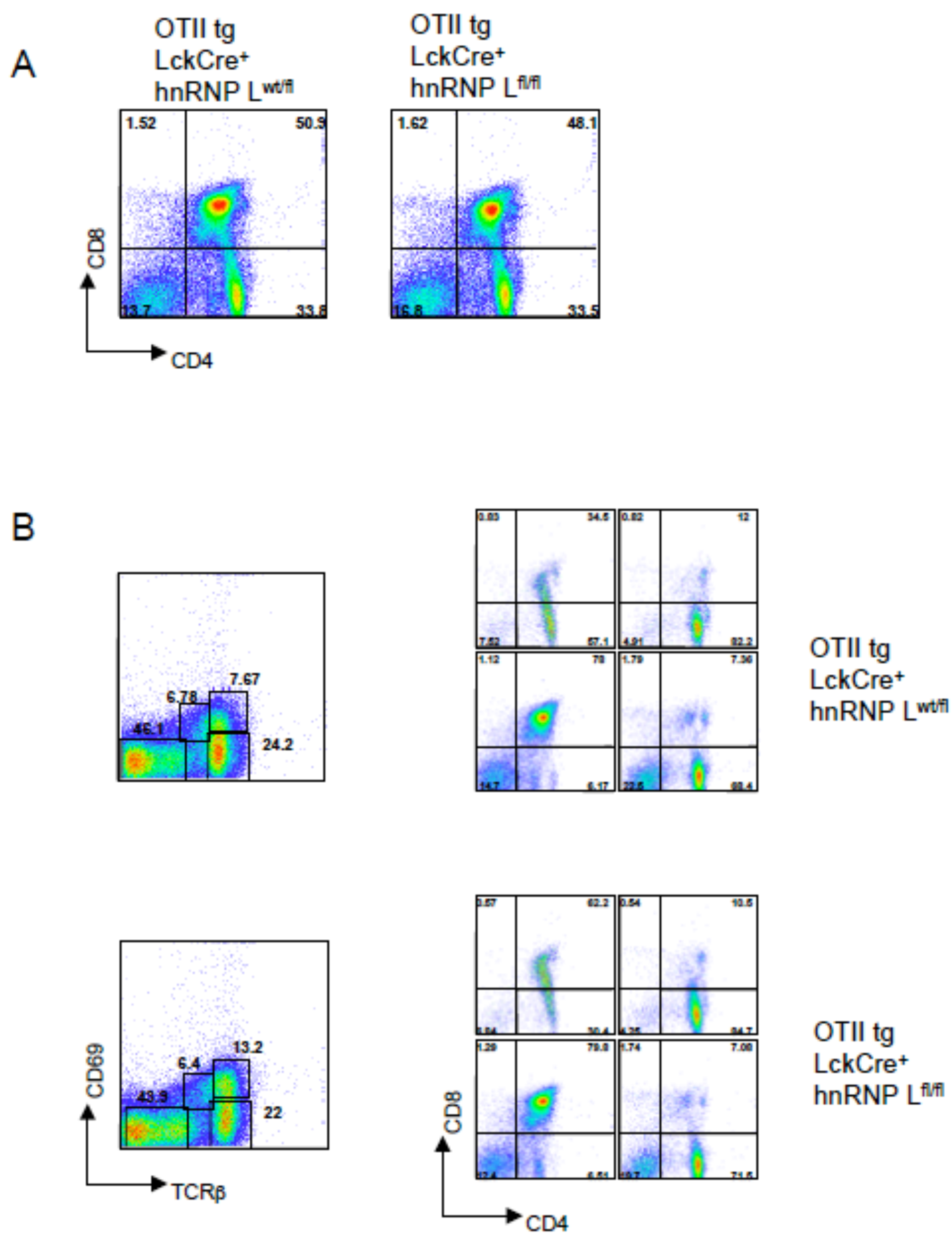
Annexe I: Positive selection



A
OTI tg
LckCre+
hnRNP Lwt/fl
OTI tg
LckCre+
hnRNP Lfl/fl
25.2
44
16.1
25.8
37.4
13.7
CD8
CD4
B
10.2
33.6
33
OTI tg
LckCre+
hnRNP Lwt/fl
CD69
TCRβ
27.7
9.69
37.7
OTI tg
LckCre+
hnRNP Lfl/fl
CD8
CD4

Annexe I Figure 1. Deletion of hnRNP L does not affect the positive selection of MHC class I restricted thymocytes. Positive selection was assessed by flow cytometry on thymus from control wt (A) or hnRNP L deleted (B) expressing both OTI transgenic TCR based on surface expression of CD4 and CD8 on gated population $TCR^{lo}CD69^{-}$, $TCR^{lo}CD69^{+}$, $TCR^{hi}CD69^{+}$ and $TCR^{hi}CD69^{-}$.



A
OTII tg
LckCre+
hnRNP L wt/fl
OTII tg
LckCre+
hnRNP L fl/fl
1.52
1.62
48.1
33.5
CD8
CD4
B
7.67
6.78
46.1
24.2
OTII tg
LckCre+
hnRNP L wt/fl
13.2
6.4
43.9
22
CD69
TCRβ
OTII tg
LckCre+
hnRNP L fl/fl
CD8
CD4

Annexe I Figure 2. Deletion of hnRNP L does not affect the positive selection of MHC class II restricted thymocytes. Positive selection was assessed by flow cytometry on thymus from control wt (A) or hnRNP L deleted (B) expressing both OTII transgenic TCR based on surface expression of CD4 and CD8 on gated population $TCR^{lo}CD69^{-}$, $TCR^{lo}CD69^{+}$, $TCR^{hi}CD69^{+}$ and $TCR^{hi}CD69^{-}$.

Other contributions

1- Khandanpour Cyrus, Krongold Joseph, Vassen Lothar, Chen Riyan, van der Reijden Bert, Janssen Joop, **Gaudreau Marie-Claude**, Peeters, Justine K. Löwenberg Bob, Patel Chandrashekhar V., Dührsen Ulrich, Möröy Tarik. A variant form of human GFI1 (GFI136N) predisposes to AML by altering epigenetic marks at the Hoxa9 locus. Blood,in revision

2- Khandanpour Cyrus *, Phelan James D. *, Vassen Lothar, Horman Shane R., **Gaudreau Marie-Claude,** Saba Ingrid, Zhu Jinfang, Paul, William E., Dührsen Ulrich, H Grimes. Leighton *, and Möröy Tarik*. Lymphoid malignancies critically require Growth factor independent 1 (Gfi1) for tumor initiation and maintenance. Cancer Cell submitted *contributed equally

3- Khandanpour Cyrus, Kosan Christian, **Gaudreau Marie-Claude,** Ulrich Dührsen, Hui Zeng and Möröy Tarik. Growth factor independence 1 (Gfi1) protects hematopoietic stem cells against apoptosis but also prevents the development of myeloproliferative-like diseases. Stem Cell 2011 Feb: 29(2):376-380

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