



# Role of the post-transcriptional regulators Pumilio1 and Pumilio2 in murine hematopoietic stem cells

Fabio Michelet

## ► To cite this version:

Fabio Michelet. Role of the post-transcriptional regulators Pumilio1 and Pumilio2 in murine hematopoietic stem cells. Human health and pathology. Université René Descartes - Paris V, 2013. English. NNT : 2013PA05S013 . tel-00911665

**HAL Id: tel-00911665**

**<https://theses.hal.science/tel-00911665>**

Submitted on 29 Nov 2013

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Université Paris Descartes

**Ecole Doctorale Biologie et Biotechnologie**

*INSERM U1016, Institut Cochin / Equipe Dusanter-Cramer*

# Role of the post-transcriptional regulators Pumilio1 and Pumilio2 in murine Hematopoietic Stem Cells

Par Fabio Michelet

Thèse de doctorat en Hématologie et Oncologie

Dirigée par Dr. Evelyne Lauret

Présentée et soutenue publiquement le 7 Novembre 2013

Devant un jury composé de :

Président:	Pr. Lacombe Catherine (PU-PH)
Rapporteur:	Dr. Simonelig Martine (DR2).
Rapporteur:	Dr. Mazurier Frédéric (DR2)
Examineur:	Dr. Cohen-Tannoudji Michel (DR2)
Directrice de thèse :	Dr. Lauret Evelyne (CR1, HDR)



# CONTENTS

ACKNOWLEDGEMENTS.....	I
ABSTRACT .....	V
LIST OF FIGURES .....	VII
LIST OF ABBREVIATIONS.....	IX

## INTRODUCTION ..... 1

### 1. STEM CELLS.....3

1.1. DEFINITION OF STEM CELL POTENTIAL .....	4
1.1.1. Totipotent stem cells .....	4
1.1.2. Pluripotent stem cells .....	4
1.1.2.1. Embryonic stem cells.....	5
1.1.3 Multipotent stem cells and Adult stem cells .....	6
1.1.4. Progenitor cells .....	7

### 2. HEMATOPOIETIC STEM CELLS .....8

2.1. MURINE HEMATOPOIETIC STEM CELL ASSAYS .....	11
2.1.1. <i>In vitro</i> assays .....	12
2.1.1.1. Colony-Forming Cell Assay .....	12
2.1.1.2. Long-term culture-initiating cell assay .....	13
2.1.2. <i>In vivo</i> Assays .....	13
2.1.2.1 Short Term <i>in vivo</i> assays .....	13
2.1.2.1.1. Colony-forming unit-spleen (CFU-S) cells assay.....	13
2.1.2.2 Long Term Repopulating Cells assay .....	14
2.1.2.2.1. Competitive repopulation assay .....	14
2.1.2.2.2. Limiting dilution assay .....	15
2.1.2.2.3. Serial transplantation assay .....	15
2.1.3. Immunophenotypical analysis of HSC/Progenitor cells.....	16
2.1.3.1. Thy1.1 <sup>lo</sup> , Lin <sup>-</sup> Sca-1 <sup>+</sup> Cells.....	16
2.1.3.2. Lin c-Kit <sup>+</sup> Sca-1 <sup>+</sup> Cells.....	16
2.1.3.3. SLAM Family Members .....	16
2.1.3.4. Fluorescent dyes .....	17
2.1.4. Phenotype and isolation of HSC based on their cycling behaviour .....	17
2.2. HUMAN HEMATOPOIETIC STEM CELL ASSAY .....	19
2.2.1. <i>In vitro</i> assays .....	19
2.2.2. <i>In vivo</i> assays .....	19
2.2.3. Immunophenotypical analysis of human HSC/Progenitor cells .....	20
2.3. HEMATOPOIETIC STEM CELLS MAINTENANCE .....	21
2.3.1. Cell-intrinsic pathways .....	21

2.3.1.1. Anti-apoptotic proteins .....	21
2.3.1.2. Transcription factors.....	22
2.3.1.3. Signal transducers .....	24
2.3.1.4. Polycomb group proteins .....	25
2.3.2. Hematopoietic stem cells niche and cell-extrinsic pathways .....	26
2.3.2.1. Cellular components of the HSC niche .....	27
2.3.2.2. Molecular regulators of HSC fate .....	29
2.3.2.2.1. Secreted ligands and their receptors .....	30
2.3.2.2.2. WNT signaling .....	32
2.3.2.2.3. Notch signaling .....	34
2.3.2.2.4. Hedgehog and TGF $\beta$ signaling .....	38
2.3.2.2.5. The extracellular matrix.....	39
2.3.2.2.6. Adhesion molecules.....	40
2.3.2.2.7. Chemical gradients .....	42
2.3.2.3. Induced HSC mobilization .....	44
2.3.2.4. The “progeny niche”: feedback regulation from HSC progeny .....	44
2.3.3. Post-transcriptional regulation in HSCs.....	45
2.3.3.1. microRNA.....	45
2.3.3.2. RNA binding proteins .....	46
<b>3. PUMILIO.....</b>	<b>49</b>
3.1. PUF FAMILY PROTEINS .....	49
3.1.1. Pumilio proteic domains .....	52
3.2. PUF PROTEINS: POST-TRANSCRIPTIONAL REPRESSORS.....	56
3.2.1. Mechanisms of action.....	56
3.2.1.1. Deadenylation recruitment .....	56
3.2.1.2. Inhibition of translation initiation .....	57
3.2.1.3. Inhibition of translation elongation.....	58
3.2.1.4. Cooperation with miRNAs .....	58
3.2.2. Non-canonical PUF mechanism of action .....	60
3.2.2.1. PUFs as activators of mRNA expression .....	60
3.2.2.2. mRNA localization by PUF proteins .....	61
3.3. PUF COMBINATORIAL REGULATION: TARGETS AND PARTNERS.....	63
3.3.1. Target mRNAs.....	63
3.3.2. Protein partners.....	64
3.4. PUF REGULATORS.....	71
3.5. PUF AND STEM CELLS MAINTENANCE IN INVERTEBRATES .....	72
3.6. MAMMALIAN PUF PROTEINS .....	73
3.6.1. Pumilio proteins and neuronal functions.....	73
3.6.2. Pumilio proteins and spermatogenesis.....	74
3.6.3. Pumilio proteins and cancer .....	75
3.6.4. Murine Pum1 and Pum2 genes: structure and localization .....	75
3.6.5. Murine Pum1 and Pum2 isoforms .....	76
3.6.6. Expression of Pum1 and Pum2 in murine HSPCs.....	76
3.6.7. Pum proteins and stem cells maintenance in mammals.....	79
<b>4. OBJECTIVES .....</b>	<b>81</b>

## RESULTS..... 83

### 5. RESULTS.....85

5.1. PUM1 and PUM2 knockdown impair murine <i>in vitro</i> HSPC potential.....	85
5.2. PUM1 expression restores the function of shMP1-transduced LSK cells.....	89
5.3. Pum1 and Pum2 KD alter the cell cycle and cell survival of murine HSPCs. ...	90
5.4. Pum KD leads to loss of immature cells.....	92
5.5. Pum1 and Pum2 KD do not lead to increased senescence .....	93
5.6. Pum1 and Pum2 are not involved in genomic stress response.....	94
5.7. Pum1 and Pum2 knockdown impair the human HSPC potential.....	96
5.8. PUM1 and PUM2 KD impair the <i>in vivo</i> reconstitution potential of murine and human HSPCs .....	98
5.9. Effects of Pum1 overexpression in murine HSCs .....	99
5.10. Implication of Pum1 and Pum2 in Delta4/Notch activity .....	101
5.11. Identification of genes implicated in Pumilio effects on HSCs.....	103
5.12. Validation of the HP7 cell line as a model for murine HSPC studies.....	104

### 6. MATERIALS AND METHODS .....108

6.1. Lentiviral constructs and transduction .....	108
6.2. Mice .....	108
6.3. Murine HSC purification .....	109
6.4. Isolation and immuno-labeling of human CD34 <sup>+</sup> cells.....	109
6.5. Culture experiments .....	110
6.6. Quantitative reverse transcriptase (RT)-PCR.....	111
6.7. Protein analysis.....	111
6.8. CFC assays.....	112
6.9. Detection of apoptotic cells .....	112
6.10. Immunophenotypical analysis of LSK cells .....	112
6.11. Senescence analysis .....	113
6.12. γH2AX foci analysis .....	113
6.13. Long-term competitive repopulation assays .....	113
6.14. Microarray .....	114
6.15. Intracellular localization of Pum1 and Pum2 in HP7 cell line.....	114
6.16. Statistical analysis.....	115
6.17. Acknowledgements .....	115

## DISCUSSION .....117

### 7. DISCUSSION .....119

7.1. Role of Pum1 and Pum2 in HSPC cell expansion.....	120
7.1.1. Impact of Pum1 and Pum2 KD on cell cycle .....	121
7.1.2. Impact of Pum1 and Pum2 KD on apoptosis .....	122
7.2. Role of Pum1 and Pum2 in HSPC hematopoietic potential.....	122
7.2.1. <i>In vitro</i> hematopoietic potential .....	122
7.2.2. <i>In vivo</i> reconstitution potential.....	123
7.3. Pum proteins and cellular stress .....	126
7.4. Are Pum1 and Pum2 effectors of the Delta4/Notch signaling? .....	127

7.5. Effects of Pum1 overexpression in KLS cells .....	128
7.6. Identification of genes implicated in Pumilio effects on HSCs. ....	129
<b>8. CONCLUSIONS AND PERSPECTIVES .....</b>	<b>131</b>
<b>ANNEX I.....</b>	<b>135</b>
<b>ANNEX II.....</b>	<b>177</b>
<b>BIBLIOGRAPHY .....</b>	<b>207</b>

## **ACKNOWLEDGEMENTS**

Je tiens tout d'abord à remercier le Professeur Catherine Lacombe de me faire l'honneur de présider le jury de cette thèse.

J'aimerais remercier également le Docteur Martine Simonelig et le Docteur Frédéric Mazurier pour avoir accepté d'être rapporteur de ce travail de thèse et pour le temps consacré à la lecture de ce manuscrit.

Je remercie le Docteur Cohen-Tannoudji Michel pour avoir accepté d'être examinateur de ce travail de thèse.

Je remercie surtout et en particulier Evelyne Lauret, ma directrice de thèse, pour m'avoir suivi pendant ces quatre années. Elle m'a enseigné tout ce que je sais aujourd'hui sur l'hématopoïèse et sur les cellules souches. J'ai énormément apprécié sa manière de travailler, toujours motivée par la curiosité de trouver des réponses à chaque nouvelle hypothèse, et toujours prête à aller de l'avant et à dépasser chaque difficulté de manière simple et positive. Travailler avec elle était tout aussi enrichissant du point de vue personnel que scientifique, j'ai beaucoup aimé ses suggestions, toujours exposées clairement et directement, ainsi que son respect total de mon opinion et de mes idées. Merci Evelyne.

Je remercie énormément Isabelle Dusanter pour m'avoir accueilli dans son équipe, pour avoir toujours été agréable et prête à m'aider mais aussi pour tous ses conseils, grâce à ses amples connaissances scientifiques et techniques dans lesquelles j'ai puisées afin de concrétiser mon projet scientifique.

Je remercie Serge Fichelson, Isabelle Vigon et Ayda Miri-Nezhad pour leur travail de recherche sur le rôle de Pumilio dans le système humain.

Je remercie Aurore Hattabi pour son travail accompli jusqu'alors, pour son aide dans les expériences, pour sa gentillesse et pour toutes nos discussions tantôt très profondes tantôt très légères qui ont égayé nos soirées au laboratoire.



Je remercie Patrycja Palikowska pour sa gentillesse, son aide et sa grande disponibilité.

Je remercie Van Thi Hong TO qui a collaboré à ce travail pendant son stage en m'offrant d'excellents résultats et en me laissant un très bon souvenir de cette période.

Je remercie donc tous les membres du laboratoire et de l'unité U1016 Inserm que j'ai connus pendant ces quatre années et qui ont rendu agréable le temps passé ici en leur compagnie.

Je remercie Azzedine, Jean-Marc et Alain pour leur sympathie sans faille, leur bonne humeur et pour toutes les histoires bizarres racontées pendant la pause déjeuner et pour toutes les discussions sur l'actualité qui m'ont aidé à mieux comprendre ce pays.

Je remercie Luana pour les beaux souvenirs des premières années.

Je remercie Katy, qui restera toujours la blonde la plus intelligente que j'ai jamais connue (après ma chef), Helen pour les moments divertissants passés ensemble, Fanny pour le « couscous » de minuit.

Je remercie Emilie pour sa sympathie, pour le « ménage » et les pauses goûter.

Je remercie les « New Entries » avec lesquels je me suis senti à l'aise dès le départ, Frédéric pour nos discussions scientifiques et non-scientifiques dont j'ai beaucoup appris, Alexandre, toujours optimiste bien qu'il parle trop tout seul, Audrey qui quant à elle parle trop aux cellules et Cécile pour sa contribution active au projet Pumilio et qui parle trop.

Un doctorat, ce n'est pas seulement une expérience de laboratoire mais aussi une expérience de vie, surtout s'il est réalisé dans une ville et un pays splendides comme Paris et la France, où j'espère revenir un jour.

C'est pour cela que je tiens à remercier toutes les personnes qui ont fait de ces quatre années une expérience incroyablement positive qui m'a fait évoluer professionnellement mais surtout d'un point de vue personnel.

Je remercie avant tout ma famille et en particulier ma mère qui a fait tant de sacrifices pour me permettre d'arriver jusqu'ici, qui m'a toujours soutenu et à qui je dois vraiment tout. Grazie Mamma!

Je remercie Chiara à qui je dois mes premiers beaux souvenirs dans cette ville et sans laquelle je me serai probablement perdu dès le premier jour en sortant du train, et qui, armée de patience, est toujours restée près de moi.

Je remercie l'équipe du CFB avec laquelle j'ai passé sans aucun doute la plus belle année de ma vie et en particulier : Riccardo et les mille soirées passées ensemble, Roberto pour ses chansons *a cappella*, Chris pour le «Ring of fire », Harry pour la « pomme » magique, Alice pour avoir démontré qu'il est possible d'escalader la fontaine de Chatelêt, Catherine pour le tango et l'escrime en attendant le RER, Luane pour l'«éponge » et Anna avec laquelle j'ai passé les soirées les plus folles et qui, sans le savoir, m'a énormément enseigné.

Je tiens aussi à remercier Sabrina avec laquelle j'ai passé des bons et amusants moments, dont j'ai un excellent souvenir et qui m'a fait me sentir un peu plus français en m'enseignant la vraie culture française... !

Merci Clara pour les gâteaux empoisonnés et pour les entraînements exténuants !

Un remerciement particulier à toutes les personnes du club de voile de Choisy pour les splendides week-ends passés tous ensemble, toujours gentils et disponibles pour me donner de nombreux conseils mais sans pitié sur l'eau ! Je remercie en particulier Ludivine, Clémentine et Chloé, vous me manquerez mais je reviendrai ! (Encore plus fort qu'avant)

Derniers remerciements enfin, mais pas des moindres, à mes amis de toujours qui sont restés près de moi malgré la distance et avec lesquels je passe d'excellents moments à chaque fois que je rentre chez moi.

Je remercie Gary et Giulio pour tout le temps passé ensemble à la recherche de nouvelles expériences et grâce à qui c'est toujours comme si j'étais parti hier !

Je remercie Davide pour toutes les discussions, toutes les soirées et les mille pensées qui nous unissent depuis des années et qui sont toujours très importantes pour moi, mais surtout pour être toujours à mes côtés dans tout ce que j'entreprends.

Je remercie Matteo qui égaye mes journées avec ses histoires, et pour son dynamisme qui a fait qu'on a passé les soirées les plus incroyables ensemble.

Je remercie Alessandro pour sa sympathie contagieuse qui me rappelle toujours que le juste style de vie prévoit une grande équité de fêtes, d'entraînements et de régime sain !!

Vous êtes tous très importants pour moi.

## ABSTRACT

The central properties of stem cells are the pluripotency and the capacity of self-renewal. Hematopoietic stem cells (HSCs) possess such common features that allows them to generate all the cells of the hematopoietic compartments, maintaining in the same time the HSC pool. We develop approaches focused on *ex vivo* HSC expansion through activation by exogenous HOXB4 (human HSCs) or Notch/Dll-4 ligand (murine HSCs). Two independent transcriptomic analyses surprisingly converged toward an increased expression of two genes never identified so far as crucial for HSC functions: Pumilio1 (Pum1) and Pumilio2 (Pum2). Pum1 and Pum2 are posttranscriptional regulators belonging to the Pumilio-FBF (PUF) family of RNA-binding proteins. Although it was established that the primordial role of PUF proteins is to sustain mitotic proliferation of stem cells in Invertebrates, so far nothing is known about the role of Pum1 and Pum2 in human and murine HSCs.

For these reasons, we have investigated the roles and mechanisms of action of Pum1 and Pum2 in murine and human HSCs through shRNA strategy. Pum1 and Pum2 knockdown (KD) in murine HSCs led to a decreased HSC expansion and clonogenic potential *ex vivo*, associated with an increased apoptosis and a cell cycle arrest in G0/G1 phase. KD of both Pum1 and Pum2 enhanced these effects, suggesting a cooperative effect. Expansion and clonogenic potential of KD Pum1 HSCs were rescued by enforced expression of Pum1 (insensitive to our shRNA), thus validating the specificity of our shRNA. Enforced expression of Pum1 could not rescue the functions of Pum2 KD HSCs, highlighting the non-redundant role of these proteins. Furthermore, when Pum1 or Pum2 KD HSCs were inoculated into lethally irradiated mice to follow the long-term hematopoietic potential, only rare bone marrow cells derived from Pum1 and Pum2 KD HSCs were evidenced after 4 months, contrary to control HSCs. Identical results were obtained with human Pum1 or Pum2 KD HSCs.

In conclusion, our results demonstrate the involvement of Pumilio factors in stemness maintenance, expansion and survival of murine and human HSCs. Identification of Pumilio factors and their targets as new regulators of HSCs expansion will allow consider them as new tools for therapeutic perspectives.



# LIST OF FIGURES

Figure 1. Differentiation of human tissues .....	5
Figure 2. Model of the hematopoietic hierarchy.....	9
Figure 3. Myeloid bypass model and Conventional model .....	11
Figure 4. The HSC microenvironment .....	27
Figure 5. Distinct cellular sources and niches for CXCL12 in bone marrow .....	31
Figure 6. The Notch signaling pathway .....	35
Figure 7. Crosstalk between HSCs and their niche .....	43
Figure 8. microRNA biogenesis and function .....	47
Figure 9. PUF proteins throughout eukaryotes.....	51
Figure 10. Diagram of the human Pum1 protein conserved domains.....	52
Figure 11. Pum Homology Domain structure and RNA binding.....	53
Figure 12. Analysis of RNA consensus sequence associated with PUM proteins .....	55
Figure 13. Deadenylase recruitment by yeast Puf5.....	57
Figure 14. Pum2 mediated translational control in <i>Xenopus</i> .....	58
Figure 15. Pumilio binding alters local p27-3' UTR structure and miR-221 and miR-222 accessibility .....	59
Figure 16. Possible mechanisms of mRNA activation by PUF proteins. ....	60
Figure 17. Working model of cooperation between FBF-2 and PGL-1 .....	62
Figure 18. mRNAs associated with human PUM proteins in HeLa S3 cells .....	63
Figure 19. Pum-NRE-Nos-Brat interaction surface in <i>Drosophila</i> .....	65
Table 1. mRNA targets and protein partners of model Puf proteins .....	68
Figure 20. Role of Pum, Nos and Brat in the Ovarian Stem Cell System .....	71
Figure 21. Pumilio1 and Pumilio2 isoforms .....	77
Figure 22. General experimental protocol for <i>in vitro</i> experiences .....	85
Figure 23. Validation of the shRNA efficiency in murine cells .....	86
Figure 24. mPum1 and mPum2 KD inhibit hematopoietic potential of murine HSPCs .....	88
Figure 25. mPum1 expression rescues the functions of shmP1-transduced LSK cells .....	89
Figure 26. PUM1 and PUM2 KD alter the cell cycle and induce cell apoptosis in murine HSPCs.....	91
Figure 27. Pum KD leads to loss of immature cells .....	92
Figure 28. Pum1 and Pum2 KD do not lead to increased senescence .....	94
Figure 29. Pum KD has no effect on protection from genomic stress or DNA repair.....	95
Figure 30. PUM1 and PUM2 KD inhibit the <i>in vitro</i> hematopoietic potential of human HSPCs .....	97
Figure 31. PUM1 and PUM2 KD inhibit the <i>in vivo</i> reconstitutive potential of murine and human HSPCs.....	99
Figure 32. <i>In vitro</i> effects of Pum1 overexpression on HSPCs.....	101

Figure 33. Delta4/Notch pro-self-renewing action is partially maintained in Pum KD cells..... 102

Figure 34. Identification of genes implicated in Pumilio effects on HSCs ..... 104

Figure 35. Validation of the HP7 cell line ..... 105

Figure 36. Intracellular localization of Pum1 and Pum2 in HP7 cell line ..... 106

Table 2. Sequences of RT-qPCR primers and list of shRNAs used to invalidate human and murine Pum1 and Pum2 ..... 116

## LIST OF ABBREVIATIONS

Ab: antibody	dpc: days post coitum
AGM: aorta-gonad mesonephros	Dpp : Decapentaplegic
Ago: Argonaute	EBF2 : early B-cell factor 2
Ang-1 : angiopoietin 1	EC: endothelial cells
ANGPT1: angiopoietin 1	ECM : extracellular matrix
ANGPTL3: angiopoietin like 3	EGF: epidermal growth factor
APC: allophycocyanin	EP: erythrocyte progenitor
BCL2: B-cell lymphoma 2	EPO: Erythropoietin
BFU-E: burst-forming units-erythroid cell	ES: embryonic stem cell
BM: bone marrow	EST: expressed sequence tag
BMP : bone morphogenetic protein	Etv6: Ets variant gene 6
Brat: Brain Tumor	FACS: fluorescence-activated cell sorting
BrdU: bromodeoxyuridine	FBF: fem-3 mRNA binding factor
CASR : $\text{Ca}^{2+}$ -sensing receptor	FITC: fluoresceine isotyocianate
CD: cluster of differentiation	FL: fetal liver
cDNA: complementary DNA	Fmi: flamingo
CDS: coding sequence	Fz: frizzled
CFC: colony-forming cell	G-CSF : granulocyte colony-stimulating factor
CFU-E: colony-forming units erythroid cell	Gfi1: growth factor independence 1
CFU-G: colony-forming units-granulocytes cell	GFP: green fluorescent protein
CFU-GEMM: colony forming units-granulocytes-erythrocytes-macrophages-megakaryocytes	GMP: granulocyte/macrophage progenitor
CFU-GM: colony forming units-granulocytes/macrophages cell	GO: gene ontology
CFU-M: colony-forming units-monocytes/macrophages cell	GP: granulocyte progenitor
CFU-Mk: megakaryocyte-restricted cell	GSC: germ stem cell
CFU-S: colony-forming unit-spleen	hb: hunchback
CLP: common lymphoid progenitor	Hes: hairy/enancher-of-split
CMP: common myeloid progenitor	HH : hedgehog
CMRP: common myeloid restricted progenitor	HIF-1 $\alpha$ : hypoxia-inducible transcription factor-1 $\alpha$
CPE: cytoplasmic polyadenylation element	Ho 33342: Hoescht 33342
CPEB: cytoplasmic polyadenylation element binding protein	Hox: homeobox
CRU: competitive repopulating unit	HP: hematopoietic progenitor
CXCL12: chemokine (C-X-C motif) ligand 12	HP7: hematopoietic precursor 7
CXCR4: C-X-C chemokine receptor type 4	HSC: hematopoietic stem cells
DAPI: 6-diamidino-2-phenylindole	HSPC : hematopoietic stem progenitor cell
DAZ: deleted in azoospermia	ICM: inner cell mass
DC:dendritic cell	IFN $\alpha$ : interferon $\alpha$
DKK1 : dickkopf homolog 1	IL:interleukine
Dll: Delta-like	IPA: ingenuity pathway analysis
DNA: Deoxyribonucleic acid	iPS: induced pluripotent stem cell
	JAK-STAT: Janus family kinase-signal transducer and activator of transcription
	KD: knockdown
	KSL: c-Kit <sup>+</sup> Lin <sup>-</sup> Sca-1 <sup>+</sup>
	KO: knockout
	KuO: Kusabira-Orange



LAP : latency-associated protein  
 Lepr : leptine receptor  
 LIF: leukemia inhibitor factor  
 Lin: lineage markers  
 LLC : large latent complex  
 LMPP: lymphoid multipotent progenitor  
 LRP5/6: LDL receptor-related proteins 5 and 6  
 LTBP1 : latent TGF- $\beta$  binding protein 1  
 LTC-IC: long-term culture-initiating cell  
 LT-HSC: long-term repopulating hematopoietic stem cells  
 LTRC: long-term reconstituting cell  
 LT-RC: long-term reconstituting cell  
 MacP: macrophage progenitor  
 MAPK: mitogen-activated protein kinase  
 mbDII4: membrane bound Delta like 4  
 Mcl1: myeloid cell leukemia 1  
 MEP: megakaryocyte/erythrocyte progenitor  
 MERP: megakaryocyte erythroid restricted progenitor  
 miRNA : microRNA  
 MkP: megakaryocyte progenitor  
 MkRP: megakaryocyte restricted progenitor  
 MPP: multipotent progenitor  
 mRNA: messenger RNA  
 MSC: mesenchymal stem cell  
 MSPC : mesenchymal stem/progenitor cell  
 mTOR: mechanistis target of rapamycin  
 Mw: molecular weight  
 MyRP: myeloid restricted progenitor  
 ncRNA : noncoding RNA  
 NK: natural killer  
 NOG: NOD/Shi-scid, IL-2Rynull  
 Nos: Nanos  
 NRE: nanos response element  
 OB : osteoblast  
 Oc : osteocalcin  
 OC : osteoclast  
 OPN: osteopontin  
 ORF: open reading frame  
 PBE: pumilio binding element  
 PcG: Polycomb group  
 PCR: polymerase chain reaction  
 PDGF: platelet derived growth factor

PDGFR $\alpha$  : platelet derived growth factor receptor  $\alpha$   
 PE: phycoerythrin  
 PGK: phosphoglycerate kinase  
 PRC1: Polycomb repression complex 1  
 Pten: phosphate and tensin homologue deleted on chromosome ten  
 PUF: Pum and FBF  
 Pum: Pumilio  
 PUM-HD: PUM homology domain  
 Raptor: regulatory-associated protein of mTOR  
 Ras: rat sarcoma protein  
 RBC: red blood cell  
 RBP: RNA binding proteins  
 Rh123: Rhodamine 123  
 RISC: RNA-induced silencing complex  
 RNA: ribonucleic acid  
 ROS : reactive oxygen species  
 RT-qPCR: Reverse transcription quantitative polymerase chain reaction  
 RU: repopulating unit  
 S17: stromal 17 cell line  
 Sca-1: stem cell antigen 1  
 SCF: stem cell factor  
 SCID: severe combined immunodeficiency  
 SDF1: stromal cell-derived factor 1  
 shRNA: short RNA  
 SILAC: stable isotope labeling by amino acids in cell culture  
 siRNA : small interfering RNA  
 SLAM: signaling lymphocyte attractant molecule  
 SP: side population  
 SPP1: secreted phosphoprotein 1  
 ST-HSC: short-term repopulating hematopoietic stem cells  
 Tel: Translocation Ets leukemia  
 TGF- $\beta$  : transforming growth factor  $\beta$   
 TPO: thrombopoietin  
 UTR: untranslated region  
 VCAM1 : vascular cell adhesion molecule 1  
 VE: vascular endothelium  
 VEGF-A : vascular endothelial growth factor A  
 wt: wild type  
 YS: yolk sac

# INTRODUCTION



# 1. STEM CELLS

During embryogenesis, a single fertilized oocyte gives rise to a multicellular organism whose cells and tissues have adopted differentiated characteristics or fates to perform the specified functions of each organ of the body. As embryos develop, cells that have acquired their particular fate proliferate, enabling tissues and organs to grow. Even after an animal is fully grown, however, many tissues and organs maintain a process known as homeostasis, where as cells die, either by natural death or by injury, they are replenished. This remarkable feature has ancient origins, dating back to the most primitive animals, such as sponges or hydrozoans. Throughout evolution, nature has exerted considerable fun and fancy in elaborating on this theme. Some amphibians, for instance, can regenerate a limb or tail when severed, and the neurons of bird brains can readily regenerate. While mammals seem to have lost at least some of this wonderful plasticity, their liver can partially regenerates providing that the injury is not too severe, and the epidermis and hair of their skin can readily repair when wounded or cut. Additionally, the epidermis, hair, small intestine, and hematopoietic system are all examples of adult tissues that are naturally in a state of dynamic flux: even in the absence of injury, these structures continually give rise to new cells, able to transiently divide, terminally differentiate and die.

The fabulous ability of an embryo to diversify and of certain adult tissues to regenerate throughout life is a direct result of stem cells properties. When a stem cell divides, each new cell has the potential either to remain a stem cell (through symmetric division) or become another type of cell (through asymmetric division) with a more specialized function. In other words stem cells are distinguished from other cell types by two important characteristics: self-renewal and potency.

Self-renewal consists in the ability to go through numerous cycle of mitotic cell division while maintaining the undifferentiated state. Potency is the capacity that allows unspecialized stem cells to differentiate into specialized cell types<sup>1</sup>.

### **1.1. DEFINITION OF STEM CELL POTENTIAL**

Many of the terms used to define stem cells depend on the behavior of the cells in the intact organism or after transplantation *in vivo* or under specific laboratory conditions *in vitro*.

#### **1.1.1. Totipotent stem cells**

The fertilized egg is considered to be totipotent because it has the potential to generate all the cells and tissues that make up an embryo and that support development in utero. The fertilized egg divides and differentiates until it produces a mature organism. Adult mammals, including humans, consist of more than 200 kinds of cells. These include nerve cells (neurons), muscle cells (myocytes), skin (epithelial) cells, blood cells (erythrocytes, monocytes, lymphocytes, etc.), bone cells (osteocytes), cartilage cells (chondrocytes) and other cells which are essential for embryonic development but are not incorporated into the body of the embryo as extraembryonic tissues, placenta, and umbilical cord. All of these cells are generated from a single, totipotent cell, the zygote, or fertilized egg (Figure 1).

#### **1.1.2. Pluripotent stem cells**

The term pluripotent, instead, is used to describe stem cells that can give rise to cells derived from all three embryonic germ layers: endoderm, mesoderm, and ectoderm. All of the many different kinds of specialized cells that make up the body are derived from one of these germ layers.

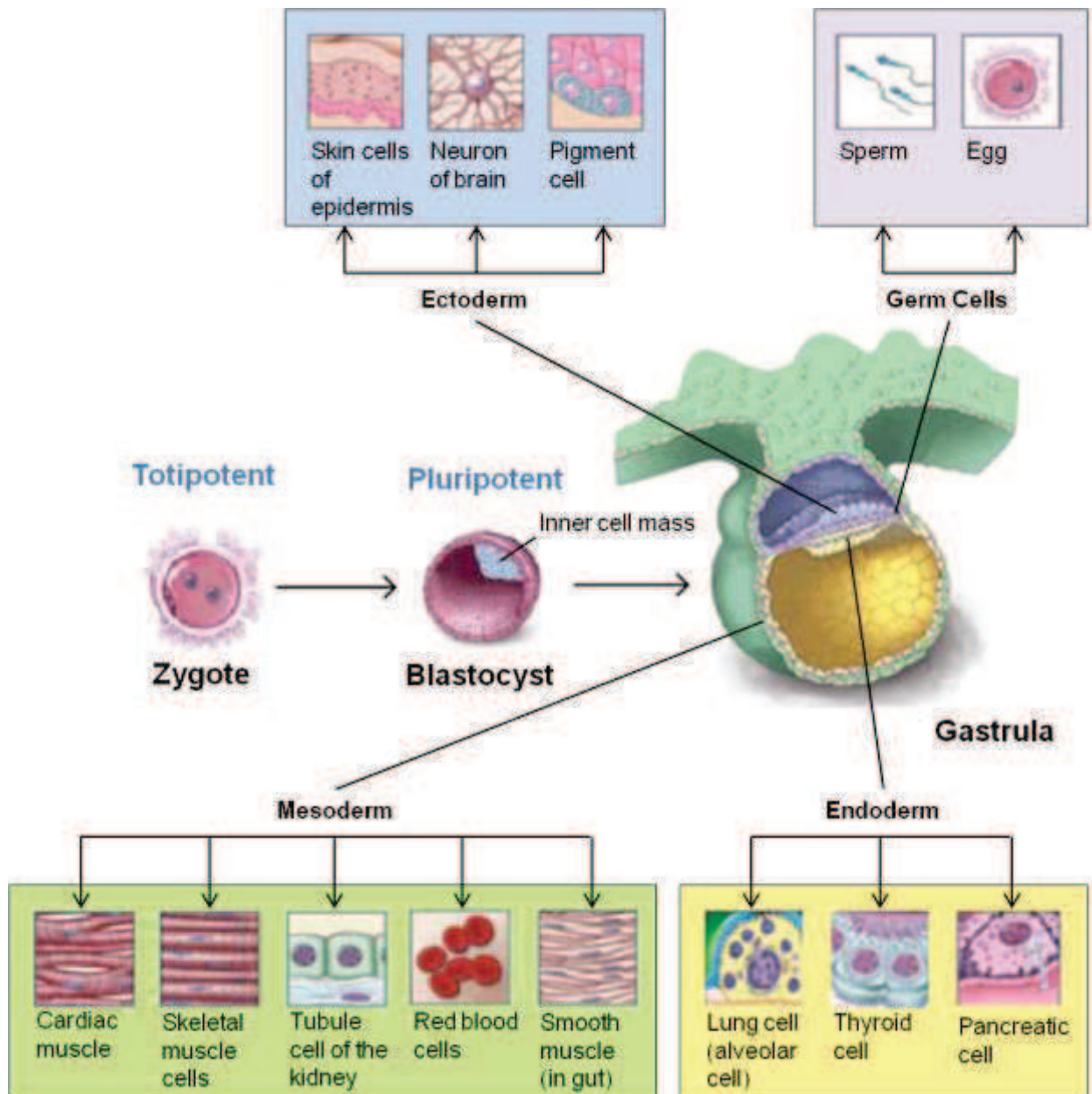


Figure 1. Differentiation of human tissues (adapted from Winslow and Duckwall, 2001)

### 1.1.2.1. Embryonic stem cells

Emanating from the pioneering research of Martin Evans in the 1970s and cumulating with the successful parallels with human tissue, cells from the inner cell mass (ICM) of mammalian blastocysts can be maintained in tissue culture under conditions where they can be propagated indefinitely as pluripotent embryonic stem (ES) cells<sup>2</sup>. If injected back into a recipient blastocyst that is then carried to term in a female host, these cells can contribute to virtually all the tissues of the chimeric

offspring, including the germ cell compartment. To maintain cultured ES cells in their relatively undifferentiated, pluripotent state, they must both express the intrinsic transcription factor Oct4, and constitutively receive the extrinsic signal from the cytokine leukemia inhibitor factor (LIF)<sup>3,4</sup>. Upon LIF withdrawal, cultured ES cells spontaneously aggregate into embryo-like bodies, where they differentiate and spawn many cell lineages, including beating heart muscle cells, blood islands, neurons, pigmented cells, macrophages, epithelia, and fat-producing adipocytes<sup>5</sup>. Similarly, when ES cells are injected into nude mice, they differentiate into multicellular masses, called teratocarcinomas. Although the programs of gene expression in these structures often bear strong resemblance to the differentiation pathways typical of developing animals, the triggering of these programs is chaotic. These examples illustrate the importance of intercellular interactions and cellular organization in orchestrating development and embryo shape.

### **1.1.3 Multipotent stem cells and Adult stem cells**

A multipotent stem cell is a stem cell that can give rise to several types of cells but it is limited in its ability to proliferate and differentiate.

Adult stem cells or somatic stem cells are considered as multipotent stem cells. They are found among differentiated cells in a tissue or organ. They can self-renew and differentiate to yield some or all of the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. The origin of adult stem cells in some mature tissues is still under investigation. Adult stem cells have been identified in many organs and tissues, including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis. They are thought to reside in a specific area of each tissue (called the “stem cell

niche”). If the differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of transplantation-based therapies.

#### **1.1.4. Progenitor cells**

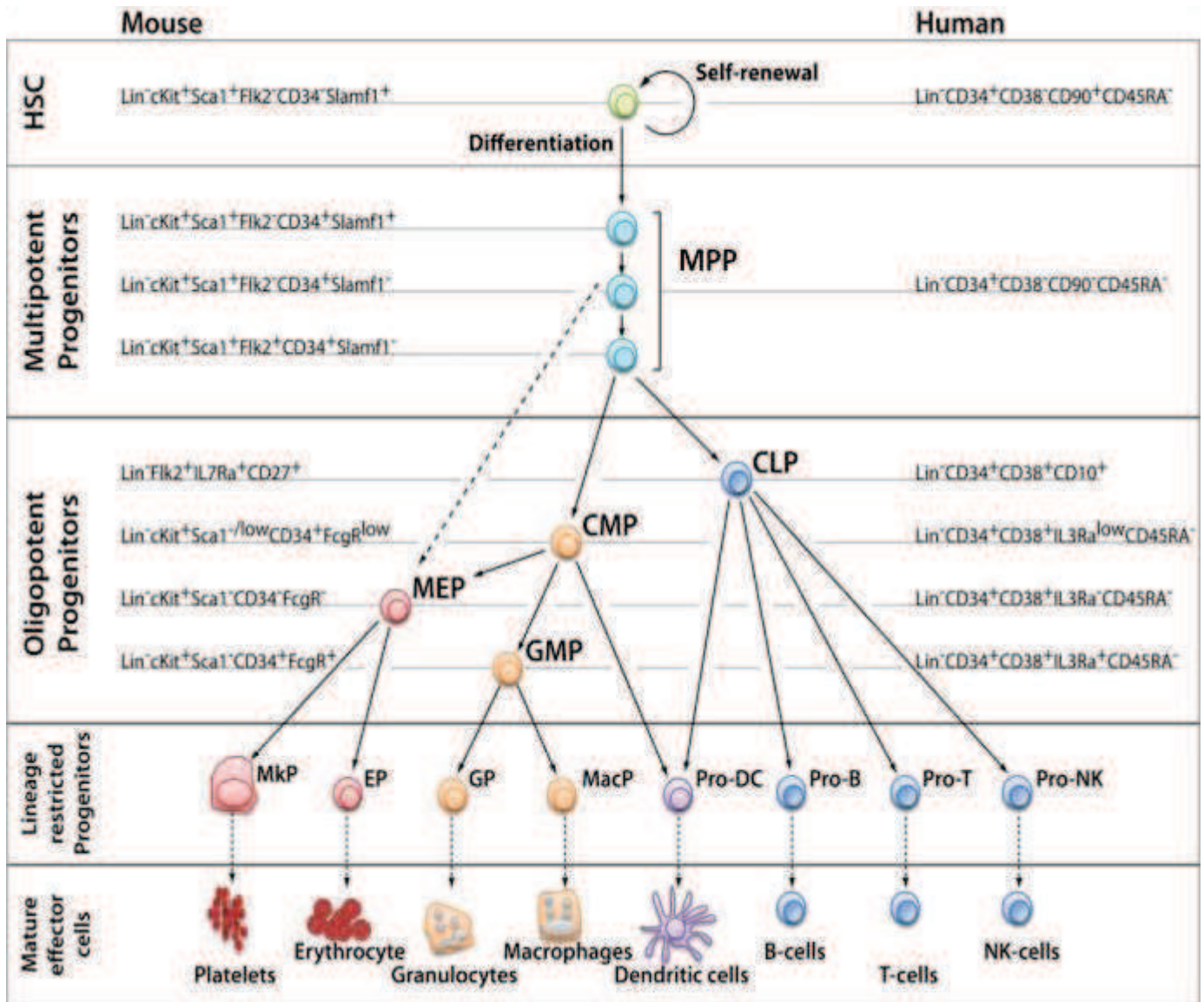
A progenitor cell occurs in fetal or adult tissues and is partially specialized; it divides and gives rise to differentiated cells. Researchers often distinguish progenitor cells from adult stem cells in the following way: when a stem cell divides, one of the two new cells is often a stem cell capable of replicating itself again. In contrast, when a progenitor cell divides, it can form more progenitor cells or it can form two specialized cells, neither of which is capable of replicating itself. Progenitor cells can replace cells that are damaged or dead, thus maintaining the integrity and functions of the tissue. Progenitor cells are often classified depending on their potency. Multipotent progenitors can give rise to several different committed oligopotent progenitors that in turn can generate even more specified lineage restricted progenitors (often called precursors) able to differentiate only in mature effector cells. So, the definition of multipotent, oligopotent and lineage-restricted progenitors is strictly related to their degree of potency along the continuum of cells between the stem cell and the mature cells. Controversy about the exact definition remains and the concept is still evolving.



## 2. HEMATOPOIETIC STEM CELLS

The hematopoietic system represents a continuum of cells with changing phenotype and properties as they progress from stem to differentiated cells. While mature blood cells are produced at a rate of more than 1 million cells per second in the human adult <sup>6</sup>, most of the hematopoietic stem cells (HSCs) from which they are derived cycle very infrequently and primarily reside in the G0 phase of the cell cycle under homeostatic conditions<sup>7</sup>. These facts present an interesting problem: how does the organism achieve a balance whereby an adequate pool of HSCs is maintained for the life of the organism, while at the same time HSCs consistently meet the organism's enormous demand for continuous replenishment of mature blood cells, most of which are short lived? To answer this question we have to think at the HSC as a cell capable to continuously provide a series of intermediate progenitors whose potency is restricted to certain lineages, allowing for an enormous amplification in the numbers of terminally differentiated cells, while properly maintaining the HSC pool homeostasis throughout life by precisely balancing self-renewal and differentiation (Figure 2). The importance of this balance is underscored by the numerous examples where aberrant HSC development causes severe disease, when HSC differentiation into committed progenitors is not accompanied by the typical loss of self-renewal capacity, or HSC derived progenitors fail to fully differentiate into mature blood cells and may enter a preleukemic progression.

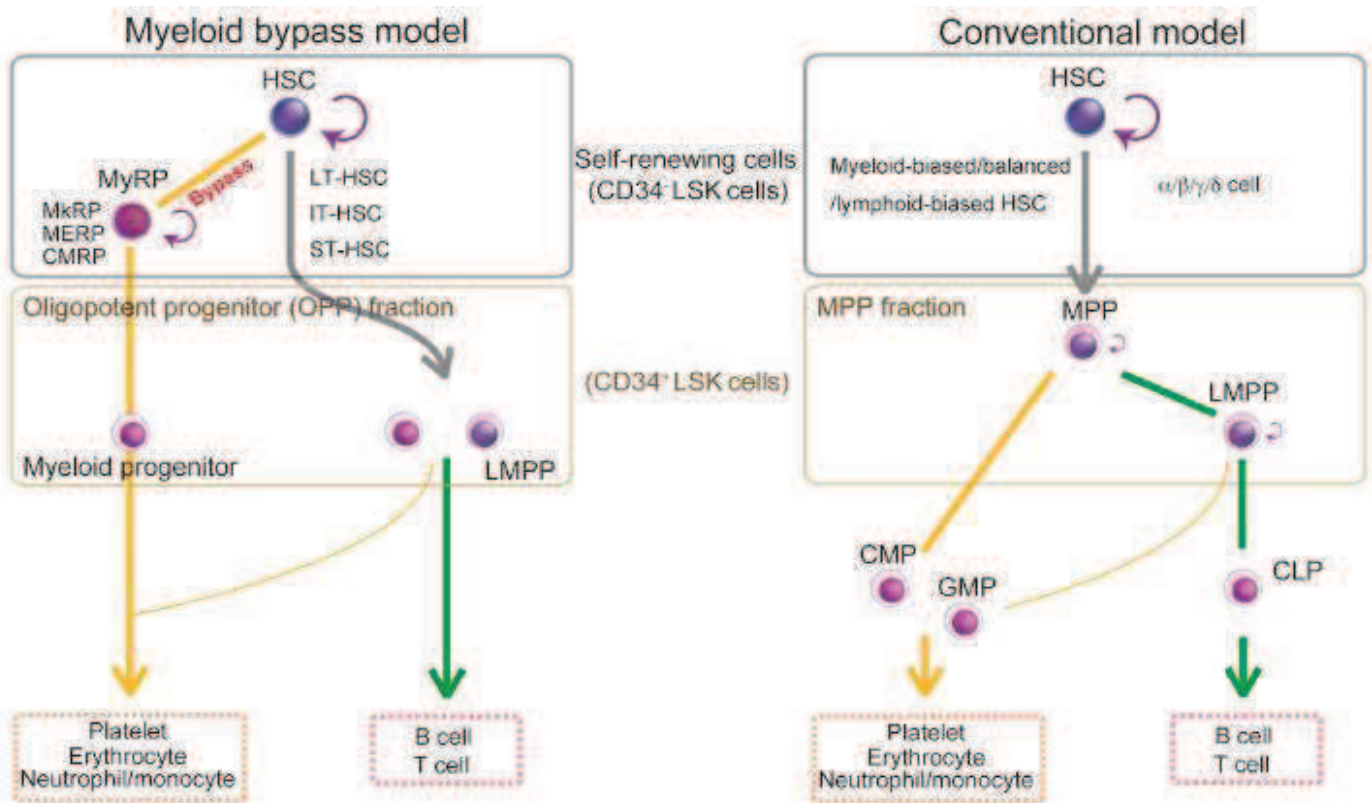
Anyway, even if consensus holds that HSCs give rise to multipotent progenitors (MPPs) of reduced self-renewal potential and that MPPs eventually produce lineage-committed progenitor cells in a stepwise manner, a recent elegant work by Yamamoto and colleagues demonstrated the existence of a differentiation pathway that suggest a revised model of hematopoietic differentiation<sup>8</sup>.



**Figure 2. Model of the hematopoietic hierarchy (Seita and Weissman, 2010)**

The HSC resides at the top of the hierarchy, and is defined as the cell that has both the self-renewal capacity and the potential to give rise to all hematopoietic cell types (multi-potency). Throughout differentiation, a HSC first loses self-renewal capacity, then loses lineage potential step-by-step as it commits to become a mature functional cell of a certain lineage. The cell surface phenotype of each population is shown for the mouse and human systems. Intermediate precursors between the first lineage committed progenitors and final mature cell, and different subsets of mature B- and T-cells are omitted. In the mouse system, heterogeneity of MPPs has been revealed by differences in cell surface marker phenotypes and functional differences of their subsets discussed. For example, evidence suggests that some of MPPs directly give rise to MEP without passing through CMP (dashed arrow). HSC: hematopoietic stem cell, CLP: common lymphoid progenitor, CMP: common myeloid progenitor, MEP: Megakaryocyte/erythrocyte progenitor, GMP: granulocyte/macrophage progenitor, MkP: Megakaryocyte progenitor, EP: erythrocyte progenitor, GP: granulocyte progenitor, MacP: macrophage progenitor, DC: dendritic cell, NK: natural killer, Lin: lineage markers

Using transgenic mice expressing Kusabira-Orange (KuO) fluorescent protein in all blood cell lineages, they unexpectedly found myeloid-restricted progenitors with long-term repopulating activity (MyRPs), which are lineage-committed to megakaryocytes, megakaryocyte-erythroid cells, or common myeloid cells (MkRPs, MERPs, or CMRPs, respectively) in the phenotypically defined HSC compartment (CD150<sup>high</sup> CD34<sup>-</sup> LSK) together with HSCs. To examine whether HSCs directly differentiate into these myeloid-restricted repopulating progenitors, they employed single cell sorting of HSCs, generating culture plates containing one HSC per well. After that, they let these cells accomplish one mitotic cycle *in vitro* to later transplant the two daughter cells in two different recipient mice. Strikingly, after single-cell transplantation, they were able to identify daughter cell pairs in which one was an HSC and the other was an MkRP as well as daughter cell pairs in which one was an HSC and the other was a CMRP, demonstrating that direct lineage commitment took place in an asymmetric manner at the HSC level and that HSCs can directly differentiate into lineage-restricted progenitors without passing through an MPP stage showing that loss of self-renewal and stepwise progression through specific differentiation stages are not essential for lineage commitment of HSCs (Figure 3). These myeloid bypass pathways could be essential for fast responses to ablation stress.



**Figure 3. Myeloid bypass model (left) and Conventional model (right) (Yamamoto et al. 2013)**

HSCs self-renew and give rise to lineage-restricted progenitor cells. In the conventional hematopoietic differentiation model (right side), HSCs in the CD34- LSK population differentiate into multipotent progenitors (MPPs, Flt3- CD34+ LSK) of reduced self-renewal potential and MPPs eventually produce lymphoid-primed multipotent progenitors (LMPPs, Flt3+ CD34+ LSK) or lineage-committed progenitor cells in a stepwise manner. All mature blood lineages are considered to pass through the MPP and/or LMPP stage in the CD34+ LSK population. In contrast, in the myeloid bypass model (left side), the CD34- LSK cell population contains CMRPs, MERPs, and MkRPs in addition to HSCs. These MyRPs are produced by HSCs (LT-HSCs, IT-HSCs or ST-HSCs). MyRPs can clonally expand via self-renewal as in HSCs, B cells, and T cells. The CD34+ LSK population, which is downstream from the CD34- LSK population, also contains lineage-committed progenitors including myeloid-restricted progenitors, whereas “true” MPPs and lymphoid-primed multipotent progenitors (LMPPs) are minor populations in the CD34+ LSK population. Together, MyRPs, rather than “MPPs and LMPPs,” are considered to be the major suppliers of myeloid cells (platelets, erythrocytes, and neutrophils/monocytes) in the hematopoietic system at a single-cell level. LMPP fraction yields cells with various differentiation potentials and these progenitors might derive from ST-HSCs in the CD34- LSK population but not from MPPs in the CD34+ LSK population. Because cells in the CD34+ LSK fraction have oligopotent differentiation potentials rather than multipotent potentials, CD34+ LSK cells are considered to be OPPs.

## 2.1. MURINE HEMATOPOIETIC STEM CELL ASSAYS

Hematopoiesis is now among the best-defined differentiation cascades in mammalian tissues due to the ease of access and morphologic distinctiveness of many of its members. The advent of antibody technology, culture capability, transplantation, and finally genetic engineering of mice has enabled a detailed understanding of processes involved in the commitment and differentiation of HSCs

into progenitor cells and ultimately mature blood cells. Lineage-committed progenitor cells differentiate in less than 3 weeks and five to 10 divisions in short-term assays; in contrast, stem cells and their immediate progeny have to accomplish a high number of divisions (>15) in “long-term assays” (>5 weeks) before they produce differentiated cells. Combining antibody-based subselection of cells with transplantation has made possible the identification of progenitors and HSCs. A range of different methods for characterizing stem and progenitor cell populations are now available for use in hematopoietic cell research. All assays measure two cardinal parameters of hematopoietic stem and progenitor cells: cell proliferation (measured by the number of cells produced) and differentiation potential (estimated by the number of different lineages represented). These two parameters can give us some “end points” that reflect the properties of these “unobservable” progenitors.

### **2.1.1. *In vitro* assays**

#### **2.1.1.1. Colony-Forming Cell Assay**

The colony-forming cell (CFC) assays measure progenitor cells in a given population using semisolid agar- or, more commonly, well-defined methylcellulose-based culture media, which are commercially available. The majority of CFCs consist of lineage-restricted colonies: colony-forming units erythroid (CFU-E) which are more mature than the erythroid restricted burst-forming units-erythroid (BFU-E); megakaryocyte-restricted CFU-Mk; colony-forming units-granulocytes (CFU-G), colony-forming units-monocytes/macrophages (CFU-M); and colony forming units-granulocytes/macrophages (CFU-GM). The most immature (multipotent) CFC measurable contains granulocytes, erythrocytes, macrophages, and often megakaryocytes (CFU-GEMM). This CFC is also often called CFU-mixed, as it may not always contain megakaryocytes but does contain erythroid and granulocyte/macrophage cells. B and T lymphocyte *in vitro* CFC potential are more

difficult to assess, usually requiring specialized coculture systems<sup>9,10</sup>, and hence are not routinely used, although there are now commercially available methylcellulose-based colony assays to measure pre-B cells. While informative about the progenitor cell content of a population of interest, the CFCs do not measure HSCs.

#### **2.1.1.2. Long-term culture-initiating cell assay**

The long-term culture-initiating cell (LTC-IC) assay is a well-established *in vitro* assay used to enumerate primitive hematopoietic stem cells (HSCs) and relies on the two cardinal functions of HSCs: ability to self-renew and differentiation capacity. LTC-ICs present in minimally processed cell suspensions or in purified cell populations and cocultured on a supportive feeder layer are detected by their sustained ability to produce hematopoietic progenitors (CFC) after  $\geq 4$  weeks in culture. Refinements including the use of a defined stromal cell line, and extending the *in vitro* culture to 6 weeks allow detection of LTC-IC at similar frequencies to transplantable HSCs quantified using *in vivo* assays. An issue with these assays is the interlaboratory variability often observed due to varying feeder layers and specific culture conditions. They are useful, however, in limiting dilution format for quantitating primitive cells when other features such as homing capacity or other functions required for *in vivo* engraftment may compromise the reliability of transplant assays.

### **2.1.2. *In vivo* Assays**

#### **2.1.2.1 Short Term *in vivo* assays**

##### **2.1.2.1.1. Colony-forming unit-spleen (CFU-S) cells assay**

Colony-forming unit-spleen (CFU-S) cells are cells that, once injected into an irradiated recipient, home to the spleen and form macroscopic colonies that provide very short-term (usually 1–3 weeks) *in vivo* repopulation of the mouse<sup>11</sup>. The CFU-S are therefore early engrafting cells, providing radioprotection to the mouse and



allowing it to survive more readily in the first 2–3 weeks posttransplantation when pancytopenia usually occurs. These progenitors are more immature than CFCs but are more mature than HSCs. The gold standard for measuring HSC is the long-term repopulating assay.

#### **2.1.2.2 Long Term Repopulating Cells assay**

Long-term reconstituting cells (LTRC) are a subpopulation of HSCs able to produce differentiated cells of multiple lymphoid and myeloid lineages for months in bone marrow and peripheral lymphoid organs. These long-lived clones are best identified by analyzing donor granulocytes, T- and B-lymphocytes in the peripheral blood of recipients 3 - 4 months after transplantation. Thus, *in vivo*, longevity, rather than multipotentiality, is the best criteria of stem cell 'activity'.

##### **2.1.2.2.1. Competitive repopulation assay**

There are various types of long-term repopulating assays. The most common assay is the competitive repopulation assay<sup>12</sup>. This assay measures the functional potential of the unknown source of HSCs against a set known number of HSCs (usually whole bone marrow cells from congenic wild-type mice). The number of repopulating units (RU) in the donor cell population (source of unknown HSC content being measured) can be determined by the following formula: donor RU = % donor cells x C/(100 - % donor cells), where C = the number of competing RU and  $1 \times 10^5$  whole bone marrow cells = 1 competing RU<sup>13,14</sup>. While providing informations about the function of HSCs in their capacity to repopulate compared to the competing bone marrow, this study provides qualitative or at best semiquantitative information about the HSCs within a given population, but it cannot distinguish between the number of HSCs or their quality (progeny produced per HSC). Furthermore, caution should be used when designing competitive repopulation assays, as it has been shown that the reliability of

this assay is critically dependent on the numbers of HSCs present in the populations being assessed: when too few or too many HSCs (recipients of  $<1 \times 10^5$  or  $>2 \times 10^7$  bone marrow cells each from donor and competing sources) are present, the data may not be meaningful<sup>13</sup>.

#### **2.1.2.2.2. Limiting dilution assay**

The frequency of HSCs (from which the number of HSCs can be calculated) is commonly measured using the limiting dilution assay, which is a variation of the competitive repopulation assay. In this assay, a series of dilutions of the unknown source (donor “test” cells) are competed against a set number of competing bone marrow cells. The number of mice negative for reconstitution in each cell dose is then measured, and the frequency of HSCs (competitive repopulating units, CRU) is estimated using Poisson statistics<sup>15,16</sup>. Note that CRU, which measures the quantity of HSCs, is distinct from RU, which measures the functional quality of HSCs.

#### **2.1.2.2.3. Serial transplantation assay**

The most stringent test of HSC potential is the serial transplantation assay. The HSC compartment has been shown to be heterogeneous, comprising a hierarchy of HSCs that can be identified by their functional capacity. The most immature HSC in this hierarchy is capable of sustaining hematopoiesis throughout serial transplantation<sup>17–19</sup>. Hence, in this assay, the source of HSCs is transplanted into sequential serial transplant recipients, and the ability of this population to sustain hematopoiesis by presumptive self-renewing divisions is determined. Limitations to this assay are its dependence upon homing and engraftment processes that may be perturbed without altering stem cell function per se, in particular mutant mouse strains.



### **2.1.3. Immunophenotypical analysis of HSC/Progenitor cells**

In the last 20 years, a number of different methods whereby HSCs and progenitor cells can be identified have emerged<sup>20–23</sup>. All rely on fluorescence-activated cell sorting (FACS)-based methods. The purity of the populations of HSCs achieved using these methods has increased within recent years, such that approximately 50%–96% of single cells in certain purified populations can give rise to long-term reconstitution after transplantation<sup>24,25</sup>. The most commonly used FACS-purified populations of HSC/progenitor cells include the following:

#### **2.1.3.1. Thy1.1<sup>lo</sup>, Lin<sup>−</sup> Sca-1<sup>+</sup> Cells**

Short-term repopulating HSCs could be isolated based on their expression of stem cell antigen-1 (Sca-1), low expression of Thy1.1, and lack of expression of lineage markers<sup>21</sup>. The same population was shown to contain long-term repopulating HSCs in a subsequent study<sup>26</sup>.

#### **2.1.3.2. Lin c-Kit<sup>+</sup> Sca-1<sup>+</sup> Cells**

The latter population was further purified by the expression of the stem cell factor receptor, c-Kit, in 1992<sup>27</sup>. This population is very heterogeneous and consists predominantly of progenitor cells with less than 10% of it representing HSCs. The lack of expression of CD34<sup>28</sup> and Flt3 (CD135)<sup>29,30</sup> has been used to further purify long-term repopulating HSCs (LSK<sup>+</sup> CD34<sup>−</sup> Flt3<sup>−</sup>) from short-term repopulating HSCs (LSK<sup>+</sup> CD34<sup>+</sup> Flt3<sup>−</sup>) and multipotent progenitors (LSK<sup>+</sup> CD34<sup>+</sup> Flt3<sup>+</sup>)<sup>30</sup>.

#### **2.1.3.3. SLAM Family Members**

SLAM proteins are a family of cell surface glycoproteins in the immunoglobulin superfamily with specific SLAM antigens (CD150<sup>+</sup> CD244<sup>−</sup> CD48<sup>−</sup> cells) identified as useful to purify a population of which approximately 50% of single cells reconstituted

lethally irradiated animals<sup>31</sup>. Unlike the limitations presented by those isolated using Thy1.1 or Sca-1 expression, the SLAM receptors appear to be expressed by many mouse strains<sup>31</sup>, and more faithfully detect HSCs in older, mobilized, or transplanted mice<sup>25</sup>.

#### **2.1.3.4. Fluorescent dyes**

Two different vital dyes, the mitochondrial-binding dye **Rhodamine 123** (Rh123) and DNA-binding dye Hoescht 33342 (Ho 33342), have also been used either alone or in combination to isolate HSCs<sup>32–36</sup>. Both of these dyes are retained at very low intensity in HSCs due to high efflux of the dyes from HSCs, as shown by studies utilizing the drug verapamil. A more common method used in laboratories today is the use of the **Hoescht 33342** (Ho 33342) to define the **side population** (SP)<sup>32</sup>. By analyzing Ho 33342 emission at two wavelengths simultaneously, the SP appear distinct from the main population in a distinct “tail” profile, which disappears with verapamil treatment<sup>32</sup>. The SP population is also not as pure as HSCs enriched by other methods such as LSK<sup>+</sup> CD34<sup>−</sup> Flt3<sup>−</sup> cells, although it can be used in combination with other markers such as Sca-1, c-Kit, and CD34 to further purify HSCs with extreme efficiency<sup>24,37</sup>. Notably, cells with an SP profile have also been detected in many other organs but with inconsistent functional correlation with stem cell-like functions<sup>38</sup>. This method should not be assumed to yield stem cells in other tissue types or even in species other than the mouse.

#### **2.1.4. Phenotype and isolation of HSC based on their cycling behaviour**

Just before birth, cycling HSCs migrate from the liver to the developing bone marrow, where they engraft in small cavities of trabecular bone. By four weeks after birth, HSCs have fully matured and acquired a dormant status. A dormant status is necessary to preserve the self-renewal capacity of HSCs and to prevent stem cell

exhaustion<sup>39–41</sup>. In the healthy adult mouse, all long-term HSC activity is a feature of a small subset of LSK bone marrow cells. In comparison to progenitor populations, HSCs are recognized as ‘slow’ cycling<sup>42–45</sup>. Classical cell cycle analyses of highly purified HSCs that measure DNA content (Hoechst 33342 or 4’, 6-diamidino-2-phenylindole (DAPI)) alone, or combined with intracellular Ki67 expression (which is absent in cells at the G0 stage), have shown that more than 70% of CD34<sup>–</sup>CD48<sup>–</sup>CD150<sup>+</sup> LSK HSCs are in the quiescent G0 stage of the cell cycle, whereas less than 10% of more differentiated multipotent progenitor cells (CD34<sup>+</sup> LSK cells) are quiescent<sup>46</sup>. Although such assays generate important informations regarding the cell cycle state of each cell following isolation, they do not reveal the cycling history of each cell over time or their precise physical location in the bone marrow. To address this, long-term label-retaining assays have been carried out, in which HSCs are labeled *in vivo* with the thymidine analogue bromodeoxyuridine (BrdU) or with a chromatin-associated green fluorescent protein (GFP) by incorporating a histone 2B–GFP fusion protein under the control of a doxycyclin-regulated transgenic allele. After labeling, BrdU incorporation is stopped by removing the BrdU source, or GFP expression is repressed by the administration of doxycyclin. These conditions are continued in the subsequent chase period (up to one year), and during this time dividing cells gradually lose the accumulated label so that after more than four cell divisions the BrdU and GFP labels, respectively, are diluted out and are no longer detectable by flow cytometry. By contrast, cells that have undergone fewer than five cell divisions during the chase period remain labeled and are therefore called label-retaining cells (LRCs). Thus, the cell cycling history can be determined by measuring the loss of the label over a long time period. For example, in the CD34<sup>–</sup>CD48<sup>–</sup>CD150<sup>+</sup> LSK HSC population, 22% still retained the label (that is, are LRCs) after 120 days of chase, 18% were LRCs after 213 days chase and some were LRCs after 380 days<sup>46</sup>. By contrast, in the hematopoietic progenitor cell populations (the CD34<sup>+</sup> LSK

cells), few, if any, LRCs were found after only 100 days of chase. Different mathematical modeling strategies using the label decay kinetics data have revealed that the CD34<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup> LSK HSC population probably comprises two subsets: a **dormant population** (~30%) dividing only every 145-193 days and a 'homeostatic' **quiescent population** (~70%) dividing every 28–36 days. Although both populations could reconstitute the hematopoietic system in primary recipients, only dormant HSCs could be serially transplanted, suggesting that long-term self-renewal capacity is retained exclusively by dormant HSCs.

## **2.2. HUMAN HEMATOPOIETIC STEM CELL ASSAY**

### **2.2.1. *In vitro* assays**

To study the properties of human HSCs we can use exactly the same kind of assays used to characterize murine HSCs. The only different parameter is the time required to culture and to differentiate human HSCs both in CFC assay and in LTC-IC assay. Murine cells cultured on methylcellulose and supplied with cytokine are able to form colonies after 7-10 days of culture, instead human clonogenic progenitors need 2-3 weeks. LTC-IC assay for human cells can be extended from 5 to 12 weeks to individuate LTC-IC or even more primitive extended-LTC-IC.

### **2.2.2. *In vivo* assays**

The study of human LT-RCs required the development of specific murine models with severe combined immunodeficiency (SCID). Immunodeficient mice are often used as recipients for human cells or tissues, because they can relatively easily accept heterologous cells due to lack of host immunity reducing rejection and allowing the engraftment of human cells, in our case HSCs. With the development of the NOD/scid mouse, an improved SCID mouse, it was possible to engraft more human cells and tissues than in the SCID mouse. However, many problems

remained before an *in vivo* humanized model could be achieved.

Mamoru Ito and his group at the Laboratory Animal Research Department in CIEA were successful in establishing an extremely severe combined immunodeficient mouse called the NOG (NOD/Shi-scid,IL-2R $\gamma$ null) mouse<sup>47</sup> by combining the NOD/scid mouse and the IL-2 receptor- $\gamma$ chain (a common receptor for several cytokines) knockout (IL2 $\gamma$ KO) mouse. NOG mice lack T and B cells, Natural Killer cells (NK) and complement activity. Furthermore, they have reduced dendritic and macrophage functions.

The NOG mouse shows markedly better engraftment of human cells and human tissues than the NOD/scid mouse and also makes possible engraftment of human cancer cells, liver cells, etc. at high rates. In addition, after transplantation of human hematopoietic stem cells, human T cells can be developed in peripheral lymphoid tissues of the NOG mouse.

### **2.2.3. Immunophenotypical analysis of human HSC/Progenitor cells**

The first CD34 surface glycoprophosphoprotein is commonly used for enrichment of human HSCs. CD34 is expressed on fetal liver hematopoietic cells, in cord blood cells and in the adult bone marrow cells. CD34<sup>+</sup> bone marrow cells comprise only 1.5% of marrow mononuclear cells, but contain precursors for all lymphohematopoietic lineages. CD34<sup>+</sup> is a very heterogeneous compartment of cells constituted mainly by multipotent and oligopotent progenitors and only 1% of this population include CD34<sup>+</sup> CD38<sup>-</sup> cells. CD34<sup>+</sup> CD38<sup>-</sup> cells are highly enriched by LT-RCs. Recently, tracking the expression of several adhesion molecules in HSC-enriched subsets; CD49f was identified as a specific HSC marker. Single CD49f<sup>+</sup> cells were highly efficient in generating long-term multilineage grafts, and the loss of CD49f expression identified transiently engrafting multipotent progenitors (MPPs).

## 2.3. HEMATOPOIETIC STEM CELLS MAINTENANCE

Hematopoiesis is a tightly regulated process in which a rare pool of hematopoietic stem cells (HSCs) gives rise to the lymphohematopoietic system. In order to maintain hematopoietic homeostasis throughout the lifetime of an animal, this pool of HSCs must be maintained. This is achieved by the processes of survival, quiescence and proliferation/self-renewal, a specialized cell division in which one or both of the daughter cells remain undifferentiated and retain essentially the same replication potential of the parent. One of the most important issues in stem cell biology and in regenerative medicine is to understand the mechanisms that regulate the properties of stem cells. Some examples of key regulators are listed below.

### 2.3.1. Cell-intrinsic pathways

#### 2.3.1.1. Anti-apoptotic proteins

Stem cell maintenance requires that proliferation pathways remain functional while differentiation, senescence and cell death pathways are repressed. There is a large body of evidence suggesting that suppression of apoptosis is required for HSC survival. Studies using transgenic mice constitutively expressing **BCL2** (*B-cell lymphoma 2*) in all hematopoietic tissues provide evidence directly supporting this theory. The forced expression of the oncogene *Bcl2* resulted in increased numbers of transgenic HSCs *in vivo* and gave these cells a competitive edge over wild type HSCs in competitive reconstitution experiments<sup>48,49</sup> suggesting that cell death plays a role in regulating the homeostasis of HSCs. **Mcl1** (Myeloid cell leukemia 1), another anti-apoptotic Bcl2 family member, has been shown to be required for HSC survival<sup>50</sup>. In this study it was shown that inducible deletion of Mcl1 in mice resulted in a severe anemic phenotype due to a drastic loss of BM cells in a cell autonomous manner.

### 2.3.1.2. Transcription factors

The transcription factor **Tel** (Translocation Ets leukemia;also known as Etv6 [Ets variant gene 6]), an Ets (E-26 transforming-specific)-related transcriptional repressor, is also required for HSC maintenance. Conditional inactivation of Tel/Etv6 in HSCs rapidly leads to the depletion of Tel/Etv6-deficient HSCs. However, Tel/Etv6 is not required for the maintenance of committed precursors. When it is conditionally inactivated in most hematopoietic lineages, it does not affect their differentiation or survival<sup>51</sup>. The mechanism by which Tel/Etv6 modulates adult HSCs survival is not known.

The **homeobox (Hox) genes** encode transcription factors that regulate embryonic body patterning and organogenesis. They play a role in the regulation of hematopoiesis. The function of HOX genes in normal hematopoiesis has been widely studied using gene expression analysis and knockin or knockout studies in HSCs and early hematopoietic progenitors. Generally the overexpression of a HOX gene leads to an expansion of stem and progenitor cell populations together with a block on differentiation. Notable examples of this include the overexpression of murine Hoxb6, which resulted in the expansion of murine HSCs and myeloid precursors, together with the inhibition of erythropoiesis and lymphopoiesis<sup>52</sup>, and overexpression of murine Hoxb3 that resulted in several hematological abnormalities, such as a block of B- and T-cell differentiation as well as a delay in myeloid precursor proliferation<sup>53</sup>. Overexpression of human HOXC4 resulted in expansion of early and committed myeloid and erythroid progenitors<sup>54</sup>, and knockin of human HOXA5 caused an increase in the number of myeloid progenitors and blocked erythroid differentiation<sup>55,56</sup>. Other HOX genes are required for the maintenance of progenitor or stem cell status and promote their proliferation, especially HOXA9 and **HOXB4**. The former is the most preferentially expressed HOX gene in human CD34<sup>+</sup> HSCs and early hematopoietic progenitors and is subsequently downregulated during

differentiation. Murine Hoxa9 and Hoxb4 overexpression enhances HSC expansion and myeloid progenitor proliferation<sup>57,58</sup>. Hoxb4 is also highly expressed in HSCs and downregulated during differentiation<sup>53,59</sup>. Our team contributed to demonstrate that its overexpression in murine and human cell lines results in a remarkable expansion of HSCs *in vivo* and *in vitro* without resulting in leukemia or lineage disturbances<sup>53,60</sup>. Indeed, the self-renewal ability of Hoxb4-transduced murine HSCs is 20–50-fold greater than untreated cells. In addition to the knockin and overexpression, knockdown and deletion studies in murine models and cell lines have also been used to evaluate the role of HOX genes in hematopoiesis. However, owing to the functional redundancy of HOX genes, the results of knockdown assays are sometimes difficult to interpret and do not always reflect the findings of studies where the gene has been overexpressed.

**Gfi1** (Growth factor independence 1), a Zinc-finger repressor, has been implicated as a regulator of HSC self-renewal. Two groups working independently determined that Gfi1 controls self-renewal of HSCs by restraining their proliferative potential<sup>61,62</sup>. They showed that *Gfi1*-deficient HSCs display increased proliferation rates and are also functionally compromised in competitive repopulation and serial transplantation assays. Gfi1 might exert its effects on HSC proliferation by regulating the cell cycle inhibitor p21<sup>CIP1/WAF1</sup>. p21 mRNA expression levels are dramatically lower in the *Gfi1*-deficient HSCs<sup>62</sup>. **p21<sup>CIP1/WAF1</sup>** itself has been implicated in the regulation of HSCs<sup>63</sup>. In its absence, HSCs have an impaired serial transplantation capacity.

The **JAK–STAT** (Janus family kinase–signal transducer and activator of transcription) pathway is a common downstream pathway of cytokine signaling that promotes hematopoiesis. Constitutive activation of the transcription factors of the Stat family, particularly **Stat3** and **Stat5**, are frequently detected in leukemias, lymphomas and solid tumors<sup>64</sup>. Activation of Stat5 in HSCs led to the dramatic



expansion of multipotent progenitors and promoted HSC self-renewal *ex vivo*<sup>65</sup>. Deletion of *Stat5* resulted in profound defects in hematopoiesis and markedly reduced ability of the mutant cells to maintain quiescence during steady-state hematopoiesis<sup>66</sup> or repopulate the bone marrow of lethally irradiated mice. Another group show that transduction of adult mouse bone marrow cells with a constitutively activated form of Stat3 increased their regenerative activity in lethally irradiated recipients, whereas the transduction of these cells with a dominant negative form of Stat3 suppressed their regenerative activity<sup>67</sup>. These studies suggest that Stat proteins play a role in HSC self-renewal and potentially in other tissues, owing to the wide range of solid tissue and blood malignancies that harbor constitutively activated Stats.

#### 2.3.1.3. Signal transducers

The **Pten** (phosphatase and tensin homologue deleted on chromosome ten) tumor suppressor, a modulator of several major signaling pathways, has very recently been implicated as a regulator of HSC self-renewal and an initiator of leukemogenesis<sup>68,69</sup>. It functions by inhibiting signaling through the AKT pathway. Although *Pten* deletion initially leads to a transient expansion of HSC numbers, the HSC pool then becomes depleted over time. *Pten*-deficient HSCs engraft normally in recipient mice, but are unable to sustain multilineage hematopoietic reconstitution. When *Pten*-deficient HSCs were transplanted into irradiated mice, they were only capable of short-term multilineage hematopoietic reconstitution and could not stably engraft irradiated recipients long term<sup>68,69</sup>.

Loss of **Lkb1**, another signal transducer involved in the Akt pathway, leads to impaired survival and escape from quiescence of HSCs, resulting in exhaustion of the HSC pool and in a marked reduction of HSC repopulating potential *in vivo*. Lkb1

deletion has an impact on cell proliferation in HSCs, but not on more committed compartments, pointing to context-specific functions for Lkb1 in hematopoiesis<sup>70–72</sup>.

The mechanistic target of rapamycin (**mTOR**) is one of the proteins regulated by Pten and Lkb1 and it serves as a key sensor of cellular-energetic state and functions to maintain tissue homeostasis. Hyperactivation of the mTOR pathway impairs HSC function and is associated with leukemogenesis. The deletion of the mTORC1 component, regulatory-associated protein of mTOR (**Raptor**), in mouse HSCs leads to an impaired HSC regeneration *in vivo*<sup>73</sup>.

#### 2.3.1.4. Polycomb group proteins

Polycomb-group proteins are a family of proteins that can remodel chromatin such that transcription factors cannot bind to promoter sequences in DNA. Recent studies have shown that Polycomb group (PcG) proteins and their interaction are important in the regulation of HSC self-renewal and lineage restriction. In particular, members of the PRC1 (Polycomb repression complex 1), such as Bmi1, Mel18 and Rae28, have been implicated. **Bmi-1** plays an important role in regulating the proliferative activity of stem and progenitor cells. It is required for the self-renewal of both adult HSCs and neural stem cells<sup>74,75</sup>. Bmi1 enhances symmetrical expansion of the stem cell pool through self-renewal, induces a marked *ex vivo* expansion of multipotent progenitors, and increases the ability of HSCs to repopulate bone marrow *in vivo*<sup>76</sup>. Leukemic cells lacking Bmi1 undergo proliferation arrest, differentiation and apoptosis, leading to failure of leukemia in a mouse transplant mode<sup>77</sup>. In Bmi1-deficient bone marrow there is an upregulation of cell cycle inhibitors p16<sup>INK4A</sup> and p19<sup>ARF</sup>, and the p53-induced gene Wig1, and a downregulation of the apoptosis inhibitor AI-6. This suggests that a mechanism exists whereby Bmi1 functions by modulating proliferation and preventing apoptosis<sup>78</sup>. Bmi1 has also been shown to

regulate the expression of Hox genes that are required for differentiation during hematopoiesis<sup>74,79</sup>.

Loss or knockdown of another Polycomb gene, **Me118**, leads to increased expression of *Hoxb4*<sup>80</sup>, and transplanted *Me118*-deficient bone marrow showed an increase in overall HSC numbers but a decrease in their activity owing to arrest in G0 phase of the cell cycle.

**Rae28**- deficient HSCs were defective in their long-term repopulating ability in serial transplantation experiments<sup>81,82</sup>. Taken together, these studies show the importance of the Polycomb proteins in HSC self-renewal and maintenance of the blood system.

### 2.3.2. Hematopoietic stem cells niche and cell-extrinsic pathways

HSCs cannot yet be maintained and expanded *in vitro* because a complex and dynamic molecular crosstalk between HSC and their endogenous microenvironment (or “niche”) directs their fate. The importance of the stem cell niche in regulating HSC function was first postulated in 1978 by Ray Schofield, when observing that the spleen is unable to support HSCs in the same way that bone marrow can<sup>83</sup>. Since then, it has become clear that not only HSCs but all somatic stem cells maintain homeostasis because they sense and respond to the need of an organism for their differentiated progeny as well as to stem cells themselves. The stem cell niche is the functional and anatomical “node” that allows integration of signals from the periphery into the appropriate stem cell behavior. As several controversial observations have led to the most recently proposed niche models and because future studies are likely to further shed more light on this subject, it is difficult to present a definitive, detailed portrait of the HSC niche but, instead, it is possible to provide an up-to-date overview integrating currently accepted niche elements and dynamic models.

### 2.3.2.1. Cellular components of the HSC niche

HSCs reside within the bone marrow, which presents a complex microenvironment that is made up of different cell types and extracellular elements. An increasing number of bone marrow lineages, structures and molecular components have been demonstrated to affect HSC fate and function (Figure 4).

The endosteal surface of the bone and cells of the osteoblastic lineage were shown first to be components of the HSC niche<sup>84–88</sup>. Whether a specific subpopulation of **osteoblastic cells** is interacting with HSCs is currently under investigation. Secreted phosphoprotein 1 [SPP1; also known as osteopontin (OPN) and one of the main extracellular proteins secreted by osteoblasts] and ALCAM (an adhesion molecule widely expressed across different lineages) were proposed as markers of a subpopulation of osteoblastic cells that affect HSC function<sup>89,90</sup>.

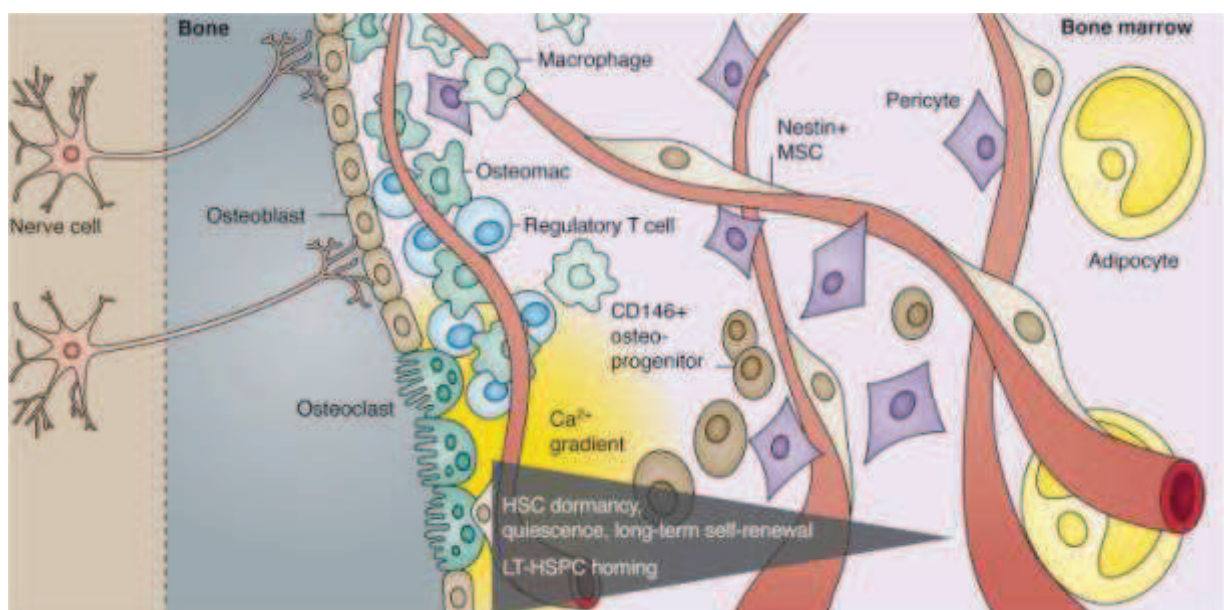


Figure 4. The HSC microenvironment (from Lo Celso and Scadden 2011)

Interestingly, hematopoiesis is dramatically affected by the conditional deletion of the ribonuclease Dicer in osteoprogenitors. However, the same deletion in fully mature, osteocalcin-positive osteoblasts does not lead to hematopoietic defects<sup>91</sup>, and the selective ablation of terminally differentiated osteoblasts does not affect hematopoiesis either<sup>91,92</sup>. The association between HSCs and osteoprogenitors might be rooted even earlier within the osteoblastic lineage, as was suggested by the fact that nestin-positive mesenchymal stromal cell-like cells are marrow stromal cells that can interact with HSCs<sup>93</sup>. Furthermore, human CD146<sup>+</sup> osteoprogenitor cells are able to direct ectopic bone formation accompanied by hematopoietic seeding<sup>94</sup>. Platelet-derived growth factor receptor alpha (PDGFR $\alpha$ ) positive, Sca1<sup>+</sup> mesenchymal progenitors have been successfully transplanted and have been shown to localize to areas of the bone marrow that are generally recognized to contain HSC niches<sup>95</sup>. Taken together, these studies show that osteoblastic cells at various stages of differentiation can support different HSC functions and states.

The endosteal HSC niche model was challenged, however, by immunofluorescence studies, which showed that the only bone marrow structure that is consistently located adjacent to HSCs is the **sinusoidal vasculature**<sup>31</sup>. Integrity and regeneration of bone marrow vasculature are, indeed, fundamental for HSC recovery from myeloablative injuries and following bone marrow transplantation<sup>96,97</sup>. Several reports agree on the location of functional, engrafting hematopoietic stem and progenitor cells (HSPCs) being near the endosteal surface but not exclusively adjacent to osteoblastic cells<sup>98–100</sup>. Since endosteal surfaces are highly vascularised, the question remains whether HSCs that are located at varying distances from osteoblastic cells are functionally distinct from those located near osteoblastic cells.

Other HSC regulators include perivascular cells, non-endothelial supportive cells<sup>101</sup>, adipocytes, which have been shown to inhibit HSC engraftment<sup>102</sup>, and the autonomous nervous system, which influences HSC mobilisation<sup>103</sup> (see the chapter “Induced HSC mobilization”). In addition, several cells of hematopoietic origin have a role in the HSC niche. For example, the activation of osteoclasts, a specialised subpopulation of endosteal macrophages that are responsible for bone resorption, leads to HSC egress from the bone marrow<sup>104</sup>, whereas their pharmacological inhibition leads to a reduction of HSPC numbers<sup>105</sup>. A different subpopulation of bone marrow macrophages, the “osteomacs”, form a canopy of cells near active osteoblasts and carry out the opposite role: their depletion leads to the loss of osteoblast activity and increased HSC mobilisation<sup>106</sup>.

The question remains whether we should think about one or several HSC niches. The observation that different HSPC localizations exist within the marrow and that a growing number of cell types are involved in HSC regulation, together with the increasing number of reports that describe the heterogeneity of even highly purified HSPC populations<sup>46,99,107</sup> could be an indication of the complex microenvironments through which HSCs navigate. One proposed model suggests that osteoblastic cells provide a context for HSC dormancy, whereas a perivascular, quiescent niche provides an intermediate niche for activated HSCs that are ready to either generate differentiating progeny or revert to dormancy, depending on the needs of the organism<sup>108,109</sup>.

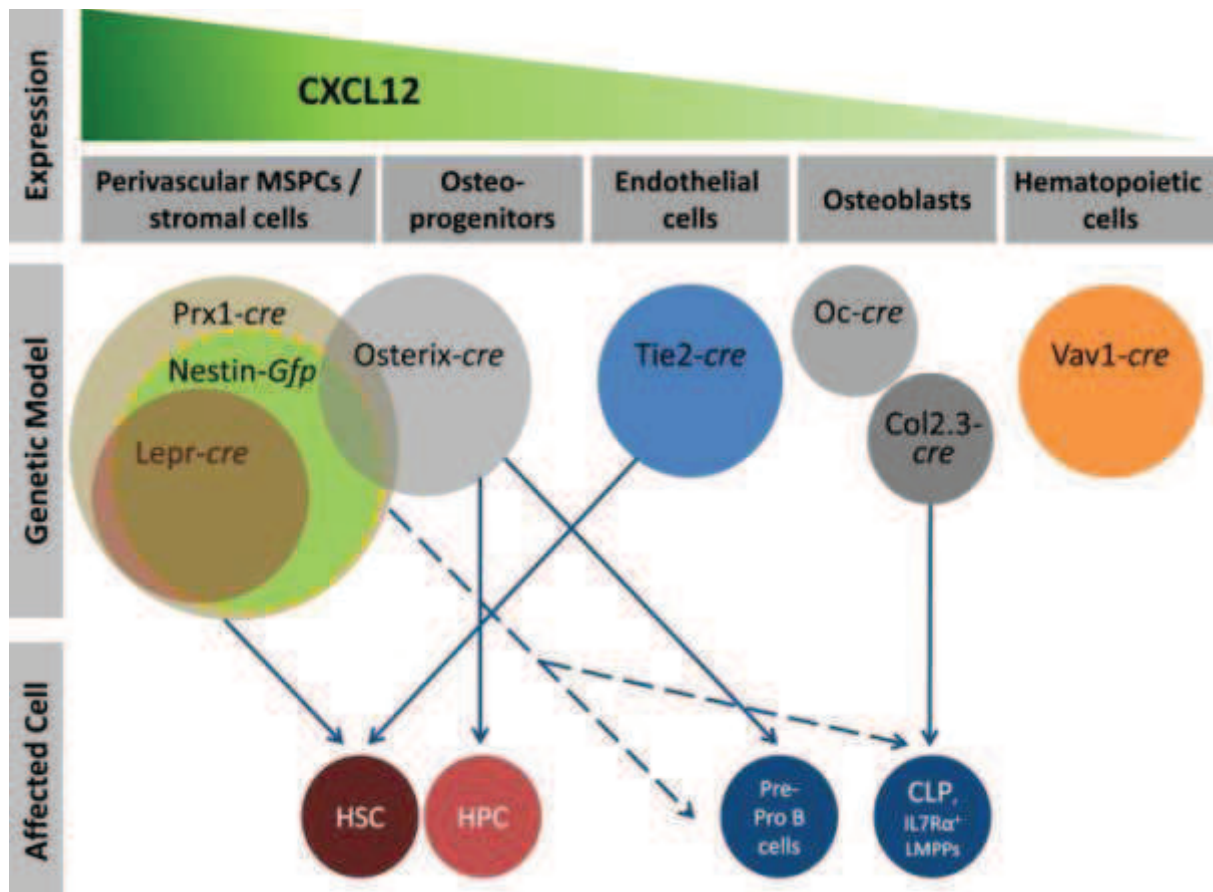
#### **2.3.2.2. Molecular regulators of HSC fate**

Independent of the identity of the niche cell that generates a signal for the HSC, a multitude of molecular regulators of HSC fate has been described. Some are known products of osteoblastic cells; however, it is possible that an increasing number of these factors are produced by multiple cell types.

#### 2.3.2.2.1. Secreted ligands and their receptors

The chemokine (C-X-C motif) ligand 12 (**CXCL12**) -C-X-C chemokine receptor type 4 (**CXCR4**) pathway is the best-defined regulator of HSC localization in the bone marrow. Osteoblastic cells produce CXCL12 (also called SDF1) and upregulate its expression in response to irradiation<sup>100</sup>. However, recent studies using conditional knockout of CXCL12 in different cellular bone marrow stromal subsets, have highlighted numerous perivascular cells throughout the marrow that are CXCL12-positive<sup>101</sup> and responsible for HSCs maintenance<sup>110</sup>, which indicates that CXCL12 directs HSC localization not only near osteoblastic cells, as it was thought for long time, but mainly throughout perivascular areas. Deletion of *Cxcl12* in osteoblasts using transgenic mice reveals no alteration in HSC or myeloerythroid progenitor cell number. However, these mice show significantly lower levels of T cell and B cell reconstitution and fewer early lymphoid progenitors in the bone marrow. These findings are consistent with the accumulation of early lymphoid progenitors adjacent to the endosteum, which reinforces the contribution of osteolineage cells to the generation of early lymphoid progenitors<sup>111</sup>. To have an idea of the emerging complexity of the different cellular niches expressing CXCL12 and affecting different HSPC populations you can refer to figure below (Figure 5).





**Figure 5. Distinct cellular sources and niches for CXCL12 in bone marrow (Hanoun and Frenette 2013).**

Most CXCL12 is derived from perivascular stromal cells that can be marked by Prx1-cre, Lepr-cre, or Nestin-Gfp that likely show significant overlap with each other. The highest levels of CXCL12 are secreted by the most immature mesenchymal stem/progenitor cells (MSPC). Osteoblasts, marked by Osteocalcin (Oc), synthesize low amounts of CXCL12 that are not essential for normal hematopoiesis, whereas deletion of *Cxcl12* in osteoblasts using Col2.3-cre leads to deficits in certain early lymphoid progenitors similar to Osterix-cre, suggesting a contribution of osteoprogenitors in the generation of lymphoid precursors. Endothelial cells, marked by Tie2-cre, also secrete CXCL12 and contribute to HSC maintenance. Although some hematopoietic cells express CXCL12, deletion in the hematopoietic system using Vav1-cre did not yield any phenotype. The effect of Prx1-targeted cells on lymphoid progenitors is indicated with a dashed line because it is likely to be derived from osteoprogenitors or perivascular stromal Prx1-cre-targeted cell fraction. The size of circles does not reflect the actual frequencies in the bone marrow, and the overlap among different models is based on estimations. HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell; CLP, common lymphoid progenitor; LMMP, lymphoid-primed multipotent progenitor.

If CXCL12 and CXCR4 are responsible for HSC localization, other cytokines, signaling pathways and adhesion molecules known to have a role in the HSC niche might, instead, regulate HSC fate.

The ligand–receptor pair stem cell factor (**SCF**) and **KIT** is known to have a pivotal role in stem cell maintenance and proliferation. A recent elegant study by Ding



et al. has highlighted the key role of the vascular niche: HSCs were depleted from bone marrow when *Scf* was deleted from endothelial cells or leptin receptor (*Lepr*)-expressing perivascular stromal cells, while HSC frequency and function were not affected when *Scf* was conditionally deleted from hematopoietic cells, osteoblasts, or nestin positive cells<sup>111</sup>.

Thrombopoietin (**TPO**), mainly secreted from osteoblast and megakaryocytes in the bone marrow, and its receptor myeloproliferative leukemia virus oncogene (**MPL**) are critically involved in postnatal steady-state HSC maintenance, reflected in a 150-fold reduction of HSCs in adult *Tpo*<sup>-/-</sup> mice<sup>112</sup>. Whereas not required for fetal HSCs expansion, the key role of TPO in postnatal HSC maintenance consists in keeping HSC quiescent to avoid postnatal HSCs exhaustion, illustrated by accelerated HSC cell-cycle kinetics in *Tpo*<sup>-/-</sup> mice. Recently it has been shown that TPO can protect HSC from genotoxic stress<sup>113</sup>.

**Angiopoietin 1** (ANGPT1) and **TIE2** tyrosine kinase (officially known as TEK) play pivotal roles in regulating *in vivo* HSC maintenance by keeping them out of the cell cycle<sup>114,115</sup>. Osteoblasts and especially endothelial cells and perivascular cells are the angiopoietin 1 expressing cell of the bone marrow. Recently, **angiopoietin-like 3** (ANGPTL3), which is primarily expressed by endothelial cells, has been shown to control HSC quiescence as well as the number of HSCs both in the steady state and following transplantation<sup>116</sup>.

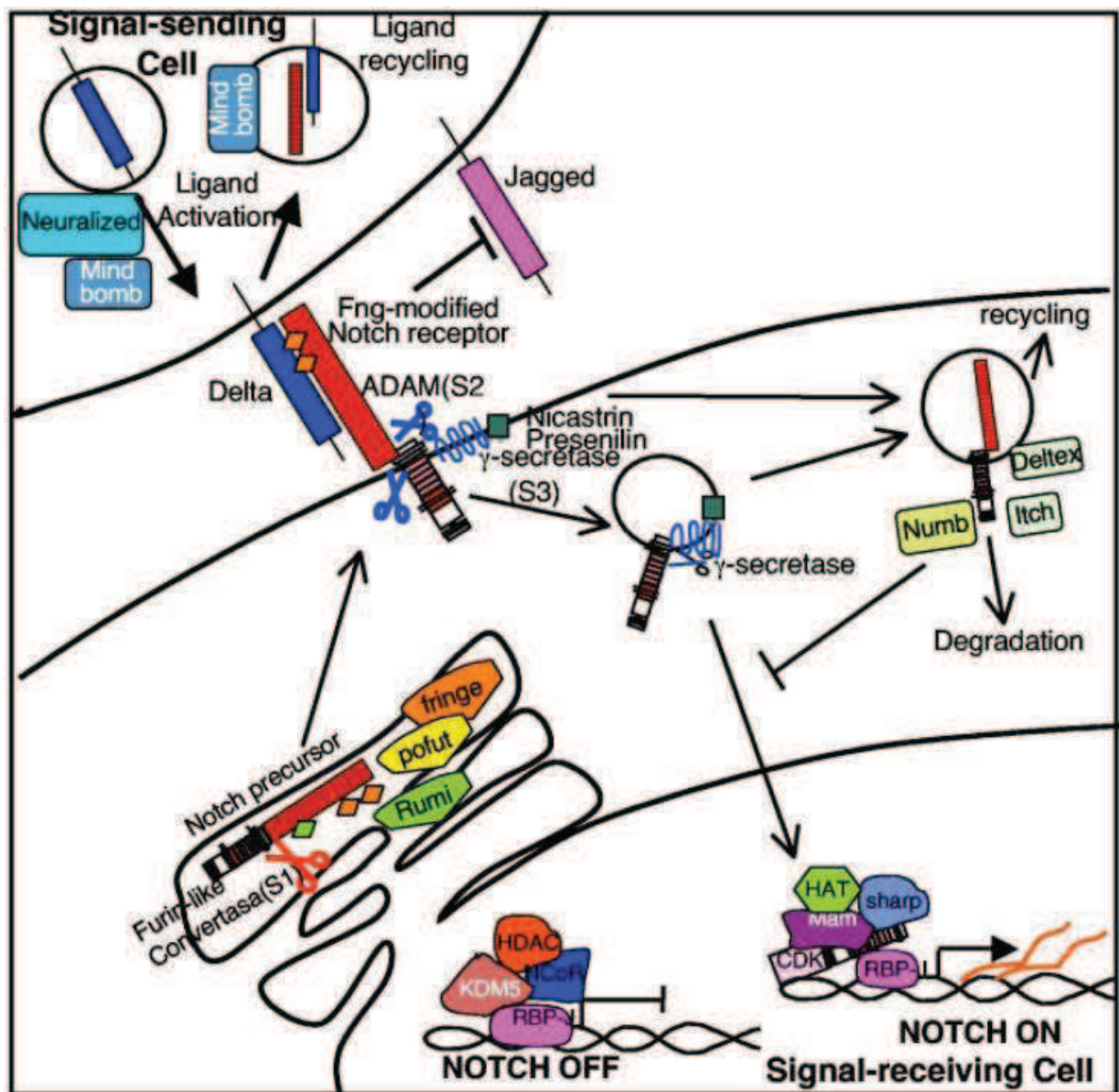
#### 2.3.2.2.2. WNT signaling

Wnt signaling begins when one of the Wnt proteins binds the N-terminal extra-cellular cysteine-rich domain of a Frizzled (Fz) family receptor. These receptors span the plasma membrane seven times and constitute a distinct family of G-protein coupled receptors. However, to facilitate Wnt signaling, co-receptors may also be required alongside the interaction between the Wnt protein and Fz receptor.

Examples include lipoprotein receptor-related protein (LRP)-5/6, receptor tyrosine kinase (Ryk), and ROR2. The **WNT** signaling cascade has been implicated with a role in HSC regulation; but this is still highly controversial, mostly because of the complexity of the signaling pathway itself<sup>117</sup>. Following initial reports that the obliteration of the canonical WNT pathway does not affect HSCs, later studies that focused on interactions between HSCs and their niche provided further detailed information on the involvement of this pathway<sup>118,119</sup>. It was shown that osteoblast-specific overexpression of the WNT inhibitor dickkopf homolog 1 (DKK1) leads to impairment of HSC self-renewal<sup>120</sup>. Similarly, the knockout of secreted frizzled-related protein 1 (SFRP1), another negative modulator of WNT signaling, leads to an initial increase in long-term reconstituting HSCs (LT-HSCs), followed by their premature exhaustion<sup>121</sup>. Interestingly, early B-cell factor 2 (EBF2, a transcription factor known to synergise with WNT signaling in certain cells and under certain conditions) knockout mice are affected by loss of HSCs<sup>122</sup>. Recently a study by Sugimura and colleagues has demonstrated the importance of the cooperation between canonical and **noncanonical Wnt** pathway in HSCs function. It was shown that Flamingo (Fmi) and Frizzled (Fz) 8, members of noncanonical Wnt signaling, are both expressed in HSCs and they functionally maintain quiescent long-term HSCs. Fmi regulates Fz8 distribution at the interface between HSCs and N-cadherin<sup>+</sup> osteoblasts (N-cad<sup>+</sup> OBs that enrich osteoprogenitors) in the niche. N-cad<sup>+</sup> OBs predominantly express noncanonical Wnt ligands and inhibitors of canonical Wnt signaling under homeostasis. Under stress conditions, noncanonical Wnt signaling is attenuated and canonical Wnt signaling is enhanced in activation of HSCs<sup>123</sup>.

### 2.3.2.2.3. Notch signaling

Notch signaling defines a conserved, fundamental pathway responsible for determination in metazoan development and is widely recognized as an essential component of lineage-specific differentiation and stem cell self-renewal in many tissues<sup>124,125</sup>, including the hematopoietic system. In mammals, there are four Notch receptors (Notch1-4), three Delta like ligands (Dll1, Dll3, and Dll4), and two ligands of the Jagged family (Jag1 and Jag2). When membrane bound receptors interact with cognate ligands on an adjacent cell, two consecutive proteolytic cleavages of the receptor are initiated, freeing the intracellular portion of Notch to enter the nucleus and activate the transcription of target genes. The first cleavage in the heterodimerization domain (HD) by a disintegrin and metalloprotease 10 generates the substrate for the second cleavage by the  $\gamma$ -secretase complex. Canonical Notch signaling requires the formation of a complex with a transcription factor of the CSL (CBF1/Su(H)/Lag1) family, CBF1/RBPJk/KBF2 in mammals. CBF1 binds DNA in a sequence specific manner and acts as a repressor of transcription in the absence of Notch signaling. Displacement of corepressors bound to CBF1 by intracellular Notch (ICN) allows the recruitment of co-activators, such as Maml1 (Mastermind Like1), and histone acetyltransferases, such as p300, to create a short-lived transcriptional activation complex. Recent genome wide chromatin immunoprecipitation arrays and sequencing have identified a large number of genes that can be regulated directly by Notch<sup>126,127</sup>. Many of these target genes may be cell type specific, but there are a few well characterized transcriptional targets of ICN-CBF1, including the *HES* (*hairy enhancer of split*) family of transcription factors, Notch-related ankyrin repeat protein (*NRARP*), *c-MYC*, and *DTX1* (Deltex1)<sup>128</sup>; Weng et al., 2006) (Figure 6). All Notch receptor paralogs and their ligands have been implicated in the regulation of diverse functions in the hematopoietic system. The best-described functions of



**Figure 6. The Notch signaling pathway. (Bigas and Espinosa, 2012).**

Signal-sending Cell: Functional Notch ligands are ubiquitinated by the E3-ubiquitin ligases mindbomb or neuralized. After ligands interact with the Notch receptor, the ligand and the extracellular part of Notch are endocytosed, and ligands may be degraded or recycled. Signal-receiving Cell: The Notch mRNA is translated as a precursor protein, which is cleaved by a furin-like convertase in the Golgi apparatus to produce a functional heterodimeric receptor. During endoplasmic reticulum/Golgi transit, Notch is modified by different glycosyltransferases (Rumi/poglut, pofut, fringes). In cells that express fringe, specific sugar moieties (diamonds) are conjugated to confer higher affinity to Delta-type ligands. After ligand binds to the EGF-like repeats of the Notch extracellular domain, an ADAM metalloprotease cleaves Notch at the S2 site, removing most of the extracellular domain. The membrane-tethered intracellular domain is then cleaved by the Presenilin complex at site S3, either in the plasma membrane or after endocytosis, freeing the Notch intracellular domain (ICN). ICN translocates to the nucleus, displaces the corepressor complex, associates with RBP-J, and recruits coactivators, such as Mastermind. ICN becomes monoubiquitinated (Ub), targeting the receptor for degradation. Several E3 ubiquitin ligases (Deltex, Nedd4, Su(Dx)/Itch, Cbl) can direct Notch receptor trafficking toward lysosomal degradation or toward recycling. Numb can also promote Notch degradation in daughters of an asymmetrically dividing cell.

Notch are in the emergence of fetal HSCs<sup>129–131</sup> and T cell commitment and early development. Indeed, the significance of Notch1 for T lymphocyte commitment, differentiation, and oncogenic transformation has been well established<sup>132–134</sup>. On the other hand, data regarding Notch involvement in non-lymphoid adult blood lineages are often controversial. Recent studies suggested a role for Notch4 in megakaryocyte differentiation<sup>135</sup>, but our group through a study in human hematopoietic progenitors challenged this conclusion<sup>136</sup>. Moreover, Oh and colleagues by tracing Notch expression in different lineages using transgenic mice, have revealed an intriguing division of labor between Notch1 and Notch2, with the former marking mainly lymphocyte progenitors and the latter reaching peak levels during early erythropoiesis<sup>137</sup>. Interestingly, Notch2-expressing progenitors were enriched for erythroid potential and upregulated the expression of an erythroid gene program. Accordingly, conditional Notch gain-of-function in hematopoietic progenitors promoted erythroid commitment and Notch loss-of-function decreased the number of erythroid progenitors and increased peripheral blood platelet counts. A recent study by Klinakis and colleagues reported that the conditional silencing of Notch signaling in the bone marrow results in the expansion of granulocyte-monocyte progenitors (GMPs) and that eventually these animals develop a chronic myelomonocytic leukemia (CMML)-like disease<sup>138</sup>, suggesting that Notch signaling might be involved in early stem/progenitor cell fate decisions.

Experiments using myeloid progenitor cell lines indicated that Notch is an essential regulator of hematopoietic differentiation, thus opening the possibility that it might function in preserving the stem cell phenotype. Nevertheless, relevance for the self renewal and maintenance of adult HSCs has been questioned<sup>139</sup>. The analysis of transgenic mice carrying a dominant negative form of the co-activator Mastermind (which specifically blocks all canonical Notch signaling) or mice deficient for *Rbpj* indicated that Notch activity was dispensable for the maintenance of HSCs in the

adult bone marrow under physiologic conditions<sup>139</sup>. Further support for these results can be found in the conditional deletion of *Notch1* or *Notch1* plus *Notch2* under the control of the interferon-dependent expression of Mx-Cre that specifically and exclusively affected lymphoid differentiation, but not other hematopoietic lineages<sup>140,141</sup>.

Recent studies have nevertheless strongly suggested a function for Notch in hematopoietic regeneration<sup>142</sup>. Our team has focused its attention on the Delta4/Notch ligand which is mainly expressed in endothelial cells in the bone marrow<sup>143</sup>. They have previously demonstrated that membrane bound Delta4/Notch ligand (mbDll4) exerts two different activities on human HSCs. CD34<sup>+</sup>CD38<sup>-</sup> cells cultured on stromal cells expressing Delta4 exhibit decreased cell expansion due to higher proportion of cells in the G0/G1 phase of the cell cycle. These cells exhibit maintenance of their LTC-IC potential even when they undergo the same number of mitosis of control cells<sup>144</sup>.



#### 2.3.2.2.4. Hedgehog and TGF $\beta$ signaling

Studies that investigate the role of **hedgehog** (HH) and Transforming Growth Factor  $\beta$  (TGF $\beta$ ) signaling in the HSC niche seem to follow a similar paradigm compared with those focusing Notch signaling<sup>145,146</sup>. The initial indication that HH signaling can cause HSC expansion through activation of bone morphogenetic protein (BMP) signaling pathways<sup>145</sup> was followed by the finding that HH signaling is dispensable for adult hematopoiesis<sup>145,146</sup>. However, stromal BMP4 was shown to contribute to HSC maintenance<sup>147</sup>.

**TGF- $\beta$**  is been reported to be able to tune down cytokine signals in HSCs by inhibiting cytokine-induced lipid raft clustering. By doing so, TGF- $\beta$  keeps the PI3K-Akt pathway suppressed and induces cyclins D1, D2, and D3 distancing from the nucleus. TGF- $\beta$  also activates Smad2 and Smad3 and regulates transcription of *p57<sup>Kip2</sup>*, the cyclin-dependent kinase inhibitor gene highly expressed in dormant HSCs<sup>148</sup>. Regulation of these machineries is critical to maintenance of the dormant state in HSCs. TGF- $\beta$ s are secreted as part of the large latent complex (LLC) consisting of TGF- $\beta$ , latency-associated protein (LAP), and latent TGF- $\beta$  binding protein-1 (LTBP-1). Because LTBP-1, a member of the LTBP/fibrillin family, covalently binds to extracellular matrix (ECM) proteins, the LLC accumulates in the ECM<sup>149</sup>. Under most conditions TGF- $\beta$  in the LLC is inactive and is thus called latent TGF- $\beta$ . Therefore, TGF- $\beta$  function is largely controlled by activation of latent TGF- $\beta$ , a process that involves dissociation of bioactive TGF- $\beta$  from LAP. Glial fibrillary acidic protein (GFAP)-expressing cells ("glial cells") were unexpectedly identified as the cells that principally process latent TGF- $\beta$  into active TGF- $\beta$  in BM. These GFAP-positive cells were **nonmyelinating Schwann** cells, which envelop sympathetic nerves in BM. They produce niche factors and are in direct contact with a significant proportion of HSCs. Autonomic nerve denervation of BM reduced the number of these TGF- $\beta$  secreting glial cells and led to the disappearance of CD34<sup>-</sup> LSK HSCs.

Thus, TGF- $\beta$  contribute to the dormancy of normal HSCs<sup>44</sup>. Interestingly, although dormant HSCs are resistant to chemotherapeutic agents, recent evidence suggests that they can be activated by cytokines, such as interferon- $\alpha$  (IFN $\alpha$ ) and granulocyte colony-stimulating factor (G-CSF), and become sensitized to chemotherapy. So, if dormancy is the main reason for the observed resistance of leukemic cells to traditional chemotherapeutic agents, TGF- $\beta$  inhibition and subsequent activation of dormant HSCs could help to eliminate resistant leukemic cells.

#### **2.3.2.2.5. The extracellular matrix**

As a result of their ability to influence stem cell fate, components of the extracellular matrix (ECM) have gained increasing attention with regards to the HSC niche<sup>150</sup>. Although it is still impossible to test how contact area, shape and matrix stiffness impact HSC fate *in vivo*, it could be shown that HSCs seeded on microwells actively produce their own ECM and undergo quiescence or proliferation depending on the size of the well<sup>151</sup>. In addition, SPP1 was the first osteoblast-derived ECM protein that was shown to influence HSC number and function<sup>152,153</sup>. Lack of SPP1 leads to a stroma-dependent increase of LT-HSCs and increased JAG1 and ANGPT1 expression in stroma cells, which perhaps explains how, in *Spp1*-deficient mice, HSC expansion is not accompanied by their exhaustion<sup>152,154</sup>.

Glycans are non-protein components of the bone marrow stroma that have a role in the HSC niche. They are likely to mediate the formation of chemokine and growth factor gradients<sup>155</sup>. Moreover, eicosanoids (including PGE2, as mentioned above) affect the strength of signaling cascades, and neurotransmitters regulate the response to HSC mobilizing agents (see below).



#### 2.3.2.2.6. Adhesion molecules

For a number of years, the role of  $\beta 1$  **integrins** in the crosstalk between HSCs and their niche has been of interest. Integrin  $\alpha 4\beta 1$  (ITGA4; also known as VLA4) mediates HSC retention within the bone marrow microenvironment<sup>156</sup>, whereas integrins  $\alpha 1\beta 1$  and  $\alpha 5\beta 1$  (ITGA3 and ITGA5; also known as VLA1 and VLA5, respectively) mediate adhesion of HSCs to, among others, SPP1<sup>152</sup>. Furthermore, these three integrins mediate CXCL12 function<sup>157</sup>. Interestingly, there is a link between WNT signaling and integrin expression in HSCs: the expression of constitutively active  $\beta$ -catenin leads to the loss of HSCs and rapid exhaustion of their progeny, but also to higher expression of integrins  $\alpha 2$ ,  $\beta 1$  and  $\beta 7$  in HSPCs<sup>158</sup>. Integrins interact with ECM proteins, but integrin  $\alpha 4\beta 1$  is also the main binding partner of vascular cell adhesion molecule 1 (VCAM1), which is expressed on the surface of endothelial cells<sup>159</sup> as well as cells of the osteoblastic lineage<sup>98</sup>. Interestingly, VCAM1 expression correlates with HSC homing<sup>160</sup> and is upregulated in response to WNT signaling<sup>108</sup>.

A new role for the adhesion molecule **E-selectin** expressed exclusively by bone marrow endothelial cells in the vascular HSC niche was recently proposed. HSC quiescence was enhanced and self-renewal potential was increased in E-selectin knockout (*Sele*<sup>-/-</sup>) mice or after administration of an E-selectin antagonist, demonstrating that E-selectin promotes HSC proliferation and is a crucial component of the vascular niche. These effects are not mediated by canonical E-selectin ligands. Deletion or blockade of E-selectin enhances HSC survival threefold to sixfold after treatment of mice with chemotherapeutic agents or irradiation and accelerates blood neutrophil recovery. As bone marrow suppression is a severe side effect of high-dose chemotherapy, transient blockade of E-selectin is potentially a promising treatment for the protection of HSCs during chemotherapy or irradiation<sup>161</sup>.

**N-cadherins**, which make up another class of adhesion molecules, might also contribute to HSC fate but their role in HSC homeostasis it has been for long time controversial<sup>162</sup>. To resolve the N-cadherin controversy, Bromberg et al<sup>163</sup> and Greenbaum et al<sup>164</sup> undertook to conditionally delete the *N-cadherin* gene in the osteoblastic lineage to determine the effect of osteoblastic N-cadherin on hematopoiesis *in vivo*. While the former group deleted the *N-cadherin* gene in maturing osteoblasts expressing collagen I, the latter group deleted *N-cadherin* much earlier in the osteoblastic lineage, in primitive osteoprogenitors at early phases of osteoblastic commitment using the promoter of the *osterix* gene, which is necessary to commit mesenchymal progenitor cells to the osteoblastic lineage. Remarkably in both studies, deletion of the *N-cadherin* gene in osteoprogenitors or more mature osteoblasts had no effect on HSC number, cycling, or differentiation potential in steady-state; no effect on HSC retention within their niche in steady-state or after mobilization with G-CSF; no effect on hematopoietic recovery after cytotoxic stress; and no effect on HSC engraftment or self-renewal after transplantation. Furthermore, neither bone formation stimulation nor the associated expansion of the HSC pool that occurs in response to para-thyroid hormone treatment was altered by deletion of the *N-cadherin* gene in maturing osteoblasts. This is in contrast with the observation that in steady-state, deletion of the *N-cadherin* gene in either osteoprogenitors or maturing osteoblasts alters osteoblast function. Deletion of N-cadherin in osteoprogenitors reduced trabecular bone density whereas deletion of N-cadherin in osteoblasts increased trabecular bone density before decreasing it in older mice. Therefore, osteoblastic N-cadherin regulates osteoblast maturation and function but has no effect on the hematopoietic system *in vivo*. These findings are congruent with the previously reported lack of effect of *N-cadherin* deletion in the hematopoietic lineage.

While closing the N-cadherin debate, these two papers fuel another controversy in respect to the role of osteoblastic niches versus perivascular HSC niches. The long quest of identifying the niche components that critically regulate HSC quiescence and self-renewal in the bone marrow is far from over, but at least we now know that N-cadherin expressed by osteoblastic cells is not necessary *in vivo*.

#### 2.3.2.2.7. Chemical gradients

Calcium and oxygen are the main chemical elements that have been studied in relation to the HSC niche.  $\text{Ca}^{2+}$  is released by osteoclasts during bone resorption, which leads to the formation of a concentration gradient that spreads out from the endosteal surface<sup>162</sup>. HSCs express the G-protein coupled  $\text{Ca}^{2+}$ -sensing receptor (**CASR**) and depend on it for their peri-endosteal localization and function<sup>165</sup>.

In contrast with the well known role of  $\text{Ca}^{2+}$  in the HSC niche, the relevance of **oxygen** tension is a controversial topic. Adaptations of the protocol for the isolation of side populations<sup>166</sup>, which use Hoechst dye *in vivo* staining to label efficiently perfused cells, suggested that HSCs reside in hypoxic areas of the bone marrow<sup>106,167</sup>. However, both endosteal and non-endosteal LT-HSCs are localized near vasculature<sup>31,99</sup>. Using the hypoxia bioprobe pimonidazole, Lévesque and colleagues showed by confocal laser scanning microscopy that the endosteum at the bone-BM interface is hypoxic, with constitutive expression of hypoxia-inducible transcription factor-1 $\alpha$  (HIF-1 $\alpha$ ) protein in steady-state mice<sup>168</sup>. Knockdown of HIF-2 $\alpha$ , and to a lesser extent HIF-1 $\alpha$ , impedes the long-term repopulating ability of human CD34<sup>+</sup> umbilical cord blood cells, inducing endoplasmic reticulum stress followed by apoptosis<sup>169</sup>. Moreover, at the peak of hematopoietic stem and progenitor cell (HSPC) mobilization induced by either granulocyte colony-stimulating factor or cyclophosphamide, hypoxic areas expand through the central BM. Furthermore, HSPC mobilization leads to increased levels of HIF-1 $\alpha$  protein and increased

expression of vascular endothelial growth factor A (VEGF-A) mRNA throughout the BM, with an accumulation of VEGF-A protein in BM endothelial sinuses. VEGF-A is a cytokine known to induce stem cell mobilization, vasodilatation, and vascular permeability *in vivo*. Thus it was proposed that the expansion in myeloid progenitors that occurs during mobilization depletes the BM hematopoietic microenvironment of O<sub>2</sub>, leading to local hypoxia, stabilization of HIF-1 $\alpha$  transcription factor in BM cells, increased transcription of VEGF-A, and accumulation of VEGF-A protein on BM sinuses that increases vascular permeability.

The HSC niche is a paramount example of the complex molecular interactions that take place in living tissues, and the evolutionary robustness of vertebrate hematopoietic systems undoubtedly relies on the intricacy of its molecular regulation (shortly resumed in Figure 7). Increasingly detailed spatio-temporal analysis of the molecular composition of the HSC niche will allow us to fit current and new data into a functional HSC niche model.

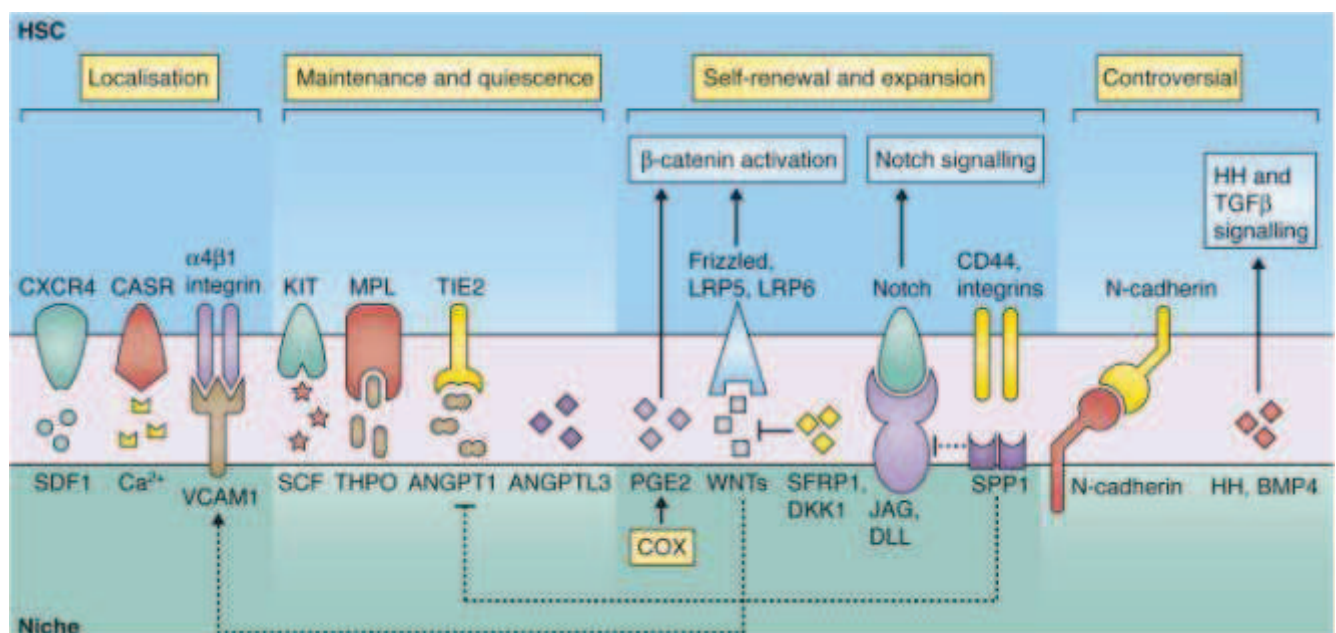


Figure 7. Crosstalk between HSCs and their niche (from Lo Celso and Scadden, 2011)

### **2.3.2.3. Induced HSC mobilization**

Granulocyte colony-stimulating factor [G-CSF, officially known as colony stimulating factor 3 (CSF3)] is the most widely used mobilizing agent in clinical practice. It has a highly multifaceted mode of action: G-CSF administration results in the transient reduction of CXCL12 expression, the activation of metalloproteases that cleave VCAM1 and CXCL12, as well as the release of norepinephrine from the sympathetic nervous system, which affects the morphology of osteoblasts and their ability to retain HSCs within the niche<sup>103,170</sup>. Endocannabinoids and reactive oxygen species (ROS) have recently been identified as non-protein mediators of the effects of G-CSF action<sup>171,172</sup>.

### **2.3.2.4. The “progeny niche”: feedback regulation from HSC progeny**

Research on the stem cell niches steers a fundamental question of whether the stem cell population is regulated by their own progeny. It is rational to believe in a feedback regulation where the number of progeny cells affects stem cells to differentiate or to remain in a quiescent state. Hematopoietic progenitors significantly produce Ang-1 and transforming growth factor (TGF)- $\beta$ , which affect HSC proliferation negatively. As described earlier, macrophages differentiate from HSCs and influence OB and Nestin<sup>+</sup> cell niche functions. Osteoclasts, which originate from hematopoietic progenitors, interact with HSC functions. A progeny of HSCs highlighted especially for feedback regulation on HSCs are megakaryocytes. During stress hematopoiesis after myeloablation or irradiation, platelet repopulation of the peripheral blood proceeds in advance to other hematopoietic components. Megakaryocytes produce high levels of TGF- $\beta$ , Ang-1 which regulate HSCs.

### 2.3.3. Post-transcriptional regulation in HSCs

Post-transcriptional regulations allow fine tuning of gene expression in every cellular context and act at different levels: mRNA maturation, mRNA stability and decay or mRNA translation. This kind of regulation is crucial in several developmental processes. Further studies on mRNA stability and translation in development and diseases will be essential to better understand the great number of processes that can't be evaluated with the classical genomic or transcriptomic-based approaches.

#### 2.3.3.1. microRNA

A remarkable discovery of whole-genome sequencing studies initiated at the turn of this century was that protein coding exons account for less than 2% of mammalian DNA<sup>173</sup>. Subsequent larger-scale transcriptome studies have established that at least two-thirds of the genome is nonetheless transcribed into RNA, exposing novel and exciting layers of genetic regulation regarding RNAs without protein-coding potential, referred to as noncoding RNAs (ncRNAs)<sup>174</sup>. It is now known that a class of ncRNAs termed microRNAs (miRNAs) exerts diverse roles in normal and pathological hematopoiesis. MicroRNAs are short non-coding RNAs of ~ 21 to 23 nucleotides in length that post-transcriptionally regulate mRNA expression. Each miRNA has the potential to target hundreds of different mRNAs and, conversely, each mRNA can be targeted by multiple miRNAs<sup>175</sup>. It is estimated that more than 60% of the mammalian transcriptome is under miRNA control<sup>176</sup>. Biogenesis of miRNAs follows a unique and highly conserved evolutionary pattern<sup>177,178</sup> (Figure 8).

Guo and colleagues demonstrated the importance of miRNA in HSCs using a conditional *Dicer* KO mouse model in which *Dicer* was selectively deleted in hematopoietic system. *Dicer* KO LSK cells showed impaired capacity to reconstitute the blood system of irradiated mice and, in an *in vitro* methylcellulose assay, the *Dicer* KO cells did not form any colonies. Together these results demonstrated that

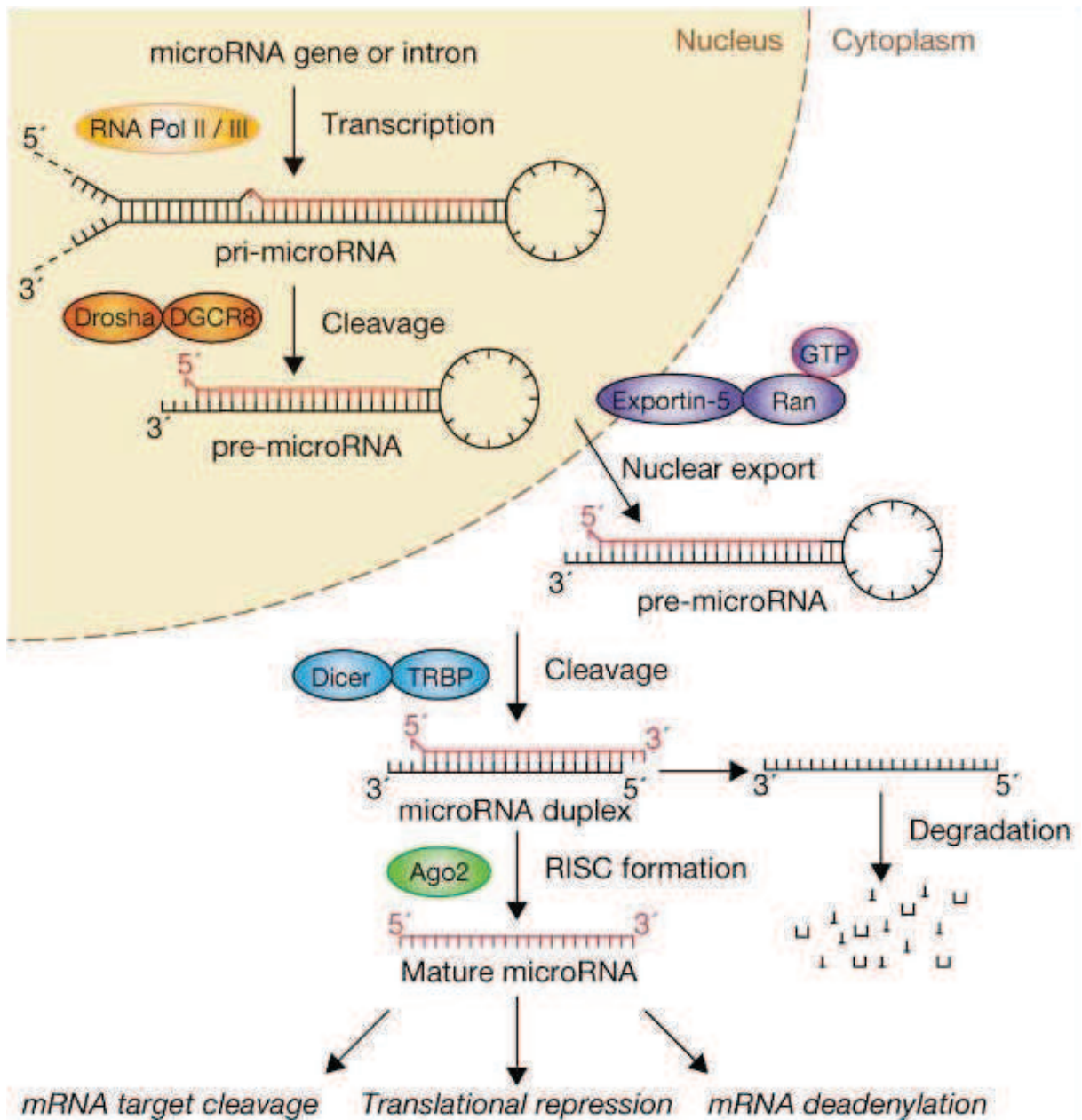
that Dicer, and by extension, its mature miRNA products, are essential for maintaining the most immature hematopoietic cell pool<sup>179</sup>. In another study measuring miRNA expression in mouse HSCs, O'Connell and colleagues examined the expression of 137 miRNAs in LSK cells and total bone marrow from C57BL/6 mice<sup>180</sup>. Of these 137 miRNAs, 11 were found to be enriched in LSK progenitors compared with total bone marrow including *miR-125a*, *miR-125b*, *miR-155*, *miR-99a*, *miR-126*, *miR-196b*, *miR-130a*, *miR-542*, *miR-181*, *miR-193*, and *miR-let7e*. More primitive HSC like LSK CD150<sup>+</sup> are further enriched for these 11 miRNAs. With the exception of miR-193b, enrichment of the same set of miRNAs was found in human CD34<sup>+</sup> human cord blood. *miR-125* family has been shown to target genes involved in apoptosis<sup>179,181</sup>, *miR-196b* regulates specific Hox family members that control differentiation<sup>182</sup>. How miRNA are integrated into molecular networks governing self-renewal and differentiation of HSCs is still under investigation.

#### **2.3.3.2. RNA binding proteins**

Another layer of complexity in the regulation of gene expression is represented by RNA binding proteins (RBPs). RBPs can selectively bind hundreds of target mRNAs affecting their stabilization, localization and translation. In this way RBPs are able to coordinate and integrate multiple control functions to achieve a higher level of harmonized outcomes. At these days the role of these RNA regulators is still not well understood.

In the next pages you will find the description of two RNA binding proteins, Pumilio1 and Pumilio2, that we have shown, for the first time, to have crucial roles in hematopoietic stem cells functions.





**Figure 8. microRNA biogenesis and function. (Winter et al. 2009)**

The miRNA processing pathway has long been viewed as linear and universal to all mammalian miRNAs. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha-DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5-Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded.





### 3. PUMILIO

*Pumilio* gene (*Pum*) was first discovered as a key component, with *Nanos* (*Nos*) and *Brain Tumor* (*Brat*) genes, of a common pathway that acts in the posterior patterning of *Drosophila* embryo. Abdominal segmentation of the *Drosophila* embryo is governed by a cascade of translational regulatory events. During oogenesis, *oskar* (*osk*) mRNA is translated selectively at the posterior pole of the oocyte. Osk protein is required to protect *Nos* mRNA from deadenylation allowing its translation specifically at the posterior pole of the oocyte and early embryo<sup>183</sup>. As is the case for *osk* mRNA, translation of *nos* mRNA is repressed in the bulk cytoplasm of the embryo but not in the specialized cytoplasm at the posterior pole. One consequence of this regulation is that *Nos* protein forms a gradient emanating from the posterior pole. In the last step of this translational regulatory cascade, *Nos* protein blocks the translation of maternal *hunchback* (*hb*) mRNA and the resulting *hb* protein gradient governs abdominal segmentation by transcriptional mechanisms<sup>184–186</sup>. Although the *Nos* gradient clearly generates the positional information critical for posterior patterning; the NREs appear to be recognized not by *Nos* but by another factor: the *Pumilio* protein. *Pumilio* binds directly the maternally supplied *hunchback* transcripts in a sequence-specific manner on the *Nanos* response elements (NREs) and serves as platform for the recruitment of brain tumor (BRAT) and posterior-localized *Nanos* proteins forming a quaternary complex that represses *hb* translation<sup>184</sup>.

#### 3.1. PUF FAMILY PROTEINS

*Drosophila Pumilio* (*Pum*) and *C. elegans* FBF (fem-3 binding factor) proteins are founder members of the evolutionary conserved family of RNA-binding proteins (Figure 9), known as the PUF family of proteins (from *Pum* and FBF)<sup>187</sup>. The genome of the budding yeast (*Saccharomyces cerevisiae*) contains six PUF genes. *C.*

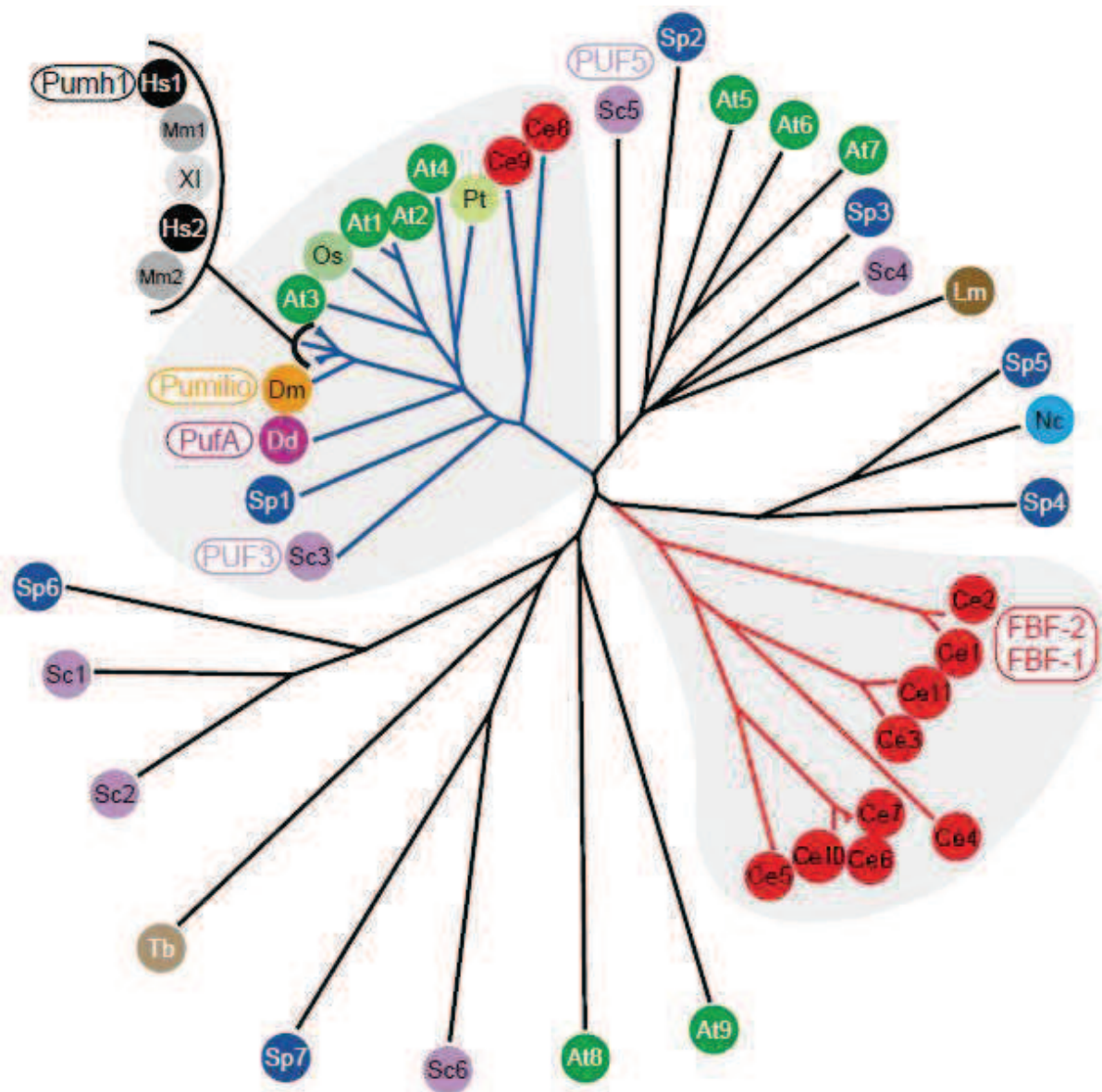
*elegans* contains eleven, whereas insects (*Drosophila* and *Anopheles* species) have only one PUF gene. Among vertebrates, zebrafish and *Xenopus* have two known PUF genes. With continuing advances in sequencing, it is likely that the number of Puf proteins is underestimated, even within an organism. For example, it has been thought that mice and humans each express only two Puf proteins. However, recent analysis of novel exons in humans, along with comparative genomics studies with mice and zebrafish, has revealed two novel classes of Puf protein, Puf-A and C14orf21. Thus, mice and humans may each actually express four Puf-related proteins: the canonical Pum1 and Pum2 proteins, as well as Puf-A and C14orf21<sup>188</sup>. A typical feature of PUF proteins is a C-terminal RNA-binding domain, composed of eight tandem repeats, known also as the *Pumilio* homology domain (PUM-HD). PUM1 and PUM2 in humans, and Pum1 and Pum2 in the mouse are closely related to *Drosophila* Pum ( ~ 80% identity in their RNA-binding domain)<sup>189</sup>. The zebrafish, human and murine Puf-A homologs are structurally distinct from the canonical 8-repeat PUM-HD on the basis of computer modeling of the human Puf-A repeat domain.<sup>188</sup> Specifically, Puf-A is modeled to contain six Puf repeats, three on each side of two repeat-like structures.

In plants is present a specific subfamily of PUF proteins. Interestingly, plants proteins are more closely related to PUM than to the FBF subfamily of PUF proteins.

Undoubtedly, further identification and cloning of PUF proteins from other invertebrate and vertebrate species will shed more light on the evolution of this extraordinarily conserved family of RNA-binding proteins.

The primary role of Puf protein is to negatively regulate target mRNA expression by stimulation of mRNA decay and/or inhibition of translation. In accordance with this role, Puf proteins are predominantly localized within the cytoplasm of cells<sup>190–196</sup>.

In neurons, mouse PUM2 and human PUM1 localize to cytoplasmic stress granules, where mRNA stalled at translational initiation are stored.



**Figure 9. PUF proteins throughout eukaryotes. (Wickens et al. 2002)**

An unrooted tree was derived by aligning only the Puf repeat regions using GCG. Partial expressed sequence tags (ESTs), which suggest many other PUF genes, were not included. PUF proteins specifically discussed in the text are highlighted. As much as possible, PUF proteins are named by their sequence relatedness within a species; thus PUF1 of yeast is more closely related to PUF2 of yeast than to the other yeast PUFs, but is not especially related to PUF1 of other organisms. Gray zones indicate the 'Pumilio cluster' (comprising *Drosophila* Pumilio, *C. elegans* PUF8 and 9, and several vertebrate PUFs) and the *C. elegans* cluster, containing nine of the 11 PUF proteins in that species. Colors indicate different species. Blue to purple – fungi (*Saccharomyces cerevisiae*, Sc; *Schizosaccharomyces pombe*, Sp; *Neurospora crassa*, Nc; *Dictyostelium discoideum*, Dd). Gray to black – vertebrates (*Homo sapiens*, Hs; *Mus musculus*, Mm; *Xenopus laevis*, XI). Green – plants (*Arabidopsis thaliana*, At; *Oryza sativa*, Os; *Populus tremula* × *P. tremuloides* (poplar), Pt). Brown – trypanosomes (*Leishmania major*, Lm; *Trypanosoma brucei*, Tb). Red – *Caenorhabditis elegans*, Ce. Orange – *Drosophila melanogaster*, Dm.

These Puf proteins are excluded from processing bodies, which are cytoplasmic sites of mRNA degradation<sup>196,197</sup>, with just one exception in which *C.elegans* FBF-2 was suggested to localize in P bodies-like structures (Figure 17)<sup>198</sup>.

### 3.1.1. Pumilio proteic domains

*Pumilio* proteins have three different domains: a glutamine/alanine rich domain<sup>199</sup>, a serine rich domain and the PUM-HD domain (Figure 10).



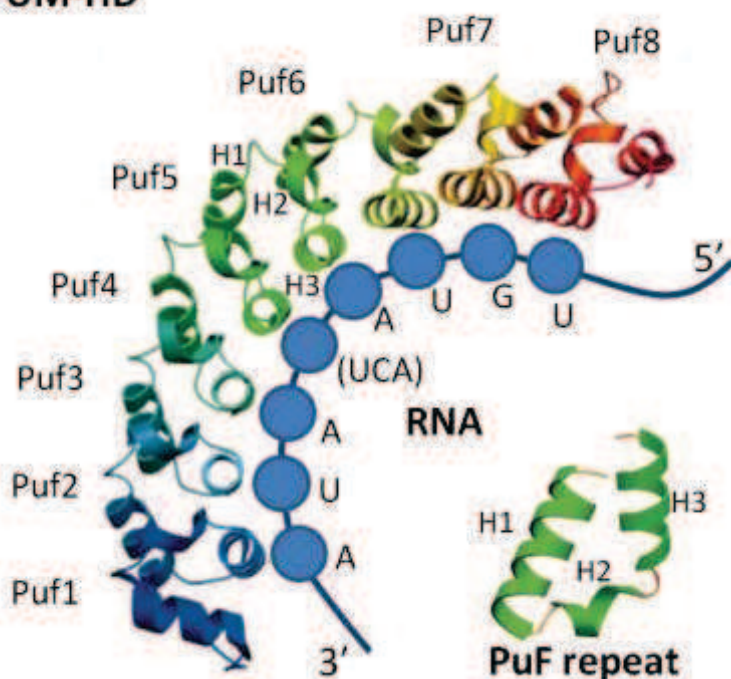
**Figure 10. Diagram of the human Pum1 protein conserved domains.**

Alanin rich, ALA RICH; Glutamine rich, GLN; Serine rich, SER RICH; Pum Homology Domain, PUM-HD; Pum repeat, PUM.

In the case of *Pumilio* protein the function of the glutamine/alanine rich and serine rich domain is still unclear, despite their conservation in human and murine *Pumilio* proteins. The Pum glutamine/alanine rich domain presents strong homologies with the prion protein domain. Similar domains are present in proteins involved in neurodegenerative disorders, where they cause macromolecular protein aggregation<sup>200</sup>. Some studies on Pum2 mouse protein at the level of hippocampal neurons have revealed the existence of Pum2 protein clusters. This finding can suggest a potential role of the glutamine/alanine rich domain in setting up these structures<sup>197</sup>. The function of the highly evolutionary conserved PUM-HD is well known. Alignment of the C-terminal part of *Drosophila Pum* with the mammalian and *Xenopus* homologs demonstrates the typical architecture of PUM-HD. The domain is composed of eight tandem imperfect repeats of 36 amino acids plus conserved N and C flanking regions. These flanking regions resemble half-repeats and therefore are also called repeat 1' and repeat 8'<sup>190,201,202,203</sup>. The most conserved amino acids reside in the middle of each repeat and interact with RNA-bases. The crystal structures of the *Drosophila Pum* and human *PUM1* RNA-binding domain have

revealed that the repeats are aligned in tandem to form an extended curved arc-like molecule<sup>204</sup>. The RNA binds to the concave surface of the molecule, where each of the eight repeats make contact with a different RNA base via three conserved amino acid residues positioned in the middle of the repeats (Figure 11).

### PUM-HD



**Figure 11. Pum Homology Domain structure and RNA binding. (Adapted from Edwards et al., 2001)**

The Pum-HD domain contains eight tandem Puf repeats (shown in different colors) that together comprise a single contiguous domain. Each repeat is composed of three  $\alpha$  helices (H1, H2 and H3). Here Pum homology domain is bound to the typical consensus sequence of the Pum binding site.

Amino acids at position 12 and 16 of the PUF repeat bind each RNA base via hydrogen bonding or van der Waals contacts with the Watson-Crick edge, whereas the amino acid at position 13 makes a stacking interaction. The recognition of RNA by naturally occurring PUF domains is base specific, such that cysteine and glutamine bind adenine, asparagines and glutamine bind uracil, and serine and glutamate bind guanine. This code has been confirmed by studies showing that the specificity of individual repeats can be switched by mutating only the amino acids that make contacts with the Watson-Crick edge of the base<sup>205</sup>. By mutating these residues it is possible to obtain artificial *Pumilio* proteins with desired different sequence specificity<sup>203</sup> and even create *Pumilio* proteins able to bind cytosine, a feature never identified before in natural occurring *Pumilio* proteins<sup>205</sup>. PUM-HD is

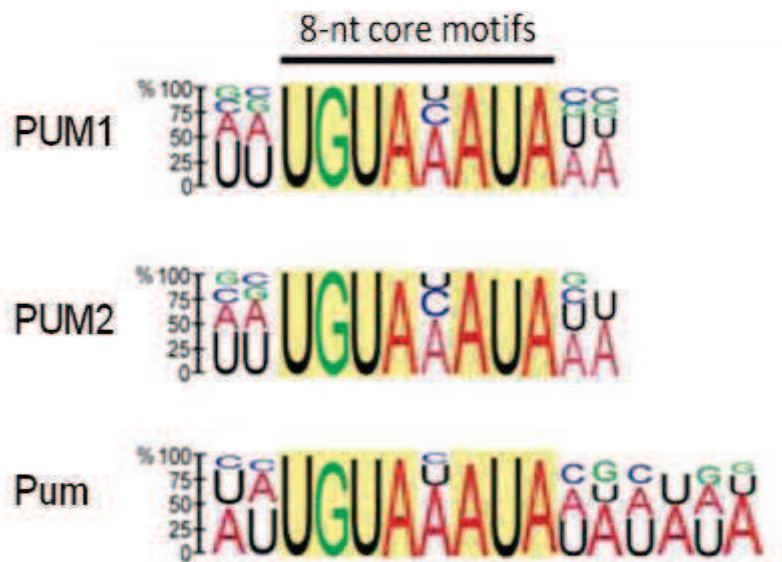


able to bind RNA targets even if every base is paired in a stem structure, albeit less efficiently. This indicates that PUF proteins can invade structured RNAs to bind their target sequences, presumably during the dynamic rearrangements intrinsic to RNA structures<sup>206</sup>.

PUF proteins recognize specific sequences, known as Nanos Response Elements (NREs) or Pumilio Binding Elements (PBE). Approximately the distribution of the NREs along the target mRNAs is for 85% exclusively in the 3'UTRs, for 3-5% solely in coding sequences (CDS) and in 17% of the cases both in CDS and 3'UTR. Just 1-2% of the NREs are found exclusively in 5'UTR of target mRNAs.

Most of the PUM bound messages have only one PUF motif. However, a substantial fraction (32%) bears at least two consensus motifs in the 3' UTR. The distance between multiple motifs ranges up to 4000 nucleotides with a median distance of 280 nucleotides. Nevertheless, a large proportion of the double motifs are located within 200 nucleotides, with a "peak" at a distance of ~20 nucleotides, indicating that the two motifs are preferentially located in close proximity. Such repetitive occurrence of PUF binding sites may affect RNA regulation: different sites could have different affinities for PUF binding leading to dose-dependent or allosteric regulation<sup>207</sup>.

The UGU triplets found in the NRE of PUF mRNA targets seem to be the core recognition sequence for PUM-HD binding. Mammals PUF proteins PUM1 and PUM2, whose RNA-binding domains are highly similar to *D. melanogaster* PUM (80% and 70% amino acid positions identical to PUM, respectively) possess the same eight nucleotides consensus recognition sequence as that of fly PUM, 5'-UGUANAUA-3' where N is A, U, or C<sup>207</sup> (Figure 12). Nevertheless, things are not as simple as they seem, and not all PUM-HD bind the same consensus motif. The identification of mRNA targets of PUF proteins has revealed more variability in mRNA sequence recognition than expected based on the prototypical 1 PUM repeat:1 RNA base binding mode observed in crystal structures of human PUM1 with *hunchback*



**Figure 12. Analysis of RNA consensus sequence associated with PUM proteins (Galgano et al. 2008).** PUF consensus motif in 3'-UTR sequences associated with human PUM1, human PUM2, *Drosophila* Pum. Height of the letters indicates the probability of appearing at the position in the motif. Nucleotides with less than 10% appearance were omitted.

RNA<sup>208</sup>. Yeast Puf4p and Puf5p use eight PUM repeats to bind to sequences containing, respectively, nine or ten bases starting from the 5' UGU<sup>194</sup>. Similarly, worm PUF proteins with eight repeat PUM-HDs recognize longer RNA sequences<sup>209–211</sup>. Crystal structure of yeast Puf4p and worm FBF-2 demonstrate that additional bases can be accommodated by direct stacking of bases or flipping bases away from the RNA-binding surface, influenced by changes in curvature of the RNA binding surfaces of these proteins<sup>212,213</sup>. Another level of complexity can be added by the fact that RNA regulatory proteins often act in complexes and their effects on one another's specificities and affinities for RNA are opaque. In *C.elegans*, for example, the PUF protein FBF-2 shows different specificity for nucleotides upstream of the UGU when bound to the CPB-1 protein partner<sup>214</sup>. Moreover, many mRNA that bind a regulatory protein *in vivo*, as judged by coimmunoprecipitation studies, lack a consensus binding site<sup>194,196,207,214</sup>.



## 3.2. PUF PROTEINS: POST-TRANSCRIPTIONAL REPRESSORS

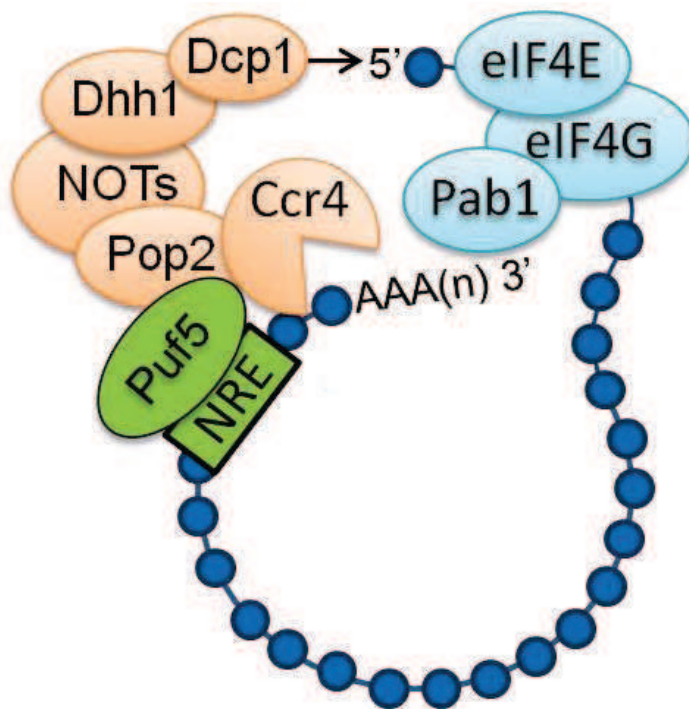
### 3.2.1. Mechanisms of action

Generally PUF proteins control gene expression by binding to the 3'-untranslated regions of specific mRNAs and triggering mRNA decay or translational repression in many different ways:

#### 3.2.1.1. Deadenylase recruitment

The first mechanism of mRNA repression by PUF proteins was evidenced by the Wickens laboratory<sup>215</sup>. Yeast Puf5 was shown to bind directly and specifically to the Pop2 subunit of the Ccr4-Pop2-NOT mRNA deadenylase complex, thereby recruiting the deadenylase to mRNAs exerting two major roles: poly(A) tail deadenylation at 3' UTR and decapping at 5' UTR (Figure 13). This major cytoplasmic exonuclease shortens mRNA poly(A) tails, influencing both mRNA stability and translation<sup>216,215</sup>. The generality of the Ccr4-Pop2-NOT recruitment mechanism is supported by the fact that yeast PUFs Puf4 and Puf3<sup>215,217,218</sup>, *Drosophila* Pumilio (Pum)<sup>219</sup>, the *C. elegans* FBF and human Pum1<sup>215,220</sup> also interact with the Ccr4-Pop2-NOT complex. In particular human PUM proteins interact with the CCR4-NOT complex subunits CNOT7 and CNOT8. CNOT7 and CNOT8 dominant negative mutants and RNA interference depletion of the deadenylase reduce PUM mediated repression and the poly(A) tail is necessary for maximal PUM mediated repression<sup>221</sup>. All these findings demonstrate a conserved mechanism of PUF mediated repression via direct recruitment of the CCR4-POP2-NOT deadenylase leading to translational inhibition and mRNA degradation. Remarkably PUF-dependent repression is observed even in the absence of deadenylation. In the closed-loop model, the 5' and 3' ends of an mRNA are in close proximity, thereby allowing a 3' UTR-binding regulator, such as Puf5, to effectively act on the 5' mRNA end as well. In fact, the decapping activator and translational repressor Dhh1 and the decapping enzyme Dcp1 interact with the

Ccr4-Pop2- NOT complex<sup>222</sup> and are also recruited to mRNAs by the Pop2–Puf5 interaction<sup>215</sup> (Figure 13).

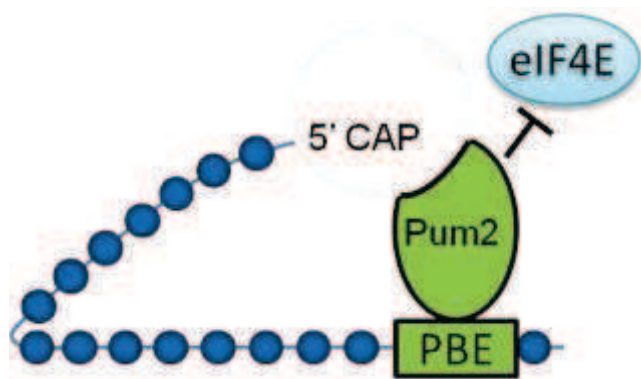


**Figure 13. Deadenylase recruitment by yeast Puf5.** PUF proteins, such as yeast Puf5, bind to the recognition sequences in the 3' UTR of their target mRNAs and recruit the Ccr4-Pop2-NOT mRNA deadenylase via their interaction with the Pop2 subunit. In addition to the deadenylase, the repressive complex recruited by Puf5 includes the decapping factors Dhh1 and Dcp1, which are associated with Ccr4-Pop2-NOT. Dhh1 and Dcp1 act on the cap to activate decapping and inhibit translation. By recruiting factors affecting both the mRNA poly(A) tail and the 5' cap, Puf5 can effectively cause deadenylation and translation repression.

Dhh1 and Dcp1 further cause mRNA repression by affecting the hydrolysis of the 5' cap (decapping), as well as functioning as translational repressors<sup>223</sup>. The recruitment of factors affecting the 5' cap could explain PUF-dependent repression observed in the absence of deadenylation.

### 3.2.1.2. Inhibition of translation initiation

The recruitment of the Ccr4-Pop2-NOT deadenylase is not the only known mechanism of mRNA post-transcriptional repression. Other mechanisms have been proposed and they include the inhibition of translation initiation. For example, *Drosophila* Pum can recruit to mRNA the translation inhibitor d4EHP, via its cofactor Brat<sup>224</sup>. d4EHP inhibits translation by competing with the translation initiation factor eIF4E for binding to the cap<sup>225</sup>. In *Xenopus*, Pum2 binds to the cap structure, competing directly with eIF4E<sup>226</sup> (Figure 14).



**Figure 14. Pum2 mediated translational control in *Xenopus*.** Pumilio 2 (Pum2) interacts with the 3' UTR-containing pumilio binding element (PBE) to repress translation in *Xenopus* oocytes. Pum2 binds directly to the 5' cap structure; in so doing, it precludes eIF4E from binding the cap and initiate translation.

### 3.2.1.3. Inhibition of translation elongation

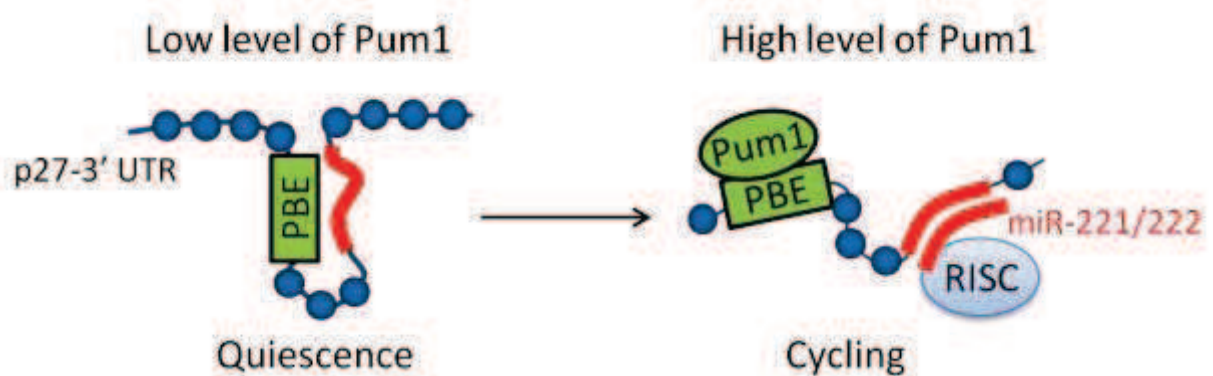
PUF and Ago proteins form an inhibitory complex with eEF1A, a GTPase required for translation elongation<sup>227</sup>. *C.elegans* FBF-1 binds CSR-1 (a *C.elegans* Ago family member) *in vitro* and *in vivo*, and *csr-1* depletion leads to increased expression of FBF target mRNAs. The FBF-1/CSR-1 heterodimer forms a complex with EFT-3 (*C. elegans* eEF1A), and this FBF-1/CSR-1/EFT-3 ternary complex has inhibited GTPase activity. Importantly, the PUF/Ago/eEF1A complex is conserved: human PUM2 associates with human AGO proteins *in vivo* and with eEF1A. Wild-type human PUM2 inhibits translation of both nonadenylated and polyadenylated mRNAs in rabbit reticulocyte lysate; however, PUM2 mutants that cannot form the PUM2/Ago/eEF1A complex or that cannot bind RNA are severely compromised for translation repression. Mechanistically, PUM2/Ago/eEF1A represses translation during elongation with ribosomes accumulating ~100–140 nts after the AUG within the open-reading frame (ORF). Thus PUF and AGO proteins form a complex with eEF1A to inhibit its GTPase activity and attenuate translation elongation

### 3.2.1.4. Cooperation with miRNAs

PUF proteins have been recently shown to be associated with miRNAs. A study on human cells has shown that PUF motifs are enriched around predicted miRNA binding sites and that high-confidence miRNA binding sites are significantly enriched

in the 3'-UTRs of experimentally determined PUM1 and PUM2 targets, strongly suggesting an interaction of human PUM proteins with the miRNA regulatory system<sup>207</sup>. In *C.elegans*, the Pumilio homolog PUF-9 is suggested to cooperate with let-7 family members to repress *hbl-1* in the hypodermis and the ventral nerve cord<sup>228</sup>. This repression requires a region of the *hbl-1* 3'-UTR that contains binding sites for PUF and let-7.

Recently, it has been proposed that PUM1 can help miRNAs to bind high structured target<sup>229</sup>, for example human PUM1 has been found to be essential for miR-221/miR-222-mediated repression of the *p27* tumor suppressor<sup>206</sup>. The binding of PUM1 induces a local conformational change in the *p27* 3'-UTR that exposes a miR-221/miR-222 binding site that is normally trapped in a hairpin structure, allowing repression of *p27* mRNA (Figure 15).



**Figure 15. Pumilio binding alters local p27-3' UTR structure and miR-221 and miR-222 accessibility.** The p27 tumour suppressor is highly expressed in quiescent cells, and its downregulation is required for cell cycle entry after growth factor stimulation. Intriguingly, p27 accumulates in quiescent cells despite high levels of its inhibitors miR-221 and miR-222. miR-221 and miR-222 are underactive towards p27-3' UTR in quiescent cells, as a result of target site hindrance. In response of growth factor stimulation PUM1 is upregulated and can bind more efficiently the p27-3' UTR. PUM1 binding induces a local change in RNA structure that favours association with miR-221 and miR-222, efficient suppression of p27 expression, and rapid entry to the cell cycle.

The same kind of cooperation is found in the regulation of the E2F3 oncogene.

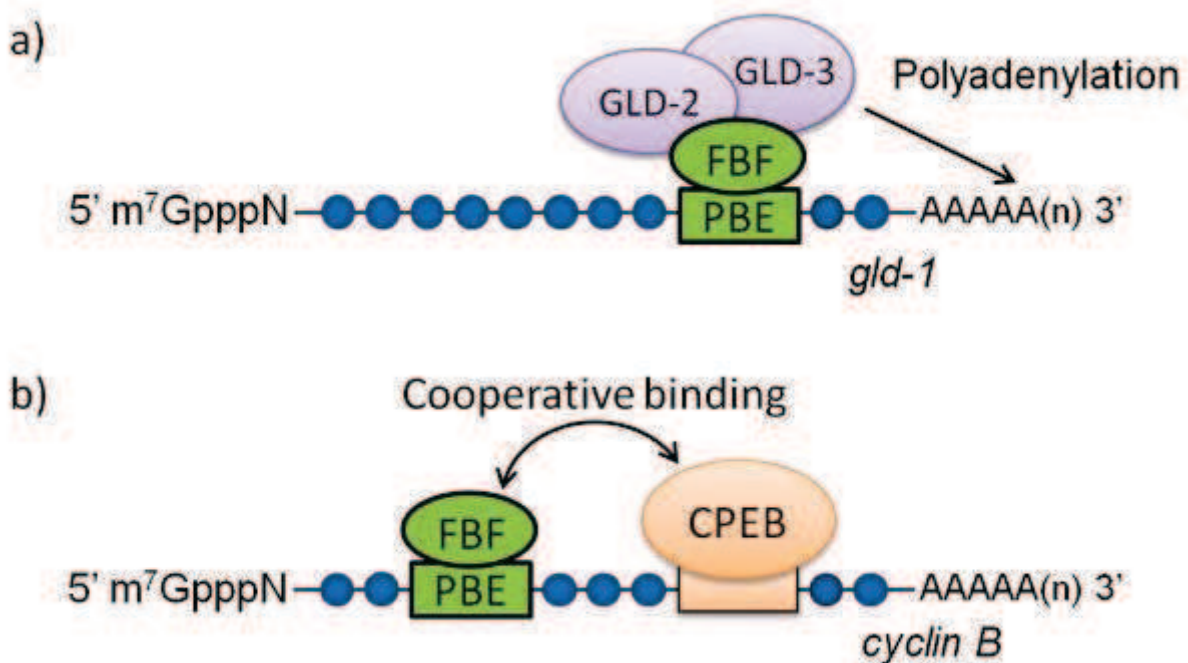
In normal cells, the E2F3 3' UTR is highly structured. Interaction of Pumilio with the PBE sequences facilitates the relaxing of the secondary structure, enabling miRNAs to gain access to their seed sequences and regulate E2F3 protein levels.

In tumors that have selectively silenced miRNAs that cooperate with Pumilio (miR-503 or miR-125b), translation control is weakened and more protein is produced.

### 3.2.2. Non-canonical PUF mechanism of action

#### 3.2.2.1. PUFs as activators of mRNA expression

An activator function for PUFs is a new concept, but one that is gaining evidence in several organism and with various PUF mRNA targets<sup>192,220,230,231</sup>. The mechanisms of PUF-dependent activation are poorly defined, but several possibilities have been proposed (Figure 16).



**Figure 16. Possible mechanisms of mRNA activation by PUF proteins.**

a) *C. elegans* FBF interacts with and stimulates the activity of the poly(A) polymerase GLD-2/GLD-3, possibly affecting mRNA polyadenylation and translational activation.  
b) In *Xenopus cyclin B* mRNA, Pum-binding sites cooperate with CPEB-binding sites to achieve translational activation. The proposed mechanism is the cooperative binding of Pum with CPEB, stabilizing CPEB on the transcript.

In *C.elegans*, FBF regulates the activation of *gld-1* in germline<sup>220</sup>. A possible mechanism is linked to cytoplasmic polyadenylation, extension of the mRNA poly(A) tail by cytoplasmic poly(A) polymerase, which represents a key mechanism for translational activation during development. A further example of translational activation by PUFs comes from *Xenopus*. In *Xenopus* oocytes, the Pum-binding

element (PBE) contributes to translational activation mediated by the cytoplasmic polyadenylation element-binding (CPEB) protein and its cognate cytoplasmic polyadenylation element (CPE)<sup>230</sup>. Whether all PUFs can be activators and repressors, or whether this is restricted to some PUFs only, remain an open question.

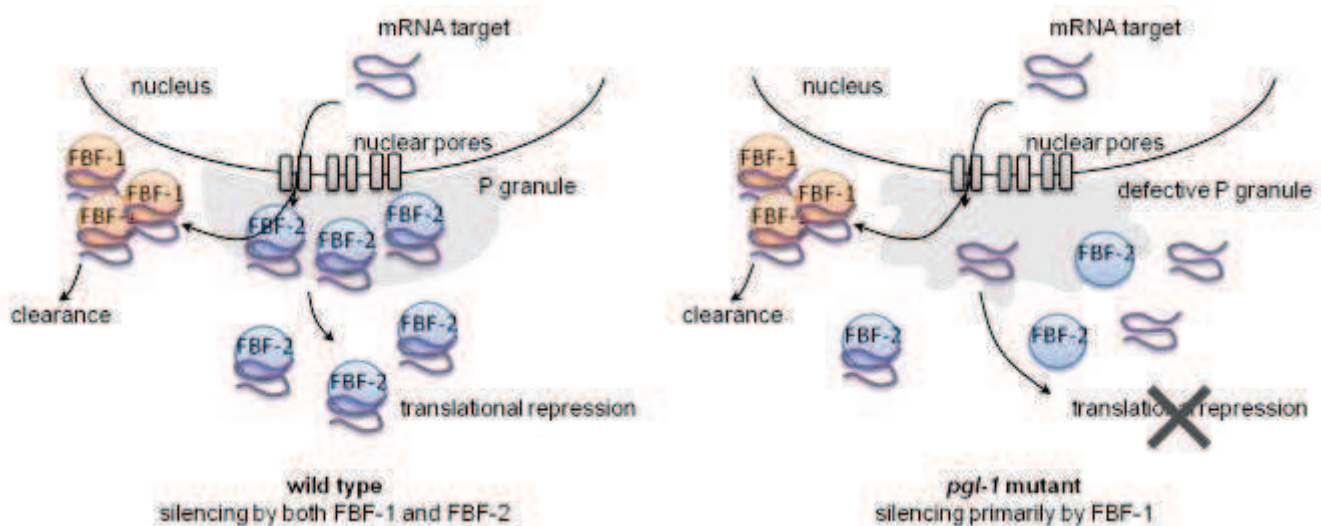
### 3.2.2.2. mRNA localization by PUF proteins

Another level of versatility is added to the function of the PUFs by recent evidence that they work as mRNA targeting factors, thereby contributing to spatial control of expression. Most examples come from yeast. Puf3 localizes mRNAs to the mitochondria<sup>232</sup>, Puf6 contributes to the asymmetric localization of *ASH1* during transport to the yeast bud<sup>233</sup> and Puf5 influences the localization of *PEX14* mRNA with peroxisomes<sup>234</sup>. Additionally, in olfactory neurons in *C.elegans*, FBF could activate the translation of *egl-4* mRNA near the cell body and sensory cilia<sup>231</sup>, and mammalian Pum2 might be involved in localize mRNA translation in neurons<sup>197,235</sup>. The mechanisms responsible for the localization activity of PUF proteins remain poorly understood.

PUF regulation is extremely complex. These proteins regulate thousand of targets by different mechanisms of action often combined together. Furthermore, association with different partners can add spatial specificity of action in different cells or in different compartments of the same cell. An example of this kind of complexity is given by a recent study by Voronina et al. in *C. elegans* germline stem cells<sup>198</sup>. Maintenance of undifferentiated stem cells depends on the PUF family RNA-binding proteins FBF-1 and FBF-2. FBF-1 and FBF-2 are 89% identical and are required redundantly to silence the expression of mRNAs that promote meiosis. Immunoprecipitation experiments suggest that FBF-1 and FBF-2 bind thousands of



mRNAs, including several meiotic mRNAs that are transcribed but silenced in the mitotic zone<sup>236,237</sup>. In the absence of both FBF-1 and FBF-2, all cells in the mitotic zone express meiotic proteins precociously, enter meiosis and differentiate into sperm<sup>193</sup>. *fbf-1 fbf-2* hermaphrodites do not make oocytes and are sterile. *fbf-1* and *fbf-2* single mutants are fertile but have smaller (*fbf-1*) or larger (*fbf-2*) mitotic zones, suggesting that, although redundant for fertility, FBF-1 and FBF-2 also have unique roles<sup>238</sup> and act with different mechanisms. Thus, despite their extensive sequence similarity, FBF-1 and FBF-2 have different effects on target mRNAs. FBF-1 promotes the degradation and/or transport and localization of meiotic mRNAs out of the stem cell region, whereas FBF-2 prevents translation only if properly localized by PGL-1 in P granules like structures situated in close proximity to the cell nucleus, where FBF-2 is able to intercept pro-meiotic targets exiting the nuclear membrane and block their translation (Figure 17).



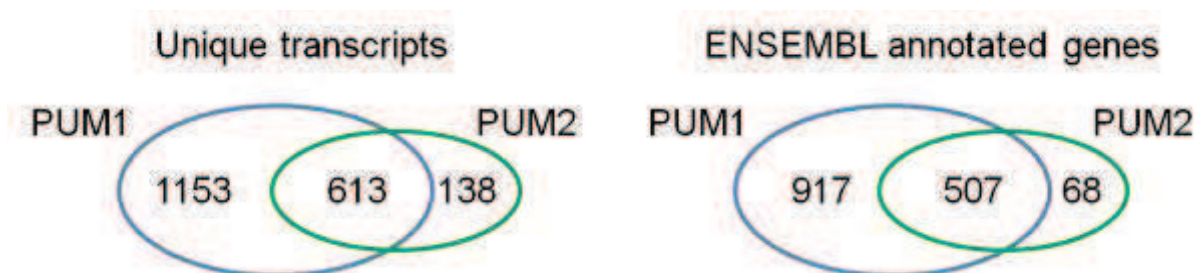
**Figure 17. Working model of cooperation between FBF-2 and PGL-1 (Voronina et al.2012).**

In the mitotic zone, upon exit from the nucleus, mRNAs encoding meiotic proteins encounter FBF-2 in P granules or FBF-1 in other perinuclear granules. mRNAs bound by FBF-2 are maintained in the cytoplasm in translationally repressed complexes. mRNAs bound by FBF-1 are cleared from the cytoplasm by an unknown mechanism. In *pgl-1* mutants, FBF-2 fails to localize to P granules and does not bind efficiently to its target mRNAs. FBF-1 is unaffected.

### 3.3. PUF COMBINATORIAL REGULATION: TARGETS AND PARTNERS

#### 3.3.1. Target mRNAs

The evidence that PUF proteins regulate multiple RNAs was first underline by studies in *Drosophila* where the only Pum protein binds at least five different mRNA (Table 1). In vertebrates PUF mediated regulation become more and more complex. Galgano et al. have systematically identified the mRNAs associated with the two human PUF proteins, PUM1 and PUM2, by the recovery of endogenously formed ribonucleoprotein complexes in HeLa cells. Then they have analyzed the associated RNAs with DNA microarrays. In this way it was shown that human PUM proteins associate with functionally related messages<sup>207</sup>. 1766 transcripts representing 1424 annotated genes were consistently associated with PUM1 and 751 with PUM2 (Figure 18).



**Figure 18. mRNAs associated with human PUM proteins in HeLa S3 cells. (Galgano et al.2008)**

Venn diagram representing overlap between PUM1 and PUM2 targeted transcripts (left) and the corresponding genes (ENSEMBL, right)

Strikingly, 507 (88%) of the PUM2 target genes were also among the experimentally defined PUM1 targets, indicating that the two human PUM paralogs have very similar substrate specificities and possibly act redundantly on common targets. Analysis of PUM1 targets revealed significant enrichment of components that regulate angiogenesis or that mediate inflammatory/immune responses (T and B cell activation). It was also found a strong enrichment of pathways important for cell-



proliferation and stress response such as the platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and the Ras (Rat Sarcoma) signaling pathways.

A similar study has been made by Chen et al. in mouse testis cells to elucidate the role of Pum1 in spermatogenesis. Pum1 knock out males exhibit a smaller average size compared to control mice and show significantly reduced sperm counts and fertility suggesting that Pum1 is essential to sustain spermatogenesis. 3687 transcripts representing 1527 Ensembl genes were consistently associated with Pum1, with a significant enrichment for mRNAs involved in pathways regulating p53, cell cycle, and MAPK signaling<sup>239</sup>. In particular, eight mRNAs encoding activators of p53 are repressed by Pum1 and deleting Pum1 results in strong activation of p53 and apoptosis which disrupt sperm production and fertility. Among these targets, Map3k1, Map2k3, and Daxx activate p38 MAPK, which in turn activates p53; Map2k7 together with Map3k1 activates JNK, which in turn activates p53; Sae1, Uba2, Pias1, and Pias2 are sumoylation ligases that prime p53 for inducing apoptosis. In human embryonic stem cells PUM2 is able to regulate MAPK1/ERK2 (mitogen-activated protein kinase 1), and MAPK14/p38- $\alpha$  (see the chapter “PUF proteins and stem cells maintenance in mammals”).

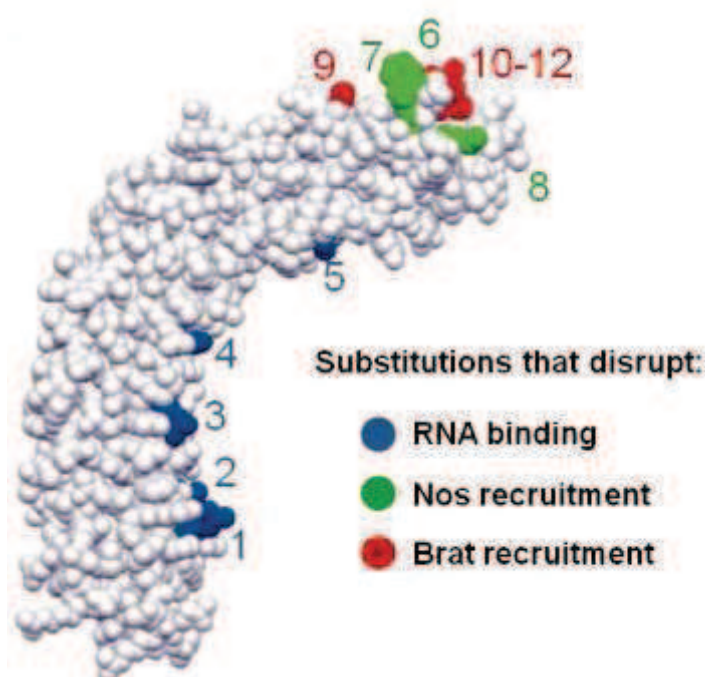
All these results suggest that PUF protein could be key players of an extremely complicated network of very meticulous and precise post-transcriptional regulation that can virtually affects every aspect of cell biology modulating thousand targets at the same time.

### **3.3.2. Protein partners**

The specificity of a PUF-mRNA interaction is often determined by other proteins that bind to the PUF polypeptide. To date, PUF proteins are known to interact physically with members of three other protein families. These interactions are thought to occur at the convex surface of the PUM-HD that appears to be a platform for protein-

protein interactions, while the concave surface of the PUM-HD provides the interface with RNA (Figure 19).

Strikingly, the families of protein interacting with PUF proteins consist of known 3'UTR regulators. The ability of PUF proteins to interact with additional 3'UTR-binding proteins allows the assembly of distinct PUF proteins complexes, which might differentiate between different mRNA sequences and lead to different biological outcomes.



**Figure 19. Pum-NRE-Nos-Brat interaction surface in *Drosophila* (Edwards et al.2001)**

Mapping onto three dimensional structure the mutations and insertions that disrupt RNA (blue), Nos (green), or Brat (red) binding. The highlighted substitutions are: 1, R1127A; 2, K1167A; 3, R1199A; 4, H1235A; 5, E1346K; 6, F1367S; 7, GPH insert at 1369; 8, QICA insert at 1372; 9, G1330D; 10, C1365R; 11, T1366D; and 12, N1368S.

- 1) **Nanos** (NOS) proteins. Nanos proteins are characterized by two distinctive CCHC zinc fingers, and bind RNA nonspecifically in the absence of a PUF protein<sup>240,241</sup>. FBF binds NOS-3, one of three *C. elegans* NOS proteins<sup>242</sup>, and Pumilio interacts with the single NOS of *Drosophila*<sup>243</sup>. The other NOS proteins of *C. elegans*, NOS-1 and NOS-2, interact with different PUF proteins, emphasizing the commonality of PUF–NOS partnerships, as well as their specificity. Similarly, a *Xenopus* PUF binds to a *Xenopus* NOS homolog, XCAT-2, in frog oocytes<sup>244</sup>. Three NOS proteins were identified in humans

(NOS1, NOS2, NOS3). In human germ cells NANOS1 and PUMILIO2 complex bind the microRNA biogenesis factor GEMIN3 to sustain spermatogenesis<sup>245</sup>. Furthermore, as say before, NANOS2 is overexpressed in self-renewing murine spermatogonial stem cells, preventing their differentiation.

2) **Cytoplasmic polyadenylation element-binding proteins** (CPEBs) are conserved among metazoans and play key roles in mRNA control<sup>246</sup>. They bind U-rich elements designated CPEs (cytoplasmic polyadenylation elements) using zinc knuckles and RRM (RNA recognition motif) domains. CPEB proteins regulate translation, localization, and poly(A) tail length, and can either activate or repress their targets<sup>246</sup>. CPEB proteins are critical in very diverse biological contexts, from synaptic plasticity to the cell cycle, cancer progression, and cellular senescence<sup>246–248</sup>. FBF-2 physically interacts with a *C.elegans* CPEB homolog CPB-1 controlling spermatogenesis<sup>214,249</sup>. CPB-1 enhances binding of FBF-2 to specific RNA sequences increasing translational repression *in vitro*. Two differences are apparent in comparison of the FBF-2/CPB-1 complex to FBF-2 alone: the ternary complex exhibits differences in preferences upstream of the UGU, and appears to be more permissive or diverse downstream. Upstream of the UGU, the most conspicuous difference is the decreased presence of a cytosine in the ternary complex as compared with FBF-2 alone. FBF-2 requires a cytosine preceding the UGU for high-affinity binding, which enhances binding ~20-fold by interacting with a specific pocket in the protein. A *Xenopus* PUF protein binds to CPEB in oocyte, though the function of the complex has yet to be determined.

3) **Brat.** *Drosophila* Brat is a member of the TRIM-NHL protein family. The family of TRIM-NHL is characterized by an N-terminal tripartite motif (TRIM; consisting of a RING domain, B-box and coiled-coil (CC) regions) and a C-

terminal NHL domain. TRIM is also found associated with other C-terminal domains and defines a superfamily of TRIM proteins, many of which are functional ubiquitin ligases. TRIM-NHL proteins are conserved among metazoan and are key regulators of development and differentiation. As cited before, *Drosophila* Brat is required for posterior repression of *hunchback*. Brat is recruited to *hunchback* mRNA through a ternary complex of Pumilio, Nanos and the mRNA<sup>250</sup>. Single amino acid substitutions in Brat compromise quaternary complex formation and disrupt regulation of *hunchback in vivo*. Furthermore Brat promotes differentiation in *Drosophila* ovarian germline stem cells and neuroblasts. Mei-P26, another protein of the NHL family found in *Drosophila*, restricts growth and proliferation in the ovarian stem cell lineage<sup>251</sup>. Two well-characterized genes in *C.elegans*, *ncl-1* and *lin-41*, also contain NHL domains: *ncl-1* regulates nucleolar size and rRNA abundance<sup>252</sup>; *lin-41* represses differentiation and promotes mitosis of specific somatic cells, a role that echoes PUF functions in stem cells. However, no genetic or physical interaction between *C.elegans* PUF and NHL proteins has been observed. Mammals express four TRIM-NHL proteins: TRIM2, TRIM3, TRIM32 and TRIM71. However, apart from *Drosophila* BRAT, which acts as a translational repressor, the molecular functions of TRIM-NHL proteins are not well-defined. In murine neural progenitors TRIM32 over-expression induces neuronal differentiation while inhibition of TRIM32 causes neural progenitor cells to retention of progenitor cell fate. TRIM32 is implicated in ubiquitination and degradation of the transcription factor c-Myc but also binds Argonaute-1 and thereby increases the activity of let-7 miRNA. This activation is sufficient for neuronal differentiation in mouse neural progenitors<sup>253</sup>. In human HEK293 and mouse ES (mES) TRIM71 is found to immunoprecipitate with PUM1 and PUM2 in a RNA-dependent manner. TRIM71 shares many targets with

miRNAs and full repression of these targets requires expression of both TRIM71 and miRNAs<sup>254</sup>. Association of mammalian TRIM71 with mRNAs results in translational repression and mRNA degradation. The NHL domain and the central part of the protein mediate RNA interaction and translational repression respectively.

Organism	Puf protein	Target transcript*	Protein Partners	Mode of regulation**	Biological Process	Number of putative targets (whole transcriptome analysis)
<i>S. cerevisiae</i>	Puf1	<i>HXK1</i>		R	Hexokinase; metabolism	40
		<i>TIF1</i>		R	eIF4A; translation initiation factor	
	Puf2	None				146
	Puf3	<i>COX17</i>	TOM-20	R,L	Cytochrome c oxydase activity; mitochondrial function	220
		<i>COX23</i>		R,L	Cytochrome c oxydase activity; mitochondrial function	
	Puf4	<i>BSC1</i>		L	Mitochondrial AAA ATPase	
		<i>HO</i>		R	Endonuclease; mating type switching	205
	Puf5	Nucleolar proteins <sup>b</sup>		R	Ribosome biogenesis factors; ribosomal subunits	
		<i>HO</i>		R	Endonuclease; mating type switching	224
		<i>TEC1</i>		R	Transcription factor; pseudohyphal growth	69
		<i>STE7</i>		R	MAP-kinase kinase; pseudohyphal growth	(25/69 found to bind to 3' UTRs)
		<i>CIN8</i>		R	Kinesi motor; mitotic spindle assembly; chromosome segregation	
		<i>LPD1</i>		R	Metabolism; lipoamide dehydrogenase	
		<i>LRG1</i>		R	GTPase activity; Pkc1-mediated signaling pathway; cell wall integrity	
		<i>DHH1</i>		R	RNA helicase; decapping	
		<i>RAX2</i>		R	Bud-site selection; cell cycle	
		<i>ASE1</i>		R	Spindle elongation	
		<i>UTR1</i>		R	ATP NADH kinase; iron homeostasis	
		<i>SWD3</i>		R	Chromatin structure; COMPASS complex	
		<i>PEX17</i>		L	Peroxisome biogenesis	
	Puf6	<i>ASH1</i>		R	Transcriptional repressor; mating type switching	
<i>Drosophila</i>	Pum	<i>hunchback</i>	Nanos, Brat	R	Transcription factor; posterior patterning	1090
		<i>Bicoid</i>		R	Transcription factor; anterior patterning	
		<i>cyclin B</i>	Nanos	R	Cyclin; germline function	
		<i>eIF4E</i>	Nanos	R	Translation initiation factor; neuronal function	
		<i>para</i>	Nanos, Brat	R	Voltage-gated sodium channel;	

<i>C. elegans</i>	FBF	<i>fbf-1</i>		R	neuronal function	1350
		<i>fbf-2</i>		R	PUF protein	
		<i>fog-1</i>		R	PUF protein	
		<i>fem-3</i>	NANOS-1, NANOS-3	R	RNA-binding protein; germline; spermatogenesis	
		<i>gld-1</i>		R,A	Stimulates the ubiquitin ligase activity of CBC/FEM1; germline; spermatogenesis	
		<i>gld-35</i>		R	RNA-binding protein; germline; entry into meiosis	
		<i>lip-1</i>		R	GLD-2/GLD-3 poly(A) polymerase; germline; entry into meiosis; spermatogenesis	
		<i>mpk-1</i> <i>egl-4</i>	NOS-1, GLD-3	R A	MAP kinase phosphatase; germline MAP kinase; germline Kinase; olfactory adaptation	
<i>Xenopus</i>	Pum1	<i>cyclin B1</i>	CPEB, Xcat-2	R,A	Oocyte maturation/differentiation	
	Pum2	<i>RINGO/Spy</i>	DAZL, ePAB	R	Oocyte maturation/differentiation	
<i>A.thaliana</i>	PUM1- PUM6	<i>WUSCHEL</i>			Somatic stem cells maintenance/self renewal	
		<i>CLAVATA-1</i>			Somatic stem cells maintenance/self renewal	
		<i>PINHEAD/ ZWILLE</i>			Somatic stem cells maintenance/self renewal	
		<i>FASCIATA-2</i>			Somatic stem cells maintenance/self renewal	
Mouse	PUM2	<i>eIF4E</i>		R	Translation initiation factor; neuronal function	
		<i>scn1a</i>		R	Dendrite morphogenesis; synapse function	
Human	Pum1	<i>Cyclin B1</i>		R	Cyclin; cell cycle	726
		<i>Cyclin E2</i>		?	Cyclin; cell cycle	1766
		<i>Cks2</i>		R	Cyclin-dependent kinase; cell cycle	
		<i>PCNA</i>		R	Cell cycle	
		<i>SLBP</i>		R	Histone mRNA binding protein; mRNA expression; cell cycle	
		<i>INTS2</i>		?	Integrator complex; transcription	
		<i>DCUN1D3</i>		?	Cullin neddylation; cell cycle	
		<i>Dll1</i>		?	Differentiation; Notch signaling pathway	
		<i>SDAD1</i>		?	Export of ribosomal subunits to the cytoplasm	
		<i>VEGF-A</i>		?	Vascular endothelial growth factor receptor A	
		<i>MET</i>		?	Hepatocyte growth factor receptor	
		<i>CDKN1B(p27- Kip1)</i>		R	Cyclin dependent kinase inhibitor; cell cycle	
	Pum2	<i>SCH1</i>		R	Voltage-gated sodium channel; neuronal function	751
		<i>eIF4E</i>		R	Translation initiation factor; neuronal function	61
		<i>DUSP6</i>		R	Phosphatase	
		<i>CEP3</i>		?	Cdc42 effector	
		<i>ERK2</i>		R	MAP kinase; human embryonic stem cells	
		<i>p38α</i>		R	MAP kinase; human embryonic stem cells	
		<i>INTS2</i>		?	Integrator complex; transcription	
		<i>DCUN1D3</i>		?	Cullin neddylation; cell cycle	
		<i>Dll1</i>		?	Differentiation; Notch signaling	

VEGF-A		?	pathway Vascular endothelial growth factor receptor A
SDAD1	DAZL/BOL	?	Export of ribosomal subunits to the cytoplasm; spermatogenesis

**Table 1. mRNA targets and protein partners of model Puf proteins (*adapted from Quenalt et al. 2011 and Miller et.al 2010*)**

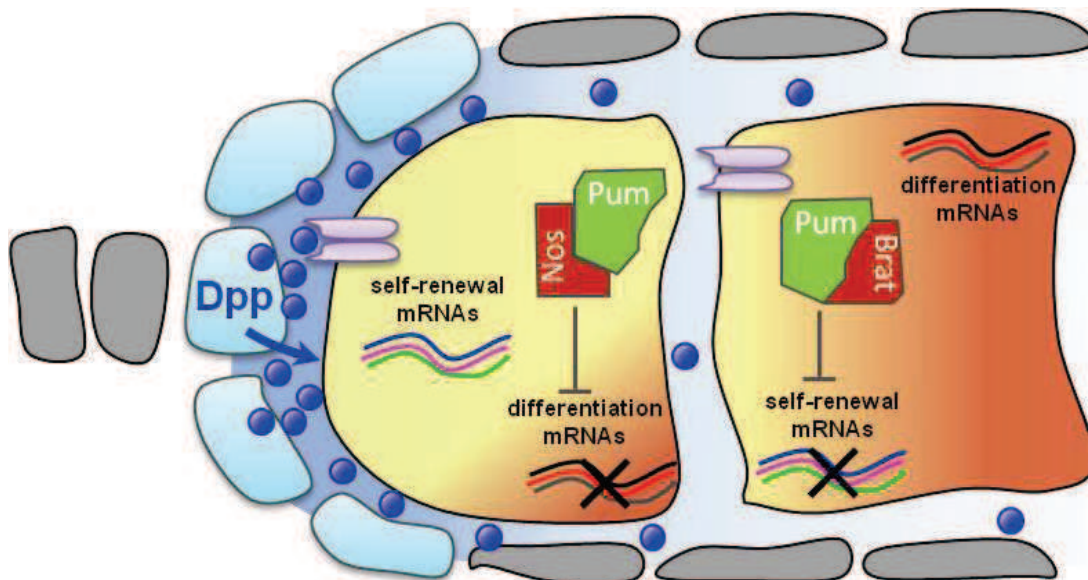
\* Target transcripts were determinate by direct assays and/or expression analysis following identification in whole transcript analysis

\*\* R – repression, L – localization, A - activation

The complexity of the interaction between Pum and its partners has been recently highlighted in an elegant work by Harris et al. in the *Drosophila* ovarian germline stem cells (GSCs). In this paper it has been shown that Pum, Nanos and Brat don't act together to regulate mRNAs fate, in the contrary, two different complexes, Pum-Nanos and Pum-Brat, act in different daughter cells to regulate and achieve two opposite functions. Thus, when anchored to the niche, the GSC receives high levels of Decapentaplegic (Dpp) signal due to its proximity to ligand-producing cells. High concentration of this factor allows Pum-Nos to repress mRNA encoding differentiation factors, including *Brat* mRNA. Following GSC division, a transient loss of Dpp signaling is sufficient to initiate a chain of events that ultimately locks one daughter cell into a differentiation fate. A reduction in Dpp signaling allows a molecular switch between the pro-self-renewing complex Pum-Nos to the pro-differentiating complex Pum-Brat<sup>255</sup> that will guide the daughter cells to differentiate in a cystoblast (Figure 20).

Besides the three canonical PUF partners Nanos, CPEB and Brat, other partners are found to cooperate directly with PUF proteins. PUM2, for example, is found to directly bind DAZ and BOULE, which are known to sustain the formation of human germline stem cells<sup>195</sup>. Moreover, PUF and Ago proteins form an inhibitory complex with eEF1A, a GTPase required for translation elongation<sup>227</sup>.





**Figure 20. Role of Pum, Nos and Brat in the Ovarian Stem Cell System** (*adapted from Harris et al. 2011*)

Schematic showing the function of Pum, Nanos and Brat in ovarian germline stem cells. See text for details.

### 3.4. PUF REGULATORS

The mechanisms responsible for PUF protein regulation are poorly understood. Nevertheless, a mechanism of negative feedback loop for self regulation of PUF expression has been highlighted in different organisms. In *C.elegans*, for example, FBF-1 mutants show increased level of FBF-2 and, vice versa, FBF-2 mutants show increased level of FBF-1. In both *fbf-1* and *fbf-2* 3'UTR are present three potential NREs and three-hybrid system assays confirm that FBF-1 and FBF-2 are able to bind their own 3'UTR<sup>238</sup>. The same kind of regulation was found in human cells. It was demonstrated by RNA-binding protein immunoprecipitation-chip (RNA-chip) that PUM1 is able to bind his own 3'UTR sequence and the 3'UTR of PUM2. This finding is strengthened by the fact that, in human fibroblast and in HEK293 cells, the level of PUM2 increases when PUM1 is suppressed by RNA-mediated interference<sup>206</sup>. The presence of two PBEs in the 3'UTR of PUM2 could explain this. Not only PUF proteins are able to control PUF proteins. In rat neuronal cells multiple miRNAs, including miR-134, are necessary for the correct elaboration of the dendritic



tree, a role common to the role of Pum2 in mice neurons<sup>197,235</sup>. RNA-binding protein Pum2 is a direct miR-134 target and a key mediator of the miR-134 growth-promoting effect on dendritogenesis<sup>256</sup>.

Another intriguing mechanism of PUF modulation is the PUM proteins phosphorylation. A study of phosphorylated proteins in HeLa cells reported an increased phosphorylation of PUM1 Ser 714 after stimulation with epidermal growth factor (EGF) and this over-phosphorylation seems to increase the RNA-binding activity of PUM1. On the contrary, mutation of Ser 714 to alanine (S714A) decreased the RNA-binding activity of PUM1<sup>206</sup>. The proteins responsible for PUM1 phosphorylation are still not known.

### 3.5. PUF AND STEM CELLS MAINTENANCE IN INVERTEBRATES

In addition to embryonic patterning *Pumilio* functions in various aspects of *Drosophila* germline development. Attenuated mutant alleles and conditional knockouts have revealed that *Drosophila Pum* supports mitotic proliferation and self-renewal of adult germline stem cells (GSCs) in fly ovary. Mutations in *Pum* lead to a failure of stem cell maintenance and promote their differentiation. In the *Drosophila* ovary 2-3 GSCs exist at the very apical tip of each ovariole<sup>257,258</sup>, where they divide asymmetrically to give rise to a cell that retains the stem cell properties and a progenitor (cytoblast) that eventually becomes a mature egg. Null mutations in the *Pum* locus are characterized by non self-renewing symmetric division of stem cells and production of two progenitors (cytoblasts) that differentiate into mature eggs<sup>257,258</sup>. Although it is not known how *Pum* supports self-renewal of GSCs, it is clear that stem cells require *Pum* intrinsically<sup>257</sup>, and that *hb* mRNA is not a downstream target of Pum in GSCs<sup>186</sup>. It was proposed that *Pum* might differentially repress the translation of an unknown protein(s) in mother and daughter cells. In fact, this might be the case because *Pum* protein is highly expressed in germline stem cells and down regulated

in early progenitors<sup>258</sup> where it binds different partners and modulates different targets<sup>255</sup> (as previously described in Figure 20).

One of the *C.elegans* PUF proteins also controls germline stem cells maintenance<sup>193</sup>. The FBF-1 and FBF-2 genes encode nearly identical proteins, which are collectively called FBF (fem-3 binding factor). The FBF-1/FBF-2 double mutant fails to maintain germline stem cells and to switch to oogenesis. Similar to *Pum*, FBF is preferentially expressed in germline stem cells and down-regulated in progenitors. What of organisms with no identifiable germ line? In this context, the role of PufA in the slime mold *Dictyostelium* is intriguing<sup>259</sup>. Normally, single *Dictyostelium* cells divide mitotically until, in response to nutrient deprivation, they aggregate and differentiate to form fruiting bodies containing stalk and spore cells. The PufA protein promotes continued vegetative divisions and inhibits differentiation. In PufA mutants, cells precociously leave mitosis and differentiate. Thus, PufA promotes proliferation in a manner analogous to *Pumilio* and FBF: proliferating single cells are viewed as stem cells that differentiate in response to an external cue. A second provocative connection between stem cell controls and PUF proteins comes from studies of aging in *S. cerevisiae*<sup>260</sup>. Strains lacking *PUF5* divide fewer times than wild type, whereas strains over expressing *PUF5* divide more times and live longer<sup>260</sup>. In this case, one can view the mother cell as a stem cell, whose mitosis is not properly sustained in the absence of the PUF protein. Thus, it was suggested that an ancestral function of *Pumilio* protein is to support proliferation and self-renewal of stem cells<sup>187,189</sup>.

### **3.6. MAMMALIAN PUF PROTEINS**

#### **3.6.1. *Pumilio* proteins and neuronal functions.**

One of the first confirmed role of *Pum* protein in mammals was the role of *Pum2* in localizing specific mRNAs at the synapse of murine neuronal cells for maintaining synapse morphology and dendritic arborization, excitability and function<sup>197</sup>.

Furthermore was recently shown by *in vivo* studies that Pum2-deficient mice have abnormal behavioral strategies in spatial and object memory test. Additionally, Pum2 deficiency is associated with increased locomotor activity and decreased body weight, and some genes, including neuronal ion channels, were differentially expressed in the hippocampus of Pum2-deficient mice. These findings demonstrate that Pum2 serves key functions in the adult mammalian central nervous system encompassing neuronal excitability and behavioral response to environmental challenges<sup>261</sup>.

### **3.6.2. Pumilio proteins and spermatogenesis**

Another important role of PUF proteins in mammalian cells is represented by their contribution to mechanisms related to spermatogenesis. Spermatogenesis in mammals is a complex process in which germline stem cells undergo 9-11 rounds of mitosis, followed by meiosis and a cellular morphogenic process called spermiogenesis that transforms round haploid spermatids into sperm. The multiple rounds of mitosis generate excess spermatogonia that must be eliminated to maintain the homeostasis of spermatogenesis and this is accomplished in part by p53-mediated apoptosis. The temporally and spatially specific activation of p53 must be precisely controlled so that not only excess spermatogonia are eliminated, but enough germ cells must also survive the elimination to generate a sufficiently large number of sperm. Pum1, through coordinated post-transcriptional regulation of multiple factors in the p53 pathway (see the chapter “target mRNAs” represses p53 activation and apoptosis after spermatogonial division<sup>239</sup>. Moreover, Pum1 null mice, show testicular hypoplasia and reduced sperm counts and fertility (Pum1<sup>-/-</sup> mice are viable with no apparent defects except they are 18% smaller than wild type). Removing p53 reduces apoptosis and rescues testicular hypotrophy in Pum1<sup>-/-</sup> mice.

### 3.6.3. Pumilio proteins and cancer

As we have seen before, Pumilio facilitate the ability of multiple miRNAs to regulate structured 3'UTR of target mRNAs. The importance of this regulation is underscored by changes that occur in human cancer cells. The oncogenic transcription factor E2F3 is often deregulated during tumorigenesis. Amplification or elevated expression from the E2F3 locus (6p22) has been identified in breast<sup>262</sup>, prostate<sup>263</sup>, and lung<sup>264</sup> cancers. Deregulated E2F3 also appears to be an important driver of proliferation in bladder carcinomas, since amplification of E2F3 is particularly common in this type of cancer<sup>263,265–267</sup>. Several miRNAs that have been shown to be selectively down-regulated in bladder carcinomas in clinical studies<sup>268</sup> target seed sequences within the E2F3 3'UTR and repress E2F3 in a manner that is strongly cooperative with Pumilio. Moreover, many cancer cell lines circumvent Pumilio-mediated regulation of E2F3 by shortening the 3' end of the E2F3 transcript, which eliminates the PRE required for Pumilio regulation. Taken together, these findings suggest that a functional cooperation between the Pumilio complex and miRNAs constrains E2F3 levels and that cancer cells consistently select changes that eliminate this regulation<sup>269</sup>.

### 3.6.4. Murine Pum1 and Pum2 genes: structure and localization

Pum1 is localized on the mouse chromosome 4. Comparison of Pum1 cDNA and mouse genome has shown that Pum1 gene spans at least 117,389 bp and is composed of 22 exons (ranging in size from 69 to 1,501 bp). Pum-HD is encoded by exons 15–22. All of the exon–intron boundaries conform to the consensus splice donor–acceptor sites (the “gt-ag” rule). Pum1 transcript (5029 bp) contains one in-frame stop codon upstream of the first ATG codon, and an open reading frame (ORF) of 3567 bp which encodes a protein of 1189 amino acids. (Mw: 127 kDa). The PUM-HD is 361 amino acids long. Pum2 has been identified on the mouse chromosome 12. Pum2 gene spans at least 75,838 bp and it is composed of 21

exons. Pum-HD is encoded by exons 14–21. All of the exon–intron boundaries conform to the consensus splice donor–acceptor sites (the “gt-ag” rule). Mouse Pum2 transcript contains one in-frame stop codon upstream of the first ATG codon and an open reading frame of 1066 amino acids (Mw: 114 kDa). Comparison of the gene structure of Pum1 and Pum2 shows highly conserved exon size and exon/intron boundaries, which reflect conservation of the gene and protein structure. Pum1 gene contains an additional exon at the most N-terminal part. Some of the exons that encode the N-terminal part of mouse Pum proteins (exons 1–14 in Pum1 and 1–13 in Pum2) have slightly different sizes due to small in frame deletions or insertions. It is noteworthy that the size of exons encoding PUM-HD is identical in Pum1 and Pum2, again reflecting the conservation of gene structure. Both Pum1 and Pum2 have long 3' UTRs, which are contained within a single large exon (exon 22 in Pum1 and exon 21 in Pum2), that also encodes the C-conserved part of PUM-HD. Human PUM1 gene is 98% identical with murine Pum1 and PUM2 is 96% identical with murine Pum2 gene<sup>270</sup>.

### **3.6.5. Murine Pum1 and Pum2 isoforms**

Alternative splicing of the Pum1 and Pum2 transcripts give rise to several different isoforms. In NCBI database five isoforms for Pum1 and three for Pum2 were validated (Figure 21). So far, different roles for different Pumilio isoforms have not been described, further studies will be necessary to shed more light in this field.

### **3.6.6. Expression of Pum1 and Pum2 in murine HSPCs**

Pum1 and Pum2 have very similar patterns of transcription in functionally defined populations of mouse fetal and adult hematopoietic stem cells and progenitors. Both genes are expressed equally in populations of fetal liver HSC (Sca-1<sup>+</sup> c-kit<sup>+</sup> AA4.1<sup>+</sup>Lin<sup>-</sup> cells) and progenitors (AA4.1<sup>-</sup> cells). This finding is in an agreement with the fact that Pum1 and Pum2 cDNAs did not match any ESTs in the Stem Cell

database, which contains transcripts preferentially expressed in fetal liver HSC (Sca-1<sup>+</sup> c-kit<sup>+</sup> AA4.1<sup>+</sup> Lin<sup>-</sup> cells)<sup>271,272</sup>. During adult hematopoiesis, Pum1 and Pum2 are highly transcribed in the rare population of purified Rho-123<sup>low</sup>Sca-1<sup>+</sup> c-kit<sup>+</sup>Lin<sup>-</sup> bone marrow cells. This population represents about 0.001% of normal BM and is highly enriched for HSC activity<sup>273,274</sup>. Interestingly, in a more heterogeneous population of

Pumilio1 isoform 1



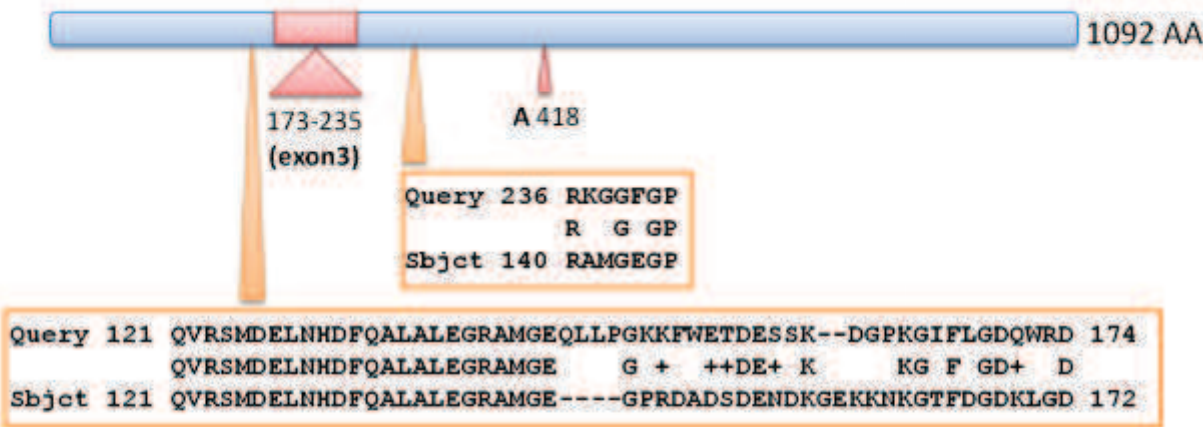
Pumilio1 isoform 2



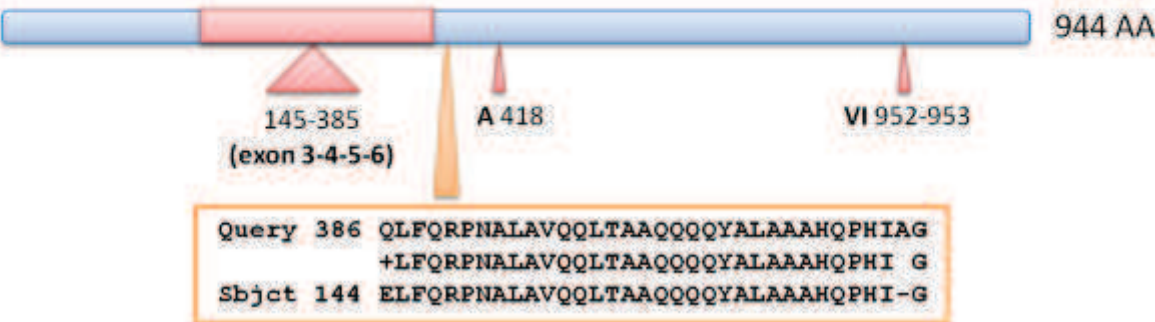
Pumilio1 isoform 3



Pumilio1 isoform 4



Pumilio1 isoform 5





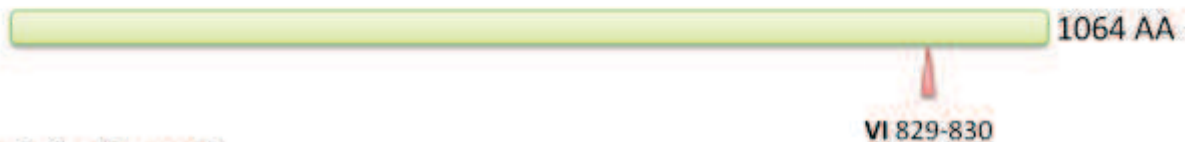
## Pumilio2 isoform 1



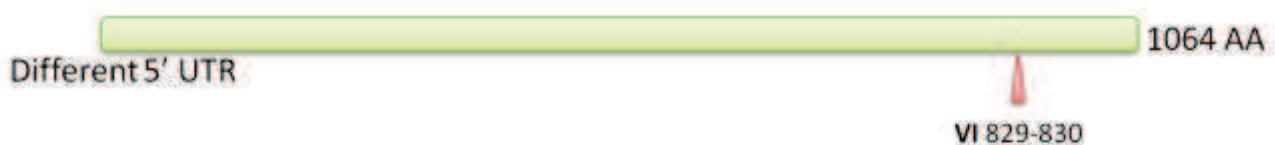
## Pumilio2 isoform 1



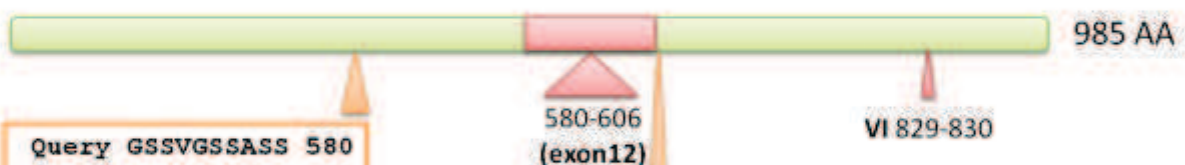
## Pumilio2 isoform 2



## Pumilio2 isoform 2



## Pumilio2 isoform 3



Query GSSVGSSASS 580  
GSS G + S  
Sbjct GSSGGLTNGS 580

Query 606 GQPFYNSLGFSSSPSPIGMPLPSQTPGHSLTPPPSLSSHGSSSSLHLGGLTNGSGRYISA 665  
GQP SLGF S S +G + S +LS GSS GGLTNGSGRYISA  
Sbjct 544 GQPGSASLGFSGSGSS-LGAAIGS-----ALSGFGSS----GGLTNGSGRYISA 586

**Figure 21. Pumilio1 and Pumilio2 isoforms**

Pumilio1 (blue) and Pumilio2 (green) isoforms validated in NCBI database. Red triangles represent deletions compared to the longest isoform (isoform1), orange rectangles represent region with different amino acid sequence if compared to the longest isoform (isoform1). Query represents the isoform1 and subject (sbjct) represents the selected isoform.

HSC (Lin<sup>-</sup> Sca-1<sup>+</sup> cells, representing 0.1-0.2% of normal bone marrow cells) and progenitors (Lin<sup>-</sup>Sca-1<sup>-</sup> cells)<sup>274-277</sup> Pum1 is not transcribed, whereas Pum2 expression is significantly down-regulated. Pum1 and Pum2 expression becomes up-regulated again in committed progenitors and mature blood cell types (Lin<sup>+</sup> bone marrow cells). Furthermore, both mouse Pumilio genes are ubiquitously expressed in

different stages of lymphoid and myeloid cell development. Both genes are transcribed in lymphoid (B cell progenitors, pro-B, pre-B, and B and T cells) and myeloid lineages (myeloid progenitors, monocytes, macrophages), as well as in the thymus and spleen. Besides, Pum1 and Pum2 transcripts are expressed at a higher level in the thymus than in the spleen, and Pum2 transcript is barely detectable in the spleen.

### **3.6.7. Pum proteins and stem cells maintenance in mammals**

Several studies have evoked an important role of mammalian PUF in stem cells maintenance and self-renewal. In human ES cells, for example, Pum2 has been found to negatively regulate the expression of two kinases of the MAPK/ERK pathway, MAPK1, and MAPK14, both known as repressors of human ES cell self-renewal<sup>278</sup>. Moreover, Pum2 interact with STAUFEN2, another RNA binding protein, to maintain mammalian neural stem cells<sup>279</sup>. Pum2 was also described as involved in the cell proliferation of cultured human adipocyte-derived stem cells, since transient knockdown of Pum2 expression by RNAi reduced their *in vitro* proliferation<sup>280</sup>. In human germline stem cells, Pum2 interacts with Deleted in Azoospermia (DAZ), DAZ-Like proteins, and the meiotic regulator BOULE, which are RNA binding proteins required for germline stem cell formation<sup>195,281,282</sup>. It has been emphasized that TRIM32, the mammalian ortholog of *Drosophila* BRAT, identified as partner of PUM for *hunchback* regulation, is a key regulator of mouse and human skeletal muscle stem cells<sup>283,284</sup>, as well as neuronal progenitor cells<sup>253</sup>. Besides TRIM32, NANOS2 (the ortholog of another partner of PUM in *Drosophila*), which is expressed in self-renewing murine spermatogonial stem cells, prevents differentiation of these cells, and appears as a key stem cell regulator<sup>285</sup>.

This raises an interesting possibility of *Pumilio* belonging to a group of candidate genes that intrinsically regulate self-renewal of various types of stem cells and possibly even murine or human hematopoietic stem cells (HSCs).





## 4. OBJECTIVES

Our team is engaged in deciphering the factors responsible for HSCs self-renewal and expansion. Because of their scarcity, expansion of HSCs represents a major challenge for improving engraftment efficiency for cell or gene therapy applications.

A part of our team has focused its attention on the self-renewing factor HOXB4, a homeoprotein identified as a major expansion factor of mouse and human HSCs. Long-term culture of human CD34<sup>+</sup> immature cells in the presence of the homeoprotein induces expansion of HSCs and hematopoietic progenitors of the myeloid and lymphoid lineages. We have demonstrated that expanded cells have an enhanced capacity to repopulate *in vivo* and to maintain their pluripotentiality.

The human genes regulated by homeoproteins during hematopoiesis are mostly unknown. So, we chose to search for potential effectors of HOXB4 using transcriptome analysis of CD34<sup>+</sup> human cells following reinforced HOXB4 signalling. Transcriptome analysis from CD34<sup>+</sup> cells exposed to HOXB4 revealed that various sets of genes encoding key hematopoietic factors and signaling pathway molecules (KLF10, HNRPDL, IKZF, and hypoxia, myc, IGF-1, 14-3-3 and angiopoietin-1 signaling) were either activated or repressed after cell exposure to this homeoprotein. Moreover, certain molecules identified (MEF2C, EZH2, DBF4, DHX9, YPEL5, Pumilio1 and Pumilio2) are involved with stem cell fate or expansion<sup>286,287</sup>.

On the other hand, another part of the team is focused on the implication of Notch signalling in stem cell maintenance. In particular, we showed that activation of the Delta4/Notch pathway in murine LSK cells cocultured on stroma expressing membrane-bound Delta4 maintained a significant proportion of the cells specifically in the G0 phase. Furthermore, LSK cells exposed to Delta4/Notch retained their LTC-IC potential for 7 days, and still displayed a long-term repopulating ability when injected into lethally irradiated recipient mice, contrary to control LSK cells. To further

decipher Notch/Delta4 mechanisms, we looked for the modulation of downstream target genes expression. We observed a decreased expression of cell cycle genes such as Cyclin D1, D2, and D3, and an upregulation of stemness gene expression such as Bmi-1, Gata-2, HoxB4 and c-Myc. In addition, the transcriptional screening has highlighted the overexpression of Pumilio-1 and -2, as part of the stem signature associated with the activation of the Delta4/Notch signalling pathway. This work is summarized in a paper actually under review in Leukemia journal (and presented in Annex I). This paper, in relation to my thesis subject, can be considered as part of preliminary results that prompted us to investigate the role of Pum1 and Pum2 in hematopoietic stem cells. My contribution to this work consisted mainly in validating the gene array results.

So, surprisingly, these two independent studies on human and murine HSC maintenance converged on increased expression levels of Pumilio1 and Pumilio2 in response to HSC self-renewing stimuli. These important results prompted us to search for direct effects of Pumilio proteins in murine and human HSCs, beginning to decipher their function with RNA interference based approach.

# RESULTS



## 5. RESULTS

In this chapter I will report the main results coming from the experiences done during my PhD (also presented as a paper presented in Annex II). The following data come from experiments mainly done on murine HSCs following purification, transduction and culture protocols and briefly summarized below (Figure 22).

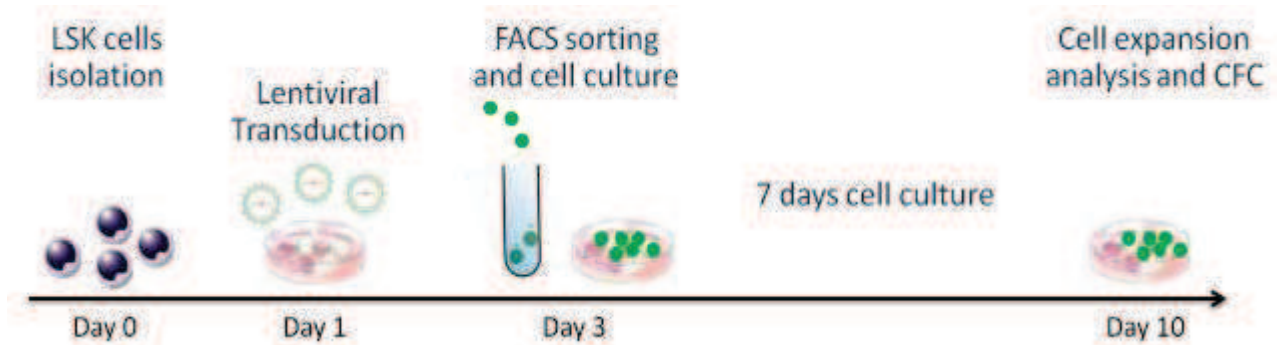
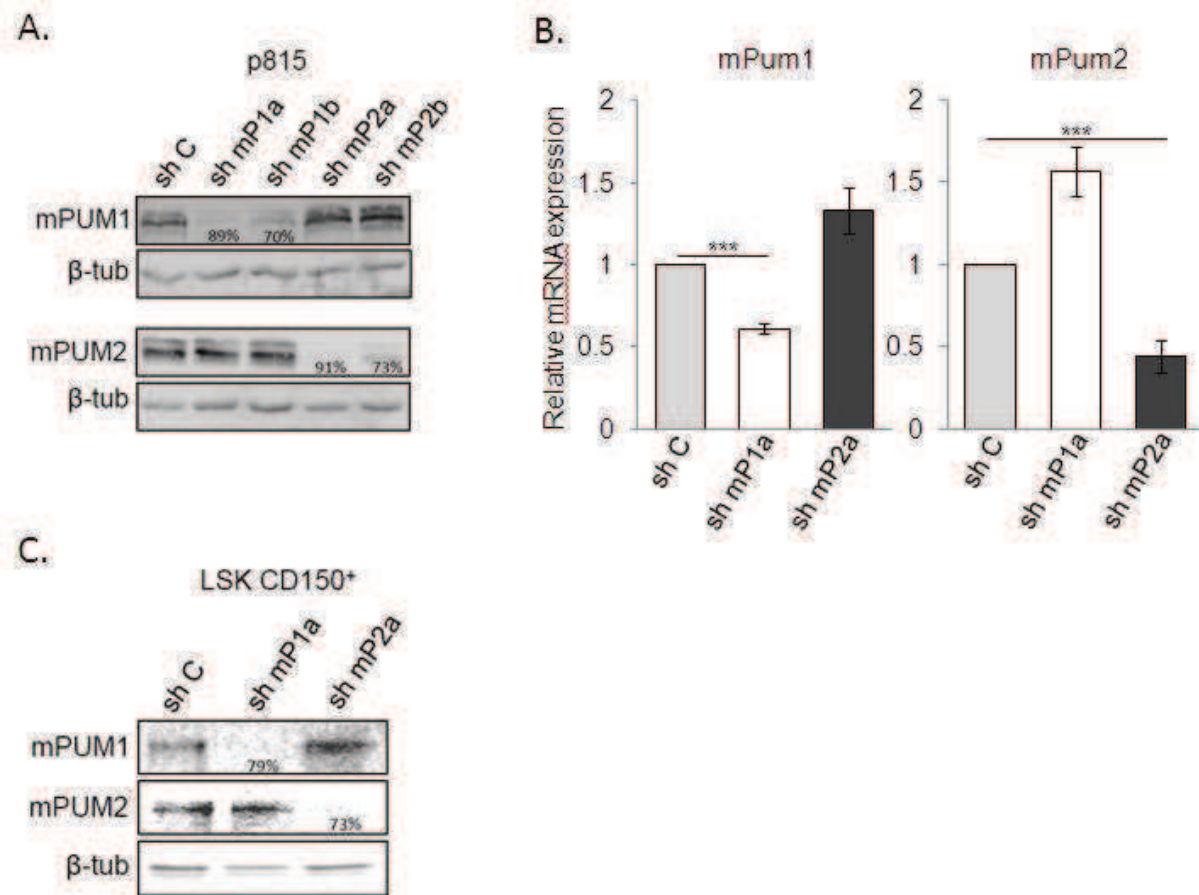


Figure 22. General experimental protocol for *in vitro* experiences

### 5.1. PUM1 and PUM2 knockdown impair murine *in vitro* HSPC potential.

We first assessed the role of PUM1 and PUM2 in murine HSPCs by performing knockdown (KD) experiments using lentiviral transduction of specific shRNAs encoding vectors. Two shRNAs specific for murine Pum1 (shmP1a and shmP1b), two shRNAs specific for murine Pum2 (shmP2a and shmP2b), and a control shRNA (shC against luciferase) were first validated into murine p815 cell line. Western blot analyses showed the effectiveness and the specificity of PUM expression KD (89% for shmP1a and 70% for shmP1b, 91% for shmP2a and 73% for shmP2b, as compared to the expression in shC cells, Figure 23A). Thereafter, these constructs were introduced into LSK cells, and Q-RT-PCR performed in shRNA-GFP<sup>+</sup> LSK cells two days after transduction revealed a 40±2% and 55±3% inhibition of Pum1 and Pum2 expression, with shmP1a or shmP2a, respectively (Figure 23B). Western blot analyses of the progeny of shRNA-transduced LSK cells confirmed that PUM1 and

PUM2 KD were almost the same as the ones observed in p815 cells (data not shown), and shRNA-transduced primitive LSK CD150<sup>+</sup> cells showed similar degree of PUM1 and PUM2 KD at the protein level (79% for shmP1a and 73% for shmP2a, as compared to the expression in shC cells, Figure 23C).

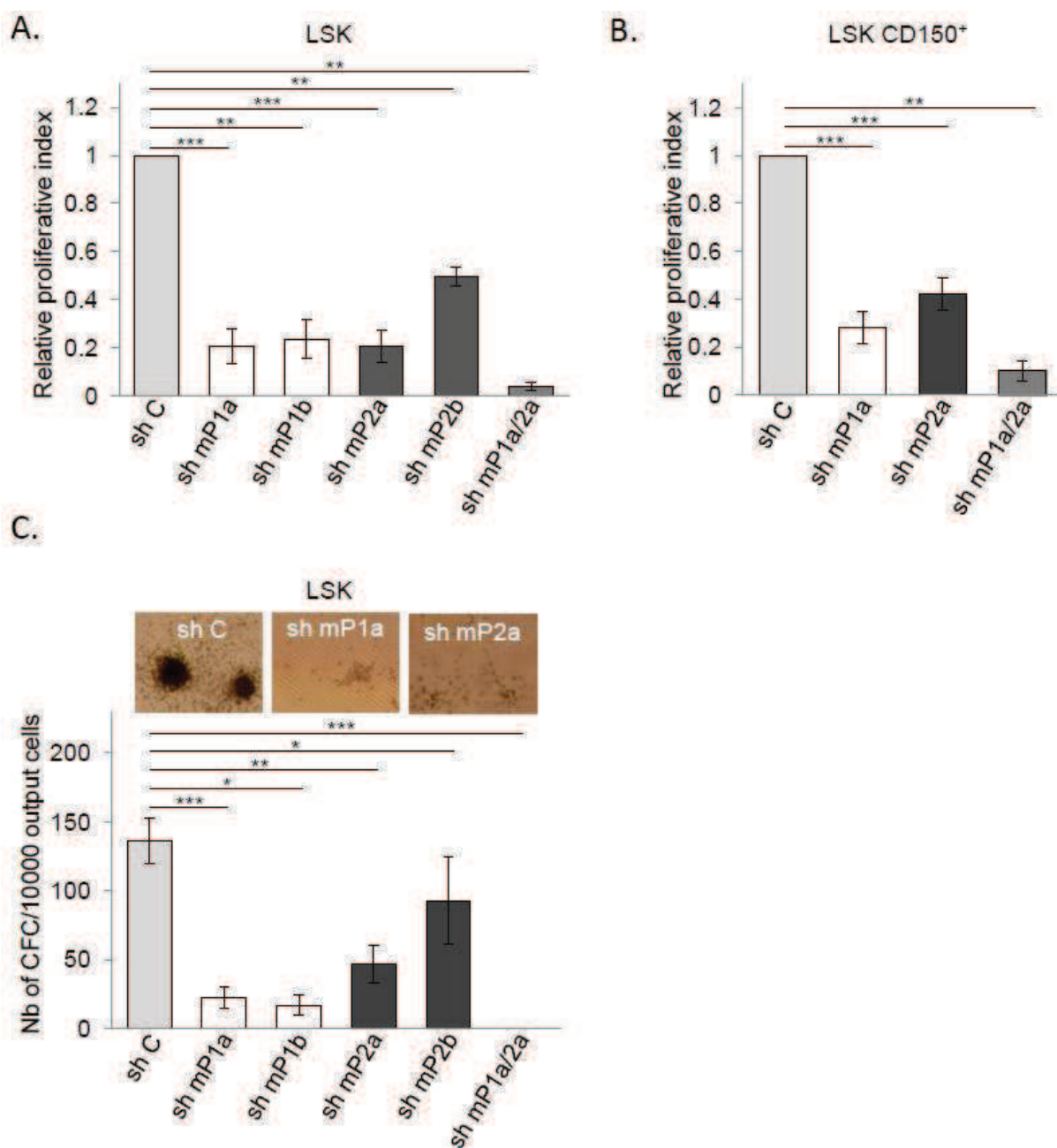


**Figure 23. Validation of the shRNA efficiency in murine cells. (A)** Immunoblot analysis of the indicated proteins in murine GFP<sup>+</sup> mastocyte p815 cells at day 7 post-shRNA/GFP lentiviral transduction. **(B)** Q-RT-PCR analysis of Pum1 and Pum2 transcripts of shRNA/GFP<sup>+</sup> cells 2 days after transduction of murine LSK cells. Relative differences in gene expression were calculated by using the 2- $\Delta\Delta$ CT method, which involves normalizing the CT value for each gene to the CT value of the mTFD2 housekeeping gene. Values are shown as the fold induction relative to shC (mean  $\pm$  SEM). **(C)** Immunoblot analysis of the indicated proteins in shRNA/GFP<sup>+</sup> cells at day 7 post-transduction of LSKCD150<sup>+</sup> cells. shC stands for shRNA directed against luciferase, shmP1a and shmP1b for shRNA directed against PUM1, and shmP2a and shmP2b for shRNA directed against PUM2.  $\beta$ -tubulin served as loading control. (\*\*\*)  $p < 0.001$

Growth properties of transduced LSK cells were analyzed upon a 7-day culture. Remarkably, we observed a major reduction in cell expansion for both PUM1 and PUM2 KD populations ( $79\pm 7\%$  and  $77\pm 8\%$  for shmP1a and shmP1b,  $80\pm 7$  and  $50\pm 4\%$  for shmP2a and shmP2b, Figure 24A). Furthermore, LSK cells simultaneously transduced with both shmP1a and shmP2a displayed a stronger reduction in cell expansion ( $96\pm 2\%$  as compared to shC). KD of Pum1 and Pum2 in LSK CD150<sup>+</sup> stem cell-enriched populations also triggered a drastic reduction of cell expansion (Figure 24B).

We next addressed the role of Pum1 and Pum2 on murine HSPC functionality. Examination of clonogenic properties (Figure 24C) revealed that Pum1 KD dramatically reduced CFC numbers. Such reduction was also evidenced after Pum2 KD. Furthermore, the few colonies generated by Pum1 or Pum2 KD cells were small-sized. Simultaneous KD of both Pum1 and Pum2 still amplified the inhibitory effects, thus suppressing any generation of colonies.

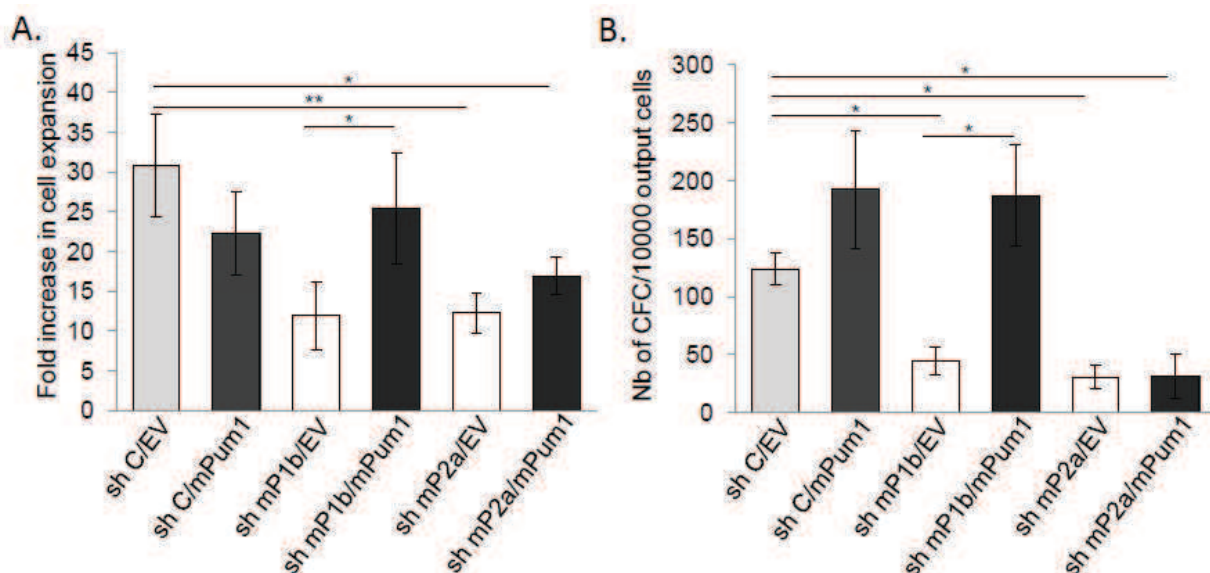




**Figure 24. mPUM1 and mPUM2 KD inhibit the hematopoietic potential of murine HSPCs.** shRNA/LSK/GFP<sup>+</sup> cells (A) or LSK CD150<sup>+</sup> cells (B) were sorted two days after transduction and maintained in culture for seven days. Fold increase in total cell number, relative to shC/GFP<sup>+</sup> cell population (n=5) at day 7. (C) CFC potential of LSK cells (n=5), and representative pictures of colonies in methylcellulose (x20). (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001)

## 5.2. PUM1 expression restores the functions of shmP1-transduced LSK cells.

The specificity of shmP1 was confirmed by conducting rescue experiments using murine PUM1, insensitive to shmP1b, which targets a sequence in the 3'UTR of endogenous PUM1 mRNA (absent from the exogenous PUM1). While enforced expression of mPUM1 in control shC-GFP<sup>+</sup> LSK cells had no major effect, the same transduction in shmP1b-transduced LSK cells restored cell expansion (Figure 25A), and also the CFC potential (Figure 25B). These results confirmed that the shmP1b activities were not “off-targets” effects, and validated mPUM1 activities. Our attempts to rescue shmP2 activity in LSK cells upon enforced expression of mPUM2 or hPUM2 was unsuccessful due to difficulties to obtain high-titered lentiviral vectors encoding murine or human Pum2. Interestingly, mPUM1 could not restore the cell expansion as well as the CFC potential of shmP2-transduced cells, suggesting that PUM1 and PUM2 exhibit non-redundant functions and thus PUM1 and PUM2 are not interchangeable (Figures 25A and 25B).

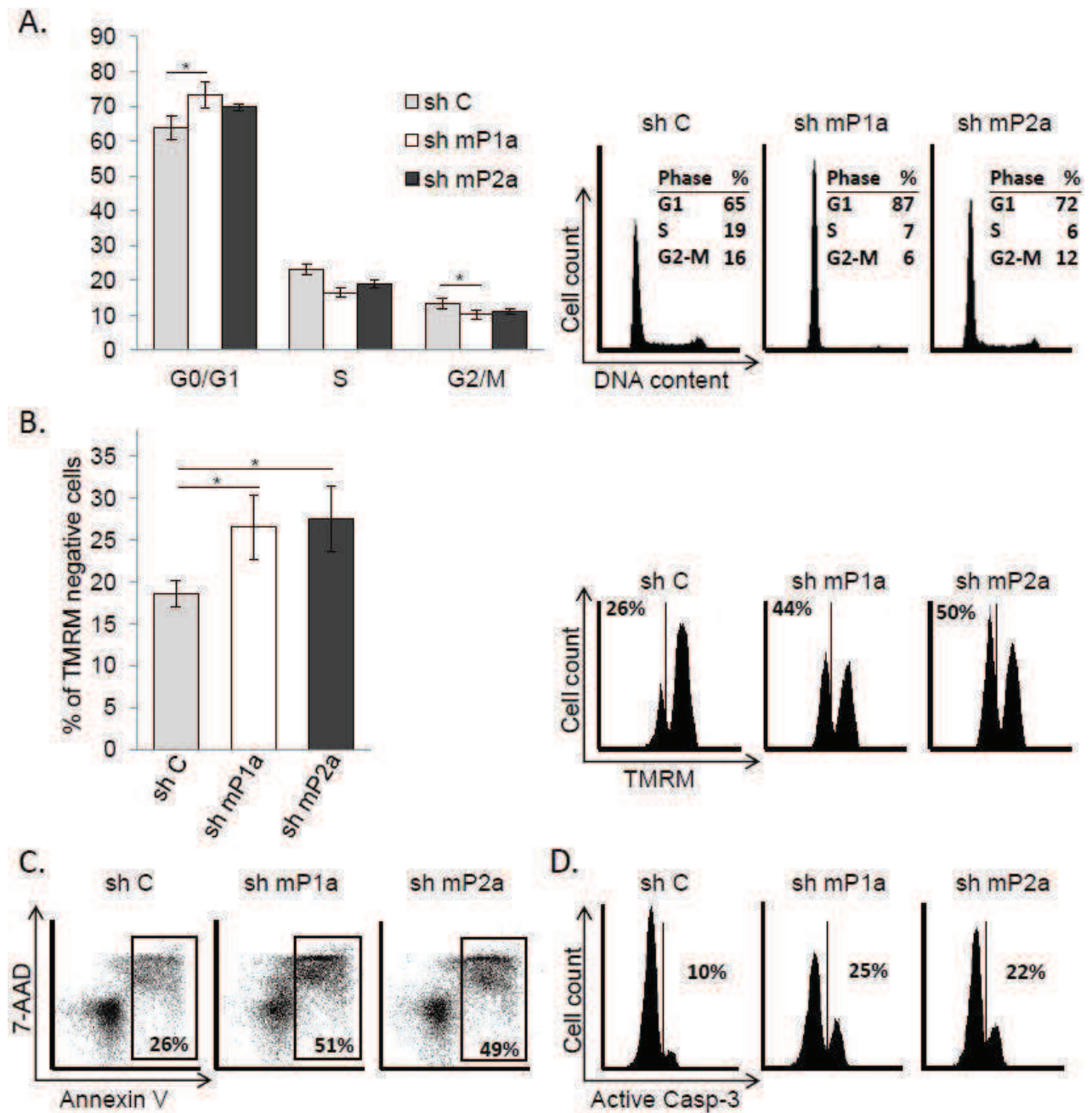


**Figure 25. mPUM1 expression rescues the functions of shmP1-transduced LSK cells.**

LSK cells, first transduced with shRNA-Tomato<sup>+</sup> constructs followed 8 hr later with mPUM1-GFP<sup>+</sup> construct transduction, were sorted two days after transduction. Tomato<sup>+</sup>/GFP<sup>+</sup> cells were maintained in culture, and 7 days later, cells were enumerated, and 10,000 output cells were plated in methylcellulose to assess the CFC potential. EV stands for empty vector. **(A)** Fold increase in total cell number (n=4). **(B)** CFC potential (n=4). (\*p<0.05, \*\*p<0.01).

### **5.3. Pum1 and Pum2 KD alter the cell cycle and cell survival of murine HSPCs.**

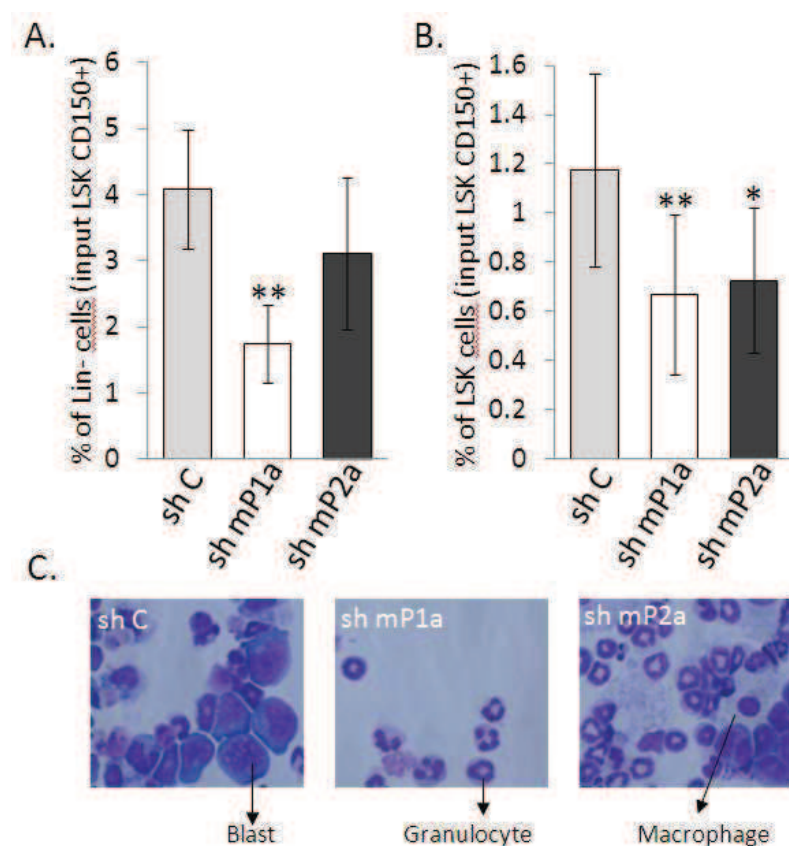
Having shown that Pum1 and Pum2 KD reduced expansion of murine HSPCs, we next aimed at deciphering the mechanisms responsible for this effect. We examined the cell cycle and apoptosis status of shRNA<sup>+</sup> cells throughout the culture. Two days after shRNA<sup>+</sup> LSK cell sorting, shmP1a/GFP<sup>+</sup> cells displayed a significantly increased proportion of cells in the G0/G1 phase ( $73\pm1$  versus  $64\pm3$  for shmP1a/GFP<sup>+</sup> cells and shC/GFP<sup>+</sup> cells, respectively) without any effect on cell apoptosis (data not shown). shmP2a/GFP<sup>+</sup> cells also showed some but not significant increase of cells in G0/G1 (Figure 26A). An enhanced proportion of apoptotic cells was then evidenced at 4 days after cell sorting, in Pum1 and Pum2 KD populations, as compared to shC populations, as assessed by measurement of mitochondrial transmembrane potential using TMRM staining (Figure 26B), annexinV/7AAD labeling (Figure 26C) and expression of active caspase-3 (Figure 26D). So, inhibition of Pum1 and Pum2 in murine HSPCs first induced a blockade of cell cycle, and thereafter an enhanced apoptosis, two biological processes that cooperatively decrease cell expansion.



**Figure 26. PUM1 and PUM2 KD alter the cell cycle and induce cell apoptosis in murine HSPCs.** Two days after transduction, shRNA/LSK/GFP<sup>+</sup> cells were sorted and maintained in culture. **(A)** After two days, cells were labeled with propidium iodide for cell cycle analysis (n=4, and one representative experiment). **(B).** After four days, apoptosis analysis was followed through the measure of the mitochondrial transmembrane potential using TMRM (n=4, and one representative experiment), **(C)** through AnnexinV-PE 7AAD (n=2, one representative experiment) and **(D)** active caspase 3 (n=2, one representative experiment) (\* p<0.05)

#### 5.4. Pum KD leads to loss of immature cells.

To further characterize the population of cells remaining at the end of the culture, we analyzed the percentage of Lin<sup>-</sup> and LSK cells still present in the progeny of purified LSK CD150<sup>+</sup> after 10 days of culture in proliferative medium. We observed that Pum1 KD significantly correlates with a decreased percentage of lin<sup>-</sup> and LSK cells at the end of the culture, and that Pum2 KD induced the same effects but with a lesser extent. To confirm these data, we performed May-Grünwald-Giemsa (MGG) staining to discriminate immature from differentiated cells (Figure 27). While immature cells were still observed in shC-transduced populations, scarce blasts were evidenced in shPum-transduced populations, confirming immunocytometric analyses. The decreased proportion of immature cells at the end of the culture is in agreement with the loss of the CFC potential in the absence of Pum1 or Pum2.



**Figure 27. Pum KD leads to loss of immature cells.**

LSK CD150<sup>+</sup> cells were used as input cells.

A) Immunophenotypical analysis of the percentage of Lin negative cells after 10 days of culture.

B) Immunophenotypical analysis of the percentage of LSK cells after 10 days of culture.

C) May-Grünwald-Giemsa staining of LSK CD150<sup>+</sup> progeny cells after 10 days of culture.

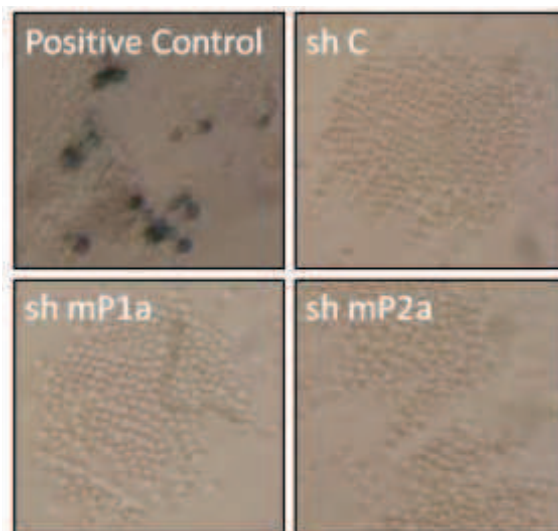
### 5.5. Pum1 and Pum2 KD do not lead to increased senescence.

After having found that Pum1 or Pum2 KDs correlated with an increased percentage of cells in G0/G1 phase of the cell cycle 4 days after shRNA transduction, followed two day later by an increased apoptosis, we wanted to evaluate other possible mechanisms that could be responsible for the decreased cell expansion.

A mechanism that could explain the decreased cell expansion in shPum1- and shPum2-transduced cells is the induction of senescence.

Cellular senescence is a phenomenon by which normal diploid cells cease to divide, normally after about 50 cell divisions *in vitro*. This phenomenon is also known as "replicative senescence". Cells can also be induced to senescence upon toxins, irradiation, or activation of some oncogenes. In response to DNA damage (including shortened telomeres) cells either age or undergo apoptosis if the damage cannot be easily repaired. Though they no longer replicate, senescent cells remain metabolically active and generally adopt phenotypes including flattened cell morphology, altered gene expression, secretion profiles (known as the senescence-associated secretory phenotype), and positive senescence-associated  $\beta$ -galactosidase staining<sup>288</sup>. We therefore evaluated the presence of senescent cells in the LSK progeny at day 10 using  $\beta$ -galactosidase staining. The results presented below (Figure 28) showed that only a negligible percentage in shC- and shPum-transduced cells were evidenced, indicating that Pum KD doesn't lead to an increase in cellular senescence.





**Figure 28. Pum1 and Pum2 KD do not lead to increased senescence.** LSK progeny cells were assessed for senescence-associated  $\beta$ -galactosidase activity at day 10 days after transduction. LSK progeny cells at 25 days of culture were used as positive control. ShC, shPum1a and shPum2a transduced populations didn't display any senescent cells.

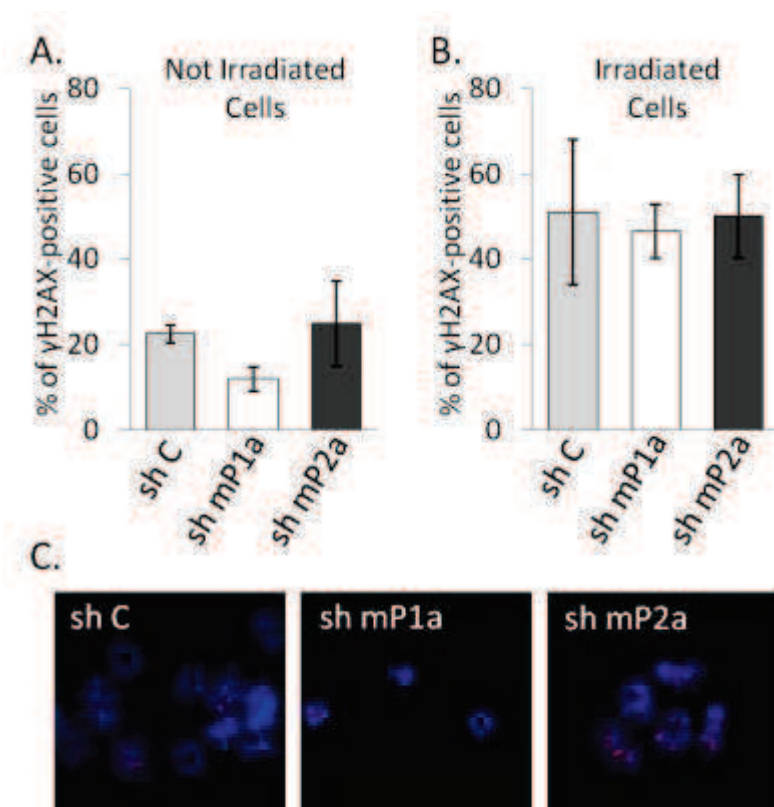
### 5.6. Pum1 and Pum2 are not involved in genomic stress response.

During our *in vitro* experiments on LSK cells, we noticed that shPum1 or shPum2 transduced-cells displayed a higher level of fragility, as compared to shC cells, each time they had to face a source of cellular stress. In fact, after problems regarding the cell culture (high temperatures, low CO<sub>2</sub> concentration, etc) or the cell sorting (anomalous pressure in the hydraulic system), shPum1- and shPum2-transduced cells showed stress intolerance such as abnormal morphology followed by a massive cell death. To examine this kind of behavior and to address additional roles of Pum proteins in HSCs biology, we studied the effects of Pum1 or Pum2 KD on genomic stress, trying to understand if the lack of Pum protein expression could be responsible responsible for enhanced DNA damage or impaired DNA repair.

Double-strand brakes (DSBs) in genomic DNA are generated endogenously during replication. In mammalian cells, DSBs are removed by two main repair pathways: homologous recombination (HR) and non homologous end-joining (NHEJ). NHEJ is regarded as the predominant mechanisms for DSB repair in vertebrates and recent data demonstrated that DSB repair through NHEJ is necessary for HSC maintenance<sup>289,290</sup>. To quantify the possible effects of Pum on DSBs, we first looked for the presence of phosphorylated H2A histone family, member X (H2AX). H2AX is



one of several genes coding for histone H2A. H2AX becomes phosphorylated on serine 139, and then called gamma-H2AX ( $\gamma$ H2AX), as a reaction on DNA double-strand breaks. Phosphorylation may mark the surrounding chromatin for recruitment of proteins required for DNA damage signaling and repair. We therefore examined by flow cytometry the presence of  $\gamma$ H2AX nuclear foci in the progeny of LSK transduced with shC, shPum1 or shPum2 at day 10. No relevant differences were detected between these three populations, suggesting no impact on basal DNA damage or DNA repair (Figure 29A). To assess the effects of Pum KD in stress condition, we irradiated the 7-day progeny of LSK cells at a dose of 2 Gy and we evaluated the presence of nuclear  $\gamma$ H2AX foci by flow cytometry and by immunofluorescence 2 hours after irradiation (Figure 29B and Figure 29C). Again, no difference between shC and shPum were evidenced, indicating that Pum1 and Pum2 are likely not involved in processes related to genomic stress or DNA damage response.



**Figure 29. Pum KD has no effect on protection from genomic stress or DNA repair.**

LSK cells were used as input cells.

A) Flow cytometry analysis of  $\gamma$ H2AX positive cells at day 7 (n=2).

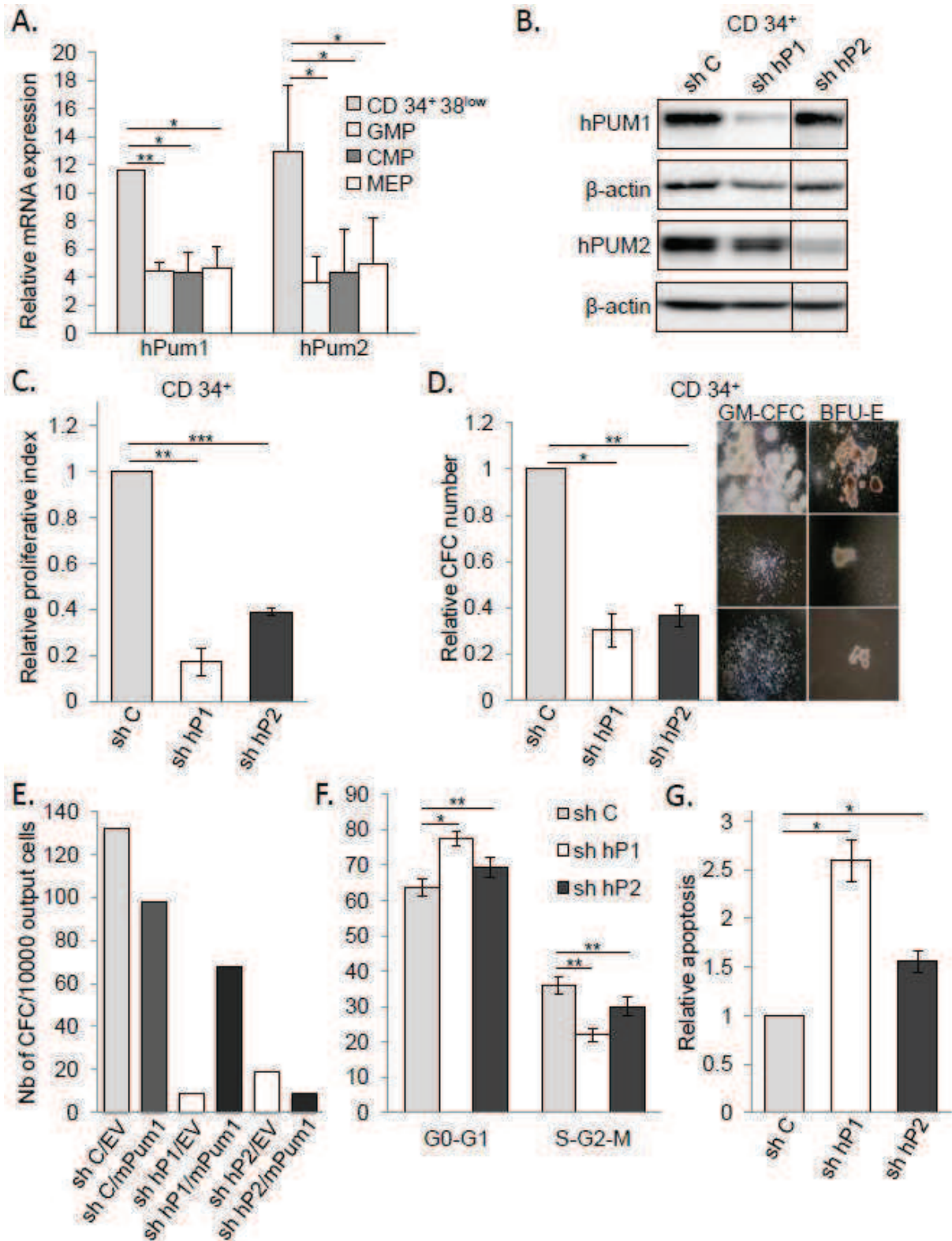
B) Flow cytometry analysis of  $\gamma$ H2AX positive cells at day 7, 2 hour after irradiation (2 Gy) (n=2).

C) Immunofluorescence staining of  $\gamma$ H2AX foci (Red) on DAPI nuclear staining (Blue). Cells with more than 3 foci were considered as positive.

### 5.7. Pum1 and Pum2 knockdown impair the human HSPC potential.

As previously mentioned, expression of Pum1 and Pum2 are upregulated in response to the self-renewing mediators HoxB4 and HoxC4 in human CD34<sup>+</sup> cells. This prompted us to also examine the role of PUM1 and PUM2 in maintaining the stem cell potential of human HSPCs. These experiments on human cells were conducted by Ayda Miri-Nezhad under the supervision of Serge Fichelson and Isabelle Vigon. The role of PUM1 and PUM2 in human HSPCs was assessed by transducing human CD34<sup>+</sup> HSPCs with shPum-containing lentiviral vectors, specific for human Pum1 (shhP1) and for human Pum2 (shhP2). Accordingly, PUM1 and PUM2 protein expression levels were decreased by 75% and 65%, respectively (Figure 30A). When human CD34<sup>+</sup> cells were kept in culture for 7 days, Pum1 KD and Pum2 KD led to 85±1% and 63±2% drop in total cell expansion, respectively (Figure 30B). As for murine HSPCs, the CFC potential of shhP1 or shhP2 CD34<sup>+</sup> cells was drastically decreased in number as well as in size, when assessed as early as two days after transduction (Figure 30C). Identical results were observed when transducing the more primitive CD34<sup>+</sup>CD38<sup>low</sup> cells (data not shown). The CFC potential of shhP1 CD34<sup>+</sup> cells was rescued upon enforced mPUM1 expression vector, and again, as for murine HSCPs, mPUM1 did not restore shhP2 activity (Figure 30D).

Furthermore, as also observed in murine HSPCs, PUM1 and PUM2 KD impaired the cell cycle of human HSPCs by enhancing the proportion of cells in G0/G1 (77±2% for shhP1 cells and shhP2 cells versus 64 for shC cells, Figure 30E) four days after cells sorting, while increased apoptosis was evidenced 2 days later, as compared to shC (Figure 30F).



**Figure 30. PUM1 and PUM2 KD inhibit the *in vitro* hematopoietic potential of human HSPCs.**

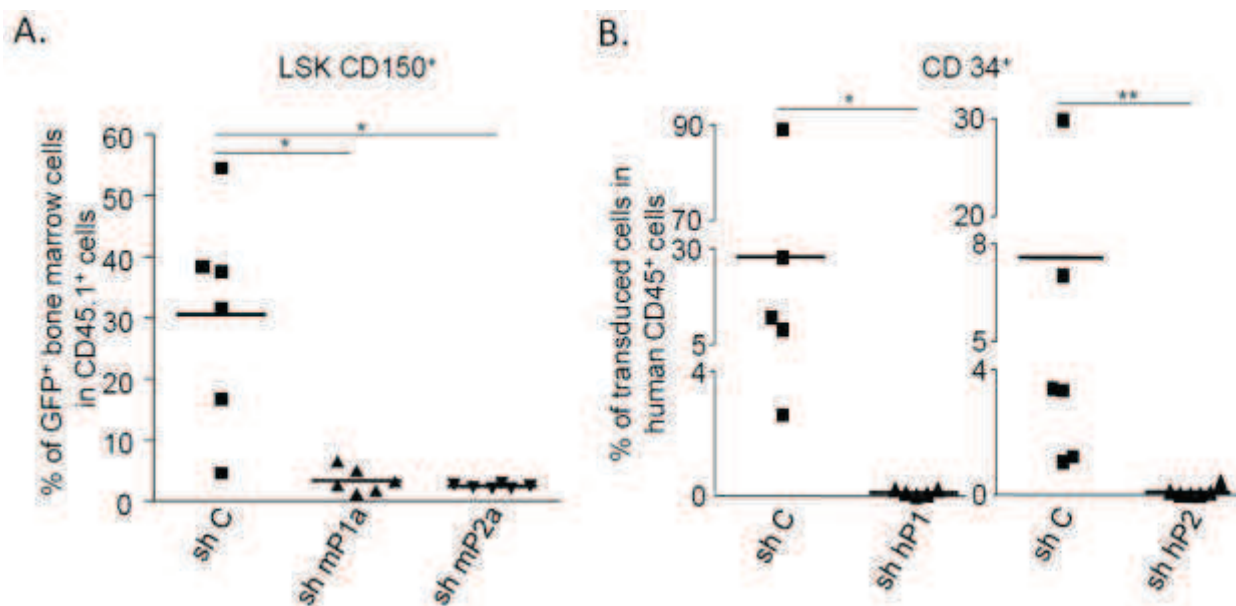
(A) Q-RT-PCR analysis of hPum1 and hPum2 transcripts in various sorted human progenitor populations: primitive CD34<sup>+</sup>CD38<sup>low</sup> cells, CMP (CD34<sup>+</sup>CD38<sup>+</sup>CD45RA<sup>low</sup>IL-3Rα<sup>low</sup>), GMP (CD34<sup>+</sup>CD38<sup>+</sup>IL-3Rα<sup>+</sup>CD45RA<sup>+</sup>), and MEP (CD34<sup>+</sup>CD38<sup>+</sup>IL-3Rα<sup>+</sup>CD45RA<sup>+</sup>). Results are normalized to GAPDH (n= 3) (B) Two days after lentiviral transduction of human CD34<sup>+</sup> cells, sorted shRNA/Tomato<sup>+</sup> cells were plated in methylcellulose to assess the CFC potential, or injected into NSG mice, or maintained in culture for 7 days. Immunoblot analysis of the indicated proteins in shRNA/Tomato<sup>+</sup> cells 7 days post-sorting. shC, control shRNA against luciferase, shhP1 for shRNA against hPum1, and shhP2 for shRNA against hPum2. β-actin served as loading control (lanes comes from the same blotting membrane but not contiguous)

(C) Fold increase in total cell number after 7 days of culture, relative to shC/GFP<sup>+</sup> cells from input shhP/Tomato<sup>+</sup>CD34<sup>+</sup> cells (n=6). (D) Relative CFC potential of shhP/Tomato<sup>+</sup>CD34<sup>+</sup> cells (to shC/Tomato<sup>+</sup> cells) plated in methylcellulose the day of sorting. (n=5, representative images of colonies in methyl cellulose, x20). (E) mPUM1 expression restored the functions of shhP1-transduced CD34<sup>+</sup> cells. CFC potential of sorted Tomato<sup>+</sup>/GFP<sup>+</sup> cells (n=1). (F) Cell cycle analysis through Hoechst dye in Tomato<sup>+</sup> cells at day 2 post-sorting of shRNA/CD34<sup>+</sup>/Tomato<sup>+</sup> cells (n= 4). (G) Apoptosis analysis through AnnexinV-FITC/7AAD labeling at day 4. Results are expressed relative to shC cells (n=4,) (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001).

## 5.8. PUM1 and PUM2 KD impair the *in vivo* reconstitution potential of murine and human HSPCs.

To further assess the effects of PUM deficiency on the functional competence of murine and human HSPCs, we performed competitive reconstitution studies. ShRNA-expressing GFP<sup>+</sup>LSK CD150<sup>+</sup> cells were inoculated into lethally irradiated mice, in competition with untransduced GFP<sup>neg</sup> LSK CD150<sup>+</sup> cells. Four months later, mice having received shC cells displayed GFP<sup>+</sup> cells numbers averaging 32±6% of BM cells, whereas mice having received shmP1- or shmP2-transduced cells harbored few bone marrow GFP<sup>+</sup> cells, with a chimerism averaging 3±0.4% and 2±1 %, respectively (Figure 31A). These results indicate that murine Pum1 and Pum2 KD HSPCs display strong impaired capacity to repopulate the bone marrow after myeloablation. The loss of the reconstitution potential after PUM KD was further confirmed in human CD34<sup>+</sup> cells by *in vivo* experiments. Indeed, a 1:1 mixture of CD34<sup>+</sup> cells transduced with either shhP1 or shhP2/Tomato vectors and with shC/GFP vector was transplanted using intra-femoral injection into immunocompromised NSG mice. Twelve weeks post-transplant, no mouse was ever positive for the presence of shhP1 or shhP2-expressing human CD45<sup>+</sup> hematopoietic cells (Figure 31B). By contrast, 5/9 and 6/8 mice were positive for the presence of GFP<sup>+</sup> shC cells in mice transplanted with shhP1 and shhP2 CD34<sup>+</sup> cells, respectively. These results indicate that Pum1 or Pum2 KD impair human HSPCs to long-term reconstitute human hematopoiesis in NSG mice, as observed for murine HSCPs.

Taken together, these results clearly establish the major role of the PUF proteins in maintaining the functions of primary murine and human HSPCs.



**Figure 31. PUM1 and PUM2 KD inhibit the *in vivo* reconstitutive potential of murine and human HSPCs.** (A) Lethally irradiated C57BL/6-Ly5.2 mice received 15,000 sorted shRNA/GFP<sup>+</sup> LSKCD150<sup>+</sup> cells from C57BL/6 (Ly5.1) donors, in competition with the same amount of GFP<sup>+</sup> LSKCD150<sup>+</sup> cells from Ly5.1 mice. Analysis of the presence of GFP<sup>+</sup> cells in CD45.1<sup>+</sup> bone marrow cells of engrafted mice was performed 4 months later by flow cytometry (at least 10<sup>5</sup> events). Each symbol represents the levels of a single chimeric mouse. (Figure representative of one experiment out of two). (B) Sublethally irradiated NSG immunodeficient mice received 40,000 sorted shPum/Tomato<sup>+</sup> CD34<sup>+</sup> cells, together with the same amount of shC/GFP<sup>+</sup> cells. Analysis of the presence of GFP<sup>+</sup> and Tomato<sup>+</sup> cells in the fraction of human bone marrow CD45<sup>+</sup> cells of engrafted mice was performed 12 weeks later. The percentage of GFP<sup>+</sup> or Tomato<sup>+</sup> cells was established from FACS analysis of at least 10<sup>5</sup> events. Each symbol represents the levels of a single chimeric mouse. (\* p<0.05, \*\*p<0.01).

### 5.9. Effects of Pum1 overexpression in murine HSCs.

Using a KD approach, we have clearly demonstrated that Pum1 or Pum2 depletion was deleterious for both *in vitro* and *in vivo* HSPC functions. Thereafter we wanted to perform mirror experiments by overexpressing Pumilio proteins to evaluate their roles as self-renewing factors.

We have worked for long time on the overexpression of Pum1 in LSK cells, and we had to face a lot of problems related to the level of Pum1 expression. In the first lentiviral vector we used, the Pum1 expression was under the control of a “strong”



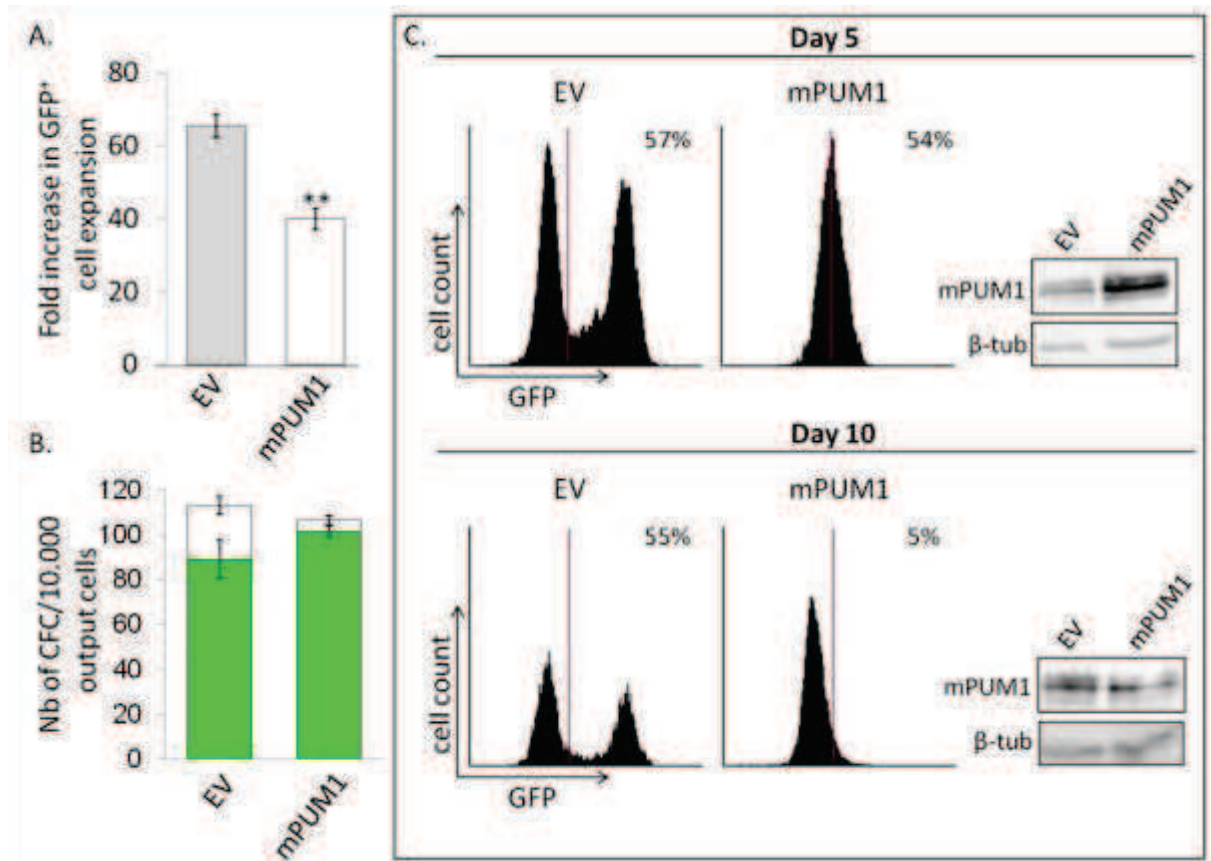
promoter (MND promoter) causing a 10-fold overexpression not compatible with HSPC cell survival. Even a “weaker” promoter such as EIF1 $\alpha$  showed toxic effects (data not shown). Finally, we engineered a lentiviral vector carrying a phosphoglycerate kinase (PGK) promoter, the weakest among the three we tested, which displayed a level of PUM1 expression compatible with HSPC survival. This construct was first used in rescue experiments as described above (§5.2).

As a first experiment to assess the effects of enforced expression of Pum1, LSK cells were transduced with an empty lentiviral vector (EV) or the vector carrying the cDNA for murine Pum1 (mPum1) placed under the control of the PGK promoter and GFP as marker gene. Two days after transduction, unsorted cells were maintained in culture for 7 days before analyzing cell expansion, % of GFP<sup>+</sup> cells and CFC potential.

mPum1-transduced LSK cells displayed a significant reduction in total cell expansion (Figure 32A), as compared to control populations. Strikingly, analysis of the percentage of GFP<sup>+</sup> cells at day 5 and 10 of the culture revealed a stable proportion of GFP<sup>+</sup> cells in the control population, whereas a drop in the percentage of GFP<sup>+</sup> cells was evidenced in Pum1-transduced population at day 10, as compared to day 5. Western blot analysis of Pum1 expression revealed a 2-fold overexpression of Pum1 in Pum-1-transduced cells at day 5, but such enforced expression disappeared at day 10 (Figure 32C), thus reinforcing the evidence of a loss of Pum-1-expressing cells during cell expansion. Analysis of the cell cycle and apoptosis at day 5 and 7 respectively, did not reveal any difference between control and mPum1-transduced populations (data not shown).

Analysis of the CFC potential at day 10 showed that control and Pum1-transduced populations gave rise to the same number of colonies (Figure 32B) but, interestingly, when 70% of the CFCs of the control cultures were GFP<sup>+</sup>, 95% of Pum1-transduced colonies were GFP<sup>+</sup> (Figure 32B). Thus, the 5% of remaining nucleated GFP<sup>+</sup> cells at

the end of the culture among the Pum1-transduced cells were highly enriched in CFC, suggesting at least a level of Pum1 expression compatible with CFC survival, and may suggest a higher CFC potential in Pum1-expressing cells.



**Figure 32. *In vitro* effects of Pum1 overexpression on HSPCs.**

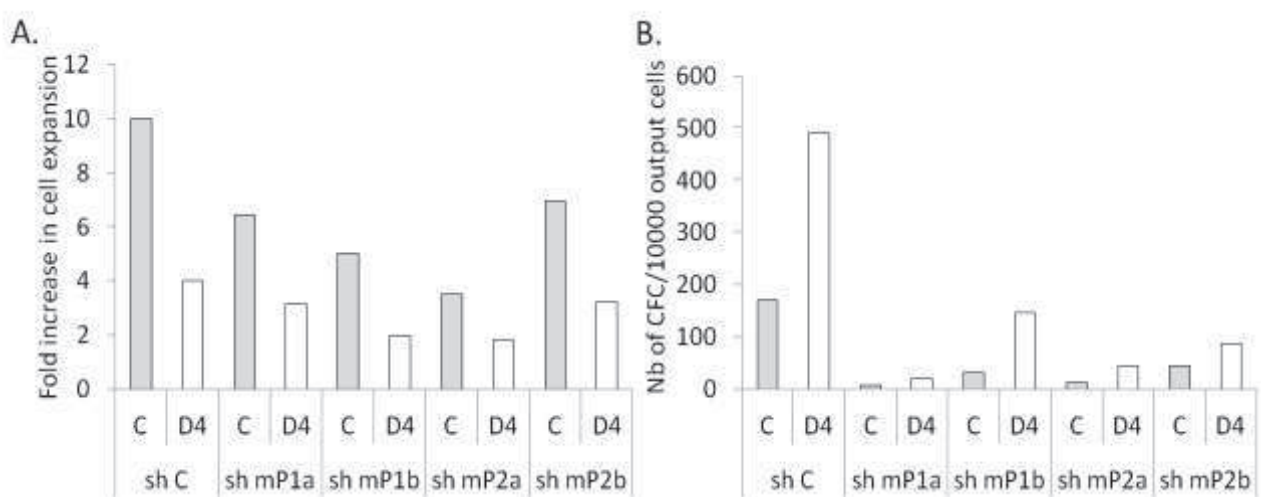
A) Cell expansion and B) CFC assay (GFP<sup>+</sup> colonies in green, GFP<sup>-</sup> colonies in white) were assessed at day 10 in cells transduced with Empty/GFP Vector (EV) or mPum1/GFP vector (mPUM1). C) From the left: GFP analysis at day 5 (up) and day 10 (down) of LSK progeny cells transduced with indicated lentiviral vectors. Immunoblot analysis of Pum1 expression at day 5 and day 10.  $\beta$ -tubulin serves as control.

## 5.10. Implication of Pum1 and Pum2 in Delta4/Notch activity

As said before, Pum1 and Pum2 were overexpressed in murine and human HSCs upon activation in culture by self-renewal factors such as Delta4 Notch ligand or HOXB4, respectively. Activation of LSK cells upon Delta4-reinforced signaling leads after a 7-day culture to a decreased cell expansion with a higher proportion of cells blocked in the G0 phase of the cell cycle, and to a higher proportion of LSK cells, and higher CFC potential. To assess whether Pum1 and Pum2 were implicated in



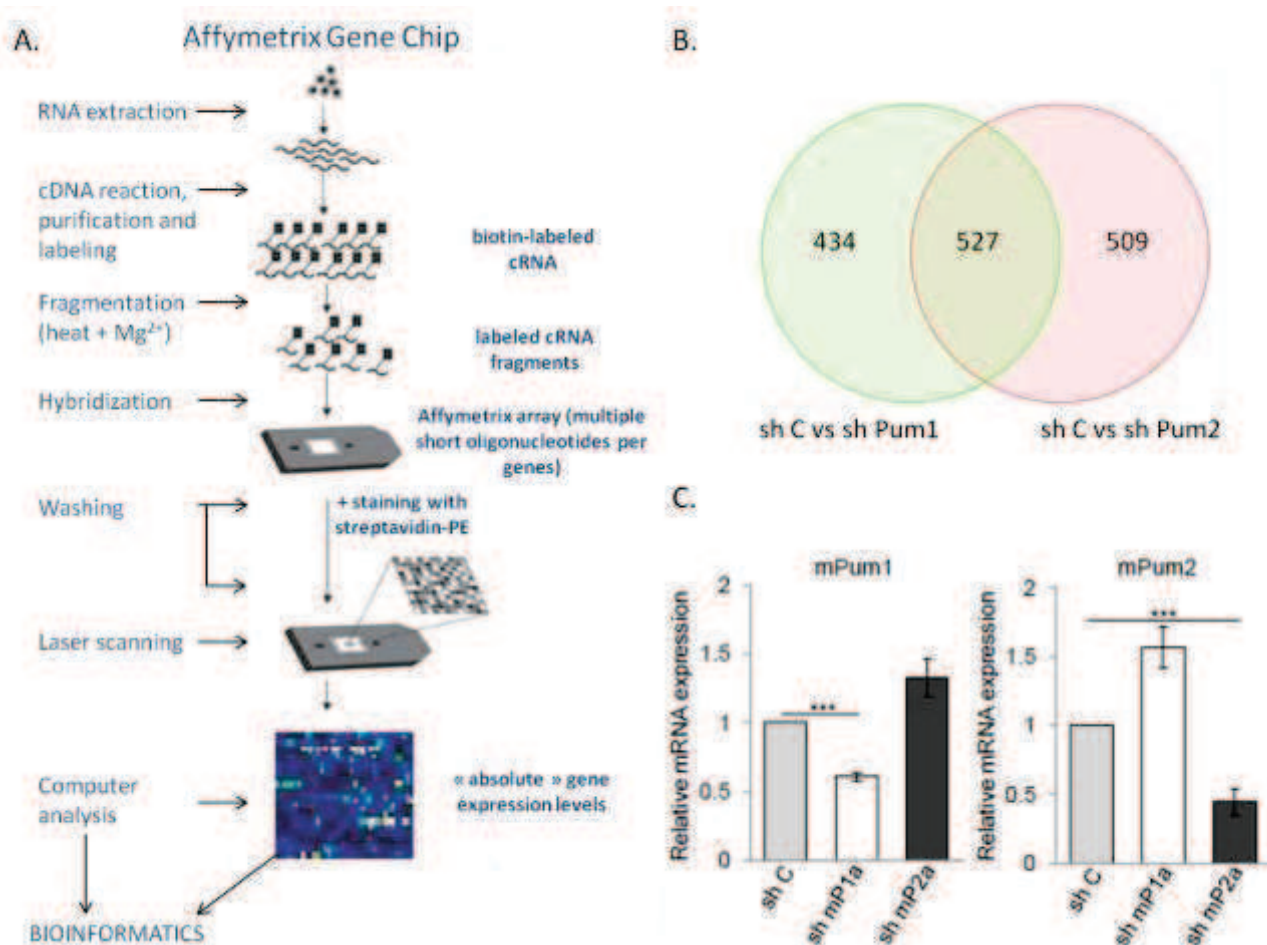
Delta4/Notch activity on murine HSCs, LSK cells were transduced with shC, shPum1 and shPum2 vectors, and plated onto murine stromal cells overexpressing the membrane bound form of Delta4 (mbDII4). We analyzed the cell expansion and the CFC potential of shRNA-transduced LSK cells cultured for 7 days onto mbDII4 stroma, as compared to LSK cells cultured onto control stroma (Figure 33A and Figure 33B). The results showed that shPum-transduced LSK cells still displayed a reduced cell expansion upon culture onto mbDII4 stroma, as compared to cells grown onto control stroma. Regarding the CFC potential, culture of shC-transduced LSK cells onto mbDII4 still enhanced the generation of CFC. In contrast, Pum1- or Pum2-KD LSK cells were strongly impaired in their capacity to generate CFC onto control stroma, and exposure to DII4 only slightly augmented these capacities (Figure 33B). These results indicate that if the absence of Pum1 or Pum2 profoundly altered the properties of LSK cells to expand and generate CFC, mbDII4 still retained some effects on LSK cells. Thus, Pum1 and Pum2 appear as front-line players necessary for the maintenance of HSC functions rather than directly contributing to mbDII4 activities.



**Figure 33. Delta4/Notch pro-self-renewing action is partially maintained in Pum KD cells.**  
A) Cell expansion. B) CFC assay. "C" indicates cells cultured in normal control condition (light gray). "D4" indicates cells cultured in presence of feeder stromal cells overexpressing the membrane bound form of Delta4 (white). Cell expansion and CFC potential were evaluated at day 10.

### 5.11. Identification of genes implicated in Pumilio effects on HSCs.

In order to identify the factors responsible for the effects obtained upon depletion of Pum1 or Pum2 in HSCs, we have devised a whole transcriptome comparative study in LSK CD150<sup>+</sup> cells using gene array (Affymetrix Gene Chip) (Figure 34A). LSK CD150<sup>+</sup> cells were transduced with shC, shPum1 or shPum2. 48 hours later, total RNA was extracted, and reverse-transcribed. Control experiments demonstrated that 48 hours after shRNAs transduction Pum1 and Pum2 mRNA were knocked down at a level of 40±2% and 55±3% respectively (Figure 34C). The transcriptome experiments were done twice with two independent biological samples. Statistical analysis revealed that 434 genes were modulated specifically by Pum1, 509 specifically by Pum2, and 527 common to both Pumilio proteins (Figure 34B). To provide a biological interpretation of these gene expression modifications, we looked for the statistical significance of the enrichment of particular functional categories using Ingenuity pathways analysis (IPA) and DAVID resources (<http://david.abcc.ncifcrf.gov/home.jsp>), focusing particularly on Gene Ontology (GO) Biological Process terms. We found that most of the genes were included in significant GO categories: “Cell Death, cell cycle, cell growth and Proliferation”. Nevertheless, the fold changes of the putative downstream effectors of Pum proteins were too weak and difficult to validate by qPCR, These data strongly suggested that the downstream Pum targets were rather modulated at a translational level without impacting the rate of mRNA.



**Figure 34. Identification of genes implicated in Pumilio effects on HSCs.**

A) Affymetrix Gene Chip. One population is used as starting material. Total RNA is extracted and cDNA is prepared. The cDNA is used in an *In vitro* Transcription (IVT) reaction to generate biotinylated cRNA. After fragmentation, this cRNA is hybridized to microarrays, washed and stained with PE-conjugated streptavidin, and subsequently scanned on a laser scanner.

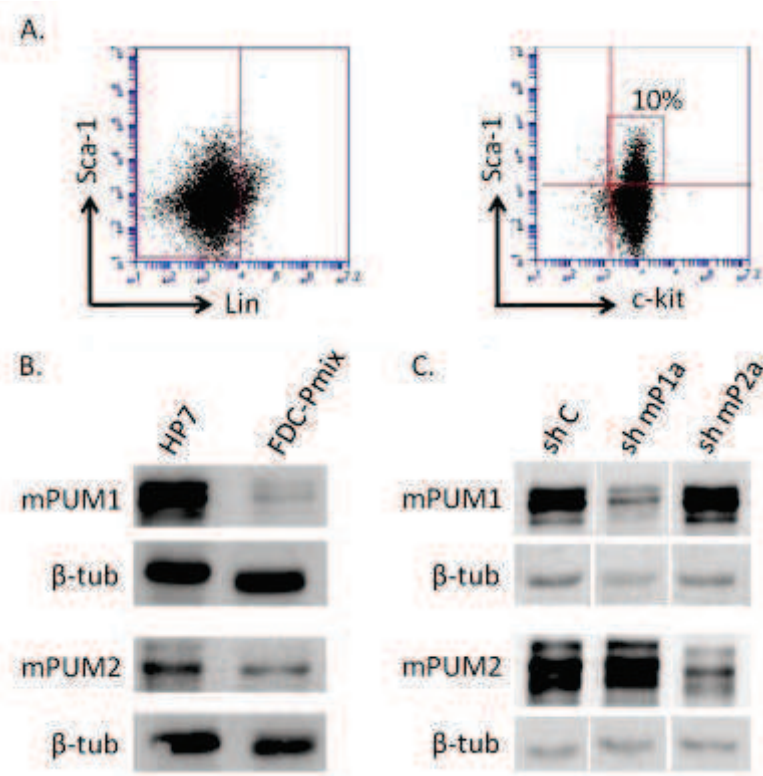
B) Number of genes found to be modulated by Pum1, Pum2 or both of them.

C) Validation of Pum1 and Pum2 KD by RT-qPCR of three different samples of LSK CD150+ cells. Two of them were used to gene array experience. RNA was extracted 48h after transduction.

## 5.12. Validation of the HP7 cell line as a model for murine HSPC studies.

After having demonstrated the key role of Pumilio proteins in HSCs function both *in vitro* and *in vivo*, we plan to identify the direct Pumilio mRNA targets (mRNA targets directly bound to Pumilio) and the protein partners of Pum1 and Pum2. For technical reasons, we switched toward a cell line in order to have homogeneous cell population capable of unlimited proliferation (the projects requiring this cell line will be

developed later on in Conclusion and Perspectives). After several attempts we chose the HP7 (Hematopoietic Precursors 7) cell line, a murine multipotent embryonic stem cell-derived cell line. HP7 cell line was created by the overexpression of LH2 protein in murine ESCs<sup>291</sup>. LH2 protein is a member of the LIM homeodomain gene family high expressed in hematopoietic fetal liver<sup>292</sup> and capable to immortalize adult HSPCs<sup>293</sup>. HP7 cells are SCF-dependent and are able to differentiate in all the myeloid lineages *in vitro* and *in vivo* in irradiated mice. HP7 cell line has recently been used to identify combinatorial control of major transcriptional regulators in HSPCs<sup>294</sup>. Our first step was to validate this cell line to ensure whether Pum exert functions similar to the ones observed in LSK cells. We have analysed the expression pattern of Lineage differentiation markers, Sca-1 and c-kit, and we found that HP7 cells are 95% Lin<sup>-</sup>, 98% c-kit positive and 10% Sca-1 positive reflecting, in part, the LSK phenotype (Figure 35A).



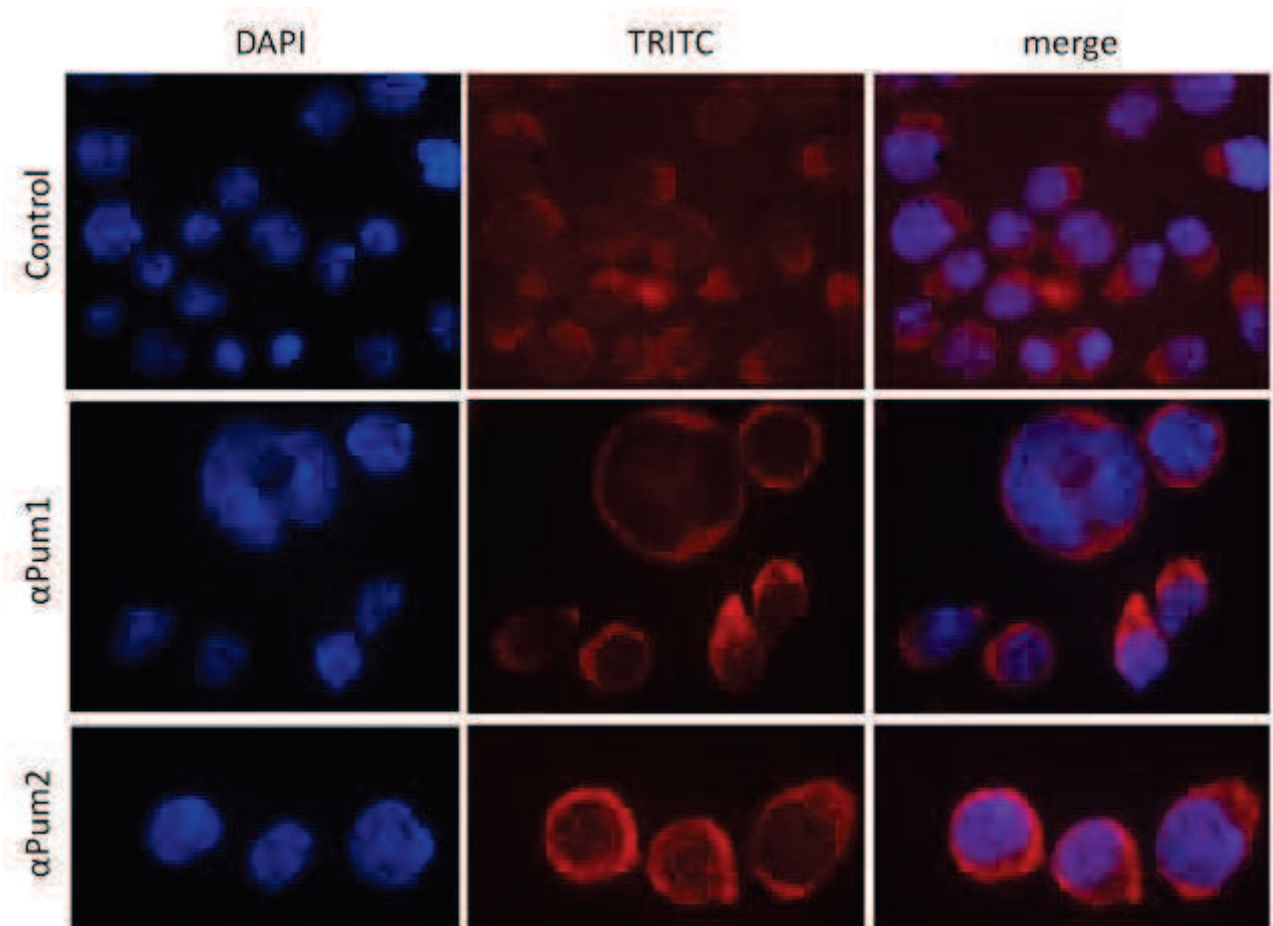
**Figure 35. Validation of the HP7 cell line.**

A) HP7 cells were incubated with biotinylated antibodies against lineage differentiation markers, Sca-1 PE and c-kit APC. After washing, biotinylated antibodies were coupled to PrCP streptavidin secondary antibodies and HP7 cells were analysed by flow cytometry.

B) Western blot analysis of Pum1 and Pum2 protein levels in HP7 and FDC-Pmix cell lines.

C) Immunoblot analysis of the indicated proteins in HP7 cells 5 days after shC, shPum1a or shPum2a transduction.

The expression level of of Pum1 and Pum2 in HP7 cells was comparable to the level found in LSK cells and higher than the one observed in another hematopoietic cell line presenting an immature phenotype, the FDC-Pmix cell line (Figure 35B). Transduction of HP7 cell line with shRNAs directed against Pum1 or Pum2 confirmed the specificity and the efficiency of each shRNAs (Figure 35C) and led to effects similar to the ones observed with LSK cells: decreased cell expansion and augmented apoptosis (data not shown), cell cycle was not assessed. Thereafter, we evaluated the intracellular localization of endogenous Pum1 and Pum2 in these cells (Figure 36).



**Figure 36. Intracellular localization of Pum1 and Pum2 in HP7 cell line.**

Pum1 and Pum2 present a cytoplasmic heterogeneous localization. HP7 cells were incubated with primary anti-Pum1 or anti-Pum2 antibodies or isotopic control (rabbit IgG). Primary antibodies were revealed by an anti-rabbit secondary antibody coupled to the AlexaFluor546 fluorochrome and visualized by the TRITC channel (red). Blue color represents the DAPI nuclear staining.

Visualization of Pum1 and Pum2 in HP7 cells by immunofluorescence indicated the cytoplasmic localization of these proteins. Ayda Miri-Nezhad has also observed that human Pum1 and Pum2 were mostly localized in the cytoplasm with a partial granular localization in human CD34<sup>+</sup> HSPCs. These results are in agreement with previous data showing cytoplasmic localization in hippocampal neurons<sup>197</sup> and HeLa cells<sup>196</sup> with granular localization in stress granules, where mRNA are stored at translational initiation or in response to cellular stress<sup>196,197</sup>. Thus, HP7 cell line appears as a good model to identify targets and partners of Pumilio proteins in HSPCs.



## 6. MATERIALS AND METHODS

### 6.1. Lentiviral constructs and transduction

For murine cells, selected shRNA sequences (Table 2) have been inserted down to H1 promoter in pSuper vector. H1-shRNA expression cassettes were next introduced into the pTRIPΔU3-GFP lentiviral vector previously described<sup>295</sup>. Alternatively the GFP sequence was replaced by a Tomato sequence. shRNA lentiviral vectors for human cells (Table 2) were generated with the pLKO.1 vectors containing the shRNA sequences and the Tomato or GFP as reporter genes. Lentiviral vector encoding anti-luciferase shRNA was used as control (shC). Murine Pum1 cDNAs were introduced into pTRIPΔU3-pgk ahead of IRES EMCV-GFP sequence<sup>296</sup>. Lentiviral particles pseudotyped by the vesicular stomatitis virus-G (VSV-G) envelope were generated by transfection of 293T cells using the Lipofectamine 2000 kit (Invitrogen) as previously described<sup>295</sup>. Viral supernatants were titrated by serial dilutions of the concentrated stocks on  $10^5$  mouse HP-7 cells<sup>293</sup> or human CD34<sup>+</sup> cells, and the titers were estimated 48-72 hours later by FACS analysis (Accuri C6); titers typically reached  $5\text{-}10 \times 10^8$  infectious particles/ml.

### 6.2. Mice

C57BL/6-Ly5.2, C57Bl/6-Ly5.1 mice (8-20 weeks old) were purchased from Charles River (l'Arbresle, France), and were maintained in the Cochin Institute facilities (Paris, France) under specific pathogen-free conditions. NOD.Cg-Prkdc(scid) Il2rg(tm1Wjl)/SzJ (NSG) (Jackson Laboratory, Bar Harbor, ME, USA) mice were housed in pathogen-free animal facilities at CEA, Fontenay-aux-Roses, France. All experimental procedures were done in compliance with French Ministry of Agriculture regulations for animal experimentation (CEA animal facility registration number: A920322) and in accordance with local ethical rules.



### **6.3. Murine HSC purification**

Lineage negative Sca-1 and c-Kit positive (LSK) cell purification was performed as described by Spangrude et al.<sup>21</sup> BM (Bone Marrow) cells were harvested in PBS containing 2% fetal bovine serum (FBS) by repeated flushing through 23-gauge needles. To enrich for primitive Lineage negative cells (Lin<sup>-</sup>), BM cells were incubated 20 min at 4°C with lineage-specific monoclonal antibodies (mAb): anti-Mac-1 (M1/70), anti-GR-1 (RB6-8C5), anti-erythroid cells (TER119), anti-B220 (RA3-6B2), and anti-CD3ε. Lin<sup>+</sup> cells were depleted by the immunomagnetic beads technique (sheep anti-rat IgG, Dynal, Invitrogen). The Lin<sup>-</sup> fraction was incubated with a phycoerythrin (PE)-conjugated goat anti-rat IgG antibody and stained with a phycoerythrin-cyanine 7 (PE-Cy7) anti-Sca-1 mAb (E13-161-7), a fluorescein-isothiocyanate (FITC) anti-c-Kit mAb (2B8), and an allophycocyanin (APC) anti-CD150 (TC15-12F12.2). Isotype-matched antibodies were used as controls. Sorting of the LSK or LSK CD150<sup>+</sup> fraction was performed using an Aria III cell sorter (Becton-Dickinson).

In some experiments, Lin<sup>-</sup> and LSK cells were analyzed as follows: cells were labeled using biotinylated mAbs raised against the differentiation markers Gr-1, Mac-1, B220, CD3 and Ter-119 specifically expressed by granulocytes, macrophages, B lymphocytes, T lymphocytes and erythrocytes, respectively (mouse lineage panel, Becton-Dickinson), revealed using streptavidin-PerCP molecules, and stained with the PE-Cy7-conjugated anti-Sca-1 mAb (E13-161-7) and an APC-conjugated anti-c-Kit mAb (2B8).

### **6.4. Isolation and immuno-labeling of human CD34<sup>+</sup> cells**

Normal cord blood units were collected according to institutional guidelines and after informed consent of the mothers (in partnership with Fondation Générale de Santé, Assistance Publique Hôpitaux de Paris). Following Ficoll separation (Lymphoprep,

Fresenius Kabi, Sèvres, France), CD34<sup>+</sup> cells were enriched with the CD34 microBead kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. For isolation of the most immature CD34<sup>+</sup>CD38<sup>low</sup> HPSCs, CD34<sup>+</sup> cells were incubated with FITC-conjugated antibody to CD34 (clone 581, Beckman Coulter, Villepinte, France) and PE-conjugated antibody to CD38 (clone T16, Beckman Coulter), then sorted. For flow cytometry analysis of CD34<sup>+</sup> cell subpopulations, IL-3R $\alpha$  (clone 7G3) and CD45RA (L48) antibodies were used.

## **6.5. Culture experiments**

Murine mastocytoma p815 cell line was cultured in DMEM containing 10% FCS. LSK cells were cultured in IMDM-defined medium containing 10% FCS (Stem Cell Technology, Vancouver, Canada). The following cytokines were added: murine Stem Cell Factor [ $\mu$ SCF], human Flt-3 ligand [HuFlt3-L], human interleukin-6 [IL-6], each at 50 ng/mL, and human interleukin-11 [IL-11] at 10 ng/mL. Human CD34<sup>+</sup> cells were cultured in IMDM-defined medium supplemented with 15% of a mixture containing Bovine Serum Albumin (BSA), Insulin and Transferrin (BIT, StemCell Technologies) and grown in the presence of human recombinant cytokines: SCF (100 ng/ml), IL-3 (60 ng/ml), Flt3-Ligand (50 ng/ml), TPO mimetic peptide (25nM).

Murine LSK and LSKCD150<sup>+</sup> or human CD34<sup>+</sup> and CD34<sup>+</sup>CD38<sup>low</sup> cells were cultured in their respective proliferation medium for one day after cell purification, and concentrated lentiviral vectors were then added once (mouse cells) or twice at 24-hr intervals (human cells), at a multiplicity of infection (MOI) of 5-20. 48hr later, percentage of transduced cells was estimated by FACS analysis. Transduction of primary cells generally ranged 60-90%. Transduced cells were sorted 48 hours after transduction, when required. For "rescue" experiments, LSK or CD34<sup>+</sup> cells were transduced with the shRNA/Tomato-encoding vectors, and transduced again 8 hr later with the cDNA/GFP rescue vectors.

## **6.6. Quantitative reverse transcriptase (RT)-PCR.**

Pellets of cells were suspended in RLT plus and RNAs were extracted using the RNeasy Plus Kit (Qiagen). Synthesis of first-strand cDNAs was performed using oligo(dT) and Superscript II reverse transcriptase (Invitrogen). PCR was performed using SYBRGreen PCR Master Mix (PE Applied Biosystems, Foster City, CA). PCR was monitored with a GeneAmp 5700 sequence detection system (PE Applied Biosystems). Quantification was performed using the  $C_t$  values method. Each PCR reaction was performed in duplicate with GAPDH and/or Beta2 Microglobuline ( $\beta 2M$ ) as internal controls to standardize the amounts of RNAs. The primers used are indexed in Table 2.

## **6.7. Protein analysis**

Total cell lysates from transduced cells were prepared in Laemmli lysis buffer. After migration in denaturing polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred onto a HybondC Extra membrane (Amersham Bioscience, Munich, Germany). The membrane was blocked for 1 h at room temperature with 5% (w/v) skimmed milk in Tris buffered saline-0.2% Tween (TBS-T), then hybridized in the same buffer with the antibodies to be tested. Membrane-bound antibody complexes were detected by Chemiluminescence (Amersham Biosciences), Images were captured using a CCD camera (Fuli-LAS4000, Fujifilm, Tokyo, Japan) and signals were quantified using a Fuji-LAS4000 luminescence image analyzer. The antibodies against the following proteins were used: Pum1 rabbit monoclonal antibody, (Abcam, ab92545), Pum2 rabbit monoclonal antibody (NOVUS Biologicals, EPR3813) or Pum2 rabbit polyclonal antibody (Abcam, ab10361),  $\beta$ -actin rabbit polyclonal antibody (Sigma Aldrich, A5441), and  $\beta$ -tubulin rabbit polyclonal antibody (Santa Cruz, sc9104).

### **6.8. CFC assays.**

For mouse assays, the CFC potential was evaluated by plating in methylcellulose medium (MethoCult® M3234, StemCell Technologies, Vancouver, BC, Canada) supplemented with cytokines. After 7 to 10 days of growth at 37°C, colonies were scored with an inverted microscope and categorized on their morphology. For human assays, similar procedure as for mouse CFC assays was used: 400 cells per ml of methylcellulose medium ([MethoCult® H4230](#), StemCell Technologies) supplemented with cytokines. After 14 and 21 days, colonies were numbered and categorized.

### **6.9. Detection of apoptotic cells**

In AnnexinV assay, cells were labeled using FITC or PE-conjugated AnnexinV detection kit (BD Pharmigen) following manufacturer's instructions. In tetramethylrhodamine methyl ester (TMRM, Invitrogen) assay, cells were labeled with TMRM at a concentration of 40 nM and incubated for 15 minutes in the dark at room temperature, as previously described<sup>297</sup>. PE-Active Caspase-3 Apoptosis Kit (BD Pharmigen) was used to detect Caspase-3-mediated apoptosis following manufacturer's instructions. In all cases, cells were analyzed by Accuri C6 flow cytometer (Becton-Dickinson).

### **6.10. Immunophenotypical analysis of LSK cells**

LSK progeny cells were labeled using biotin-conjugated mouse lineage panel (BD Biosciences) following manufacturer's instructions. After PBS wash LSK progeny cells were incubated 30 minutes at 4°C with streptavidin PerCP, anti-Sca-1 PE and anti-cKit APC. Once washed in PBS, cells were analyzed by FACS (C6 Accuri, BD).

### **6.11. Senescence analysis**

$\beta$ -galactosidase activity was assessed using senescence  $\beta$ -galactosidase staining kit (Cell Signaling) following manufacturer's instructions.

### **6.12. $\gamma$ H2AX foci analysis**

$\gamma$ H2AX presence was assessed by FACS and by immunofluorescence. For the first approach H2A.X Phosphorylation Assay Kit (Merck Millipore) was used following manufacturer's instructions. In immunofluorescence assay cells were fixed on glass coverslips with 3.7% paraformaldehyde (15 min at room temperature (RT)), washed with PBS and permeabilized with ice-cold methanol followed by incubation in blocking buffer (10% horse serum, 1% BSA) for 1h at RT and with mouse monoclonal anti- $\gamma$ H2AX and anti-GFP (Millipore, Billerica, MA, USA) primary antibodies (in PBS with 0.1% Triton X-100) overnight in 4°C. Alexa Fluor 555 F(ab')<sub>2</sub> fragment of goat anti—mouse IgG was then added for 1h at RT. Nuclei were counterstained with DAPI. All slides were visualized using Leica DMI 6000 microscope (Wetzlar, Germany) equipped with a 63 × 1.6 oil-immersion objective and a MicroMAX 1300Y camera (Princeton Instruments, Trenton, NJ, USA). Pictures were analyzed using ImageJ software (developed at the National Institute of Health, Bethesda, MD, USA).

### **6.13. Long-term competitive repopulation assays.**

Animal experiments were performed following the conditions defined by the Ethical Committee of the French Agriculture Department. Lethally irradiated C57BL/6-Ly5.2 mice have received 15,000 sorted shRNA/GFP<sup>+</sup> LSKCD150<sup>+</sup> cells from C57BL/6-Ly5.1 donors, in competition with the same amount of GFP<sup>-</sup> LSKCD150<sup>+</sup> cells from Ly5.1 mice, together with 1.5x10<sup>5</sup> Ly5.2 BM cells. Hematopoietic reconstitution was assessed 4 months after transplantation through analysis of peripheral blood or BM

cells. Transduced cells were identified through GFP detection and after staining with anti-CD45.1-PE by flow cytometry.

For human cell competitive transplantations, 3-Gy-irradiated NSG mice (8-12 weeks old) were injected with a mixture of  $7 \times 10^4$  shPum/Tomato<sup>+</sup> CD34<sup>+</sup> cells and  $7 \times 10^4$  shC/GFP<sup>+</sup> CD34<sup>+</sup> cells using intrafemoral injection to ensure maximal engraftment. Human hematopoietic reconstitution was assessed 12 weeks after transplantation in the BM by flow cytometry using GFP fluorescence and labeling with PC7-conjugated antibody to human CD45. Percentages of Tomato<sup>+</sup>shPUM or GFP<sup>+</sup>shC in CD45<sup>+</sup> cells were calculated and mice were considered positive when at least 0.5% of human cells were detected among mouse bone marrow cells.

#### **6.14. Microarray**

LSK CD150<sup>+</sup> cells were transduced with shC-, shPum1- or shPum2-GFP<sup>+</sup> lentiviral vectors. 48 hours after shRNA transduction 50.000 cells for each sample were sorted and RNA was extracted using PureLink RNA Mini Kit (Ambion). Biotinylated cDNAs were prepared following standard Affymetrix protocol (GeneChip whole transcript (WT) Sense Target labeling assay kit). Transcriptomic study was done using Affymetrix Mouse Gene 1.1 ST array strip. Affymetrix gene arrays were analyzed through Affymetrix Fluidics Station 450 and GCS 3000 scanner (Affymetrix). Quality controls, normalization and statistical analysis were done using Partek software (version 6.6, Partek Inc., St. Luis, MO, USA).

#### **6.15. Intracellular localization of Pum1 and Pum2 in HP7 cell line**

Cells grown on Lab-Tek coverglass were fixed with 4% paraformaldehyde (30min at room temperature (RT)), washed with PBS and permeabilized with 0,1% Triton (15 min at room temperature) and incubated in blocking buffer (tris Buffer Saline (TBS), 0,1X Tween20, 1% BSA, 1% goat serum) for 1h at RT. Anti-Pum1 (Abcam, rabbit,

EPR3795) or anti-Pum2 (Bethyl, rabbit, A300-202A) primary antibodies (in PBS with 0.1% Triton X-100) were incubated 1 hour at room temperature. Alexa Fluor 546 coupled goat anti-rabbit antibodies (Invitrogen) were incubated 1 hour at room temperature. Nuclei were counterstained with DAPI. All slides were visualized using Leica DMI 6000 microscope (Wetzlar, Germany) equipped with a 63 × 1.6 oil-immersion objective and a MicroMAX 1300Y camera (Princeton Instruments, Trenton, NJ, USA). Pictures were analyzed using ImageJ software (developed at the National Institute of Health, Bethesda, MD, USA).

#### **6.16. Statistical Analysis**

All measurements were performed at least three times. Continuous variables were analyzed using Student's t-test for paired values. The measured values were expressed as mean  $\pm$  SEM. p-values of less than 0.05 were considered to be statistically significant. Chi square or Wilcoxon tests were used.

#### **6.17. Acknowledgements**

We acknowledge the different Cochin Research Facilities such as the Animal facilities, Cytometry and Immunobiology, and Genomic Facilities (Inserm U1016).



A.

Primer	Sequence
hPum1	TTGGAGCTCCTGTTCTGACTT (Forward) GAACTGGATGCCAGGTTGTT (Reverse)
hPum2	TACTCCCAATCAGGGTCAGC (Forward) CCAACCACTAAGGCACCAGT (Reverse)
GAPDH	GGAGGAGTGGGTGTCGCTGTT (Forward) GGGAAAGTGTGGCGTGAT (Reverse)
mPum1	AATCCTCCAGGCTGCGTACCAAC (Forward) ACATCTGCAGTGCCAATGATAGGACA (Reverse)
mPum2	GGATGCCGATTGCAAAGATT (Forward) CGCTCAACTACT TCAGTTGGAGAG (Reverse)
TFIID	ACGGACAACCTGCGTTGATTTT (Forward) ACTTAGCTGGGAAGCCCAAC (Reverse)

B.

shRNA	Sequence
sh hP1	CCGGCAGTTCTTTCTACGGCAACAACCTCGAGTTGTTGCCGTAGAAAGAACTGTTTTTTTG
sh hP2	CCGGGCTCCCAGAGTAGTTCTTTATCTCGAGATAAAGAACTACTCTGGGAGCTTTTTTG
sh mP1a	GTATTATGGAGTCACGCCAT TTCAAGAGA ATGGCGTGACTCCATAATATTTTTT
sh mP1b	GTTGCCCTGCTGTTTACTGG TTCAAGAGA CCAGTAAACAGCAGGGCAATTTTTT
sh mP2a	GCACCTGTCTTAATAAGTTC TTCAAGAGA GAACTTATTAAGACAGGTGTTTTTT
sh mP2b	GAGAGTGGGCTTTCTTTGTG TTCAAGAGA CACAAAGAAAGCCCACTCTTTTTTT
sh C	GCGTACGCGGAATACTTCGA TTCAAGAGA TCGAAGTATTCCGCGTACGTTTTT

**Table 2.**

A) Sequence of primer used for RT-qPCR

B) List of the sequences of shRNA used to invalidate human and murine PUM1 and PUM2

## **DISCUSSION**



## 7. DISCUSSION

The PUF family of RNA binding proteins, with the typical 3D structure of the functional domain of its members, is extremely conserved in nature and, strikingly, even its role seems to be conserved. When we read about biological roles of PUF proteins in the literature, we have a strong probability to find a link with stem cells, and the function exerted by PUF proteins in stem cells is most of the time the same: to sustain their self-renewal. This amazing conservation of structure and roles is observed in different organisms. In Invertebrates, for example, the role of PUF proteins in *D. melanogaster* and *C. elegans* germ stem cell maintenance is well established<sup>193,257,258</sup>. Among plants, PUF proteins of *Arabidopsis Thaliana* are overexpressed in the meristem, a tissue containing undifferentiated cells (meristematic cells) found in zone of the plants where the growth can take place<sup>298</sup>. Even if we can't properly speak of stem cells, PUF proteins exert pro-self-renewing and anti-differentiating effects even in the slime mold *Dictyostelium*, in which PUF overexpression prevents the ameboid undifferentiated cells from aggregation and differentiation in fruit bodies<sup>259</sup>. Furthermore, in yeast, strains lacking *PUF5* divide fewer times than the wild type, while strains overexpressing *PUF5* extend their number of mitosis and their life span<sup>260</sup>. Thus, if we accept the comparison between the mother cell of a unicellular organism and a stem cell, we could imagine that PUF proteins are essential in sustaining properly yeast mitosis in the same way they sustain stem cell self-renewal.

In mammals, only few studies were focused on studying the role of PUF proteins in stem cells. The two canonical members of PUF family, PUM1 and PUM2, were suspected to sustain embryonic stem cells<sup>278</sup> and neural stem cells<sup>279</sup> self-renewal, and germ stem cell maintenance through indirect evidence. All these examples can

give us an idea about the importance of these proteins all along the phylogenetic tree, supporting the hypothesis for their ancestral role in stem cell functions.

All these elements could be sufficient to prompt researchers to examine the role of PUF proteins in different subtypes of stem cells. Nevertheless, two new arguments were put forward by our team. Interestingly, two independent transcriptomic studies to identify target genes of two regulators of HSPC self-renewal, HOXB4 in human CD34<sup>+</sup>CD38<sup>-</sup> cells and Delta4/Notch pathway in murine LSK cells, converged toward a strong upregulation of both Pum1 and Pum2<sup>286,287</sup>, reinforcing again the link between stem cell self-renewal and PUF proteins.

For these reasons, we choose to assess the role of PUM1 and PUM2 in human and murine HSPCs, starting with a RNA interference-based approach to knockdown Pum expression upon transduction of HSPCs with lentiviral vectors carrying the proper shRNA sequences.

Our data demonstrate that PUM proteins have similar effects in both murine and human system and in both case, they play a crucial role in supporting HSPC maintenance and growth. To our knowledge, these results are the first demonstration of a direct participation of PUF proteins on the long-term functions of mammal primary HSPCs. As told before, relatively few studies have evoked that mammalian PUF proteins could exert the same functions as those identified with non-vertebrate PUF homologues, and most of them rely upon indirect evidences.

### **7.1. Role of Pum1 and Pum2 in HSPC cell expansion**

In our experiments, we first purified HSPCs at day 0, we transduced them at day 1, and we let the shRNAs act for 48 hours in order to sort the cells at day 3 upon the proper expression of the GFP or tomato marker gene. Sorted cells are plated at day 3

and let proliferate for 7 days until day 10.

In our first assay, we measured the cell expansion along the 7-day culture (from day 3 to day 10). Murine and human shPum1- or shPum2-transduced HSPCs displayed a significant reduced cell expansion, while murine cells transduced concomitantly with both shPum1/GFP and shPum2/tomato displayed a massive drop in cell expansion.

#### **7.1.1. Impact of Pum1 and Pum2 KD on cell cycle**

The first mechanisms that in part explain this phenotype appeared at day 5, thus 4 days after shRNA transduction, and consisted in the blockade of cell cycle in the G0/G1 phase.

This result is in agreement with previous studies. Human adipocyte-derived stem cells were described to proliferate less than control cells when transfected with small interfering RNA knocking down Pum2, as assessed by BrdU incorporation, and the proportion of cells in the S phase was decreased<sup>280</sup>. Human BJ primary fibroblasts were found to be retained in G0 phase when KD for PUM1<sup>206</sup>. The mechanism responsible for this phenotype was the interaction between PUM1 and the miRNA system. In these cells, human PUM1 has been found to be essential for miR-221/miR-222-mediated repression of the cell cycle inhibitor *p27<sup>kip1</sup>*. The binding of PUM1 induces a local conformational change in the *p27* 3'-UTR that exposes a miR-221/miR-222 binding site allowing repression of *p27<sup>kip1</sup>* mRNA. When PUM1 is absent, the miR-221/miR-222 binding site is trapped in a hairpin structure, protecting *p27<sup>kip1</sup>* from degradation and blocking the cell cycle in G0 phase.

Regulation of the cell cycle by PUF proteins has been emphasized also in *Xenopus* oocytes, in which PUM negatively regulate the translational activation of the mRNA of the cell cycle regulator cyclin B1<sup>244</sup>. In the *D.Melanogaster* maternal germ line, PUM repress the translation of cyclin B mRNA<sup>219</sup>.

To identify the genes early involved in the cell cycle arrest, we evaluated the expression of human p21<sup>cip1</sup>, p27<sup>kip1</sup> and p57<sup>kip2</sup> and murine *p21<sup>cip1</sup>* by RT-qPCR at day 4, just one day before the cell cycle arrest begins. No major difference between shPum1-2-transduced and control cells were found, suggesting that the putative regulation of these cell cycle genes (if it occurs) does not take place at the level of mRNAs (not detected by transcriptomic approaches), but rather at the translational level. This problem will be recurrent throughout our work and will be discussed later in this chapter.

### **7.1.2. Impact of Pum1 and Pum2 KD on apoptosis**

Another mechanism that explains the reduction of cell expansion is the increased apoptosis evidenced two days after the cell cycle arrest in the G0/G1 phase. Both human and murine HSPCs displayed about a 2-fold increase in apoptotic cells 6 days after shPum1 and/or shPum2 transduction (day 7). Regulation of apoptosis by PUM1 has been clearly established during murine spermatogenesis: deleting PUM1 was consistently associated with a significant upregulation for mRNAs involved in pathways regulating p53, cell cycle, and MAPK signaling in the late spermatocyte stage<sup>239</sup>. In particular, eight mRNAs-encoding activators of p53 were overexpressed in absence of Pum1 and deletion of Pum1 resulted in strong activation of p53 and apoptosis, which disrupt sperm production and fertility.

## **7.2. Role of Pum1 and Pum2 in HSPC hematopoietic potential.**

### **7.2.1. *In vitro* hematopoietic potential**

We next addressed the role of PUM1 and PUM2 on HSPC functionality. Examination of the clonogenic properties revealed that Pum1 KD dramatically reduced CFC numbers. Such reduction was also evidenced after Pum2 KD. Furthermore, the few



colonies generated by Pum1 or Pum2 KD cells were small-sized. Simultaneous KD of both Pum1 and Pum2 in murine HSPCs amplified the inhibitory effects, thus suppressing any generation of colonies. The impaired CFC potential of LSK and CD34<sup>+</sup> cells could be considered as the natural consequence of the augmented proportion of cells displaying cell cycle arrest and apoptosis upon the transduction with shPum1 and/or shPum2, nevertheless, another hypothesis can be evaluated, for example the selective loss of progenitors.

This hypothesis was supported by further experiments in which we screened by flow cytometry the percentage of undifferentiated (Lin negative) and LSK cells in the progeny of shC, shPum1 and shPum2 LSK cells at the end of the culture. We found in shPum1- and shPum2-transduced populations a 2-fold reduction in LSK and Lin<sup>-</sup> cells compared to the control. MGG staining at the same day highlighted blast cells (immature cells) in shC and, in a minor extent, in shPum2 populations. In contrast, in shPum1-transduced cultures, all the cells reached a terminal granulocytic or macrophagic differentiation. Whether the cause of these results was an early differentiation or a selective apoptosis of immature cells still remains an open question. Two arguments are in favor in a role of PUM proteins in the more primitive HSPCs. Spassov and colleagues have observed that murine *Pum1* and *Pum2* expression is high in LSK cells and downregulated (50%) in Lin<sup>-</sup>Sca-1<sup>+</sup> and Lin<sup>-</sup> cells<sup>270</sup>. Ayda Miri-Nezhad in our group has found that human *PUM1* and *PUM2* are highly expressed in CD34<sup>+</sup>CD38<sup>-</sup> cells and down-regulated (40-50%) in Megakaryocyte-Erythroid progenitors (MEPs), common-myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs) (data not shown).

### **7.2.2. *In vivo* reconstitution potential**

In addition to the role of PUM1 and PUM2 *in vitro*, we demonstrated that PUM1 and PUM2 play major roles in the functions of HSPCs *in vivo*. Our results indicate that

murine Pum1 and Pum2 KD HSPCs display strong impaired capacity to repopulate the bone marrow after myeloablation. The loss of the reconstitution potential after PUM KD was further confirmed in human CD34<sup>+</sup> cells by *in vivo* experiments performed in immunodeficient mice. Taken together, these results clearly establish the major role of the PUF proteins in maintaining the functions of primary murine and human HSPCs.

An ancestral role for PUF proteins in the maintenance and self-renewal of human ES cells was proposed since Pum2 has been found to negatively regulate the expression of two kinases of the MAPK/ERK pathway, MAPK1 (mitogen-activated protein kinase 1), and MAPK-14, both known as repressors of human ES cell self-renewal<sup>278</sup>. In human germline stem cells, Pum2 interacts with Deleted in Azoospermia (DAZ), DAZ-Like proteins, and the meiotic regulator BOULE, which are RNA-binding proteins required for germline stem cell formation<sup>195</sup>. It can be emphasized that TRIM32, the mammalian ortholog of *Drosophila* BRAT, identified as partner of PUM for *hunchback* regulation, is a key regulator of mouse and human skeletal muscle stem cells as well as neuronal progenitor cells<sup>283,284</sup>. Besides TRIM32, NANOS2 (the ortholog of another partner of PUM in *Drosophila*), which is expressed in self-renewing murine spermatogonial stem cells, prevents differentiation of these cells, and also appears as a key stem cell regulator<sup>285</sup>. However, all these data are solely indirect arguments to propose a role of PUF proteins in the maintaining of the functions of primary mammal stem cells.

Description of knockout mice deprived of Pum1 or Pum2 has been reported. Pum1<sup>-/-</sup> mice are viable and grow to adulthood without apparent major defects except their smaller size and sterility of males. Pum2<sup>-/-</sup> mice display significantly smaller testes although the mutants are otherwise viable and fertile<sup>239,282</sup>. These studies do not

mention obvious disturbance in hematopoiesis after disruption of any one of the PUF genes, thus suggesting that the lack of expression of either PUM1 or PUM2 proteins has no major incidence on baseline blood and HSPC homeostasis *in vivo*.

Here we demonstrated unambiguously that, while possibly not required under homeostatic conditions, PUM1 and PUM2 play critical role in HSPC functionality after myeloablation, as demonstrated either in mouse as in human models. Similar phenomenon was previously described in mouse gene disruption models: mice invalidated for the receptor of Thrombopoietin c-mpl just display a slight defect of hematopoiesis (reduced HSPC number) but compatible with life. However, these HSPCs are unable to reconstitute hematopoiesis after myeloablation<sup>300,301</sup>. Discrepancies have been also reported between the phenomenal *in vitro* self-renewal activity of HOXB4 as compared to the mild phenotype observed in HOXB4 deficient mice<sup>302</sup>. Furthermore, in KO organism, during development, the lack of a specific protein can be supplied by the activation of “parallel” pathway of signalization. In mammals, four Pumilio homologs exist, two canonical members (Pum1 and Pum2) and two non-canonical members (Puf-A and C14orf21) and we can't exclude that mechanisms of compensation could act during development.

It will be interesting to use the *Pum1* or *Pum2* KO cells and mice to devise long-term hematopoietic reconstitution experiments, reconstituting wild type mice with KO HSCs, getting them away from the probable compensative alterations present in the KO organism where they have developed. Vice versa we could reconstitute KO mice with wild type HSCs, evaluating possible perturbed mechanisms in the hematopoietic niche. Anyway, to shed more light on Pum action, a double KO for Pum1 and Pum2 could be really useful to elucidate the presence of possible compensations.

However, as demonstrated by our rescue experiments, one Pum protein is not able to compensate the lack of the other protein, at least *in vitro*. In fact, we were able to

rescue *Pum1* KD induced loss of cell expansion and CFC potential by reintroducing murine PUM1 cDNA, insensitive to the sh*Pum1*. On the opposite, we weren't able to rescue *Pum2* KD phenotypes by overexpressing *Pum1*. These results confirmed that the sh*Pum1* activities were not "off-targets" effects, and validated mPUM1 activities suggesting that PUM1 and PUM2 exhibit non-redundant functions and thus PUM1 and PUM2 are not interchangeable. These last results are in agreement with our work in which we compared, by gene array, the transcripts modulated upon KD of *Pum1* and *Pum2* in human and murine HSCs and with the work of Galgano and colleagues. In our work we identified different subsets of gene regulated by *Pum1* and *Pum2* both in human and murine HSCs, in which just about the 50% of the modulated genes were common to PUM1 and PUM2 KD. In the work of Galgano and colleagues in HeLa cells, researchers have purified and sequenced all the mRNAs directly bound to human PUM1 or PUM2, demonstrating that 1153 different targets were specifically bound by PUM1, 138 specifically by PUM2 and 613 by both PUM1 and PUM2<sup>207</sup>. These results suggest that PUM proteins, at least for a part of their tasks, are not redundant, and that the proper action of the two proteins is required.

### **7.3. Pum proteins and cellular stress**

As we mentioned, even if *Pum1* and *Pum2* KO didn't display any obvious defect in hematopoiesis under homeostatic conditions, we demonstrated that they have an important role in stressed hematopoiesis. Different studies have shown that in normal conditions, Pum proteins are localized in the cytoplasm with a feeble granular localization. When cells undergo cellular stress as oxidative stress or heat shock, Pum proteins acquire a more pronounced granular localization. Vessey and colleagues demonstrated that these granules are stress granules<sup>197</sup>. Stress granules are dense aggregation in the cytosol, composed of proteins and RNAs, appearing

when the cells are under stress<sup>303,304</sup>. The RNA molecules stored are stalled at translation in pre-initiation complexes. The purpose of stress granules might be to protect RNAs from harmful conditions. The accumulation of RNAs into dense globules could keep them from reacting with harmful chemicals and safe-guard the information coded in their RNA sequence. Stress granules might also function as a decision point for untranslated mRNAs. Molecules can go down one of three pathways: further storage, degradation, or re-initiation of translation.

During our *in vitro* experiments on LSK cells, we noticed that shPum1 or shPum2 transduced LSK cells displayed a higher level of fragility, as compared to shC cells, each time they had to face a source of cellular stress. To examine this kind of behavior and to address additional roles of Pum protein in HSC biology, we studied the effects of Pum1 or Pum2 KDs on genomic stress, and in particular in mechanisms related to double strand break DNA repair. We didn't notice any evident role of Pumilio on DNA damage response. Further studies on Pum proteins and oxidative stress are now in progress in our team.

#### **7.4. Are Pum1 and Pum2 effectors of the Delta4/Notch signaling?**

As presented in Paper 1, murine *Pum1* and *Pum2* have been found to be overexpressed in HSCs upon activation in culture by the self-renewal factor Delta4 Notch ligand. Activation of LSK cells upon Delta4-reinforced signaling leads, after a 7-day culture: to a decreased cell expansion with a higher proportion of cells blocked in the G0 phase of the cell cycle, to a higher proportion of LSK cells, and a higher CFC potential. To assess to which extent Pum1 and Pum2 contribute to the Delta4/Notch activity in murine HSCs, LSK cells were transduced with shC, shPum1 or shPum2, and sorted GFP+ cells were plated onto mbDII4 stroma. The results showed that shPum-transduced LSK cells still displayed a reduced cell expansion upon culture onto mbDII4 stroma, as compared to cells grown onto control stroma.

Culture of shC-transduced LSK cells onto mbDII4 still enhanced the generation of CFC. In contrast, Pum1- or Pum2-KD LSK cells were impaired in their capacity to generate CFC onto control stroma, and exposure to mbDII4 only slightly augmented these capacities. These results indicate that if the absence of Pum1 or Pum2 profoundly altered the properties of LSK cells to expand and form CFC, mbDII4 still retained some effects on LSK cells. Thus, Pum1 and Pum2 appear as front-line players necessary for the maintenance of HSCs functions, but does not seem directly implicated in mbDII4 activity. It would be interesting to establish whether Pumilio proteins are master regulators responsible for the establishment of the genetic program that maintain the primitive function of stem cells, or whether PUM members are just a part of this genetic program and they are essential for stem cell self-renewal even without being able to reprogram stem cell fate by them self. One way to answer these questions is to overexpress *Pum1* in murine LSK cells.

### **7.5. Effects of Pum1 overexpression in LSK cells**

After having demonstrated that Pum1 or Pum2 depletion was deleterious for both *in vitro* and *in vivo* HSPC functions, we wanted to perform mirror experiments by overexpressing Pumilio proteins to evaluate their hypothetic roles as self-renewing factors.

mPum1-transduced LSK cells displayed a reduction in total cell expansion, as compared to control populations. Strikingly, analysis of the percentage of GFP<sup>+</sup> cells throughout the culture revealed a drop in the percentage of GFP<sup>+</sup> cells in Pum1-transduced populations during the culture. This loss of GFP expression suggests a proliferation disadvantage or an enhanced apoptosis of Pum1/GFP positive cells. Nevertheless we didn't found difference in apoptosis between *Pum1* overexpressing and control cells, leaving open the hypothesis of cell cycle arrest in more mature Pum1 expressing cells (evaluation is now in progress in our team).

Thus, analysis of the CFC potential of control and Pum1-transduced cells at day 10 revealed an identical CFC potential but, interestingly, when 70% of colonies of control population were GFP<sup>+</sup>, reflecting somehow the 60% of GFP<sup>+</sup> cells at the end of the culture, 95% of Pum1-transduced colonies were GFP positive although they derived from a population with only 5% of GFP<sup>+</sup> cells. These data indicate that the 5% of remaining GFP<sup>+</sup> cells at the end of the culture were incredibly enriched in CFC, suggesting not only that a well defined level of Pum1 expression is compatible with CFC survival and expansion, but also that these cells are capable to retain primitive CFC potential.

Further studies, now in progress in our team, will shed more light on this striking effect exerted by Pum1 overexpression.

#### **7.6. Identification of genes implicated in Pumilio effects on HSCs.**

In order to identify the factors responsible for the effects obtained upon depletion of Pum1 or Pum2 in HSCs, we have devised a whole transcriptome comparative study in murine LSK CD150<sup>+</sup> and in human CD34<sup>+</sup> CD38<sup>-</sup> cells using gene array. Statistical analysis revealed that: in mouse cells, 434 genes were modulated specifically by Pum1, 509 specifically by Pum2, and 527 common to both Pumilio proteins, while in human cells, 725 genes were specifically modulated by *PUM1*, 572 by *PUM2*, and 542 by both. Biological interpretation of these gene expression modifications using Ingenuity pathways analysis (IPA), focusing particularly on Gene Ontology (GO) Biological Process terms converge, for the majority of the genes, in three GO categories: “Cell Death, cell cycle, cell growth and Proliferation”. Nevertheless, the fold changes of the putative downstream effectors of Pum proteins were too weak and difficult to validate by RT-qPCR. These data strongly suggested that the downstream Pum targets early modulation happens at a translational level without impacting the rate of mRNA.



Classical Western blot analysis of PUM targets in LSKCD150+ cells is not appropriate. We need at least 30-40 µg of proteins to screen for proteins, and it is very difficult to recover these amounts from an homogeneous population of LSKCD150+ cells because of the limited starting material (around 5.000-6.000 CD150<sup>+</sup> LSK cells per mouse). Furthermore, we cannot wait for the cells to proliferate (and we have to keep in mind that shPum1 and shPum2 cells display reduced cell expansion, adding another technical obstacle) since at this time the resulting populations will become quite differentiated, deprived of HSCs, and extremely heterogeneous. Furthermore, 5 days post transduction would be too late to check for changes in protein levels for phenotypes that appear at day 3 or 4 post shRNAs transduction. All this technical problems prompted us to search for an alternative way to study the modification of the protein levels upon transduction with shPum1 and shPum2. These techniques will be discussed below.

## 8. CONCLUSIONS AND PERSPECTIVES

Activation of endogenous HOXB4 signaling (in human HSCs) or Notch signaling by Delta-like (Dll) -4 ligand (in murine HSCs) leads to an increased maintenance of the primitive potential of HSCs. Transcriptomic analysis of these cells converged on an increased expression level of Pum1 and Pum2 both in murine and human HSCs.

We have demonstrated unambiguously that Pum1 and Pum2 are crucial for stem cells functions in murine and human system both *in vitro* and *in vivo*. KD of Pumilio proteins leads to reduced cell expansion due to cell cycle arrest and increased apoptosis. Furthermore, lack of Pum1 or Pum2 impaired CFC potential *in vitro* and long-term reconstitution potential *in vivo*. Rescue experiments suggested that Pum1 and Pum2 are not interchangeable although they have similar roles.

Pum1 and Pum2 can modulate thousands of targets and these targets are mostly regulated at the translation level. This fine combinatorial regulation of thousand of mRNAs is often achieved upon interaction with several partners that can be different and responsible for changes in Pum proteins affinity for their targets, depending on the cellular context. Another level of complexity is added by new evidences suggesting that Pum proteins act often in cooperation with the miRNA system.

This complicated and fascinating level of control, after the transcriptional one, could be used to modulate large genetic programs, regulating strength and speed of protein translation. Pumilio proteins could be master regulators, together with other RNA binding proteins and miRNAs, of the so called “RNA operons”: sets of mRNAs with coherent function that could be modulated at the same time and that are responsible for precise functions at determined time during development. In this case we can imagine that the emerging properties of Pumilio regulation consist in sustained stem cells function and maintenance.

However, the nodal points to understand the principles of Pum proteins action are:

- 1) To identify the targets indirectly or directly modulated,
- 2) To identify the partners involved in Pum1 and Pum2 actions.

To assess the modulations of protein levels induced by Pum1 or Pum2 KD in HSPC context, we can use two techniques.

The first, the NanoPro, can allow us to quantify protein levels in really small samples, theoretically consisting of just hundreds of cells. The disadvantage of this approach is that it is based on immunolabeling and we have to assess the expression of proteins decided *a priori*.

For global analysis of protein status, instead, we are devising experiments involving the application of the SILAC (stable isotope labeling by amino acids in cell culture) method. SILAC is an approach for incorporation of a label into proteins for mass spectrometry (MS)-based quantitative proteomics. With such approach we can compare even three different populations and we can have a semi-quantitative evaluation of the whole proteome of a given population compared to another one. To realize this approach, we need at least 200 µg of proteins for each population tested. This prompted us to use this technique on the HP7 (Hematopoietic Precursors 7) cell line as surrogate for murine cells and UT7 cell line (a cell line constituted by myeloid progenitors-like cells) for human cells.

To identify the targets directly recognized by PUM1 and PUM2, we will use a technique based on immunoprecipitation of PUM proteins followed by purification and sequencing of associated mRNAs (PAR-CLIP). The PUM/mRNA complexes will be purified. mRNAs bound to these complexes will be sequenced in order to identify the

direct Pum targets. This approach was already used by Galgano and colleagues to find Pum related targets in HeLa cervical cancer derived cells but we want to find Pum targets specifically involved in the stem cell context.

To seek for Pum partners, the complexes isolated after immunoprecipitation of PUM1 or PUM2 will be used to employ liquid-chromatography coupled to mass-spectrometry (LC-MS) assay, allowing us to identify Pum-related proteins.

Given that the function of Pum proteins is related to stem cell maintenance and given that abnormal stem cells seems to be at the origin of cancer-related disease<sup>305</sup>, Pum functions will be evaluated in different cancer cellular samples. At the moment, preliminary results on acute myeloid leukemia patients seem to be encouraging, prompting us to validate Pum function in other stem cells related hematopoietic diseases.



## **ANNEX I**





## **The Notch Delta-4 ligand helps to maintain quiescence and primitive potential of Hematopoietic Stem/Progenitor Cells through activation of a key gene network.**

Cyril Catelain<sup>1,2,3</sup>, Fabio Michelet<sup>5,6</sup>, Sonia Poirault-Chassac<sup>1,2,3</sup>, Thierry Kortulewski<sup>4</sup>, Diana Tronik-Le Roux<sup>4</sup>, William Vainchenker<sup>1,2,3</sup> and Evelyne Lauret<sup>5,6</sup>

1. INSERM, U1009, 114 rue E. Vaillant, Villejuif, F-94805 ;
2. Institut Gustave Roussy, Villejuif, F-94805 ;
3. Université Paris-Sud 11, Orsay, F-91405 ;
4. CEA, 18 rue du Panorama, BP15, Fontenay-aux Roses, F92265
5. Institut Cochin, Université Paris Descartes, CNRS (UMR 8104), Paris, France.
6. Inserm, U1016, Paris, France.

Corresponding author : E. Lauret

Address U1016 INSERM, Institut Cochin, DRC Dep<sup>t</sup>  
22 rue Méchain 75014 PARIS FRANCE  
Tel : 33 1 40 51 64 05 (office)  
Fax : 01 43 25 11 67  
E mail : [evelyne.lauret@inserm.fr](mailto:evelyne.lauret@inserm.fr)

Fabio Michelet is fellow from the French Research Ministry. This work was supported by grants from INSERM, Institut Gustave Roussy (Contrat de Recherche Clinique, n°2000.10 and CRI-SPS-2003-02), and ATC Cellules Souches, LNC, and ARC. We thank Yann Lécuse for cell sorting, and Dr Isabelle Vigon, Dr Isabelle Dusanter, and Gillian Butler-Browne for critical input regarding this work.

## Abstract

Understanding mechanisms regulating hematopoietic stem cell function represents a major challenge in improving cell therapy protocols. Our previous work has shown that the Delta4/Notch pathway maintained a higher proportion of human CD34<sup>+</sup>CD38<sup>low</sup> cells in the G0/G1 phase, and retained their LTC-IC potential, independently of cell divisions. To try to discover the mechanisms underlying such activities, we have developed a murine model. We show that activation of the Delta4/Notch pathway in murine Lin-Sca<sup>+</sup>ckit<sup>+</sup> (LSK) cells cocultured on stroma expressing membrane-bound Delta4 maintained a significant proportion of the cells specifically in the G0 phase. Furthermore, LSK cells exposed to Delta4/Notch retained their LTC-IC potential for 7 days, and still displayed a long-term repopulating ability when injected into lethally irradiated recipient mice, contrary to control LSK cells. Both effects were independent of the presence of p21<sup>Cip1/Waf1</sup>. To further decipher Notch/Delta4 mechanisms, we looked for the modulation of downstream target gene expression. We observed a decreased expression of cell cycle genes such as Cyclin D1, D2, and D3, and an upregulation of stemness gene expression such as Bmi-1, Gata-2, HoxB4 and c-Myc. In addition, the transcriptional screening has also highlighted new downstream posttranscription factors, named Pumilio-1 and -2, as part of the stem signature associated with the Delta4/Notch signaling pathway.

## Introduction

Notch is a well-conserved signaling pathway and its function in cell fate determination is crucial during embryonic development and in the maintenance of tissue homeostasis during adult life. Its activation depends on cell-cell interactions that are essential for the generation of cell diversity from initially equivalent cell populations. Accumulating evidence suggests that Notch signaling is active at multiple points during hematopoiesis (1). Notch signaling is essential for the emergence of intraembryonic hematopoietic stem/progenitor cells (HSPCs) in the aorta-gonad mesonephros in the developing embryo. It appears dispensable for the maintenance of HSPCs in the adult bone marrow compartment through two complementary approaches blocking the canonical Notch signaling within HSPCs (one with a dominant negative form of the mastermind-like protein, and the other inactivating the Rbp-J gene) (2, 3). While it is well established that Notch signaling controls HSPC differentiation toward the T cell lineage, some controversies exist regarding its role on megakaryocytic (4, 5) or erythrocytic commitment (6). Notch1 is a major oncogene, as most patients with T-cell lymphoblastic leukemia harbor activating Notch1 mutations (7) while the Notch pathway exerts a tumor-suppressor function in myeloid leukemia (8). Despite its dispensable role on the *in vivo* maintenance of adult HSPCs, multiple studies support a role for Notch in the maintenance of HSPC functions in culture. Overexpression of Notch1 or Notch2 or their downstream target gene Hes1 in bone marrow HSPCs resulted in increased HSPC numbers and/or enhanced self-renewal (9-11). Coculture experiments of murine HSPCs with Notch ligand-expressing stromas (12) also increased the number of HSPC's while immobilized Notch ligands promoted early T cell differentiation and generation of multilineage increases in the number of HSPCs with short-term lymphoid and myeloid repopulating activity (13). A recent report describes Notch-mediated expansion of human cord blood HSPCs capable of rapid myeloid reconstitution (14).

We have previously reported that, when membrane-bound, the Notch ligand Dll4 (mbDll4) counteracts the proliferation of human CD34<sup>+</sup> cells induced by cytokines, by maintaining a higher proportion of cells in the G0/G1 state. Furthermore, mbDll4 preserves a high LTC-IC potential in output CD34<sup>+</sup> cells, even in cells having performed a similar number of divisions, indicating that LTC-IC retention was mediated by mechanisms independent of the mitotic history (15). These effects of mbDll4 on human CD34<sup>+</sup> cells required the  $\gamma$ -secretase activity, and were correlated with an overexpression of some well-known target genes of the canonical Notch signaling (Hes-1, Hey-1 and -2) (16). In this study We further wanted to decipher the mechanisms underlying mbDll4 activity on HSPCs. We show that mbDll4 counteracts proliferation of murine Lin<sup>-</sup>Sca-1<sup>+</sup>ckit<sup>+</sup> (LSK) cells by specifically keeping a higher fraction of the cells in the G0 state. Furthermore, mbDll4 limits the loss of their *in vitro* and *in vivo* reconstitutive potential. Both effects were independent of the presence of p21<sup>Cip1/Wap1</sup>. Transcriptome analysis of LSK cells activated by the Dll4/Notch pathway revealed a decreased expression of cell cycle genes such as Cyclin D1/2/3, an enhanced expression of the Notch target gene Hes-1, of self-renewal genes (Bmi-1, HoxB4, Gata-2 and c-Myc) and of Pumilio-1 and -2, genes involved in stem cell expansion in other models. These genes might constitute important targets for further studies.

## **Materials and methods**

### **Mice**

C57BL/6-Ly5.2, C57BL/6-Ly5.1 and CF1 mice (8-20 weeks old) were purchased from Janvier CERJ (Le Genest-St-Isle, France). p21<sup>Cip1/Waf1-/-</sup> mice (17) were provided by P. Leder. All mice were maintained in the Gustave-Roussy Institute facilities under specific pathogen-free conditions.

### **LSK cell purification**

Lineage negative Sca-1 and c-Kit positive (LSK) cell purification was performed as described by Spangrude et al. (18). Primitive Lineage negative cells (Lin<sup>-</sup>) cells were enriched by incubating bone marrow cells with lineage-specific monoclonal antibodies (mAb): anti-Mac-1 (M1/70), anti-GR-1 (RB6-8C5), anti-erythroid cells (TER119), anti-B220 (RA3-6B2), anti-CD4 (GK1.5), and anti-CD5 (Lyt-1), followed by a depletion of Lin<sup>+</sup> cells by the immunomagnetic bead technique (sheep anti-rat IgG, Dynal, Oslo, Norway). The Lin<sup>-</sup> fraction was incubated with a phycoerythrin (PE)-conjugated goat anti-rat IgG antibody and stained with a fluorescein-isothiocyanate (FITC) anti-Sca-1 mAb (E13-161-7) and an allophycocyanin (APC) anti-c-Kit mAb (2B8). In some experiments, Lin<sup>-</sup> and LSK cells were analyzed using biotinylated mAbs raised against differentiation markers (Gr-1, Mac-1, B220, CD3 and Ter-119, mouse lineage panel, Becton Dickinson), revealed using streptavidin-APC-Cy7 molecules, and stained with the (FITC) anti-Sca-1 mAb and an (APC) anti-c-Kit mAb. Isotype-matched antibodies were used as controls. Sorting of the LSK fraction was performed using a FACSvantage (Becton-Dickinson).

### **Culture experiments**

Each of the S17 stroma cells (C/S17 and mbD114/S17) were obtained and cultured as previously described (15). LSK cells were cultured in 24-well plates coated with confluent

C/S17 or mbDl14/S17 in  $\alpha$ -MEM containing 10% FCS (Stem Cell Technology, Vancouver, Canada). The following cytokines were added: murine Stem Cell Factor [ $\mu$ SCF], human Flt-3 ligand [HuFlt3-L], human interleukin-6 [IL-6], each at 100 ng/mL, and human interleukin-11 [IL-11] at 10 ng/mL. Some LSK cells were cultured on immobilized Dll4Fc (10  $\mu$ g/ml) on plastic as previously described (15), irrelevant human IgG1 serving as a negative control. Some cultures were performed in the presence of 10mM DAPT (a gamma-secretase inhibitor: N-{3,5-difluorophenacetyl)-L-alanyl}-S-phenylglycine-t-butyl ester; Calbiochem, San Diego) or DMSO as vehicle.

### **Cell-cycle analysis**

*Carboxyfluorescein Succinimidyl Ester (CFSE) Staining.* Before coculture, sorted LSK cells were incubated with CFSE 1  $\mu$ M for 10 minutes at 37°C allowing protein staining (Oostendorp, Blood, 2000). Cell divisions were analyzed on a FACSort cytometer after two days in culture (Becton Dickinson). *Pyronin/Hoescht analysis.* FITC-anti-CD45 mAb labelled cells were incubated with 10  $\mu$ g/ml Hoechst 33342 and 1  $\mu$ g/ml Pyronin Y (RNA dye) (Sigma Chemical Co, St Louis, MO) at 37°C for 45 minutes in Hanks balanced salt solution (HBSS) medium supplemented with 2mM Hepes, 10% FBS and 1mg/l glucose (19). *Ki-67 analysis.* Cells were labelled with APC-anti-CD45 mAb, incubated with a cytofix-cytoperm solution (Becton Dickinson), washed with a PermWash solution (Becton Dickinson), and finally incubated with FITC-anti-Ki-67 mAb and DAPI before LSRII cytometer analysis (BD).

### **Long-Term Culture-Initiating Cell (LTC-IC) and CFC assay**

Long-term culture-initiating cell (LTC-IC) assay was performed as described by Sutherland (20). Briefly, sorted LSK cells were placed on a MS-5 feeder layer in microwell plates, and incubated at 33°C in a humidified incubator with 5% CO<sub>2</sub> in air. Cultures were fed weekly by half-media change. The cultures were recovered after 4 weeks and assayed for the presence of myeloid culture colony-forming unit (CFU-C) using a methylcellulose medium (M3234; Stem

Cell Technologies, Vancouver, BC, Canada) supplemented with cytokines. After 7 to 10 days of growth at 37°C, colonies were scored with an inverted microscope based on their morphology.

### **Long-term competitive repopulation assay**

C57Bl/6 (Ly5.2) mice were used as recipients, whereas Lin<sup>-</sup> or LSK cells were prepared from C57Bl/6 (Ly5.1) donors. Lethally irradiated recipients were cotransplanted with  $1.5 \times 10^5$  Ly5.2 BM cells together with various amounts of cells exposed to S17 stromas or immobilized Dll4Fc protein for 7 days, injected into the retroorbital sinus of mice. Hematopoietic reconstitution was assessed 20 weeks after transplantation through analysis of BM. Cells were stained with anti-CD45.1-PE, and analyzed by flow cytometry. Animals displaying greater than 0.1% of CD45.1-PE positive cells were considered as positive for repopulation.

### **Microarray**

Total RNAs from sorted cell populations were isolated using the RNeasy kit (Qiagen) and further amplified using the MessageAmp aRNA kit (Ambion, Austin, TX). The integrity of the RNA samples was verified using an Agilent Bioanalyzer (Palo Alto, CA). For each hybridization, 2 µg of amplified RNA were reverse-transcribed using the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and aminoallyl-dUTP (Sigma). The cDNAs were labelled and hybridized to the murine microarray manufactured in CEA microarray platform as described (21).

Slides were scanned with a Genepix 4000 microarray scanner (Axon Instruments, Molecular Devices, Sunnyvale, CA). Images were processed to acquire Cy5 and Cy3 fluorescence intensities for each hybridized spot using Genepix Pro 4.0 software (Axon Instruments). Spots or areas of the array with obvious blemishes were flagged and excluded from subsequent analysis. Result files were imported into GeneSpring 6.1 software (Silicon Genetics, Agilent)



for further analyzes. To eliminate dye-related artefacts in 2-color experiments, intensity-dependent Lowess normalization was performed. The results represent the average of 8 independent measures and were deposited in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) with the GSE 5135 accession number. Differentially expressed genes were obtained by analysis of variance using ANOVA parametric test ( $P < 0.01$ ) followed by Benjamini and Hochberg multiple test correction. For stringent comparisons, only probes with a hybridization value in at least 6 of 8 replicates were considered for further analysis.

### **Quantitative reverse-transcriptase (RT)-PCR**

Total RNA was extracted from cells using the Trizol reagent (Invitrogen). Synthesis of first-strand cDNAs was performed using oligo(dT) and Superscript II reverse transcriptase (Invitrogen). PCR was performed using SYBRGreen PCR Master Mix (PE Applied Biosystems, Foster City, CA). PCR was monitored with a GeneAmp 5700 sequence detection system (PE Applied Biosystems). Quantification was performed using the  $C_t$  values method. The primers used are indexed in Supplementary data Table S1.

### **Statistical Analysis**

All measurements were performed at least three times. Continuous variables were analyzed using Student's t-test for paired values. The measured values were expressed as mean  $\pm$  SEM. p-values of less than 0.05 were considered to be statistically significant.

## Results

### **mbDll4/S17 maintained a higher fraction of cells in the G0 phase**

We previously showed that exposure of human CD34<sup>+</sup>CD38<sup>low</sup> cells to stroma expressing membrane-bound Delta4 (mbDll4/S17) reduced cell expansion by keeping a fraction of cells in the G0/G1 state, and limited the loss of the primitive potential by mechanisms independent of the mitotic history (15). To generalize these mechanisms, we assess whether mbDll4 activities described for human cells, could be reproduced with murine HSPCs. To this end, murine bone marrow LSK cells were cultivated with cytokines on control C/S17 and mbDll4/S17 stromas. After 7-day of culture, the fold increase in total cell number was significantly reduced (1200±90 onto C/S17 versus 600±72 onto mbDll4/S17,  $p=0.00002$ , Figure 1A), indicating that mbDll4/S17, as for human CD34<sup>+</sup> cells, reduced murine nucleated cell expansion. Apoptosis analysis did not revealed any difference in the % of apoptotic cells between C/S17 and mbDll4/S17 population at day 2 (12±5 for C/S17 versus 13±4 for mbDll4/S17 culture) nor at 7 days of culture (data not shown), thus precluding a potential role of apoptosis in the reduced cell expansion. Labeling of LSK cells with CFSE to simultaneously monitor cell divisions at 2 days of culture showed that the proportion of cells having completed 0 or 1 division was significantly higher in the population exposed to mbDll4/S17 as compared to C/S17 (3±0 for C/S17 versus 6±0 for mbDll4/S17 culture at 0 division,  $p=0.02$  and 19±0 for C/S17 versus 15±0 for mbDll4/S17 culture at one division,  $p=0.04$ , respectively), while the proportion of cells having performed 4 divisions was lower in the population exposed to mbDll4/S17, as compared to cells grown on C/S17 (18±2 for C/S17 versus 12±2 for mbDll4/S17 culture,  $p=0.03$ , Figure 1B). To precisely decrypt the modifications in the cell cycle, RNA and DNA contents of LSK cells were measured by Pyronin-Y (PY) and Hoechst 33342 (Ho) staining, respectively, and the fraction of cells in G0 or G1 or S/G2/M was determined (Figure 1C). In the population exposed to mbDll4/S17,

we observed a higher proportion of cells in G0 ( $14 \pm 4$  % for LSK cells exposed to mbD114/S17 versus  $6 \pm 3$  % for cells exposed to C/S17) at the expense of cells in G1, without any significant modification in the proportion of cells in S/G2/M. These data confirmed that, as for human CD34<sup>+</sup> cells, mbD114 reduced LSK cell proliferation, and highlighted the fact that mbD114 counteracts the stimulatory proliferative effects of cytokines by keeping a fraction of LSK cells out of the cell cycle.

### **mbD114 inhibited myeloid differentiation and maintained primitive potential of HSPCs.**

The primitive potential was first assessed by following the % of LSK cells throughout 7 days of culture on C/S17 and mbD114/S17. A progressive decline in the % of LSK cells in the control culture became significant at day 2, and this difference increased progressively to reach at day 7, 5-fold more LSK cells on mbD114/S17, as compared to C/S17 ( $38 \pm 0$ % on mbD114/S17 versus  $7 \pm 4$ % of LSK cells on C/S17,  $p=0.003$ , Figure 2A). Such maintenance of LSK cells led to a 3.5-enhanced production of output LSK in mbD114 cultures at day 7 (Figure 2B). We did not notice any significant difference in the proportion of the different lineages among Lin<sup>+</sup> cells, indicating that in our culture conditions, mbD114 did not favour differentiation toward a particular myeloid lineage (Figure S1A).

To further characterize the primitive potential of cells generated after 7 days of culture on both stromas, we monitored their *in vitro* CFC and LTC-IC as well as their *in vivo* potential. mbD114/S17 maintained a higher proportion of clonogenic colonies ( $61 \pm 11$  CFC per 500 output Lin<sup>-</sup> cells on mbD114/S17 versus  $37 \pm 18$  CFC per 500 Lin<sup>-</sup> on C/S17,  $p=0.002$ , Figure S1B). The increased CFC number was the result of a general increase in all types of colonies (CFU-GM, BFU-E, and mixed colonies). Furthermore, colonies from Lin<sup>-</sup> cells generated on mbD114/S17 displayed a larger size, as compared to those derived from Lin<sup>-</sup> cells on C/S17 (data not shown).

The LTC-IC potential of output LSK cells generated on mbD114/S17 was preserved at day 7,

compared to the one of input bone marrow LSK cells ( $18 \pm 3.5$  LTC-IC-derived CFC/1000 input LSK versus  $27 \pm 28$  for 1000 output LSK cells from mbDII4 culture) while this potential was strongly impaired in output LSK cells generated onto C/S17 ( $2 \pm 3$  LTC-IC-derived CFC/1000 output LSK cells,  $p=0.03$ , Figure 2C). This leads to a strongly enhanced expansion of LTC-IC-derived CFC ( $338 \pm 27$  LTC-IC-derived CFC for mbDII4/S17 versus  $8 \pm 2.5$  LTC-IC-derived CFC for C/S17 from 1,000 input LSK cells,  $p=0.002$ , Figure 2D). The *in vivo* repopulating activity was assessed through two series of experiments, in which we examined the long-term reconstitution in the bone marrow ( $> 16$  weeks, Figure 2E). In the first experiment, to cast off the expansion difference between control and mbDII4 cultures, we injected all the progeny of Ly5.1 LSK cells grown for 7 days on C/S17 and mbDII4/S17, whatever the number of cells, together with a fixed number of Ly5.2 bone marrow competitor cells into irradiated Ly5.2 recipients. While no chimerism was detected at 16 weeks in mice infused with cells grown on C/S17 (independent of the number of cells injected), the persistence of a low chimerism (ranging from 0.1 to 1%) was observed with the progeny of 30 and 100 LSK cells cultivated on mbDII4/S17. In a second series of experiments, we examined the reconstitutive potential of sorted output Lin<sup>-</sup> cells from control and mbDII4 cultures. The long-term reconstitution potential was higher in output Lin<sup>-</sup> cells grown on mbDII4/S17, as demonstrated by the absence of chimeric mice for control population versus 60 and 30% of chimeric mice (chimerism comprised between 0.1 and 0.5 %). These data indicated that exposure to mbDII4 also limits the loss of the *in vivo* long-term primitive potential. Thus, as for human CD34<sup>+</sup> cells, exposure of murine LSK cells to mbDII4 maintains their primitive potential.

To confirm the implication of Notch signaling in mbDII4 activity, we analyzed the requirement of the  $\gamma$ -secretase complex. When LSK cells were cultured on mbDII4/S17 for 7 days in the presence of DAPT (an inhibitor of the  $\gamma$ -secretase), cell expansion was restored ( $594 \pm 142$  for LSK cells onto mbDII4/S17 in the presence of DAPT versus  $393 \pm 79$  for LSK cells cultured on C/S17 with DMSO, Figure S2A), and the maintenance of a high proportion

of LSK cells on mbDll4/S17 was abrogated ( $4\pm 1$  versus  $26\pm 5$  % of LSK cells, respectively, Figure S2B).

### **p21<sup>Cip1/Waf1</sup> is dispensable for mbDll4 activity**

To evaluate the impact of the cell cycle regulator p21<sup>Cip1/Waf1</sup> on the activity of mbDll4 on HSPCs, we used LSK cells from control (WT) and p21<sup>Cip1/Waf1</sup>-deficient mice (p21<sup>-/-</sup>), and cultivated them for 7 days on C/S17 and mbDll4/S17. In the absence of p21<sup>Cip1/Waf1</sup>, while expansion of nucleated cells was slightly enhanced, as previously described (22) ( $630\pm 92$  fold increase versus  $527\pm 66$  for p21<sup>-/-</sup> and WT, respectively, Figure 3A), a reduced cell expansion on mbDll4/S17 was still observed with p21<sup>-/-</sup> HSPCs, indicating that p21<sup>Cip1/Waf1</sup> was not implicated in the antiproliferative activity of mbDll4. We next addressed the implication of p21<sup>Cip1/Waf1</sup> in maintaining the primitive potential on mbDll4/S17. A 7-day culture of p21<sup>-/-</sup> LSK cells on mbDll4/S17 still maintained a higher proportion of LSK cells ( $41\pm 2$  versus  $19\pm 2$  for p21<sup>-/-</sup> grown on mbDll4/S17 and C/S17, respectively,  $p=0.02$ , Figure 3B), a higher CFC potential in output Lin<sup>-</sup> cells (Figure 3C), and an enhanced expansion of LTC-IC-derived CFC ( $889\pm 23$  for mbDll4/S17 versus  $31\pm 3$  for C/S17,  $p=0.001$ , Figure 3D). Thus, the absence of p21<sup>Cip1/Waf1</sup> did not alter the response of LSK cells to mbDll4, thus ruling out a potential role of p21<sup>Cip1/Waf1</sup> in the inhibition of cell proliferation as well as in the maintenance of the primitive potential conferred by mbDll4.

### **Identification of downstream target genes modulated by mbDll4**

To identify potential mbDll4 target genes independent of the S17 cellular context, we used a chimeric Dll4Fc protein consisting of the extracellular domain of human Dll4 fused to the Fc portion of a human IgG1 immunoglobulin (Dll4Fc). The similarity of the LSK cell response between immobilized Dll4Fc and mbDll4, previously observed with human C34<sup>+</sup>CD38<sup>low</sup> cells (15) was confirmed for several criteria. LSK cells cultured for 7 days on immobilized Dll4Fc displayed a 2-fold reduction in cell expansion (Figure S3A), a higher proportion of

LSK cells in the G0 state ( $13 \pm 2$  % for LSK cells exposed to Dll4Fc versus  $7 \pm 1$  % for control LSK,  $p=0.001$ , Figure S3B), maintained a higher proportion of LSK cells ( $22 \pm 7$  % for Dll4Fc cultures versus  $5 \pm 2$  for control cultures,  $p=0.001$ ), retained a primitive potential, evidenced by an LTC-IC potential identical to the input cells, leading to an enhanced expansion of LTC-IC-derived CFC ( $16 \pm 3$  for control culture versus  $323 \pm 19$  for Dll4Fc cultures,  $p=0.04$ , Figure S3D), and a higher *in vivo* reconstitution potential when inoculated into irradiated mice (78% of chimeric mice for Dll4Fc cultures versus 12% for control culture, Figure S3E).

To reveal signaling pathways involved in the effects of Dll4Fc on LSK cells, we performed a differential transcriptomic analysis of LSK cells cultured for 6 or 12 h with or without Dll4Fc. Using the ANOVA parametric test ( $p < 0.01$ ) and Benjamini and Hochberg multiple testing correction we found 559 and 64 probes differentially expressed in LSK cells, twelve hours after exposure or not to Dll4Fc, respectively. Very few genes were modulated at 6hr (data not shown). Interestingly, one of the upregulated genes at this early time was HoxB4 ( $1.8 \pm 0.4$ ,  $p=0.01$ ). Changes in gene expression observed at 12h were confirmed by RT-qPCR on a series of 10 selected genes encompassing all the scale of modulations (Figure 4A), validating thus the microarray data. Genes such as Gata-2 ( $2.4 \pm 0.4$ ,  $p=0.04$ ), Hes-1 ( $4.7 \pm 1.8$ ,  $p=0.01$ ), Bmi-1 ( $5.1 \pm 2.3$ ,  $p=0.01$ ), and c-Myc ( $13.9 \pm 1.5$ ,  $p=0.007$ ) were already known to be involved in *Notch signaling*; the chaperone Hsp90 protein ( $1.4 \pm 0.1$ ,  $p=0.01$ ), the polycomb Eed protein ( $1.7 \pm 0.2$ ,  $p=0.002$ ), and the transcription factor Mnt ( $6.9 \pm 1.2$ ,  $p=0.03$ ) were not previously identified as involved in this process. In addition, the post-transcriptional repressor Pumilio-2 identified in Invertebrates and previously described in other models to be involved in stem cell fate or expansion, was also found highly upregulated ( $6.8 \pm 1.1$ ,  $p=0.04$ ). Since PUM1 and PUM2 genes share ~83% overall similarity, the microarray probe could not discriminate between both transcripts. Therefore, we measure the expression levels of Pum-1 by RT-qPCR. The results show that Pum-1 was also upregulated in response to Dll4 ( $2.9 \pm 0.7$ ,  $p=0.04$ ), suggesting that both mammalian Pumilio proteins might potentially play an important role in Notch-ligand Dll4-dependent cell development and differentiation. We also

confirmed by RT-qPCR the down regulation of osteopontin (OPN) ( $0.3 \pm 0.04$ ,  $p=0.01$ ) and the CCL12 chemokine ( $0.3 \pm 0.06$ ,  $p=0.04$ ).

To provide a biological interpretation of these gene expression modifications, we looked for the statistical significance of the enrichment of particular functional categories using Ingenuity pathways analysis (IPA) and DAVID resources (<http://david.abcc.ncifcrf.gov/home.jsp>), focusing particularly on Gene Ontology (GO) Biological Process terms. We found that most of the genes were included in three significant GO categories: “Cell Growth and Proliferation”, “Cell Cycle Process” and “Translation Process” (Figure S4). Based on this and consistent with previous studies, we aimed to enlarge the analysis of cell cycle genes involved in this process by assessing the expression of some key downstream cell cycle target genes modulated by Dll4Fc. As shown in Figure 4B, we found the upregulation of p130 ( $1.33 \pm 0.11$ ,  $p=0.02$ ), E2F4 ( $2.4 \pm 0.18$ ,  $p=0.02$ ) and Rb ( $2.7 \pm 0.41$ ,  $p=0.04$ ) and a downregulation of Cyclin D1 ( $0.26 \pm 0.01$ ,  $p=0.02$ ), Cyclin D2 ( $0.27 \pm 0.02$ ,  $p=0.03$ ) and Cyclin D3 ( $0.38 \pm 0.05$ ,  $p=0.04$ ) after exposure to Dll4-Fc, as compared to the level in control LSK cells. In contrast, the level of expression of p18<sup>INK4c</sup>, p19<sup>INK4d</sup>, p21<sup>Cip1/Waf1</sup>, p27<sup>Kip1</sup>, p57<sup>Kip2</sup>, p107, p130 and E2 cyclin were not modified after exposure of LSK cells to mbDll4 (data not shown).

## Discussion

In accordance with our previous work performed on human CD34<sup>+</sup> cells (15), the development of the murine model confirms that HSPC cultured on mbDll4-expressing stroma displayed a reduction in cell expansion, and maintained their primitive potential. The present study further reveals that the reduced cell proliferation triggered by mbDll4 was mainly due to the maintenance of a larger proportion of HSPC in the G0 state. It also shows that cell culture on mbDll4-expressing stroma limited the loss of their long-term repopulating capacity, and finally leads to the identification of the gene signature of HSPC in response to Notch/Dll4 signaling.

### **Maintenance of a higher proportion of LSK cells in the G0 phase**

The maintenance of quiescence is a key feature for regulating HSPC homeostasis. Using the signaling lymphocyte attractant molecule (SLAM) family markers to identify quiescent HSPCs, several studies report that most HSPCs are localized adjacent to sinusoidal blood vessels in the bone marrow (23) (24) (25). The role of the vascular niche has been demonstrated to maintain HSPC (26) and more recently, endothelial and perivascular cells have been shown to play a key role as main producers of SCF (25), and CCL12 (27). Only a few of the external factors governing HSC quiescence in the vascular niche have been identified among which Tie2/Angiopoietin-1 play a major role (28). Dll4 has been shown to be expressed in epithelial thymic cells (29), and more specifically in endothelial cells (30). We have previously shown that exposure of human CD34<sup>+</sup> cells to mbDll4 maintains a high proportion of cells out of the cell cycle (G0/G1). The present data has shown that this enhancement in the proportion of non-cycling cells is due to a higher proportion of the cells being in G0. Therefore, in spite of our conditions of culture based on strong cytokine activation, activation of the Notch pathway by mbDll4 still maintains a significant proportion



of cells in G0, suggesting that Dll4-expressing endothelial cells participate to the maintenance of HSPC out of the cell cycle.

To get insight into the mechanisms of Dll4 actions, we have analyzed the molecular regulation of the cell cycle machinery after the onset of culture onto Dll4Fc. The role of p21<sup>Cip1</sup> was assessed using p21<sup>Cip1/Waf1</sup>-deficient mice and showed unambiguously that p21<sup>Cip1/Waf1</sup> is not involved in mbDll4 activity. We next examined the transcriptional modulation of some cell cycle genes. The D-cyclin family, cyclin D1, cyclin D2, and cyclin D3, were all expressed, albeit at different levels, in HSPCs (31). Cyclin D1-/-D2-/-D3-/- embryos displayed a reduced numbers of fetal HSPCs, with impairment in their ability to proliferate, revealing a unique requirement for the D-cyclins in the hematopoietic lineage (32). The downmodulation of the 3 D-cyclins observed in response to Dll4 is therefore in agreement with the key role of D-cyclins in HSPC proliferation. E2F4, which can bind to all 3 pRB family members to form the majority of cellular pRB family complexes, has been proposed to play a critical role in coordinating cell cycle exit (33). The retinoblastoma (Rb) family of transcriptional repressors, including the pRb, p107, and p130 proteins, restricts cell cycle entry by regulating E2F gene transcription of positive cell cycle regulators. Conditional deletion of all three Rb family members in adult mice resulted in a robust cell-intrinsic myeloproliferation phenotype, with an increase in HSPC proliferation, and severe defects in self-renewal (34). Therefore, the upregulation of E2F4 and Rb in response to Dll4 is consistent with their role in the cell cycle. Taken together, these findings indicate that Cyclins D and Rb family members may play a critical role in the activity of the Dll4/Notch pathway for the maintenance of the quiescent state.

#### **mbDll4 limits the loss of HSPC potential *in vitro* and *in vivo*.**

Exposure of LSK cells to mbDll4, not only preserves their *in vitro* CFC and LTC-IC potential, but also limits the loss of the *in vivo* long-term reconstitutive potential, as compared

to LSK cells cultured in control conditions. A comparable study has been previously performed using a chimeric Dll1Fc, immobilized on plastic (14). Contrary to Dll4, *in vitro* exposure of LSK cells to Dll1Fc induces a proliferation comparable to control populations.

Exposure of LSK cells to Dll4 was correlated with an enhanced expression of Hes-1, the well-known Notch target gene. A recent study suggests that  $\gamma$ SE complex/Notch signaling controls early HSPC commitment decisions in bone marrow, partly through the Hes family of transcriptional repressors (8). Furthermore, overexpression of Hes-1 in LSKCD34<sup>-</sup> cells leads *in vivo* to an accumulation of the primitive SP cells and LSKCD34<sup>-</sup> cells in marrow of recipient mice, while maintaining the production of the differentiated blood cells (9). However, its role on the cell cycle is still under discussion: an overexpression of Hes-1 in human CD34<sup>+</sup> cells reduced their proliferation (35), while a comparable study led to an increase in the incorporation of BrdU in Hes-1-overexpressing cells, with a reduction in apoptosis and an increase in their capacity to expand in NOD-SCID mice (36). Different levels of Hes-1 expression may be at the origin of these contrasting effects.

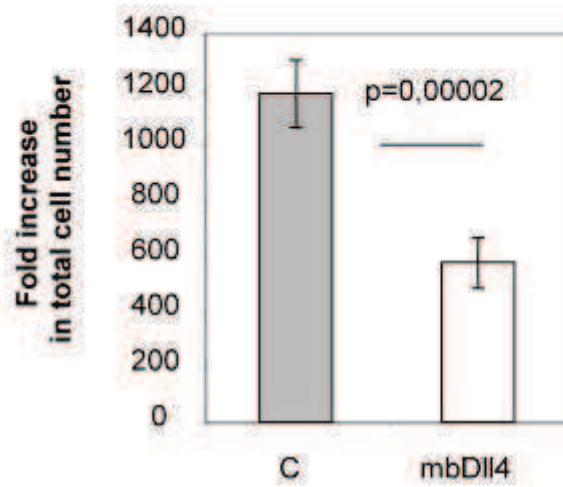
To identify the downstream mechanisms involved in Dll4 activity, we have compared the transcriptional response of LSK cells exposed to Dll4 to control LSK cells. Our comparative study of the transcriptome has provided us with a skeleton of an answer by revealing the upregulation of some genes already identified to be implicated in the regulation of HSPC self-renewal such as Bmi-1 (37), HoxB4 (38), Gata-2 (39) and c-Myc (40, 41). Transcriptional screening has also highlighted new up-regulated candidates, Pumilio-1 and -2, downstream of the Dll4/Notch signaling pathway. Among the post-transcriptional regulators of stem cells, Pum proteins, which belong to the evolutionary highly conserved family of PUF proteins, have been shown to be central for the maintenance of germinal and somatic stem cells in Invertebrates. They are able to bind specific motifs mainly present in the 3'-untranslated region (3'UTR) of target mRNAs, thus favoring sets of mRNA's to be degraded or kept

untranslated. The fact that PUF genes are targets of Notch signaling in HSPC was previously observed in *C. elegans* germline stem cells, the expression of the *fbf-2* gene being under the control of GLP-1/Notch signaling (42). Activation of PUF genes by Notch signaling in both *C. elegans* germ stem cells and murine HSPCs enforces the link between these two major mechanisms regulating stem cell mitoses. However the implication of Pum proteins in the function of mammalian stem cells has been rarely addressed, only few reports have highlighted a possible role of Pum-2 and two Invertebrate PUM partners (NANOS and TRIM32) in the primitive potential of murine embryonic and adult spermatogonial and neural stem cells, respectively (43-45). Interestingly, a connection between PUF proteins and another main regulator of HSC maintenance such as HoxB4/C4 was stressed through a previous study (46). Comparative transcriptome analyzes of human CD34<sup>+</sup> cells subjected or not to HOXB4 or HOXC4 have revealed that both homeoproteins upregulate Pum-1- and Pum2-encoding genes. The link between Pumilio and Dll4/Notch pathway in the function of HSC will be further investigated.

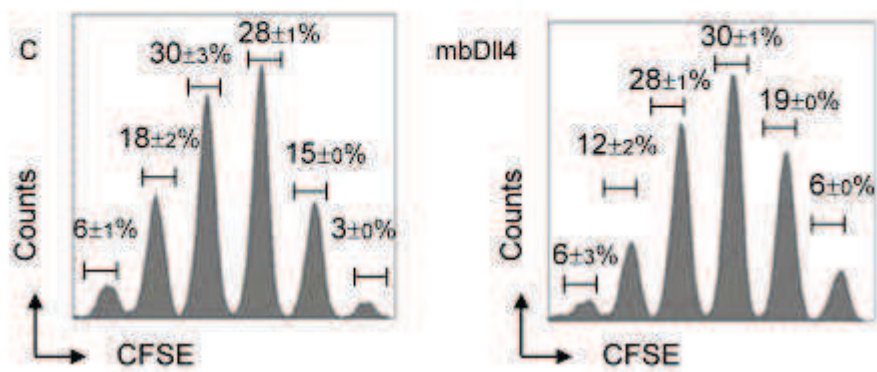
In conclusion, we have demonstrated that the Notch/Dll4 pathway displays clear-cut positive effects on the maintenance of the primitive functions of human and murine HSPC in culture. Target genes of Notch/Dll4 may represent good candidates for the development of new stem cell therapy strategies. These newly identified factors could be safer and more potent for the expansion, self-renewal, and maintenance of human HSPCs.

Figure 1

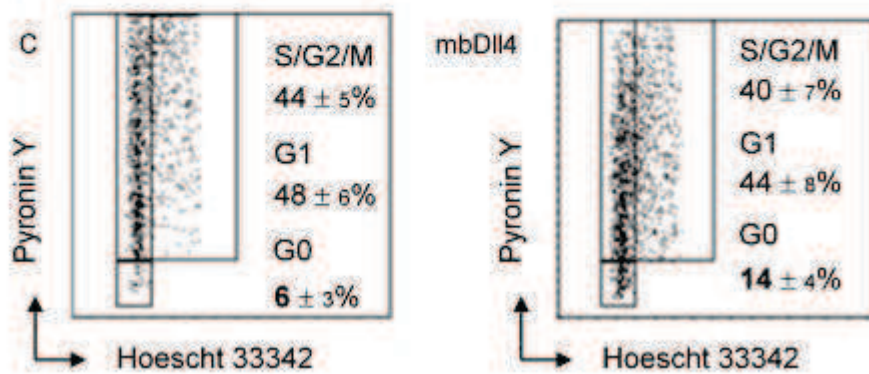
A.



B.



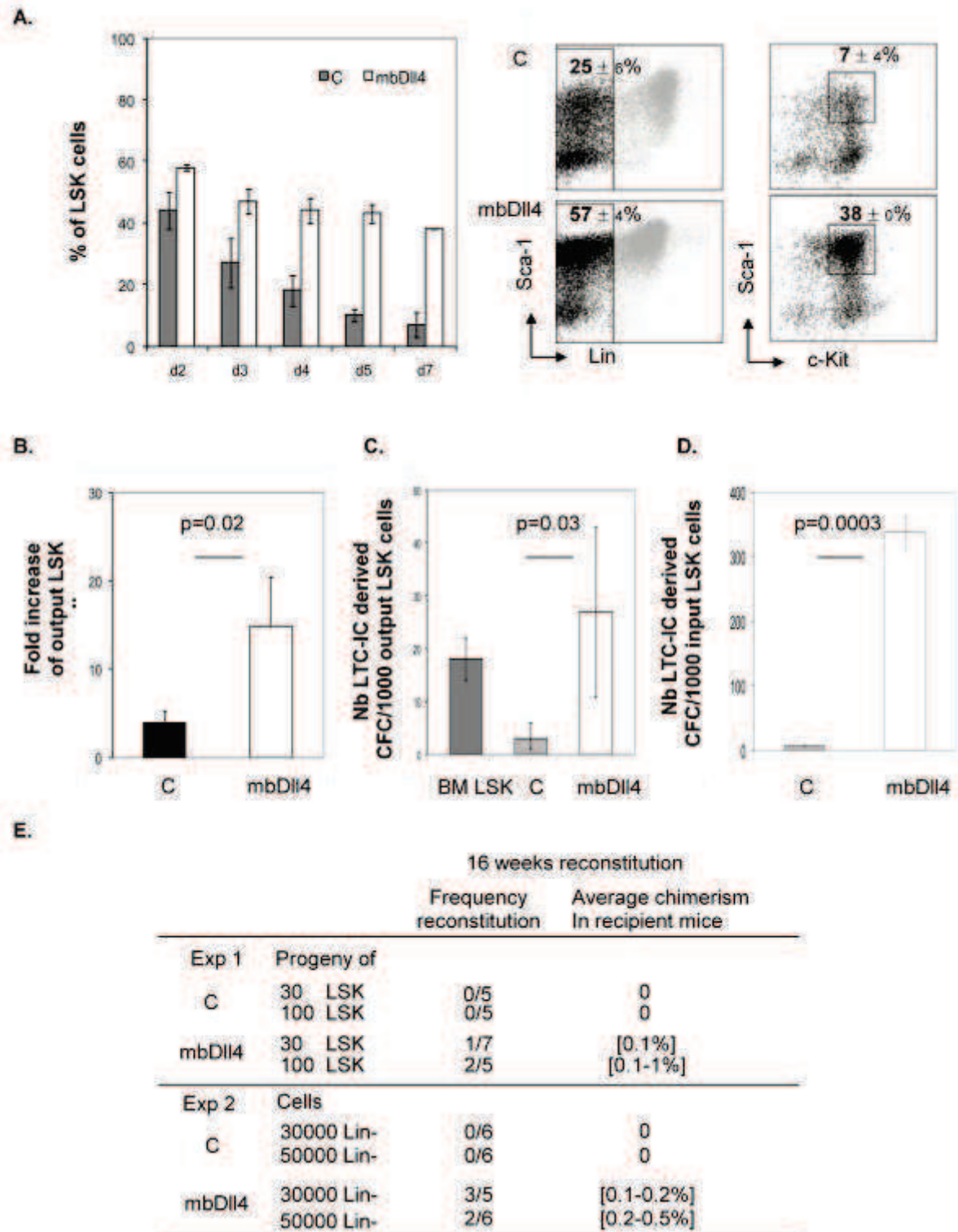
C.



**Figure 1 : mbDl14/S17 reduces LSK cell proliferation by keeping a fraction of cells in**

**G0.** (A) *Fold increase in total cell number.* Sorted BM LSK cells were cultivated in wells coated with C/S17 or mbDl14/S17 in the presence of mSCF, hFlt3-L, hIL-6 and mIL-11. Hematopoietic cells were harvested at day 7 and the fold increase in total cell number was calculated by dividing the number of output cells at day 7 by the number of input LSK cells. (B) *High resolution tracking of cell division.* CFSE-labelled LSK cells were cultured in wells coated with C/S17 or mbDl14/S17 in the presence of the same cocktail of cytokines. Flow cytometry analysis of CFSE<sup>+</sup> cells was made after 2 days of culture (FACS analysis of one representative experiment). The percentages represent the mean of 3 experiments. (C) *Distribution of cells in G0 versus G1 and S/G2M phase of the cell cycle.* Sorted BM LSK cells were cultivated as previously described. Cells harvested at day 2 were stained with PyroninY (RNA dye) and Hoescht (DNA dye). Cells residing in G0 appear at the bottom of the G0/G1 peak, G1 cells are in the upper part and SG2M cells are at the right hand side of the G1 peak as indicated. Data are expressed as mean  $\pm$  SEM, n=3, p< 0.05.

Figure 2

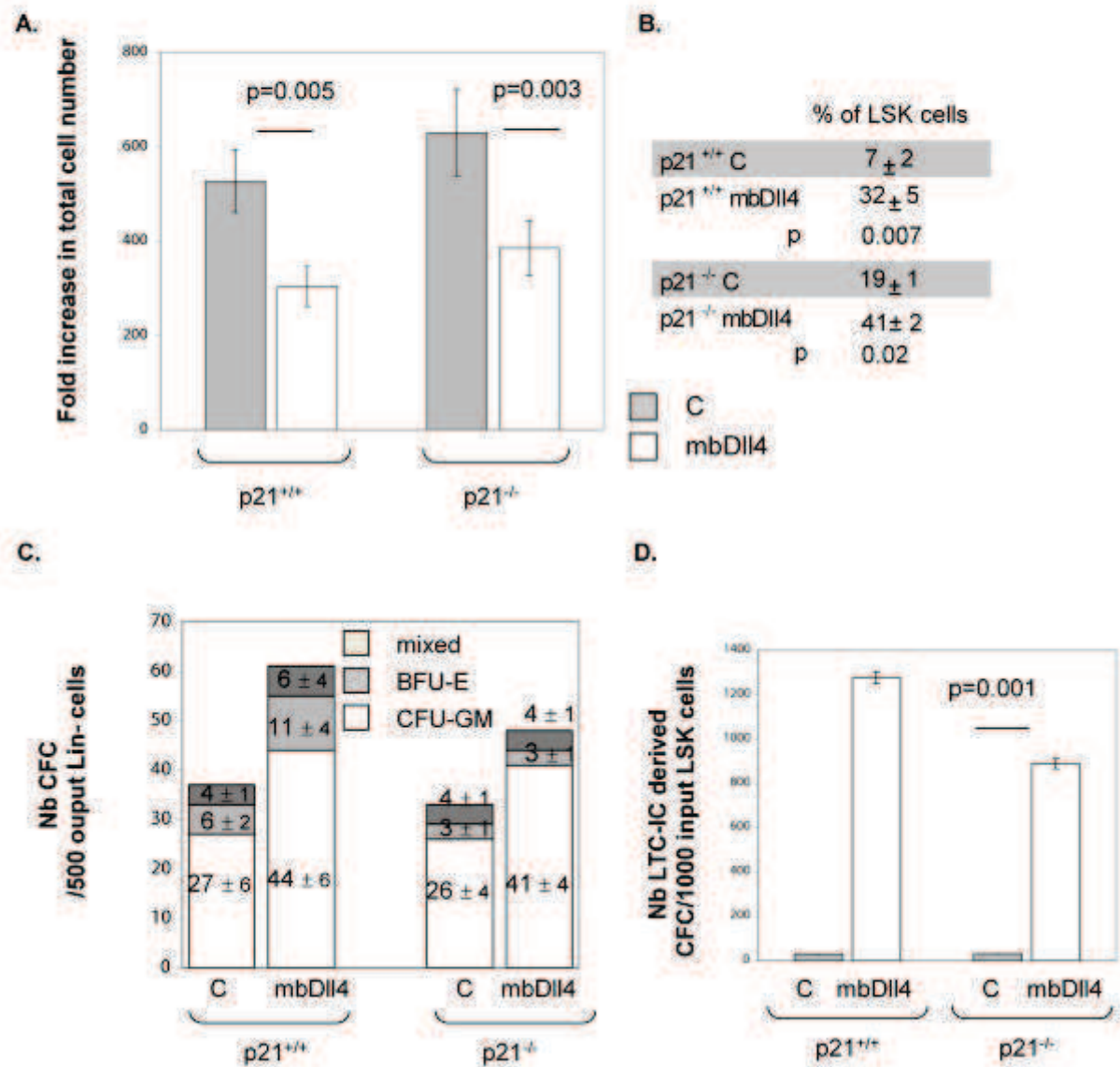


**Figure 2 : mbDII4/S17 limits *in vitro* the loss of the primitive potential.**

LSK cells were cultivated on mbDII4/S17 and C/S17 stromas in the presence of mSCF, hFlt3-L, hIL-6 and mIL-11. (A) Cells were harvested at the indicated days throughout the culture and were labelled to identify the percentage of LSK cells. (B) The fold increase in LSK cell number was calculated by dividing the number of output LSK cells at day 7 by the number of input LSK cells. (C) *LTC-IC potential of LSK cells*. 200 input and output LSK cells from each condition were cultured for 5 weeks on MS-5 cells, and cells from each well were then plated in methylcellulose and the number of CFC-derived LTC-IC was counted after 7 days (mean  $\pm$  SEM of four independent experiments). (D) *Expansion of LTC-IC derived CFC*. Histogram represents the number of LTC-IC derived CFC per 1000 input LSK cells. The total number of LTC-IC-derived CFC cells was calculated using the total cell count, and the percentages of LSK cells. Data are expressed as mean  $\pm$  SEM, n=3, p<0.05. (E) *In vivo reconstitution potential*. Exp 1. Progeny of 30 or 100 sorted Ly5.1 LSK cells cultivated in the conditions previously described, were harvested at day 7, mixed with fresh  $1.5 \times 10^5$  Ly5.2 BM cells and transplanted into lethally irradiated C57B6 Ly5.2 mice (5 to 7 mice per group). Analysis of the presence of CD45.1<sup>+</sup> cells in bone marrow (>16 weeks reconstitution) of reconstituted mice (5 mice per group) was performed using flow cytometry (FACS Sort). Exp 2. 30.000 and 50.000 output Lin<sup>-</sup> cells were sorted at day 7 and transplanted using the same conditions as Exp1 (5 to 10 mice per group). The Table presents the frequency of reconstitution and the average chimerism observed in the recipient mice.



Figure 3



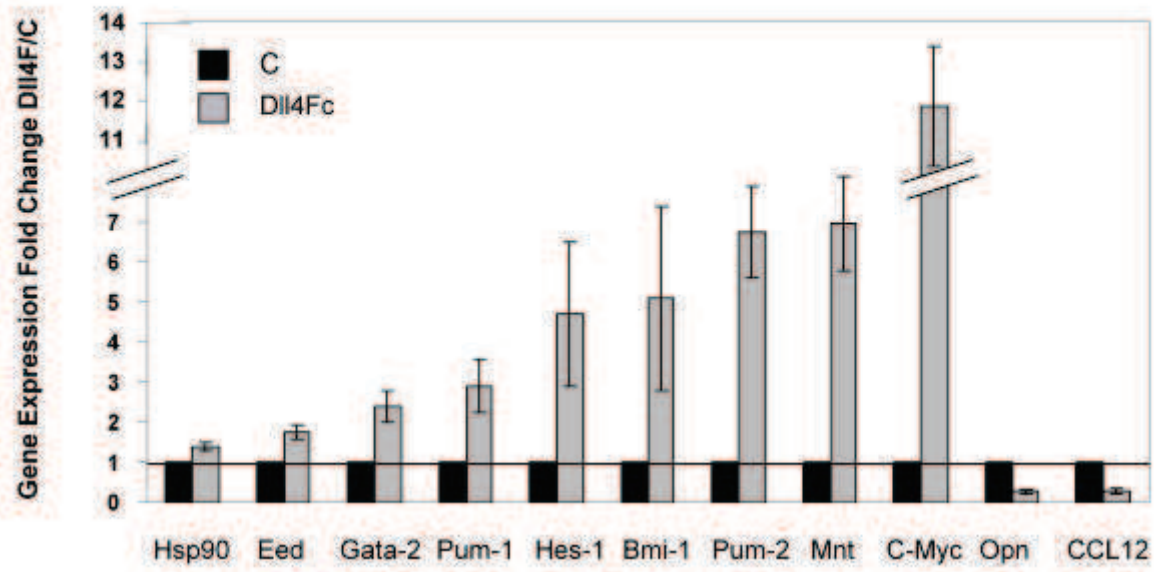


**Figure 3. Absence of p21<sup>Cip1/Waf1</sup> does not alter the response of LSK cells to Dll4.**

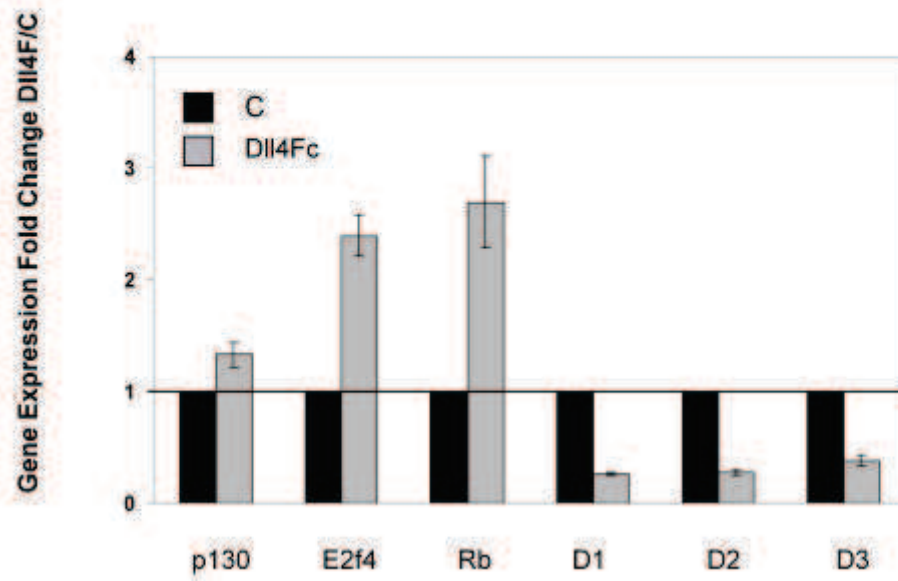
Sorted BM LSK cells from p21<sup>+/+</sup> and p21<sup>-/-</sup> mice were cultivated on C/S17 and mbDll4/S17 in the presence of mSCF, hFlt3-L, hIL-6, and mIL-11. Hematopoietic cells were harvested 7 days later and several parameters were examined. (A) The fold increase in the total cell number was calculated by dividing the number of output cells at day 7 by the number of input LSK cells (results are expressed as means  $\pm$  SEM of 4 experiments). (B) Percent of output LSK cells at day 7 of culture (results are expressed as means  $\pm$  SEM of 4 experiments). (C) Sorted Lin<sup>-</sup> cells were cultured in 1% methylcellulose, and the number of BFU-E, GM-CFC, and mixed colonies was counted (results are expressed as means  $\pm$  SEM of 4 experiments). (D) 200 output LSK cells from each condition were cultivated for 5 weeks on MS-5 cells, and cells from each well were then plated in methylcellulose and the number of CFC-derived LTC-IC was counted after 7 days. The total number of LTC-IC-derived CFC was calculated using the total cell number and the percent of LSK cells, and histograms represent the number of LTC-IC derived CFC per 1000 input LSK cells (results are expressed as means  $\pm$  SEM of 4 experiments).

Figure 4

A.



B.



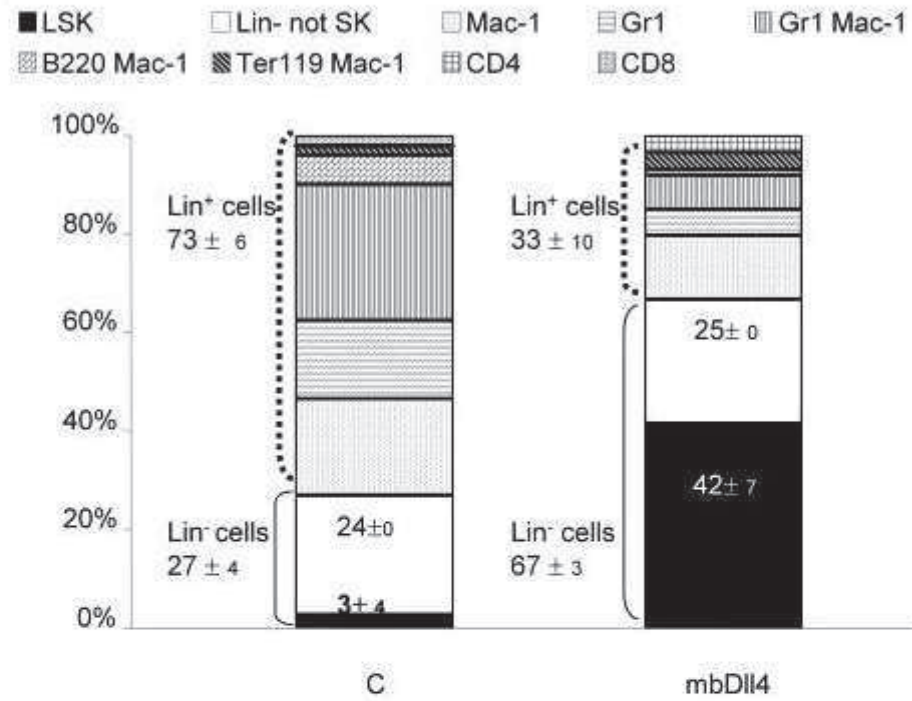
**Figure 4. Gene expression modulations in response to Dll4.**

Quantitative real time PCR was performed to validate the microarray data. Target genes were involved in self-renewal (A) and cell cycle (B). PCR amplifications were carried out in triplicate, on RNA prepared from LSK cells exposed or not to Dll4Fc for 12 hours (3 independent experiments). The mRNA expression of each gene was normalized to that of TF2D mRNA.

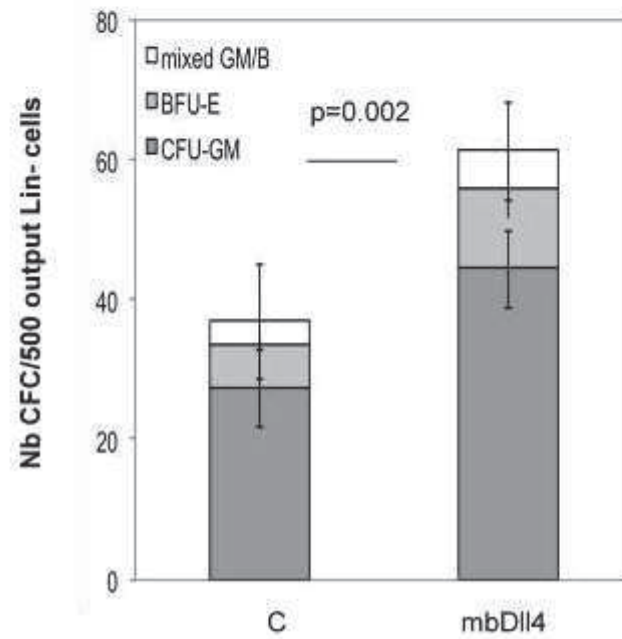
The final ratios, expressed as a fold change, were generated by comparing expression levels of input LSK cells exposed to Dll4Fc compared to unexposed controls. Results are presented as the mean  $\pm$  SEM in triplicate assays,  $p < 0.05$ .

Figure S1

A.



B.

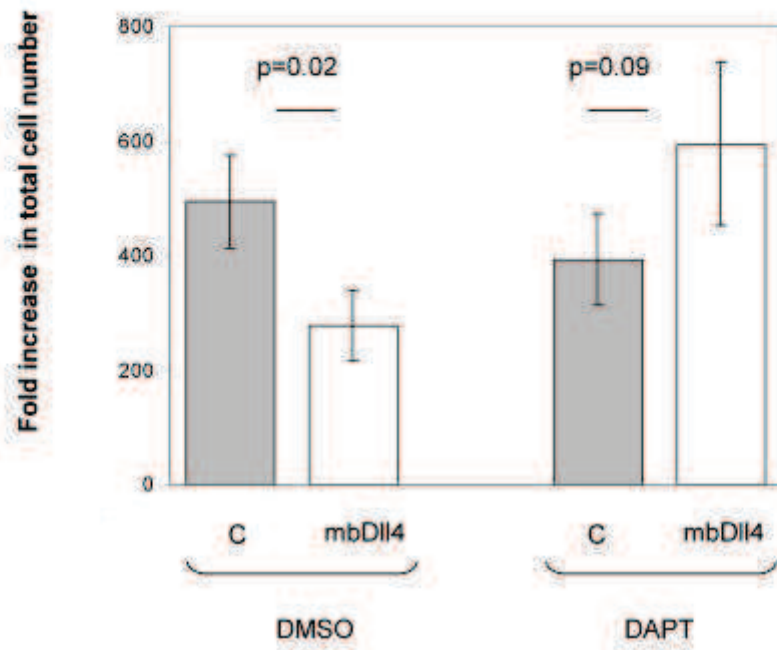


**Figure S1: Effects of mbDll4/S17 on myeloid differentiation.**

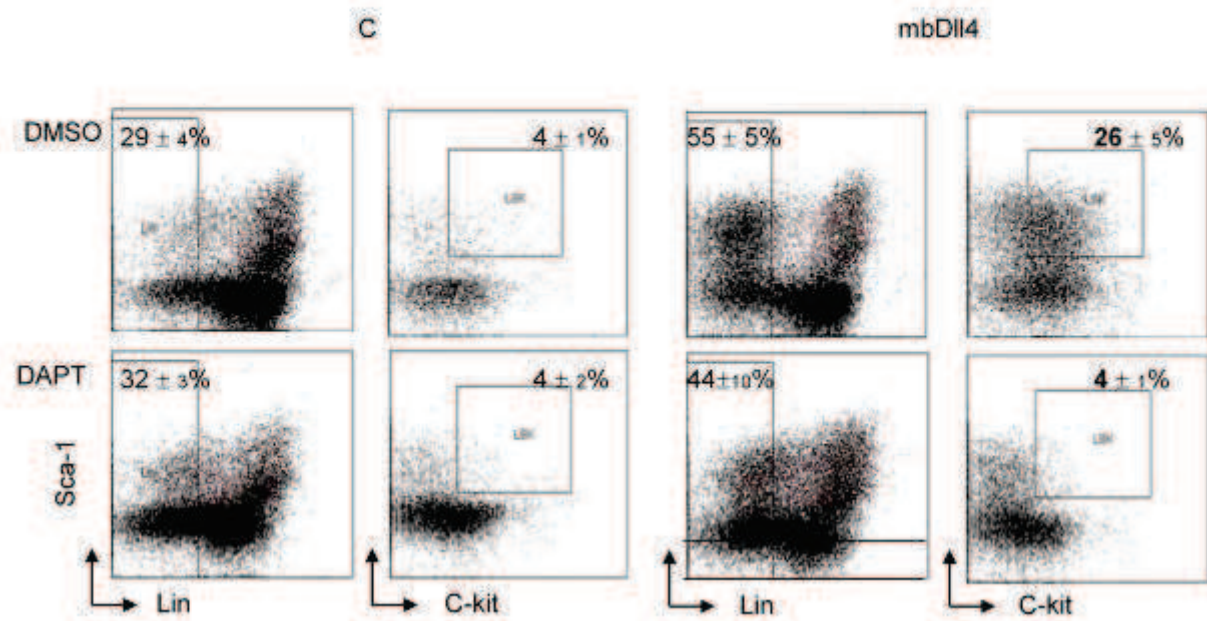
BM LSK cells were cultured in wells coated with C/S17 or mbDll4/S17 in the presence of mSCF, hFlt3-L, hIL-6, and hIL-11 for 7 days. (A) Phenotype of nucleated cells after 7 days of culture on control C/S17 and mbDll4/S17 stromas was performed using monoclonal -FITC, -PE, -APC-conjugated or biotinylated mAb, raised against differentiation markers GR-1, MAC-1, B220, CD4, CD8 and TER-119 specifically expressed by granulocytes, macrophages, B lymphocytes, T lymphocytes and erythrocytes, respectively, and against immature markers: Sca-1 and c-Kit. Results are presented as the mean  $\pm$  SEM in 3 experiments. (B) Sorted Lin<sup>-</sup> cells were cultured in 1% methylcellulose, and the number of BFU-E, GM-CFC, and mixed colonies was counted (results are expressed as means  $\pm$  SEM of 4 experiments).

Figure S2

A.



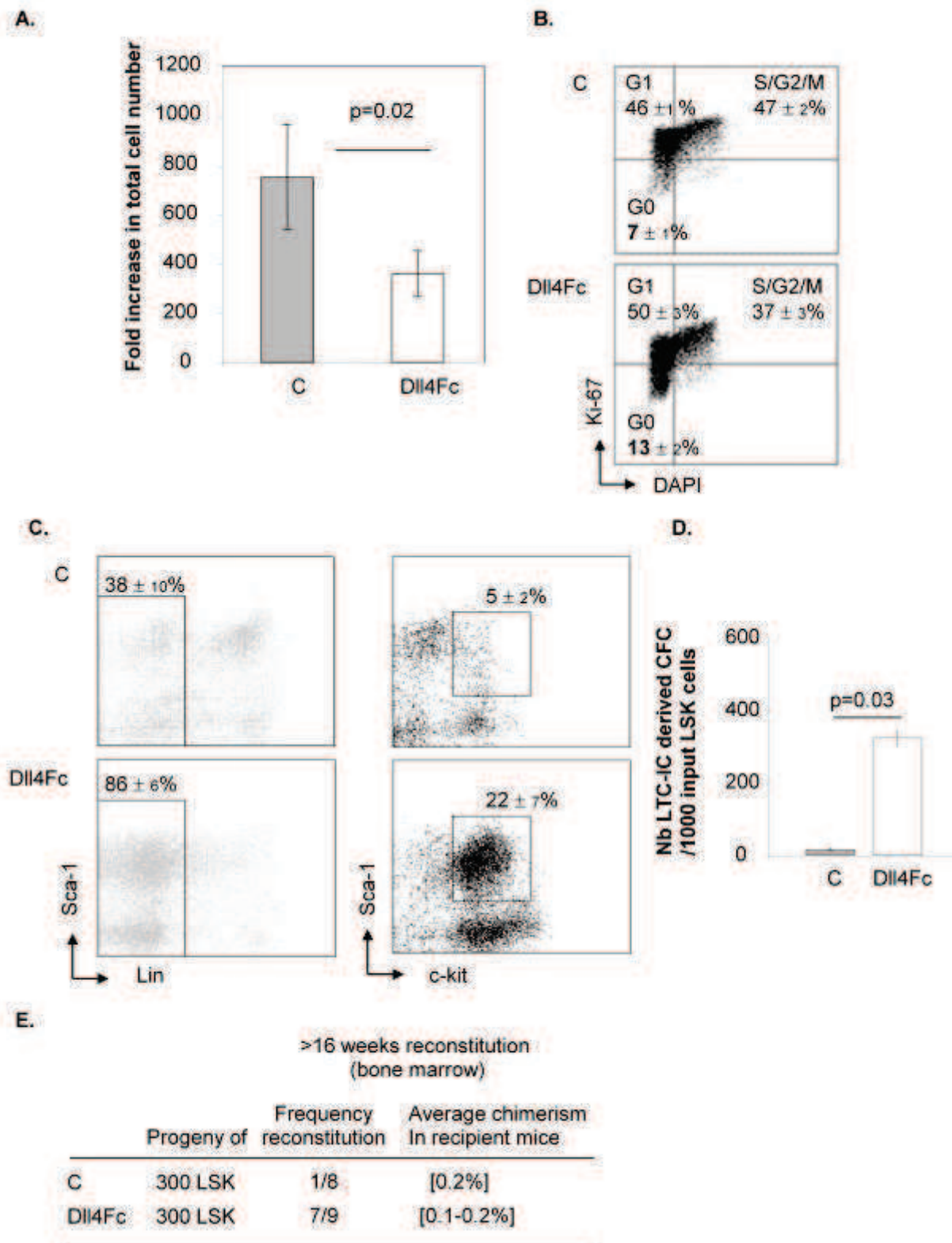
B.



**Figure S2. mbDll4/S17 activates the Notch pathway.**

Sorted LSK cells were cultivated with or without DMSO or DAPT (30  $\mu$ M). Cells were harvested at day 7, and counted. (A) Effect of the presence of DAPT on the fold increase in total number of cells at day 7. (B) The percentage of LSK cells was estimated after 7 days of cultures on C/S17 and mbDll4/S17 with or without DAPT. All data represent the mean  $\pm$  SEM, n=3, p< 0.05.

Figure S3





**Figure S3. Dll4Fc reproduces mbDll4 activities.**

LSK cells were cultured for seven days in wells precoated with immobilized Dll4Fc as previously described (15). Cultures with IgG1 served as a negative control. (A) Fold increase in total cell number after seven days of culture (n=3). (B) Distribution of cells in G0 versus G1 and S/G2/M phase of the cell cycle at day 2 (n=3). (C) FACS profiles of output LSK cells in one representative experiment (out of three) after seven days of culture. (D) Expansion of LTC-IC derived CFC after seven days of culture (n=3). (E) Reconstitution potential of cells exposed to Dll4Fc. Progeny of 300 Ly5.1 LSK cells cultivated in the conditions previously described were harvested at day 7, mixed with  $1.5 \times 10^5$  Ly5.2 BM cells, and transplanted into lethally irradiated C57Bl6/J Ly5.2 mice (5 to 7 per group). Analysis of the presence of CD45.1<sup>+</sup> cells in bone marrow (>16 weeks reconstitution) of reconstituted mice was performed using flow cytometry (FACS Sort).

Figure S4

Overrepresented functional categories	Numbers of involved genes	%
<b>Cell Growth and Proliferation</b>	<b>161</b>	<b>33.1</b>
Cell Growth and/or Maintenance	122	25.1
Cell Proliferation	39	8
<b>Cell Cycle Process</b>	<b>129</b>	<b>24.8</b>
Cell Cycle	30	6.2
M Phase of Mitotic cell Cycle	16	3.2
Regulation of Cell Cycle	16	3.3
M Phase of Mitotic cell Cycle	16	3.2
Mitosis	16	3.2
Mitotic cell Cycle	12	2.5
M Phase	9	1.8
Cell Cycle Checkpoint	5	1
Mitotic Checkpoint	3	0.6
Regulation of Mitotic Cell Cycle	3	0.6
Regulation of Mitosis	3	0.6
<b>Translation Process</b>	<b>113</b>	<b>22</b>
mRNA Processing	28	5.8
RNA Metabolism	28	4.7
RNA Processing	22	4.5
Translation	15	3
RNA Splicing	8	1.6
RNA Modification	7	1.4
Translational Initiation	5	1
<b>Other Process</b>	<b>92</b>	<b>18.7</b>
Intracellular Transport	38	7.5
Protein Biosynthesis	30	6.2
Nucleocytoplasmic Transport	11	2.3
Response to Endogenous Stimulus	10	2.1
Intra-Golgi Transport	3	0.6

**Figure S4. Overrepresented functional categories modulated in response to Dll4.**

Functional analysis of genes modulated in LSK cells exposed to Dll4Fc for 12 hr, using GO, terms from DAVID database (<http://david.abcc.ncifcrf.gov/home.jsp>) and IPA.

Table S1

Table S1. Sequences of qRT-PCR primers

Gene	5' position sequence (Forward)	3' position sequence (Reverse)
cyclin D1	5'-TGTTACTTGTAGCGGCCTGTTG-3'	5'-CCGGAGACTCAGAGCAAA TCC-3'
cyclin D2	5'-CACGACTTCATTGAGCACATCCT-3'	5'-GCGGATCAGGGACAGCTTCT-3'
cyclin D3	5'-TGCCAAAACGCCCCAGTAC-3'	5'-CGGGATGCCCGAAGGA-3'
cyclin E1	5'-GCAGCGAGCAGGAGACAGA-3'	5'-GCTGCTTCCACACCACTGTCTT-3'
cyclin E2	5'-CGCAGCCGTTT ACAAGCTAAG-3'	5'- TGGGTTTCTTGCAGAGAGTCT-3'
p18	5'-AACCA TCCCAGTCCTTCTGTCA-3'	5'-CCCCTTTCCTTTGCTCCT AA TC-3'
p19	5'-CGGT A TCCACT A TGCTTCTGGAA-3'	5'-CCGCTGCGCCACTCAA-3'
p21	5'-GCGCAGATCCACAGCGATAT-3'	5'-CGGACATCACCAGGATTGG-3'
p27	5'-GGAGCAGTGTCCAGGGATGA-3'	5'-AACCGTCTGAAACATTTTCTTCTGT-3'
p57	5'-TGCTGCGGCCAATGC-3'	5'-CGTTCGACGCCTTGTTCTC-3'
Rb	5'-TCTACCTCCCTTGCCCTGTTT-3'	5'-CAGAAGGCGTGACAGAGTGT-3'
p107	5'-AGCTTCAGCCACTCAAAGTGTAAG-3'	5'-GCTCACTTGGTGCGCTTTTT-3'
p130	5'-TGATGGCAAAGGTCACAAAAGA-3'	5'-GGCCTGTGGCTGAGTCCTGTA-3'
E2F4	5'-AAGCTGGCAGCCGACACT-3'	5'-AGCACGTTGGTGATGTCGTAGAT-3'
Opn	5'-GGCATTGCCTCCTCCCTC-3'	5'-TGCAGGCTGTAAGCTTCTCCT-3'
Hsp90	5'-ACCATTGCTAAGTCTGGCACG-3'	5'-GATCATGGAGATGTCTGCACCA-3'
Gata2	5'-CCCTAAGCAGAGAAGCAAGGC-3'	5'-TGGCACCACAGTTGACACACT-3'
Pum2	5'-GGATGCCGATTGCAAAGATT-3'	5'-CGCTCAACTACTTCAGTTGGAGAG-3'
Eed	5'-GTTTGGGATTTAGAAGTAGAAGATCCTC-3'	5'-GCCGCGCCACATTTATG-3'
Mnt	5'-GGGATGTGAAGGACTCGGG-3'	5'-TGTAGTCAATTCATAGTTACAAATGCTTTT-3'
CCL12	5'-CGGTAAACCAGTCAGCCTGAG-3'	5'-CTCTGGCGATGTGGCTCTC-3'
c-Myc	5'-CTGGATTTCTTTGGGCGT-3'	5'-TGGTGAAGTTCACGTTGAGGG-3'
Bmi-1	5'-CCAGCAAGTATTGTCCTATTTGTGA-3'	5'-ATATCTTGAAGAGTTTTATCTGACCTTATGTT-3'
HoxB4	5'-CCTGGATGCGCAAAGTTCA-3'	5'-CGTCAGGTAGCGGTTGTAGTGA-3'
Hes-1	5'-CGCGGTATTTCCCAACAC-3'	5'-AAGGCAGACATTCTGGAAATGACT-3'
Pum1	5'-AATCCTCCAGGCTGCGTACCAAC-3'	5'-ACATCTGCAGTGCCAATGATAGGACA-3'
TFIID	5'-CGGACAACCTGCGTTGATTTT-3'	5'-ACTTAGCTGGGAAGCCCAAC-3'

**Table S1. Sequences of RT-qPCR primers.**

## References

1. Bigas A, Espinosa L. Hematopoietic stem cells: to be or Notch to be. *Blood*. 2012 Apr 5;119(14):3226-35.
2. Duncan AW, Rattis FM, Dimascio LN, Congdon KL, Pazianos G, Zhao C, et al. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat Immunol*. 2005;23:23.
3. Maillard I, Koch U, Dumortier A, Shestova O, Xu L, Sai H, et al. Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. *Cell stem cell*. 2008 Apr 10;2(4):356-66.
4. Mercher T, Cornejo MG, Sears C, Kindler T, Moore SA, Maillard I, et al. Notch signaling specifies megakaryocyte development from hematopoietic stem cells. *Cell stem cell*. 2008 Sep 11;3(3):314-26.
5. Poirault-Chassac S, Six E, Catelain C, Lavergne M, Villeval JL, Vainchenker W, et al. Notch/Delta4 signaling inhibits human megakaryocytic terminal differentiation. *Blood*. 2010 Dec 16;116(25):5670-8.
6. Oh P, Lobry C, Gao J, Tikhonova A, Loizou E, Manet J, et al. *In vivo* Mapping of Notch Pathway Activity in Normal and Stress Hematopoiesis. *Cell stem cell*. 2013 Jun 19.
7. Grabher C, von Boehmer H, Look AT. Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia. *Nat Rev Cancer*. 2006 May;6(5):347-59.
8. Klinakis A, Lobry C, Abdel-Wahab O, Oh P, Haeno H, Buonamici S, et al. A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. *Nature*. 2011 May 12;473(7346):230-3.
9. Kunisato A, Chiba S, Nakagami-Yamaguchi E, Kumano K, Saito T, Masuda S, et al. HES-1 preserves purified hematopoietic stem cells *ex vivo* and accumulates side population cells *in vivo*. *Blood*. 2003 Mar 1;101(5):1777-83.
10. Varnum-Finney B, Xu L, Brashem-Stein C, Nourigat C, Flowers D, Bakkour S, et al. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nature medicine*. 2000;6(11):1278-81.
11. Varnum-Finney B, Halasz LM, Sun M, Gridley T, Radtke F, Bernstein ID. Notch2 governs the rate of generation of mouse long- and short-term repopulating stem cells. *The Journal of clinical investigation*. 2011 Mar;121(3):1207-16.
12. Varnum-Finney B, Purton LE, Yu M, Brashem-Stein C, Flowers D, Staats S, et al. The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood*. 1998 Jun 1;91(11):4084-91.
13. Varnum-Finney B, Brashem-Stein C, Bernstein ID. Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood*. 2003 Mar 1;101(5):1784-9.
14. Delaney C, Heimfeld S, Brashem-Stein C, Voorhies H, Manger RL, Bernstein ID. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nature medicine*. 2010 Feb;16(2):232-6.
15. Lauret E, Catelain C, Titeux M, Poirault S, Dando JS, Dorsch M, et al. Membrane-bound Delta-4 Notch ligand reduces the proliferative activity of primitive human hematopoietic CD34(+)CD38(low) cells while maintaining their LTC-IC potential. *Leukemia*. 2004;18:788-97.
16. Lahmar M, Catelain C, Poirault S, Dorsch M, Villeval JL, Vainchenker W, et al. Distinct effects of the soluble versus membrane-bound forms of the notch ligand delta-4 on human CD34+CD38low cell expansion and differentiation. *Stem cells*. 2008 Mar;26(3):621-9.
17. Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell*. 1995;82(4):675-84.



18. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science* (New York, NY. 1988;241:58-62.
19. Gothot A, Pyatt R, McMahon J, Rice S, Srour EF. Functional heterogeneity of human CD34(+) cells isolated in subcompartments of the G0 /G1 phase of the cell cycle. *Blood*. 1997 Dec 1;90(11):4384-93.
20. Sutherland HJ, Eaves CJ, Eaves AC, Dragowska W, Lansdorp PM. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis *in vitro*. *Blood*. 1989;74:1563-.
21. Godefroy N, Bouleau S, Gruel G, Renaud F, Rincheval V, Mignotte B, et al. Transcriptional repression by p53 promotes a Bcl-2-insensitive and mitochondria-independent pathway of apoptosis. *Nucleic Acids Res*. 2004;32(15):4480-90.
22. Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, et al. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* (New York, NY. 2000;287(5459):1804-8.
23. Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*. 2005 Jul 1;121(7):1109-21.
24. Kiel MJ, He S, Ashkenazi R, Gentry SN, Teta M, Kushner JA, et al. Hematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. *Nature*. 2007 Sep 13;449(7159):238-42.
25. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain hematopoietic stem cells. *Nature*. 2012 Jan 26;481(7382):457-62.
26. Butler JM, Nolan DJ, Vertes EL, Varnum-Finney B, Kobayashi H, Hooper AT, et al. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell stem cell*. 2010 Mar 5;6(3):251-64.
27. Ding L, Morrison SJ. Hematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*. 2013 Mar 14;495(7440):231-5.
28. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell*. 2004;118(2):149-61.
29. Hozumi K, Mailhos C, Negishi N, Hirano K, Yahata T, Ando K, et al. Delta-like 4 is indispensable in thymic environment specific for T cell development. *The Journal of experimental medicine*. 2008 Oct 27;205(11):2507-13.
30. Shutter JR, Scully S, Fan W, Richards WG, Kitajewski J, DeBlandre GA, et al. DLL4, a novel Notch ligand expressed in arterial endothelium. *Genes & development*. 2000;14(11):1313-8.
31. Passegue E, Wagers AJ, Giuriato S, Anderson WC, Weissman IL. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *The Journal of experimental medicine*. 2005 Dec 5;202(11):1599-611.
32. Kozar K, Ciemerych MA, Rebel VI, Shigematsu H, Zagozdzon A, Sicinska E, et al. Mouse development and cell proliferation in the absence of D-cyclins. *Cell*. 2004 Aug 20;118(4):477-91.
33. Trimarchi JM, Lees JA. Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol*. 2002 Jan;3(1):11-20.
34. Viatour P, Somervaille TC, Venkatasubrahmanyam S, Kogan S, McLaughlin ME, Weissman IL, et al. Hematopoietic stem cell quiescence is maintained by compound contributions of the retinoblastoma gene family. *Cell stem cell*. 2008 Oct 9;3(4):416-28.
35. Yu X, Alder JK, Chun JH, Friedman AD, Heimfeld S, Cheng L, et al. HES1 inhibits cycling of hematopoietic progenitor cells via DNA binding. *Stem cells*. 2006 Apr;24(4):876-88.
36. Shojaei F, Trowbridge J, Gallacher L, Yuefei L, Goodale D, Karanu F, et al. Hierarchical and ontogenic positions serve to define the molecular basis of human hematopoietic stem cell behavior. *Developmental cell*. 2005 May;8(5):651-63.

37. Iwama A, Oguro H, Negishi M, Kato Y, Morita Y, Tsukui H, et al. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. *Immunity*. 2004;21(6):843-51.
38. Amsellem S, Pflumio F, Bardinet D, Izac B, Charneau P, Romeo PH, et al. *Ex vivo* expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. *Nature medicine*. 2003 Nov;9(11):1423-7.
39. Ezoe S, Matsumura I, Nakata S, Gale K, Ishihara K, Minegishi N, et al. GATA-2/estrogen receptor chimera regulates cytokine-dependent growth of hematopoietic cells through accumulation of p21(WAF1) and p27(Kip1) proteins. *Blood*. 2002 Nov 15;100(10):3512-20.
40. Satoh Y, Matsumura I, Tanaka H, Ezoe S, Sugahara H, Mizuki M, et al. Roles for c-Myc in self-renewal of hematopoietic stem cells. *The Journal of biological chemistry*. 2004;279(24):24986-93. Epub 2004 Apr 02.
41. Wilson A, Murphy MJ, Oskarsson T, Kaloulis K, Bettess MD, Oser GM, et al. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes & development*. 2004;18(22):2747-63.
42. Lamont LB, Crittenden SL, Bernstein D, Wickens M, Kimble J. FBF-1 and FBF-2 regulate the size of the mitotic region in the *C. elegans* germline. *Developmental cell*. 2004 Nov;7(5):697-707.
43. Lee MH, Hook B, Pan G, Kershner AM, Merritt C, Seydoux G, et al. Conserved regulation of MAP kinase expression by PUF RNA-binding proteins. *PLoS genetics*. 2007 Dec 28;3(12):e233.
44. Schwamborn JC, Berezikov E, Knoblich JA. The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. *Cell*. 2009 Mar 6;136(5):913-25.
45. Sada A, Suzuki A, Suzuki H, Saga Y. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. *Science (New York, NY)*. 2009 Sep 11;325(5946):1394-8.
46. Auvray C, Delahaye A, Pflumio F, Haddad R, Amsellem S, Miri-Nezhad A, et al. HOXC4 homeoprotein efficiently expands human hematopoietic stem cells and triggers similar molecular alterations as HOXB4. *Hematologica*. 2012 Feb;97(2):168-78.





## **ANNEX II**



# **Pumilio1 and Pumilio2 RNA-binding factors control the functions of mammalian hematopoietic stem cells.**

Fabio Michelet<sup>1,2\*</sup>, Ayda Miri-Nezhad<sup>1,2\*</sup>, Aurore Hattabi<sup>1,2</sup>, Cyril Catelain<sup>4</sup>, Aïssa Ben Youcef<sup>3</sup>, Azzedine Yacia<sup>1,2</sup>, Françoise Pflumio<sup>3</sup>, Isabelle Dusanter-Fourt<sup>1,2</sup>, Serge Fichelson<sup>1,2</sup>, Isabelle Vigon<sup>1,2#</sup>, and Evelyne Lauret<sup>1,2#</sup>

1. Institut Cochin, Université Paris Descartes, Sorbonne Paris Cité, CNRS (UMR 8104), Paris, France.

2. Inserm, U1016, Paris, France.

3. UMR967, INSERM/CEA/Université Paris Diderot/Université Paris Sud, 18 rue du Panorama, BP15, Fontenay-aux Roses, F92265

4. Inserm, U1009, Villejuif, France.

## **Additional Footnotes**

\* These authors contribute equally to this work

# These authors contribute equally to this work

## **Author contribution**

**Fabio Michelet:** Collection and/or assembly of data, data analysis and interpretation, manuscript writing.

**Ayda Miri-Nezhad:** Collection and/or assembly of data, data analysis and interpretation.

**Aurore Hattabi:** Collection and/or assembly of data, data analysis and interpretation.

**Cyril Catelain:** Collection and/or assembly of data, data analysis and interpretation.

**Aïssa Ben Youcef:** Collection and/or assembly of data, data analysis and interpretation.

**Françoise Pflumio:** Provisions of study material or patients, data analysis and interpretation, manuscript writing.

**Isabelle Dusanter-Fourt:** Manuscript writing.

**Serge Fichelson:** Conception and design, data analysis and interpretation, manuscript writing.

**Isabelle Vigon:** Conception and design, data analysis and interpretation, manuscript writing,

final approval of manuscript.

**Evelyne Lauret:** Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

## **Contact**

Corresponding author : E. Lauret

Address U1016 INSERM, Institut Cochin, Dept Immuno/Hématologie  
22 rue Méchain 75014 Paris  
Tel : +33 1 40 54 64 05 (office)  
Fax : 01 43 25 11 67  
E mail : [evelyne.lauret@inserm.fr](mailto:evelyne.lauret@inserm.fr)

## **Running Title**

PUM1 and -2 are required for maintenance of HSC functions.

## **Acknowledgments**

We acknowledge the different Cochin Research Facilities such as the Animal facilities, and Cytometry and Immunobiology, and Genomic Facilities (Inserm U1016). Aïssa Ben Youssef, Aurore Hattabi, and Fabio Michelet were fellows from the French Research Minister, the “Ligue Nationale contre le Cancer”, the « Fondation pour la Recherche Médicale » and Ayda Miri-Nezhad from the “Société Française d’Hématologie”. This work was supported by grants from Institut National de la Santé et de la Recherche Médicale, (INSERM), Centre National de la Recherche Scientifique (CNRS), Paris-Descartes University, the Ligue Nationale contre le Cancer (RS13/75-84), Association de Recherche contre le Cancer (ECL2010R01075). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

## Summary

The cellular mechanisms that regulate self-renewal of mammalian hematopoietic stem cells (HSCs) are still poorly known. We have recently found that two HSC self-renewing factors, namely HOXB4 and Delta-like 4/Notch, regulate expression of Pumilio1 and -2 (PUM) RNA-binding proteins in murine and human HSCs. Here, we analyzed the role of the two PUMs in the functions of HSCs using a lentiviral RNAi-based strategy. We show that PUM1 or PUM2 knockdown causes loss of mammalian HSC *in vitro*, by inhibiting cell cycle progression, cell survival and clonogenic properties. Also, inhibition of PUM1 or PUM2 expression strongly decreases the long-term hematopoietic potential of both murine and human HSCs *in vivo*, following transplantation in animals. Notably, PUM1 and PUM2 exhibit similar but non-redundant activities in HSCs. Our data support a model where the two post-transcriptional regulators PUM1 and PUM2 provide key components that maintain the functions of mammalian HSCs.

## INTRODUCTION

Adult stem cells are undifferentiated, long-lived cells, unique in their abilities to produce differentiated daughter cells and to retain their stem cell identity by self-renewing [1]. The molecular events that keep them functional are controlled by dynamic interplays between extrinsic signaling, epigenetic, transcriptional and posttranscriptional regulations, which are still poorly understood. Through two independent approaches in human and murine hematopoietic stem/progenitors cells (HSPCs) to identify target genes of two regulators of their self-renewal, namely HOXB4 [2] and Dll4-activated Notch receptor [3] respectively, we observed an upregulation of *PUM1* and *PUM2* [4] (Catelain et al., submitted). These genes are the two major members of the posttranscriptional regulators Pumilio and FBF (PUF) family in mammals.

Interestingly, studies mostly performed in model systems recently emphasized the importance of members of this RNA-binding proteins family in regulating stem cell fate [5, 6]. PUF proteins in complexes with other partner proteins such as BRAT, NANOS, BOULE, STAUFEN or DAZL regulate target mRNAs expression by binding specific nucleotide motif mostly located in mRNA 3' untranslated region through their PUM Homology Domain (PUM-HD). This, leads to mRNA degradation, changes in mRNA intracellular localization and/or inhibition of their translation [7-11].

Few studies have been conducted to examine the role of PUM1 or PUM2 on mammalian stem cell properties. Some of them have proposed PUM2 as implicated in the self-renewal of human embryonic stem (ES) [12], whereas others underlined the importance of BRAT, NANOS, and STAUFEN, PUF partners identified in Invertebrates in the regulation of the spermatogonial and neural stem cells in mice [11, 13, 14]. In murine hematopoietic system, *Pum1* and *Pum2* are highly transcribed in populations of adult murine hematopoietic Rho<sup>-</sup> 123<sup>low</sup>Sca-1<sup>+</sup>cKit<sup>+</sup>Lin<sup>-</sup> hematopoietic stem cells (HSCs), while significantly down-regulated in more committed progenitors (Lin<sup>-</sup>Sca1<sup>+</sup> cells) [15]. These data prompted us to elucidate the roles of PUM1 and PUM2 in primary murine and human HSPCs. In the present study, we

demonstrated, that, in murine Lin<sup>-</sup>Sca<sup>+</sup>c-kit<sup>+</sup> (LSK) as well as in human CD34<sup>+</sup> HSPCs, PUM knockdown (KD) led to a dramatic drop of cell expansion and clonogenic potential *in vitro*, and a loss of *in vivo* long-term reconstitution potential when inoculated into irradiated mice. Decreased cell expansion was associated with blockage of cell cycle progression at G0/G1 cell phase, and enhanced apoptosis. Furthermore, our data showed that PUM1 and PUM2 exhibit non-redundant functions and are not interchangeable. Altogether our results underpin for the first time the major role of both PUM1 and PUM2 proteins in the maintenance and growth of murine and human primary HSCs, opening the way for PUM participation in the functions of other adult tissue stem cells.

## **Materials and Methods**

### **Mice**

C57BL/6-Ly5.2, C57Bl/6-Ly5.1 mice (8-20 weeks old) were purchased from Charles River (l'Arbresle, France), and maintained in the Cochin Institute facilities (Paris, France) under specific pathogen-free conditions. NOD.Cg-Prkdc(scid) Il2rg(tm1Wjll)/SzJ (NSG) (Jackson Laboratory, Bar Harbor, ME, USA) mice were housed in pathogen-free animal facilities at CEA, Fontenay-aux-Roses, France. All experimental procedures were done in compliance with French Ministry of Agriculture regulations for animal experimentation (CEA animal facility registration number: A920322) and in accordance with local ethical rules.

### **Isolation of murine and human HSPCs.**

Murine lineage negative Sca-1 and c-Kit positive (LSK) cell purification was performed as previously described [16]. Normal cord blood units were collected according to institutional guidelines and after informed consent of the mothers (in partnership with Fondation Générale de Santé, Assistance Publique Hôpitaux de Paris). Following Ficoll separation (Lymphoprep, Fresenius Kabi, Sèvres, France), CD34<sup>+</sup> cells were enriched with the CD34 microBead kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions.



Isolation of the most immature CD34<sup>+</sup>CD38<sup>low</sup> HSPCs was performed with FITC-conjugated antibody to CD34 (clone 581) and PE-conjugated antibody to CD38 (clone T16). Cell sorting was performed using an Aria III cell sorter (Becton-Dickinson).

### **Lentiviral constructs and transduction**

For murine cells, selected shRNA sequences (Supplemental Table 1) have been inserted into the pTRIPΔU3-GFP lentiviral vector as previously described [17]. Alternatively the GFP sequence was replaced by a Tomato sequence. shRNA lentiviral vectors for human cells were generated with the pLKO.1 vectors containing the shRNA sequences and the Tomato or GFP as reporter genes. A lentiviral vector encoding anti-luciferase shRNA was used as control (shC). Murine *Pum1* cDNAs was introduced into pTRIPΔU3-pgk ahead of IRES EMCV-GFP sequence [18]. Lentiviral particles pseudotyped by the vesicular stomatitis virus-G (VSV-G) envelope were generated as previously described [19].

### **Culture experiments**

Murine p815 mast cell line was cultured in DMEM containing 10% FCS. LSK cells were cultured in IMDM-defined medium containing 10% FCS (Stem Cell Technology, Vancouver, Canada), and the following cytokines: murine Stem Cell Factor [muSCF], human Flt-3 ligand [HuFlt3-L], human interleukin-6 [IL-6], each at 50 ng/mL, and [IL-11] at 10 ng/mL. Human CD34<sup>+</sup> cells were cultured in IMDM-defined medium supplemented with 15% of a mixture containing Bovine Serum Albumin (BSA), Insulin and Transferrin (BIT, StemCell Technologies, Vancouver, BC, Canada) and grown in the presence of human SCF (100 ng/ml), IL-3 (60 ng/ml), Flt3-L (50 ng/ml), TPO mimetic peptide (25nM).

Murine LSK and LSKCD150<sup>+</sup> or human CD34<sup>+</sup> and CD34<sup>+</sup>CD38<sup>low</sup> cells were cultured in their respective medium for one day after cell purification, and concentrated lentiviral vectors were then added at a multiplicity of infection (MOI) of 5-20. 48hr later, percentage of transduced cells was estimated by FACS analysis (C6 Accuri, BD), generally ranged 60-90%.

Transduced cells were sorted 48 hours after transduction. For “rescue” experiments, LSK or CD34<sup>+</sup> cells were transduced with the shRNA/Tomato-encoding vectors, and transduced again 8 hr later with the cDNA/GFP rescue vectors.

### **CFC assays.**

For mouse and human assays, the CFC potential was evaluated by plating in methylcellulose medium according to manufacturer’s instructions (MethoCult® M3234 for murine cells and MethoCult® H4230 for human cells, StemCell Technologies).

### **Long-term competitive repopulation assays.**

Animal experiments were performed following the conditions defined by the Ethical Committee of the French Agriculture Department. Lethally irradiated C57BL/6-Ly5.2 mice have received 15,000 sorted shRNA/GFP<sup>+</sup> LSKCD150<sup>+</sup> cells from C57BL/6-Ly5.1 donors, in competition with the same amount of GFP<sup>neg</sup> LSKCD150<sup>+</sup> cells from Ly5.1 mice, together with 1.5x10<sup>5</sup> Ly5.2 BM cells. Hematopoietic reconstitution was assessed 4 months after transplantation through analysis of peripheral blood or BM cells. Transduced cells were identified through GFP detection and after staining with anti-CD45.1-PE by flow cytometry.

For human cell competitive transplantations, 3-Gy-irradiated NSG mice (8-12 weeks old) were injected with a mixture of 7x10<sup>4</sup> shPum/Tomato<sup>+</sup> CD34<sup>+</sup> cells and 7x10<sup>4</sup> shC/GFP<sup>+</sup> CD34<sup>+</sup> cells using intrafemoral injection to ensure maximal engraftment. Human hematopoietic reconstitution was assessed 12 weeks after transplantation in the BM by flow cytometry using GFP fluorescence and labeling with PC7-conjugated antibody to human CD45. Percentages of Tomato<sup>+</sup>shPUM or GFP<sup>+</sup>shC in CD45<sup>+</sup> cells were calculated and mice were considered positive when at least 0.5% of human cells were detected among mouse bone marrow cells.

## **Protein analysis**

Total cell lysates from transduced cells were prepared in Laemmli lysis buffer. After migration in denaturing polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred onto a HybondC Extra membrane (Amersham Bioscience, Munich, Germany). Membrane-bound antibody complexes were detected by Chemiluminescence (Amersham Biosciences), Images were captured using a CCD camera (Fuji-LAS4000, Fujifilm, Tokyo, Japan) and signals were quantified using a Fuji-LAS4000 luminescence image analyzer. The antibodies used are listed in Supplemental Table 1.

## **Detection of apoptotic cells**

In tetramethylrhodamine methyl ester (TMRM, Invitrogen) assay, cells were labeled with TMRM at a concentration of 40 nM and incubated for 15 minutes as previously described [20]. In AnnexinV assay, cells were labeled using FITC or PE-conjugated AnnexinV detection kit (BD Pharmingen) following manufacturer's instructions. PE-Active Caspase-3 Apoptosis Kit (BD Pharmingen) was used to detect Caspase-3-mediated apoptosis following manufacturer's instructions.

## **Statistical Analysis**

All measurements were performed at least three times. Continuous variables were analyzed using Student's t-test for paired values. The measured values were expressed as mean  $\pm$  SEM. p-values of less than 0.05 were considered to be statistically significant. Wilcoxon and Chi-square tests were used for murine and human *in vivo* assays, respectively.

## Results

### **PUM1 and PUM2 KD impair murine HSPC potential *in vitro*.**

We first assessed the role of PUM1 and PUM2 in murine HSPCs by performing KD experiments using transduction of specific shRNAs encoding lentivirus. Two shRNAs specific for murine *Pum1* (shmP1a, shmP1b), two shRNA for murine *Pum2* (shmP2a and shmP2b), and a control shRNA (shC) against luciferase were first validated into the p815 murine mast cell line. Western blot analyses showed efficient PUM KD in shRNA expressing cells (Figure 1A). HSC-enriched LSK CD150<sup>+</sup> cells showed similar PUM1 and PUM2 KD upon shRNA transduction (Figure 1B). Growth properties of transduced LSK HSPCs were analyzed over a 7-day culture. Remarkably, we observed a major reduction in cell expansion upon either PUM1 or PUM2 KD (Figure 2A). LSKs simultaneously transduced with both shmP1a and shmP2a displayed even stronger cell expansion reduction. KD of PUM1 and PUM2 in the more primitive LSKCD150<sup>+</sup> HSCs similarly triggered drastic reduction of cell expansion (data not shown). We next addressed the role of PUM1 and PUM2 on murine HSPC function. Examination of clonogenic properties (Figure 2B) revealed that PUM1 and PUM2 KD dramatically reduced the CFC number as well as their size. Simultaneous KD of both PUM again amplified the inhibitory effects, suppressing any colony generation.

The specificity of shmP1 was confirmed by conducting rescue experiments using murine *Pum1* cDNA, insensitive to shmP1b that targets a sequence in the 3'UTR of *Pum1* mRNA. While enforced expression of m*Pum1* in control shC tomato<sup>+</sup>-LSK cells had no major effect, the same m*Pum1* transduction in shmP1b tomato<sup>+</sup>-LSK cells restored cell expansion (Figure 2C), and also the clonogenic potential (Figure 2D), thus validating specific mPUM1 activities. Our efforts to rescue PUM2 activity in shmP2 LSK cells upon enforced expression of hPUM2 were unsuccessful due to difficulties to obtain high-titer of PUM2 encoding lentiviral vector. Interestingly, mPUM1 could not restore cell expansion or CFC potential of shmP2-transduced cells, suggesting that PUM1 and PUM2 have non-redundant functions in

murine HSPCs.

### **PUM1 and PUM2 KD alter the cell cycle and cell survival of murine HSPCs.**

Having shown that PUM1 and PUM2 KD impaired expansion of murine HSPCs, we next aimed at deciphering the mechanisms responsible for this effect. We examined the cell cycle and apoptosis status of shRNA<sup>+</sup> cells along the culture. Four days after shRNA transduction, shmP1a/GFP<sup>+</sup> LSK cells displayed significant increased proportion of cells in the G0/G1 phase (73±1% versus 64±3% for shmP1a/GFP<sup>+</sup> and shC/GFP<sup>+</sup> cells, respectively) without any effect on cell apoptosis (data not shown); shmP2a/GFP<sup>+</sup> cells also showed some but no significant increase of cells in G0/G1 (Figure 2E and S1A). Enhanced proportion of apoptotic cells was then evidenced 6 days after shRNA transduction, in PUM1 and PUM2 KD populations, as compared to shC populations, which was assessed by measuring mitochondrial transmembrane potential using TMRM staining (Figure 2F and S1B), by annexinV/7AAD labelling (Figure S1C) and by expression of active caspase-3 (Figure S1D). So, inhibition of PUM1 and PUM2 in murine HSPCs first induced a blockade of cell cycle, followed by an enhanced apoptosis, two biological processes that cooperatively decrease cell expansion.

### **PUM1 and PUM2 KD impair human HSPC functions.**

As previously mentioned, expression of *PUM1* and *PUM2* are up-regulated in response to the self-renewing mediators HoxB4 and HoxC4 in human CD34<sup>+</sup> cells [4]. This prompted us to similarly examine the role of PUM in human HSPCs. shPum-containing lentiviral vectors specific for human *PUM1* or *PUM2* (shhP1 and shhP2) decreased PUM1 and PUM2 protein expression levels by 75% and 65%, respectively, as compared to controls human shC<sup>+</sup> cells (Figure 3A). When these CD34<sup>+</sup> HSPCs were kept in culture for 7 days, PUM1 and PUM2 KD led to 85±1% and 63±2% drop in total cell expansion, respectively (Figure 3B). As for murine HSPCs, the CFC potential of shhP1 or shhP2 CD34<sup>+</sup> cells was drastically

decreased in number and size of colonies, when assessed as early as two days after transduction (Figure 3C). Identical results were observed when transducing the more primitive CD34<sup>+</sup>CD38<sup>low</sup> cells (data not shown). The CFC potential of shhP1 CD34<sup>+</sup> cells was rescued upon enforced *mPum1* expression (which is insensitive to shhP1) assessing the specificity of shhP1. Again, as for murine HSPCs, mPUM1 did not restore shhP2 activity (Figure 3D).

Furthermore, as observed in murine HSPCs, hPUM1 and hPUM2 KD enhanced the proportion of hHSPCs in G0/G1 cell cycle phase (Figure 3E) 4 days after transduction, while increased apoptosis was evidenced after two more days, as compared to shC (Figure 3F).

### **PUM1 and PUM2 KD impair the *in vivo* reconstitution potential of murine and human HSCs.**

To further assess the effects of PUM KD on the functional competence of murine and human HSCs, we performed long-term hematopoietic reconstitution assays. Lethally irradiated Ly5.2 mice were engrafted with untransduced (GFP<sup>neg</sup>) LSKCD150<sup>+</sup> Ly5.1 cells in competition either with shmP1a, shmP2a or shC/GFP<sup>+</sup>LSKCD150<sup>+</sup> Ly5.1 cells. Four months later, mice having received shC cells displayed 32±6% GFP<sup>+</sup> cells among bone marrow mononuclear Ly5.1 cells, whereas mice having received shmP1a or shmP2a-transduced cells harbored quite a few bone marrow GFP<sup>+</sup> Ly5.1 cells, with a chimerism averaging 3±0.4% and 2±1%, respectively (Figure 4A). These results indicate that murine PUM1 and PUM2 KD inhibit the long-term HSC capacity to reconstitute hematopoiesis after myeloablation. The *in vivo* reconstitution potential of PUM KD human HSCs was also investigated by xenogenic competitive cells transplantation in immunocompromised NSG mice. In that case, a 1:1 mixture of shhP1/ or shhP2/Tomato<sup>+</sup> CD34<sup>+</sup> cells and shC/GFP<sup>+</sup> CD34<sup>+</sup> cells was transplanted in sublethally irradiated NSG mice using intra-femoral injection. While 5/9 and 6/8 mice were positive for the presence of human bone marrow shC/GFP<sup>+</sup> CD45<sup>+</sup> cells twelve weeks post-transplant, none of the transplanted mice was ever positive for the presence of shhP1 or

shhP2/Tomato<sup>+</sup> human CD45<sup>+</sup> cells (Figure 4B). These results indicate that PUM1 or PUM2 KD impair human HSC ability to *in vivo* maintain human hematopoiesis in NSG mice. Taken together, these results clearly establish the major role of the PUF proteins in retaining the functional properties of primary murine and human HSCs.

## Discussion

In this study we have demonstrated that the two RNA-binding proteins PUM1 and PUM2 play critical role in the functions of mammalian HSPCs. We focused on both murine and HSPCs where PUM1 or PUM2 expression was selectively knockdown through lentiviral RNAi-based strategy. Our studies support four major conclusions. First, we show that loss of either PUM1 or PUM2 proteins block cell cycle progression and inhibit cell survival of murine and human HSPCs *in vitro*, thus leading to robust reduction of cell expansion. Second, the CFC assay demonstrates that PUM1 and PUM2 are essential for the maintenance of murine and human HSPC clonogenic properties. Third, we show that PUM1 or PUM2 KD render murine and human HSC unable to reconstitute hematopoiesis *in vivo*, when transplanted into irradiated mice. Fourth, we provide evidence that PUM1 and PUM2 exhibit similar but non-redundant properties. In particular we show that *Pum1* cDNA is able to rescue cell expansion and clonogenic properties of PUM1- but not PUM2 KD HSPCs. Altogether our data indicate that the post-transcriptional regulators PUM1 and PUM2 control the maintenance of functional HSCs *in vivo*.

While PUF factors are clearly important to regulate tissue stem cells in model organisms, only few studies have evoked a role of PUF in stem cell functionality in mammals. Most of these studies involved *in vitro* approaches. An ancestral role for PUF proteins in the maintenance and self-renewal of human ES cells was proposed since PUM2 has been found to negatively regulate the expression of the mitogen-activated protein kinase (MAPK)-1 and 14, both

known as repressors of human ES cell self-renewal [12]. PUM2 was also described as regulating proliferation of cultured human adipocyte-derived stem cells [21]. Furthermore, PUM-associated proteins are known to participate to tissue stem cell maintenance. In human germline stem cells, PUM2 interacts with DAZ proteins, and the meiotic regulator BOULE, which are two RNA-binding proteins required for germline stem cell formation [22, 23]. NANOS2, an ortholog of PUM partner NANOS in *Drosophila*, is essential for the self-renewal of murine spermatogonial stem cells [13], whereas TRIM32, a mammalian ortholog of BRAT, another *Drosophila* PUM partner, is a key regulator of both human skeletal muscle stem cells [24, 25] as well as neuronal progenitor cells [14]. A Stauf/PUM2/RNA complex was further shown to play an important role in regulating the maintenance versus differentiation of neuronal stem progenitor cells [11]. Collectively, our data provide the first evidence for a role of PUM factors in the maintenance of mammalian HSPCs both *in vitro* and *in vivo*.

*Pum1* and *Pum2* knockout mice did not exhibit obvious alterations of hematopoiesis upon loss of one of the two mammalian PUM, which seems contradictory with our present data [26, 27]. However, there is growing number of examples in which genetic deletion of a gene in embryonic stem cells results in compensatory changes during animal development that mask relevant phenotypes observed with the corresponding inhibition in adult cells [28, 29]. In HSC for instance, mice invalidated for the Thrombopoietin c-mpl receptor or for the display only slight defect of hematopoiesis compatible with life, despite the fact that Thrombopoietin c-mpl receptor-deficient HSCs are unable to reconstitute hematopoiesis after myeloablation [30]. Alternatively, PUM1 and PUM2 may predominantly regulate stress hematopoiesis *in vivo*, following myeloablation.

In the present work, we show that both human and murine HSPCs exhibit enhanced proportion of cells in the G0/G1 phase followed by induction of apoptosis upon PUM1 or PUM2 KD. Regulation of cell cycle progression by PUF proteins has been emphasized in previous studies. PUM regulate the translational activation of the maternal cyclin B mRNA in

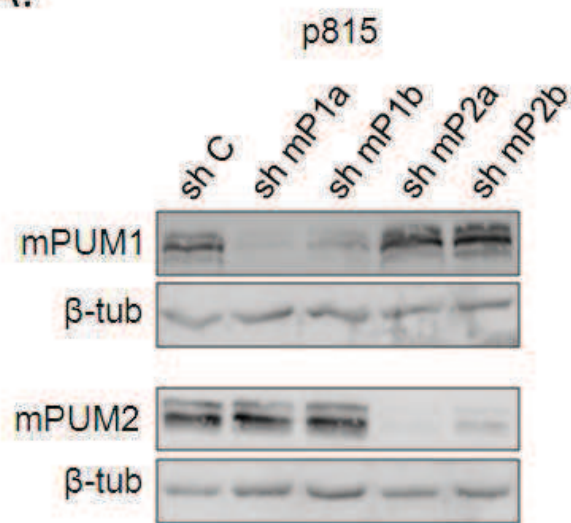


*Drosophila* germ line [31] as well as of cyclin B1 in *Xenopus* oocytes [32, 33]. Furthermore KD of PUM2 reduces cell proliferation in cultured human adipose-derived stem cells without impairing cell survival [21]. Conversely, PUM1 is known to regulate cell apoptosis of murine spermatocytes, leading to decreased sperm production and infertility [26]. Our data show that both PUM1 and PUM2 regulate cell cycle progression and cell survival in mammalian HSPC. While KD of PUM1 or PUM2 had similar consequences on cell cycle and apoptosis in murine or human HSPCs, KD of both proteins triggered additive effects. Remarkably, we were unable to restore PUM2 activity by co-expressing *Pum1* cDNA in murine or human shPUM2+ cells, whereas the same *Pum1* cDNA expression vector was active and reverse shPUM1 phenotype under similar experimental conditions. These observations suggest non overlapping activities of PUM1 and PUM2 in mammalian HSPC. These data extend studies performed in human HeLa cell model, which showed that PUM1 and PUM2 share a number of common in addition to distinct sets of mRNA targets [34].

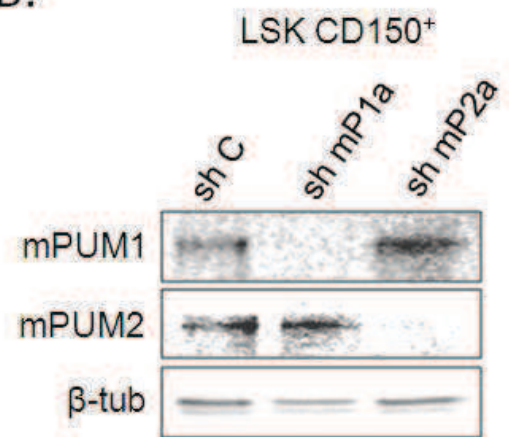
In conclusion, our study reveals that PUM1 and PUM2 posttranscriptional regulators are crucial for the maintenance of functional HSPCs, which are considered as a paradigm for other adult tissue stem cells. A number of other posttranscriptional regulators which include well-depicted miRNA have already been identified that preserve HSC functionality [35]. Thus, an appealing idea from our work is that broad-spectrum DNA and RNA regulators complement each other to ensure maintenance of functional stem cells. PUF-RNA complexes should allow identification of a new panel of key stem cell genes that were overlooked by common analyses of transcriptional regulators. These may provide new targets to favor stem cell maintenance and improve stem cell-based therapies.

Figure 1

A.

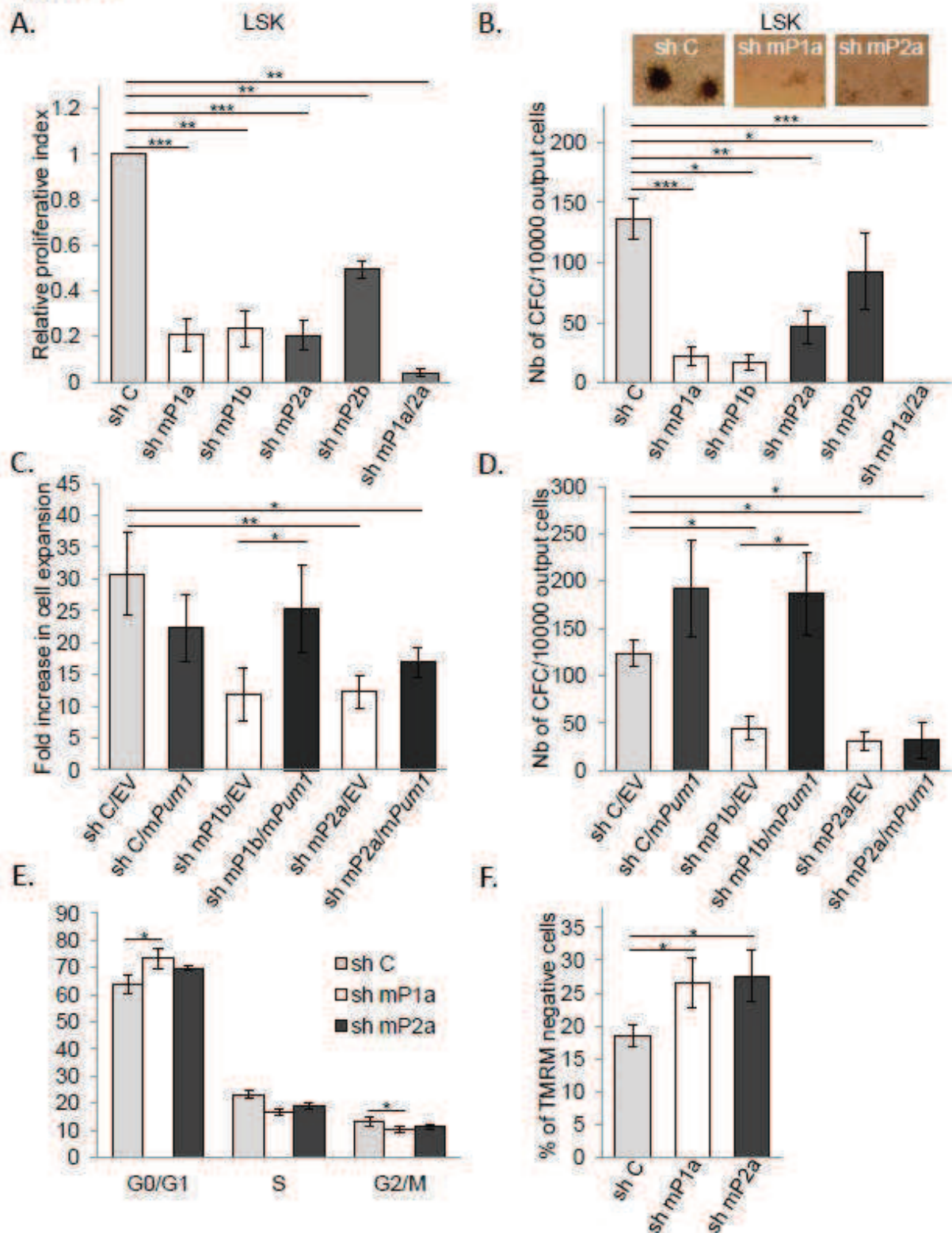


B.



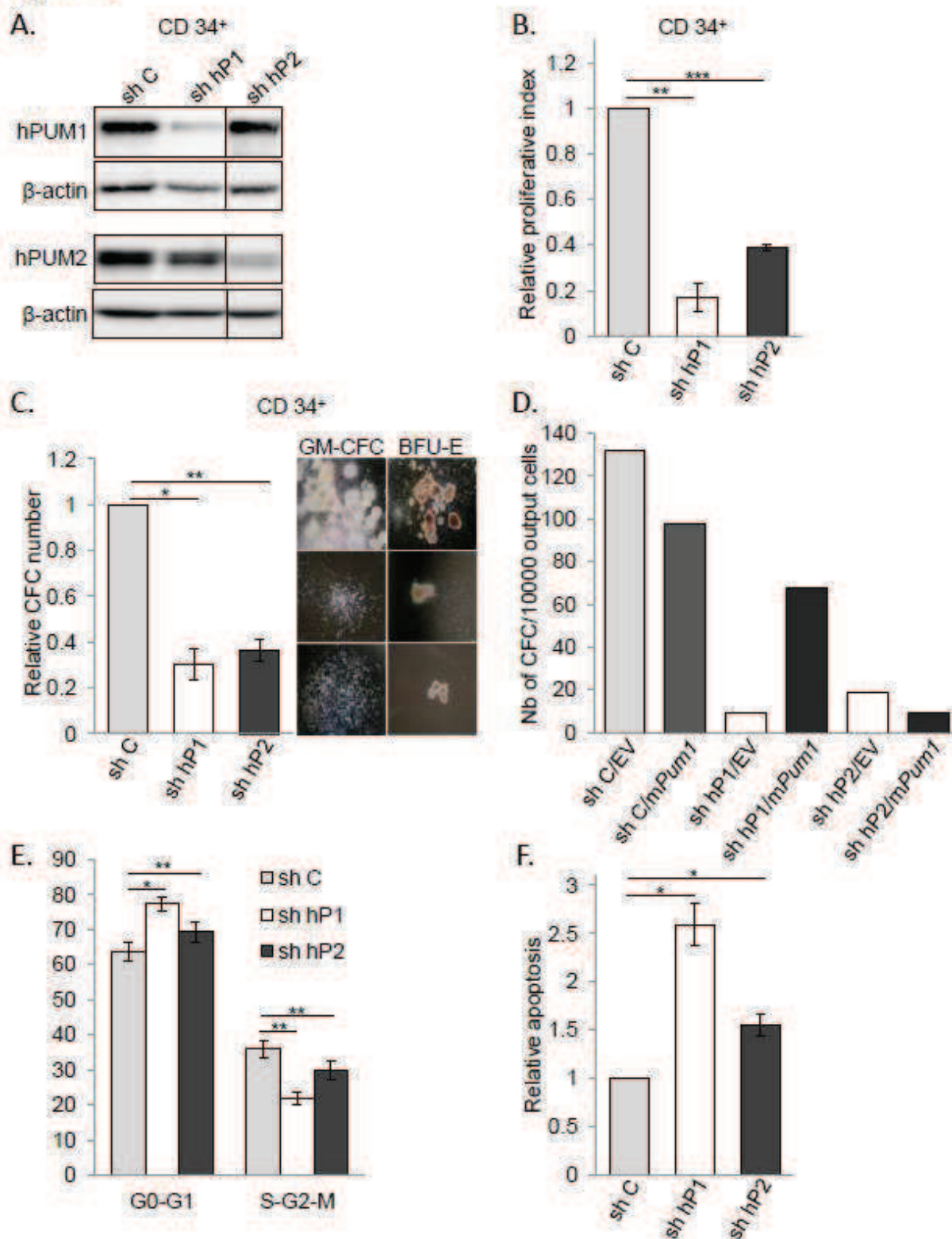
**Figure 1. Validation of shRNA efficiency in murine cells.** (A) Immunoblot analysis of the indicated proteins in murine GFP<sup>+</sup> mast p815 cells at day 7 post-shRNA/GFP lentiviral transduction or (B) in shRNA/GFP<sup>+</sup> cells at day 7 post-transduction of LSKCD150<sup>+</sup> cells. shC stands for shRNA against luciferase, shmP1a and shmP1b for shRNA against *Pum1*, and shmP2a and shmP2b for shRNA against *Pum2*.  $\beta$ -tubulin serves as control.

Figure 2



**Figure 2: mPUM1 and mPUM2 KD inhibit the hematopoietic potential of murine HSPCs.** shRNA/LSK/GFP<sup>+</sup> cells sorted 2 days after transduction were maintained in culture for 7 days. **(A)** Fold increase in total cell number, relative to shC cell population (n=5) at day 7. **(B)** CFC potential of LSK cells at day7 of culture (n=5), and representative pictures of colonies (x20). **(C)** Rescue experiments. LSK cells, first transduced with shRNA/Tomato<sup>+</sup> constructs followed 8 hr later with m*Pum1*/GFP<sup>+</sup> construct, were sorted 2 days later. Tomato<sup>+</sup>/GFP<sup>+</sup> cells maintained in culture for 7 days were enumerated, and cells were plated to assess the CFC potential. EV: empty vector. Fold increase in total cell number (n=4). **(D)** CFC potential (n=4). **(E)** Cell cycle analysis through propidium iodide labeling after 2 days in culture (n=4). **(F).** Apoptosis analysis through measurement of the mitochondrial transmembrane potential using TMRM after 4 days in culture (n=4). (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001)

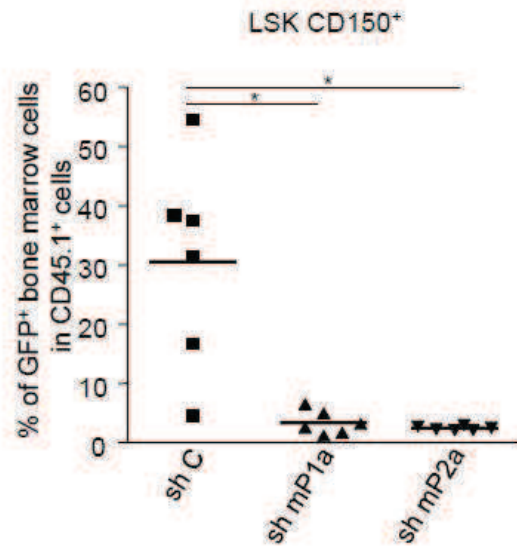
Figure 3



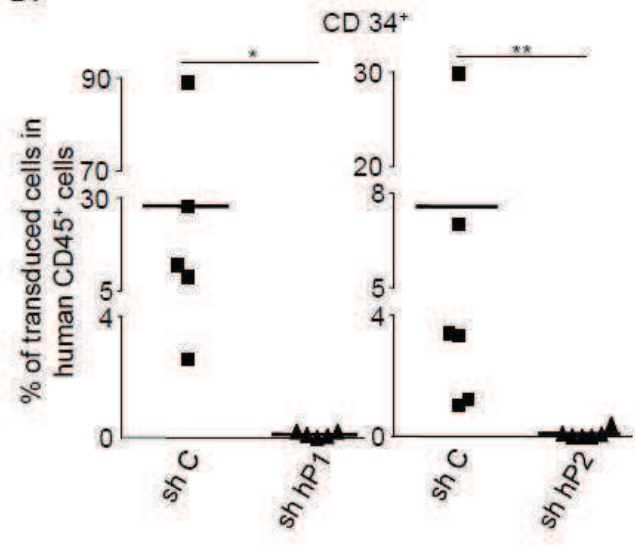
**Figure 3. PUM1 and PUM2 KD inhibit the *in vitro* hematopoietic potential of human HSPCs.** 2 days after transduction of human CD34<sup>+</sup> cells, sorted shRNA/Tomato<sup>+</sup> cells were maintained in culture for 7 days, or plated in methylcellulose. **(A)** Immunoblot analysis of the indicated proteins in shRNA/Tomato<sup>+</sup> cells at day 7. shC stands for control shRNA against luciferase, shhP1 for shRNA against hPUM1, and shhP2 for shRNA against hPUM2. β-actin serves as loading control (lanes come from same but not contiguous blotting membrane) **(B)** Fold increase in total cell number at day 7, relative to shC/tomato<sup>+</sup> cells from input shhP/Tomato<sup>+</sup>CD34<sup>+</sup> cells (n=6). **(C)** Relative CFC potential of shhP/Tomato<sup>+</sup>CD34<sup>+</sup> cells (to shC/Tomato<sup>+</sup> cells) plated in methylcellulose the day of sorting (n=5, representative images of colonies, x20). **(D)** mPum1 expression restored the functions of shhP1-transduced CD34<sup>+</sup> cells. CFC potential of sorted Tomato<sup>+</sup>/GFP<sup>+</sup> cells (n=1). **(E)** Cell cycle analysis through Hoechst dye in Tomato<sup>+</sup> cells at day 4 post-transduction (n=4). **(F)** Apoptosis analysis through AnnexinV-FITC labeling at day 6 post-transduction. Results are expressed relative to shC cells (n=4, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001).

Figure 4

A.



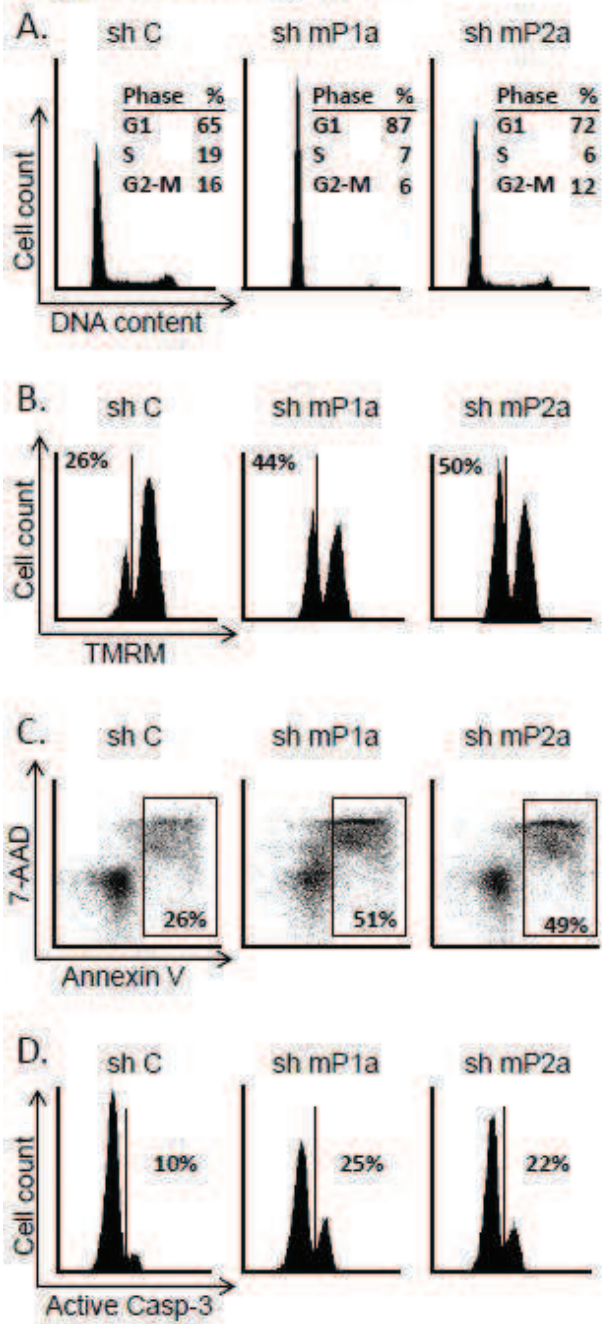
B.





**Figure 4. PUM1 and PUM2 KD inhibit the *in vivo* reconstitutive potential of murine and human HSPCs.** **(A)** Lethally irradiated C57BL/6-Ly5.2 mice received 15,000 sorted shRNA/GFP<sup>+</sup> LSKCD150<sup>+</sup> cells from C57Bl/6 (Ly5.1) donors, in competition with the same amount of GFP<sup>neg</sup> LSKCD150<sup>+</sup> cells from Ly5.1 mice. Analysis of the presence of GFP<sup>+</sup> cells in CD45.1<sup>+</sup> bone marrow cells of engrafted mice was performed 4 months later (at least 10<sup>5</sup> events). Each symbol represents the levels of a single chimeric mouse (Figure representative of 1 experiment out of 2). **(B)** Sublethally irradiated NSG mice received 40,000 sorted shPum/Tomato<sup>+</sup> CD34<sup>+</sup> cells, together with the same amount of shC/GFP<sup>+</sup> cells. Analysis of the presence of GFP<sup>+</sup> and Tomato<sup>+</sup> cells in the fraction of human bone marrow CD45<sup>+</sup> cells of engrafted mice was performed 12 weeks later. The percentage of GFP<sup>+</sup> or Tomato<sup>+</sup> cells was established from FACS analysis of at least 10<sup>5</sup> events. Each symbol represents the levels of a single chimeric mouse. (\* p<0.05, \*\*p<0.01).

# Supplemental Figure 1



### **Supplemental Figure 1. Cell cycle and apoptosis analysis in LSK progeny**

Two days after transduction, shRNA/LSK/GFP<sup>+</sup> cells were sorted and maintained in culture.

**(A)** After two days, cells were labeled with propidium iodide for cell cycle analysis (n=4, and one representative experiment). **(B)**. After four days, apoptosis analysis was followed through the measure of the mitochondrial transmembrane potential using TMRM (n=4, and one representative experiment), **(C)** through AnnexinV-PE 7AAD (n=2, one representative experiment) and **(D)** active caspase 3 (n=2, one representative experiment)

## Supplemental Table 1

A.

shRNA	Sequence
sh hP1	CCGGCAGTTCTTTCTACGGCAACAACCTCGAGTTGTTGCCGTAGAAAGAACTGTTTTTTG
sh hP2	CCGGGCTCCCAGAGTAGTTCTTTATCTCGAGATAAAGAACTACTCTGGGAGCTTTTTG
sh mP1a	GTATTATGGAGTCACGCCAT TTCAAGAGA ATGGCGTGACTCCATAATATTTTTT
sh mP1b	GTTGCCCTGCTGTTTACTGG TTCAAGAGA CCAGTAAACAGCAGGGCAATTTTTT
sh mP2a	GCACCTGTCTTAATAAGTTC TTCAAGAGA GAACTTATTAAGACAGGTGTTTTT
sh mP2b	GAGAGTGGGCTTTCTTTGTG TTCAAGAGA CACAAAGAAAGCCCACTCTTTTTT
sh C	GCGTACGCGGAATACTTCGA TTCAAGAGA TCGAAGTATTCCGCGTACGTTTTT

B.

Antibody	Description
anti-PUM1	rabbit monoclonal antibody (Abcam, ab92545)
anti-PUM2	rabbit monoclonal antibody (NOVUS Biologicals, EPR3813)
anti-PUM2	rabbit polyclonal antibody (Abcam, ab10361)
anti- $\beta$ -ACTIN	rabbit polyclonal antibody (Sigma Aldrich, A5441)
anti- $\beta$ -TUBULIN	rabbit polyclonal antibody (Santa Cruz, sc9104)

**Supplemental Table 1. List of shRNA sequences and antibodies used.**

## References

1. Weissman IL. Stem cells : units of development, units of regulation and units in evolution. *Cell*. 2000;100:157-168.
2. Amsellem S, Pflumio F, Bardinet D et al. *Ex vivo* expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. *Nature medicine*. 2003;9:1423-1427.
3. Lauret E, Catelain C, Titeux M et al. Membrane-bound Delta-4 Notch ligand reduces the proliferative activity of primitive human hematopoietic CD34(+)CD38(low) cells while maintaining their LTC-IC potential. *Leukemia*. 2004;18:788-797.
4. Auvray C, Delahaye A, Pflumio F et al. HOXC4 homeoprotein efficiently expands human hematopoietic stem cells and triggers similar molecular alterations as HOXB4. *Haematologica*. 2012;97:168-178.
5. Wickens M, Bernstein DS, Kimble J et al. A PUF family portrait: 3'UTR regulation as a way of life. *Trends Genet*. 2002;18:150-157.
6. Quenault T, Lithgow T, Traven A. PUF proteins: repression, activation and mRNA localization. *Trends Cell Biol*. 2010;21:104-112.
7. Goldstrohm AC, Hook BA, Seay DJ et al. PUF proteins bind Pop2p to regulate messenger RNAs. *Nature structural & molecular biology*. 2006;13:533-539.
8. Cao Q, Padmanabhan K, Richter JD. Pumilio 2 controls translation by competing with eIF4E for 7-methyl guanosine cap recognition. *RNA (New York, NY)*. 2010;16:221-227.
9. Kedde M, van Kouwenhove M, Zwart W et al. A Pumilio-induced RNA structure switch in p27-3' UTR controls miR-221 and miR-222 accessibility. *Nature cell biology*. 2010;12:1014-1020.
10. Miles WO, Tschop K, Herr A et al. Pumilio facilitates miRNA regulation of the E2F3 oncogene. *Genes & development*. 2012;26:356-368.
11. Vessey JP, Amadei G, Burns SE et al. An asymmetrically localized Stauf2-dependent RNA complex regulates maintenance of mammalian neural stem cells. *Cell stem cell*. 2012;11:517-528.
12. Lee MH, Hook B, Pan G et al. Conserved regulation of MAP kinase expression by PUF RNA-binding proteins. *PLoS genetics*. 2007;3:e233.
13. Sada A, Suzuki A, Suzuki H et al. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. *Science (New York, NY)*. 2009;325:1394-1398.
14. Schwamborn JC, Berezikov E, Knoblich JA. The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. *Cell*. 2009;136:913-925.
15. Spassov DS, Jurecic R. Mouse Pum1 and Pum2 genes, members of the Pumilio family of RNA-binding proteins, show differential expression in fetal and adult hematopoietic stem cells and progenitors. *Blood cells, molecules & diseases*. 2003;30:55-69.
16. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science (New York, NY)*. 1988;241:58-62.
17. Casetti L, Martin-Lannere S, Najjar I et al. Differential contributions of STAT5A and STAT5B to stress protection and tyrosine kinase inhibitor resistance of chronic myeloid leukemia stem/progenitor cells. *Cancer Res*. 2013;73:2052-2058.
18. Zennou V, Petit C, Guetard D et al. HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell*. 2000;101:173-185.
19. Sirven A, Ravet E, Charneau P et al. Enhanced transgene expression in cord blood CD34(+)-derived hematopoietic cells, including developing T cells and NOD/SCID

- mouse repopulating cells, following transduction with modified trip lentiviral vectors. *Mol Ther.* 2001;3:438-448.
20. Zand MS, Vo T, Pellegrin T et al. Apoptosis and complement-mediated lysis of myeloma cells by polyclonal rabbit antithymocyte globulin. *Blood.* 2006;107:2895-2903.
  21. Shigunov P, Sotelo-Silveira J, Kuligovski C et al. PUMILIO-2 is involved in the positive regulation of cellular proliferation in human adipose-derived stem cells. *Stem Cells Dev.* 2012;21:217-227.
  22. Moore FL, Jaruzelska J, Fox MS et al. Human Pumilio-2 is expressed in embryonic stem cells and germ cells and interacts with DAZ (Deleted in AZoospermia) and DAZ-like proteins. *Proceedings of the National Academy of Sciences of the United States of America.* 2003;100:538-543.
  23. Urano J, Fox MS, Reijo Pera RA. Interaction of the conserved meiotic regulators, BOULE (BOL) and PUMILIO-2 (PUM2). *Molecular reproduction and development.* 2005;71:290-298.
  24. Frosk P, Weiler T, Nysten E et al. Limb-girdle muscular dystrophy type 2H associated with mutation in TRIM32, a putative E3-ubiquitin-ligase gene. *Am J Hum Genet.* 2002;70:663-672.
  25. Nicklas S, Otto A, Wu X et al. TRIM32 Regulates Skeletal Muscle Stem Cell Differentiation and Is Necessary for Normal Adult Muscle Regeneration. *PloS one.* 2012;7:e30445.
  26. Chen D, Zheng W, Lin A et al. Pumilio 1 Suppresses Multiple Activators of p53 to Safeguard Spermatogenesis. *Curr Biol.* 2012;22:420-425.
  27. Xu EY, Chang R, Salmon NA et al. A gene trap mutation of a murine homolog of the *Drosophila* stem cell factor Pumilio results in smaller testes but does not affect litter size or fertility. *Molecular reproduction and development.* 2007;74:912-921.
  28. Knight ZA, Shokat KM. Chemical genetics: where genetics and pharmacology meet. *Cell.* 2007;128:425-430.
  29. Rossi L, Lin KK, Boles NC et al. Less is more: unveiling the functional core of hematopoietic stem cells through knockout mice. *Cell stem cell.* 2012;11:302-317.
  30. Yoshihara H, Arai F, Hosokawa K et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell stem cell.* 2007;1:685-697.
  31. Kadyrova LY, Habara Y, Lee TH et al. Translational control of maternal Cyclin B mRNA by Nanos in the *Drosophila* germline. *Development (Cambridge, England).* 2007;134:1519-1527.
  32. Nakahata S, Kotani T, Mita K et al. Involvement of *Xenopus* Pumilio in the translational regulation that is specific to cyclin B1 mRNA during oocyte maturation. *Mechanisms of development.* 2003;120:865-880.
  33. Pique M, Lopez JM, Foissac S et al. A combinatorial code for CPE-mediated translational control. *Cell.* 2008;132:434-448.
  34. Galgano A, Forrer M, Jaskiewicz L et al. Comparative analysis of mRNA targets for human PUF-family proteins suggests extensive interaction with the miRNA regulatory system. *PloS one.* 2008;3:e3164.
  35. O'Connell RM, Baltimore D. MicroRNAs and hematopoietic cell development. *Curr Top Dev Biol.* 2012;99:145-174.

## **BIBLIOGRAPHY**



1. Fuchs, E. & Segre, J. A. Stem Cells: A New Lease on Life. *Cell* **100**, 143–155 (2000).
2. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
3. Nichols, J. *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379–391 (1998).
4. Niwa, H., Burdon, T., Chambers, I. & Smith, A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev.* **12**, 2048–2060 (1998).
5. Bradley, A. Embryonic stem cells: proliferation and differentiation. *Curr. Opin. Cell Biol.* **2**, 1013–1017 (1990).
6. Ogawa, M. Differentiation and proliferation of hematopoietic stem cells. *Blood* **81**, 2844–2853 (1993).
7. Rossi, D. J., Seita, J., Czechowicz, A., Bhattacharya, D. & Weissman, D. B. and I. L. Hematopoietic Stem Cell Quiescence Attenuates DNA Damage Response and Permits DNA Damage Accumulation During Aging. *Cell Cycle* **6**, 2371–2376 (2007).
8. Yamamoto, R. *et al.* Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* **154**, 1112–1126 (2013).
9. Schmitt, T. M. & Zúñiga-Pflücker, J. C. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* **17**, 749–756 (2002).
10. Whitlock, C. A. & Witte, O. N. Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proc. Natl. Acad. Sci. U. S. A.* **79**, 3608–3612 (1982).
11. Till, J. E. & McCulloch, E. A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. 1961. *Radiat. Res.* **175**, 145–149 (2011).
12. Harrison, D. E. Competitive repopulation: a new assay for long-term stem cell functional capacity. *Blood* **55**, 77–81 (1980).
13. Harrison, D. E., Jordan, C. T., Zhong, R. K. & Astle, C. M. Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. *Exp. Hematol.* **21**, 206–219 (1993).
14. Yuan, R., Astle, C. M., Chen, J. & Harrison, D. E. Genetic regulation of hematopoietic stem cell exhaustion during development and growth. *Exp. Hematol.* **33**, 243–250 (2005).
15. Szilvassy, S. J., Humphries, R. K., Lansdorp, P. M., Eaves, A. C. & Eaves, C. J. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 8736–8740 (1990).
16. Taswell, C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol. Baltim. Md* 1950 **126**, 1614–1619 (1981).
17. Lemischka, I. R., Raulet, D. H. & Mulligan, R. C. Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell* **45**, 917–927 (1986).
18. Purton, L. E. *et al.* RARgamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation. *J. Exp. Med.* **203**, 1283–1293 (2006).
19. Rosendaal, M., Hodgson, G. S. & Bradley, T. R. Organization of haemopoietic stem cells: the generation-age hypothesis. *Cell Tissue Kinet.* **12**, 17–29 (1979).
20. Goldschneider, I., Gordon, L. K. & Morris, R. J. Demonstration of Thy-1 antigen on pluripotent hemopoietic stem cells in the rat. *J. Exp. Med.* **148**, 1351–1366 (1978).
21. Spangrude, G. J., Heimfeld, S. & Weissman, I. L. Purification and characterization of mouse hematopoietic stem cells. *Science* **241**, 58–62 (1988).
22. Visser, J. W. & Bol, S. J. A two-step procedure for obtaining 80-fold enriched suspensions of murine pluripotent hemopoietic stem cells. *Stem Cells* **1**, 240–249 (1982).
23. Visser, J. W., Bol, S. J. & van den Engh, G. Characterization and enrichment of murine hemopoietic stem cells by fluorescence activated cell sorting. *Exp. Hematol.* **9**, 644–655 (1981).
24. Matsuzaki, Y., Kinjo, K., Mulligan, R. C. & Okano, H. Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. *Immunity* **20**, 87–93 (2004).

25. Yilmaz, O. H., Kiel, M. J. & Morrison, S. J. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. *Blood* **107**, 924–930 (2006).
26. Uchida, N. & Weissman, I. L. Searching for hematopoietic stem cells: evidence that Thy-1.1<sup>lo</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. *J. Exp. Med.* **175**, 175–184 (1992).
27. Ikuta, K. & Weissman, I. L. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 1502–1506 (1992).
28. Osawa, M., Hanada, K., Hamada, H. & Nakauchi, H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**, 242–245 (1996).
29. Adolfsson, J. *et al.* Upregulation of Flt3 expression within the bone marrow Lin<sup>-</sup> Sca1<sup>+</sup> c-kit<sup>+</sup> stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity* **15**, 659–669 (2001).
30. Yang, L. *et al.* Identification of Lin<sup>-</sup> Sca1<sup>+</sup> kit<sup>+</sup> CD34<sup>+</sup> Flt3<sup>-</sup> short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood* **105**, 2717–2723 (2005).
31. Kiel, M. J. *et al.* SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**, 1109–1121 (2005).
32. Goodell, M. A., Brose, K., Paradis, G., Conner, A. S. & Mulligan, R. C. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* **183**, 1797–1806 (1996).
33. Li, C. L. & Johnson, G. R. Rhodamine123 reveals heterogeneity within murine Lin<sup>-</sup>, Sca-1<sup>+</sup> hemopoietic stem cells. *J. Exp. Med.* **175**, 1443–1447 (1992).
34. McAlister, I. *et al.* Transplantation of hematopoietic stem cells obtained by a combined dye method fractionation of murine bone marrow. *Blood* **75**, 1240–1246 (1990).
35. Phillips, R. L., Reinhart, A. J. & Van Zant, G. Genetic control of murine hematopoietic stem cell pool sizes and cycling kinetics. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 11607–11611 (1992).
36. Wolf, N. S., Koné, A., Priestley, G. V. & Bartelmez, S. H. In vivo and in vitro characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. *Exp. Hematol.* **21**, 614–622 (1993).
37. Lin, K. K. & Goodell, M. A. Purification of hematopoietic stem cells using the side population. *Methods Enzymol.* **420**, 255–264 (2006).
38. Challen, G. A. & Little, M. H. A side order of stem cells: the SP phenotype. *Stem Cells Dayt. Ohio* **24**, 3–12 (2006).
39. Wilson, A. & Trumpp, A. Bone-marrow haematopoietic-stem-cell niches. *Nat. Rev. Immunol.* **6**, 93–106 (2006).
40. Arai, F. *et al.* Niche regulation of hematopoietic stem cells in the endosteum. *Ann. N. Y. Acad. Sci.* **1176**, 36–46 (2009).
41. Kiel, M. J. & Morrison, S. J. Uncertainty in the niches that maintain haematopoietic stem cells. *Nat. Rev. Immunol.* **8**, 290–301 (2008).
42. Morrison, S. J. & Weissman, I. L. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* **1**, 661–673 (1994).
43. Cheshier, S. H., Morrison, S. J., Liao, X. & Weissman, I. L. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3120–3125 (1999).
44. Yamazaki, S. *et al.* TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. *Blood* **113**, 1250–1256 (2009).

45. Passegué, E., Wagers, A. J., Giuriato, S., Anderson, W. C. & Weissman, I. L. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *J. Exp. Med.* **202**, 1599–1611 (2005).
46. Wilson, A. *et al.* Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* **135**, 1118–1129 (2008).
47. Ito, M., Kobayashi, K. & Nakahata, T. NOD/Shi-scid IL2rgamma(null) (NOG) mice more appropriate for humanized mouse models. *Curr. Top. Microbiol. Immunol.* **324**, 53–76 (2008).
48. Domen, J., Gandy, K. L. & Weissman, I. L. Systemic overexpression of BCL-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. *Blood* **91**, 2272–2282 (1998).
49. Domen, J., Cheshier, S. H. & Weissman, I. L. The role of apoptosis in the regulation of hematopoietic stem cells: Overexpression of Bcl-2 increases both their number and repopulation potential. *J. Exp. Med.* **191**, 253–264 (2000).
50. Opferman, J. T. *et al.* Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* **307**, 1101–1104 (2005).
51. Hock, H. *et al.* Tel/Etv6 is an essential and selective regulator of adult hematopoietic stem cell survival. *Genes Dev.* **18**, 2336–2341 (2004).
52. Fischbach, N. A. *et al.* HOXB6 overexpression in murine bone marrow immortalizes a myelomonocytic precursor in vitro and causes hematopoietic stem cell expansion and acute myeloid leukemia in vivo. *Blood* **105**, 1456–1466 (2005).
53. Sauvageau, G. *et al.* Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. *Genes Dev.* **9**, 1753–1765 (1995).
54. Daga, A. *et al.* The retroviral transduction of HOXC4 into human CD34(+) cells induces an in vitro expansion of clonogenic and early progenitors. *Exp. Hematol.* **28**, 569–574 (2000).
55. Crooks, G. M. *et al.* Constitutive HOXA5 expression inhibits erythropoiesis and increases myelopoiesis from human hematopoietic progenitors. *Blood* **94**, 519–528 (1999).
56. Fuller, J. F. *et al.* Characterization of HOX gene expression during myelopoiesis: role of HOX A5 in lineage commitment and maturation. *Blood* **93**, 3391–3400 (1999).
57. Thorsteinsdottir, U. *et al.* Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. *Blood* **99**, 121–129 (2002).
58. Kroon, E. *et al.* Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. *EMBO J.* **17**, 3714–3725 (1998).
59. Pineault, N., Helgason, C. D., Lawrence, H. J. & Humphries, R. K. Differential expression of Hox, Meis1, and Pbx1 genes in primitive cells throughout murine hematopoietic ontogeny. *Exp. Hematol.* **30**, 49–57 (2002).
60. Amsellem, S. *et al.* Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. *Nat. Med.* **9**, 1423–1427 (2003).
61. Zeng, H., Yücel, R., Kusan, C., Klein-Hitpass, L. & Möröy, T. Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells. *EMBO J.* **23**, 4116–4125 (2004).
62. Hock, H. *et al.* Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. *Nature* **431**, 1002–1007 (2004).
63. Cheng, T. *et al.* Hematopoietic stem cell quiescence maintained by p21<sup>cip1</sup>/waf1. *Science* **287**, 1804–1808 (2000).
64. Yu, H. & Jove, R. The STATs of cancer — new molecular targets come of age. *Nat. Rev. Cancer* **4**, 97–105 (2004).
65. Kato, Y. *et al.* Selective activation of STAT5 unveils its role in stem cell self-renewal in normal and leukemic hematopoiesis. *J. Exp. Med.* **202**, 169–179 (2005).

66. Wang, Z., Li, G., Tse, W. & Bunting, K. D. Conditional deletion of STAT5 in adult mouse hematopoietic stem cells causes loss of quiescence and permits efficient nonablative stem cell replacement. *Blood* **113**, 4856–4865 (2009).
67. Chung, Y.-J. *et al.* Unique effects of Stat3 on the early phase of hematopoietic stem cell regeneration. *Blood* **108**, 1208–1215 (2006).
68. Yilmaz, O. H. *et al.* Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* **441**, 475–482 (2006).
69. Zhang, J. *et al.* PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature* **441**, 518–522 (2006).
70. Gan, B. *et al.* Lkb1 regulates quiescence and metabolic homeostasis of haematopoietic stem cells. *Nature* **468**, 701–704 (2010).
71. Gurumurthy, S. *et al.* The Lkb1 metabolic sensor maintains haematopoietic stem cell survival. *Nature* **468**, 659–663 (2010).
72. Nakada, D., Saunders, T. L. & Morrison, S. J. Lkb1 regulates cell cycle and energy metabolism in haematopoietic stem cells. *Nature* **468**, 653–658 (2010).
73. Kalaitzidis, D. *et al.* mTOR Complex 1 Plays Critical Roles in Hematopoiesis and Pten-Loss-Evoked Leukemogenesis. *Cell Stem Cell* **11**, 429–439 (2012).
74. Park, I. *et al.* Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423**, 302–305 (2003).
75. Molofsky, A. V. *et al.* Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* **425**, 962–967 (2003).
76. Iwama, A. *et al.* Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. *Immunity* **21**, 843–851 (2004).
77. Lessard, J. & Sauvageau, G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* **423**, 255–260 (2003).
78. Park, I.-K., Morrison, S. J. & Clarke, M. F. Bmi-1, stem cells, and senescence regulation. *J. Clin. Invest.* **113**, 175–179 (2004).
79. Van der Lugt, N. M., Alkema, M., Berns, A. & Deschamps, J. The Polycomb-group homolog Bmi-1 is a regulator of murine Hox gene expression. *Mech. Dev.* **58**, 153–164 (1996).
80. Kajiume, T., Ninomiya, Y., Ishihara, H., Kanno, R. & Kanno, M. Polycomb group gene mel-18 modulates the self-renewal activity and cell cycle status of hematopoietic stem cells. *Exp. Hematol.* **32**, 571–578 (2004).
81. Kim, J. Y. *et al.* Defective long-term repopulating ability in hematopoietic stem cells lacking the Polycomb-group gene rae28. *Eur. J. Haematol.* **73**, 75–84 (2004).
82. Ohta, H. *et al.* Polycomb group gene rae28 is required for sustaining activity of hematopoietic stem cells. *J. Exp. Med.* **195**, 759–770 (2002).
83. Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* **4**, 7–25 (1978).
84. Calvi, L. M. *et al.* Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425**, 841–846 (2003).
85. Grassinger, J., Haylock, D. N., Williams, B., Olsen, G. H. & Nilsson, S. K. Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential. *Blood* **116**, 3185–3196 (2010).
86. Lord, B. I., Testa, N. G. & Hendry, J. H. The relative spatial distributions of CFUs and CFUc in the normal mouse femur. *Blood* **46**, 65–72 (1975).
87. Visnjic, D. *et al.* Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* **103**, 3258–3264 (2004).
88. Zhang, J. *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425**, 836–841 (2003).
89. Mayack, S. R. & Wagers, A. J. Osteolineage niche cells initiate hematopoietic stem cell mobilization. *Blood* **112**, 519–531 (2008).



90. Nakamura, Y. *et al.* Isolation and characterization of endosteal niche cell populations that regulate hematopoietic stem cells. *Blood* **116**, 1422–1432 (2010).
91. Raaijmakers, M. H. G. P. *et al.* Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* **464**, 852–857 (2010).
92. Corral, D. A. *et al.* Dissociation between bone resorption and bone formation in osteopenic transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13835–13840 (1998).
93. Méndez-Ferrer, S. *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466**, 829–834 (2010).
94. Sacchetti, B. *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131**, 324–336 (2007).
95. Morikawa, S. *et al.* Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J. Exp. Med.* **206**, 2483–2496 (2009).
96. Hooper, A. T. *et al.* Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell Stem Cell* **4**, 263–274 (2009).
97. Kobayashi, H. *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat. Cell Biol.* **12**, 1046–1056 (2010).
98. Jiang, Y., Bonig, H., Ulyanova, T., Chang, K. & Papayannopoulou, T. On the adaptation of endosteal stem cell niche function in response to stress. *Blood* **114**, 3773–3782 (2009).
99. Lo Celso, C. *et al.* Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* **457**, 92–96 (2009).
100. Xie, Y. *et al.* Detection of functional haematopoietic stem cell niche using real-time imaging. *Nature* **457**, 97–101 (2009).
101. Sugiyama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* **25**, 977–988 (2006).
102. Naveiras, O. *et al.* Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* **460**, 259–263 (2009).
103. Katayama, Y. *et al.* Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell* **124**, 407–421 (2006).
104. Kollet, O. *et al.* Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat. Med.* **12**, 657–664 (2006).
105. Lymperi, S., Ersek, A., Ferraro, F., Dazzi, F. & Horwood, N. J. Inhibition of osteoclast function reduces hematopoietic stem cell numbers in vivo. *Blood* **117**, 1540–1549 (2011).
106. Winkler, I. G. *et al.* Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* **116**, 4815–4828 (2010).
107. Dykstra, B. *et al.* Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell* **1**, 218–229 (2007).
108. Malhotra, S. & Kincade, P. W. Canonical Wnt pathway signaling suppresses VCAM-1 expression by marrow stromal and hematopoietic cells. *Exp. Hematol.* **37**, 19–30 (2009).
109. Trumpp, A., Essers, M. & Wilson, A. Awakening dormant haematopoietic stem cells. *Nat. Rev. Immunol.* **10**, 201–209 (2010).
110. Greenbaum, A. *et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* **495**, 227–230 (2013).
111. Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* **481**, 457–462 (2012).
112. Qian, H. *et al.* Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell* **1**, 671–684 (2007).
113. De Laval, B. *et al.* Thrombopoietin-increased DNA-PK-dependent DNA repair limits hematopoietic stem and progenitor cell mutagenesis in response to DNA damage. *Cell Stem Cell* **12**, 37–48 (2013).

114. Arai, F. *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**, 149–161 (2004).
115. Barker, J. E. Early transplantation to a normal microenvironment prevents the development of Steel hematopoietic stem cell defects. *Exp. Hematol.* **25**, 542–547 (1997).
116. Zheng, J., Huynh, H., Umikawa, M., Silvany, R. & Zhang, C. C. Angiopoietin-like protein 3 supports the activity of hematopoietic stem cells in the bone marrow niche. *Blood* **117**, 470–479 (2011).
117. Malhotra, S. & Kincade, P. W. Wnt-related molecules and signaling pathway equilibrium in hematopoiesis. *Cell Stem Cell* **4**, 27–36 (2009).
118. Cobas, M. *et al.* Beta-catenin is dispensable for hematopoiesis and lymphopoiesis. *J. Exp. Med.* **199**, 221–229 (2004).
119. Koch, U. *et al.* Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. *Blood* **111**, 160–164 (2008).
120. Fleming, H. E. *et al.* Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell* **2**, 274–283 (2008).
121. Renström, J. *et al.* Secreted frizzled-related protein 1 extrinsically regulates cycling activity and maintenance of hematopoietic stem cells. *Cell Stem Cell* **5**, 157–167 (2009).
122. Kieslinger, M., Hiechinger, S., Dobрева, G., Consalez, G. G. & Grosschedl, R. Early B cell factor 2 regulates hematopoietic stem cell homeostasis in a cell-nonautonomous manner. *Cell Stem Cell* **7**, 496–507 (2010).
123. Sugimura, R. *et al.* Noncanonical Wnt Signaling Maintains Hematopoietic Stem Cells in the Niche. *Cell* **150**, 351–365 (2012).
124. Artavanis-Tsakonas, S., Matsuno, K. & Fortini, M. E. Notch signaling. *Science* **268**, 225–232 (1995).
125. Kopan, R. & Ilagan, M. X. G. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* **137**, 216–233 (2009).
126. Palomero, T. *et al.* NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 18261–18266 (2006).
127. Hamidi, H., Gustafson, D., Pellegrini, M. & Gasson, J. Identification of novel targets of CSL-dependent Notch signaling in hematopoiesis. *PloS One* **6**, e20022 (2011).
128. Weng, A. P. *et al.* c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev.* **20**, 2096–2109 (2006).
129. Clements, W. K. *et al.* A somitic Wnt16/Notch pathway specifies haematopoietic stem cells. *Nature* **474**, 220–224 (2011).
130. Dzierzak, E. & Speck, N. A. Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat. Immunol.* **9**, 129–136 (2008).
131. Kumano, K. *et al.* Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* **18**, 699–711 (2003).
132. Ciofani, M. & Zúñiga-Pflücker, J. C. Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat. Immunol.* **6**, 881–888 (2005).
133. Grabher, C., von Boehmer, H. & Look, A. T. Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia. *Nat. Rev. Cancer* **6**, 347–359 (2006).
134. Tanigaki, K. *et al.* Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nat. Immunol.* **3**, 443–450 (2002).
135. Mercher, T. *et al.* Notch signaling specifies megakaryocyte development from hematopoietic stem cells. *Cell Stem Cell* **3**, 314–326 (2008).
136. Poirault-Chassac, S. *et al.* Notch/Delta4 signaling inhibits human megakaryocytic terminal differentiation. *Blood* **116**, 5670–5678 (2010).

137. Oh, P. *et al.* In Vivo Mapping of Notch Pathway Activity in Normal and Stress Hematopoiesis. *Cell Stem Cell* **13**, 190–204 (2013).
138. Klinakis, A. *et al.* A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. *Nature* **473**, 230–233 (2011).
139. Maillard, I. *et al.* Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. *Cell Stem Cell* **2**, 356–366 (2008).
140. Radtke, F. *et al.* Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547–558 (1999).
141. Varnum-Finney, B. *et al.* Notch2 governs the rate of generation of mouse long- and short-term repopulating stem cells. *J. Clin. Invest.* **121**, 1207–1216 (2011).
142. Butler, J. M. *et al.* Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell* **6**, 251–264 (2010).
143. Shutter, J. R. *et al.* Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev.* **14**, 1313–1318 (2000).
144. Lauret, E. *et al.* Membrane-bound delta-4 notch ligand reduces the proliferative activity of primitive human hematopoietic CD34+CD38low cells while maintaining their LTC-IC potential. *Leukemia* **18**, 788–797 (2004).
145. Bhardwaj, G. *et al.* Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat. Immunol.* **2**, 172–180 (2001).
146. Hofmann, I. *et al.* Hedgehog signaling is dispensable for adult murine hematopoietic stem cell function and hematopoiesis. *Cell Stem Cell* **4**, 559–567 (2009).
147. Goldman, D. C. *et al.* BMP4 regulates the hematopoietic stem cell niche. *Blood* **114**, 4393–4401 (2009).
148. Yamazaki, S. *et al.* Cytokine signaling, lipid raft clustering, and HSC hibernation. *Ann. N. Y. Acad. Sci.* **1106**, 54–63 (2007).
149. Annes, J. P., Munger, J. S. & Rifkin, D. B. Making sense of latent TGFbeta activation. *J. Cell Sci.* **116**, 217–224 (2003).
150. Connelly, J. T. *et al.* Actin and serum response factor transduce physical cues from the microenvironment to regulate epidermal stem cell fate decisions. *Nat. Cell Biol.* **12**, 711–718 (2010).
151. Kurth, I., Franke, K., Pompe, T., Bornhäuser, M. & Werner, C. Hematopoietic stem and progenitor cells in adhesive microcavities. *Integr. Biol. Quant. Biosci. Nano Macro* **1**, 427–434 (2009).
152. Nilsson, S. K. *et al.* Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* **106**, 1232–1239 (2005).
153. Stier, S. *et al.* Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J. Exp. Med.* **201**, 1781–1791 (2005).
154. Staal, F. J. T. & Clevers, H. C. WNT signalling and haematopoiesis: a WNT-WNT situation. *Nat. Rev. Immunol.* **5**, 21–30 (2005).
155. Haylock, D. N. & Nilsson, S. K. The role of hyaluronic acid in hemopoietic stem cell biology. *Regen. Med.* **1**, 437–445 (2006).
156. Priestley, G. V., Scott, L. M., Ulyanova, T. & Papayannopoulou, T. Lack of alpha4 integrin expression in stem cells restricts competitive function and self-renewal activity. *Blood* **107**, 2959–2967 (2006).
157. Peled, A. *et al.* The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood* **95**, 3289–3296 (2000).
158. Kirstetter, P., Anderson, K., Porse, B. T., Jacobsen, S. E. W. & Nerlov, C. Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat. Immunol.* **7**, 1048–1056 (2006).

159. Ulyanova, T., Priestley, G. V., Nakamoto, B., Jiang, Y. & Papayannopoulou, T. VCAM-1 ablation in nonhematopoietic cells in MxCre<sup>+</sup> VCAM-1<sup>f/f</sup> mice is variable and dictates their phenotype. *Exp. Hematol.* **35**, 565–571 (2007).
160. Lewandowski, D. *et al.* In vivo cellular imaging pinpoints the role of reactive oxygen species in the early steps of adult hematopoietic reconstitution. *Blood* **115**, 443–452 (2010).
161. Winkler, I. G. *et al.* Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nat. Med.* **18**, 1651–1657 (2012).
162. Lévesque, J.-P., Helwani, F. M. & Winkler, I. G. The endosteal ‘osteoblastic’ niche and its role in hematopoietic stem cell homing and mobilization. *Leukemia* **24**, 1979–1992 (2010).
163. Bromberg, O. *et al.* Osteoblastic N-cadherin is not required for microenvironmental support and regulation of hematopoietic stem and progenitor cells. *Blood* **120**, 303–313 (2012).
164. Greenbaum, A. M., Revollo, L. D., Woloszynek, J. R., Civitelli, R. & Link, D. C. N-cadherin in osteolineage cells is not required for maintenance of hematopoietic stem cells. *Blood* **120**, 295–302 (2012).
165. Adams, G. B. *et al.* Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature* **439**, 599–603 (2006).
166. Goodell, M. A. Stem cell identification and sorting using the Hoechst 33342 side population (SP). *Curr. Protoc. Cytom. Editor. Board J Paul Robinson Manag. Ed. A1 Chapter 9*, Unit9.18 (2005).
167. Parmar, K., Mauch, P., Vergilio, J.-A., Sackstein, R. & Down, J. D. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 5431–5436 (2007).
168. Lévesque, J.-P. *et al.* Hematopoietic progenitor cell mobilization results in hypoxia with increased hypoxia-inducible transcription factor-1 alpha and vascular endothelial growth factor A in bone marrow. *Stem Cells Dayt. Ohio* **25**, 1954–1965 (2007).
169. Rouault-Pierre, K. *et al.* HIF-2 $\alpha$  Protects Human Hematopoietic Stem/ Progenitors and Acute Myeloid Leukemic Cells from Apoptosis Induced by Endoplasmic Reticulum Stress. *Cell Stem Cell* (2013). doi:10.1016/j.stem.2013.08.011
170. Brouard, N., Driessen, R., Short, B. & Simmons, P. J. G-CSF increases mesenchymal precursor cell numbers in the bone marrow via an indirect mechanism involving osteoclast-mediated bone resorption. *Stem Cell Res.* **5**, 65–75 (2010).
171. Jiang, S. *et al.* Endocannabinoids are expressed in bone marrow stromal niches and play a role in interactions of hematopoietic stem and progenitor cells with the bone marrow microenvironment. *J. Biol. Chem.* **285**, 35471–35478 (2010).
172. Tesio, M. *et al.* Enhanced c-Met activity promotes G-CSF-induced mobilization of hematopoietic progenitor cells via ROS signaling. *Blood* **117**, 419–428 (2011).
173. Lander, E. S. *et al.* Initial sequencing and analysis of the human genome. *Nature* **409**, 860–921 (2001).
174. Carninci, P. *et al.* The transcriptional landscape of the mammalian genome. *Science* **309**, 1559–1563 (2005).
175. Lim, L. P. *et al.* Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433**, 769–773 (2005).
176. Friedman, R. C., Farh, K. K.-H., Burge, C. B. & Bartel, D. P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **19**, 92–105 (2009).
177. Ambros, V. *et al.* A uniform system for microRNA annotation. *RNA New York N* **9**, 277–279 (2003).
178. Kim, V. N., Han, J. & Siomi, M. C. Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell Biol.* **10**, 126–139 (2009).
179. Guo, S. *et al.* MicroRNA miR-125a controls hematopoietic stem cell number. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 14229–14234 (2010).



180. O'Connell, R. M. *et al.* MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 14235–14240 (2010).
181. Ooi, A. G. L. *et al.* MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. *Proc. Natl. Acad. Sci.* **107**, 21505–21510 (2010).
182. Yekta, S., Shih, I.-H. & Bartel, D. P. MicroRNA-directed cleavage of HOXB8 mRNA. *Science* **304**, 594–596 (2004).
183. Zaessinger, S., Busseau, I. & Simonelig, M. Oskar allows nanos mRNA translation in *Drosophila* embryos by preventing its deadenylation by Smaug/CCR4. *Dev. Camb. Engl.* **133**, 4573–4583 (2006).
184. Murata, Y. & Wharton, R. P. Binding of pumilio to maternal hunchback mRNA is required for posterior patterning in *Drosophila* embryos. *Cell* **80**, 747–756 (1995).
185. Wharton, R. P., Sonoda, J., Lee, T., Patterson, M. & Murata, Y. The Pumilio RNA-binding domain is also a translational regulator. *Mol. Cell* **1**, 863–872 (1998).
186. Parisi, M. & Lin, H. The *Drosophila* pumilio gene encodes two functional protein isoforms that play multiple roles in germline development, gonadogenesis, oogenesis and embryogenesis. *Genetics* **153**, 235–250 (1999).
187. Wickens, M., Bernstein, D. S., Kimble, J. & Parker, R. A PUF family portrait: 3'UTR regulation as a way of life. *Trends Genet. TIG* **18**, 150–157 (2002).
188. Kuo, M.-W. *et al.* A Novel puf-A Gene Predicted from Evolutionary Analysis Is Involved in the Development of Eyes and Primordial Germ-Cells. *PLoS ONE* **4**, e4980 (2009).
189. Spassov, D. S. & Jurecic, R. The PUF family of RNA-binding proteins: does evolutionarily conserved structure equal conserved function? *IUBMB Life* **55**, 359–366 (2003).
190. Macdonald, P. M. The *Drosophila* pumilio gene: an unusually long transcription unit and an unusual protein. *Dev. Camb. Engl.* **114**, 221–232 (1992).
191. Zhang, B. *et al.* A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. *Nature* **390**, 477–484 (1997).
192. Archer, S. K., Luu, V.-D., de Queiroz, R. A., Brems, S. & Clayton, C. Trypanosoma brucei PUF9 Regulates mRNAs for Proteins Involved in Replicative Processes over the Cell Cycle. *PLoS Pathog.* **5**, (2009).
193. Crittenden, S. L. *et al.* A conserved RNA-binding protein controls germline stem cells in *Caenorhabditis elegans*. *Nature* **417**, 660–663 (2002).
194. Gerber, A. P., Herschlag, D. & Brown, P. O. Extensive association of functionally and cytologically related mRNAs with Puf family RNA-binding proteins in yeast. *PLoS Biol.* **2**, E79 (2004).
195. Moore, F. L. *et al.* Human Pumilio-2 is expressed in embryonic stem cells and germ cells and interacts with DAZ (Deleted in AZoospermia) and DAZ-like proteins. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 538–543 (2003).
196. Morris, A. R., Mukherjee, N. & Keene, J. D. Ribonomic analysis of human Pum1 reveals cis-trans conservation across species despite evolution of diverse mRNA target sets. *Mol. Cell. Biol.* **28**, 4093–4103 (2008).
197. Vessey, J. P. *et al.* Dendritic localization of the translational repressor Pumilio 2 and its contribution to dendritic stress granules. *J. Neurosci. Off. J. Soc. Neurosci.* **26**, 6496–6508 (2006).
198. Voronina, E., Paix, A. & Seydoux, G. The P granule component PGL-1 promotes the localization and silencing activity of the PUF protein FBF-2 in germline stem cells. *Dev. Camb. Engl.* **139**, 3732–3740 (2012).
199. White, E. K., Moore-Jarrett, T. & Ruley, H. E. PUM2, a novel murine puf protein, and its consensus RNA-binding site. *RNA* **7**, 1855–1866 (2001).

200. Zoghbi, H. Y. & Orr, H. T. Glutamine repeats and neurodegeneration. *Annu. Rev. Neurosci.* **23**, 217–247 (2000).
201. Barker, D. D., Wang, C., Moore, J., Dickinson, L. K. & Lehmann, R. Pumilio is essential for function but not for distribution of the *Drosophila* abdominal determinant Nanos. *Genes Dev.* **6**, 2312–2326 (1992).
202. Zamore, P. D., Bartel, D. P., Lehmann, R. & Williamson, J. R. The PUMILIO-RNA interaction: a single RNA-binding domain monomer recognizes a bipartite target sequence. *Biochemistry (Mosc.)* **38**, 596–604 (1999).
203. Wang, X., McLachlan, J., Zamore, P. D. & Hall, T. M. T. Modular Recognition of RNA by a Human Pumilio-Homology Domain. *Cell* **110**, 501–512 (2002).
204. Edwards, T. A., Pyle, S. E., Wharton, R. P. & Aggarwal, A. K. Structure of Pumilio reveals similarity between RNA and peptide binding motifs. *Cell* **105**, 281–289 (2001).
205. Filipovska, A., Razif, M. F. M., Nygård, K. K. A. & Rackham, O. A universal code for RNA recognition by PUF proteins. *Nat. Chem. Biol.* **7**, 425–427 (2011).
206. Kedde, M. *et al.* A Pumilio-induced RNA structure switch in p27-3' UTR controls miR-221 and miR-222 accessibility. *Nat. Cell Biol.* **12**, 1014–1020 (2010).
207. Galgano, A. *et al.* Comparative analysis of mRNA targets for human PUF-family proteins suggests extensive interaction with the miRNA regulatory system. *PloS One* **3**, e3164 (2008).
208. Lu, G. & Hall, T. M. T. Alternate modes of cognate RNA recognition by human PUMILIO proteins. *Struct. Lond. Engl.* **19**, 361–367 (2011).
209. Koh, Y. Y. *et al.* Stacking interactions in PUF–RNA complexes. (2011). at <<http://130.203.133.150/viewdoc/summary?jsessionid=5DA97BBB34EDCD214E942C82579884B7?doi=10.1.1.221.779>>
210. Opperman, L., Hook, B., DeFino, M., Bernstein, D. S. & Wickens, M. A single spacer nucleotide determines the specificities of two mRNA regulatory proteins. *Nat. Struct. Mol. Biol.* **12**, 945–951 (2005).
211. Stumpf, C. R., Kimble, J. & Wickens, M. A *Caenorhabditis elegans* PUF protein family with distinct RNA binding specificity. *RNA New York N* **14**, 1550–1557 (2008).
212. Wang, Y., Opperman, L., Wickens, M. & Hall, T. M. T. Structural basis for specific recognition of multiple mRNA targets by a PUF regulatory protein. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 20186–20191 (2009).
213. Miller, M. T., Higgin, J. J. & Hall, T. M. T. Basis of altered RNA-binding specificity by PUF proteins revealed by crystal structures of yeast Puf4p. *Nat. Struct. Mol. Biol.* **15**, 397–402 (2008).
214. Campbell, Z. T. *et al.* Cooperativity in RNA-Protein Interactions: Global Analysis of RNA Binding Specificity. *Cell Reports* **1**, 570–581 (2012).
215. Goldstrohm, A. C., Hook, B. A., Seay, D. J. & Wickens, M. PUF proteins bind Pop2p to regulate messenger RNAs. *Nat. Struct. Mol. Biol.* **13**, 533–539 (2006).
216. Preiss, T., Muckenthaler, M. & Hentze, M. W. Poly(A)-tail-promoted translation in yeast: implications for translational control. *RNA New York N* **4**, 1321–1331 (1998).
217. Hook, B. A., Goldstrohm, A. C., Seay, D. J. & Wickens, M. Two yeast PUF proteins negatively regulate a single mRNA. *J. Biol. Chem.* **282**, 15430–15438 (2007).
218. Goldstrohm, A. C., Seay, D. J., Hook, B. A. & Wickens, M. PUF Protein-mediated Deadenylation Is Catalyzed by Ccr4p. *J. Biol. Chem.* **282**, 109–114 (2007).
219. Kadyrova, L. Y., Habara, Y., Lee, T. H. & Wharton, R. P. Translational control of maternal Cyclin B mRNA by Nanos in the *Drosophila* germline. *Dev. Camb. Engl.* **134**, 1519–1527 (2007).
220. Suh, N. *et al.* FBF and Its Dual Control of *gld-1* Expression in the *Caenorhabditis elegans* Germline. *Genetics* **181**, 1249–1260 (2009).
221. Van Etten, J. *et al.* Human Pumilio proteins recruit multiple deadenylases to efficiently repress messenger RNAs. *J. Biol. Chem.* **287**, 36370–36383 (2012).

222. Coller, J. M., Tucker, M., Sheth, U., Valencia-Sanchez, M. A. & Parker, R. The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes. *RNA* **7**, 1717 (2001).
223. Coller, J. & Parker, R. General translational repression by activators of mRNA decapping. *Cell* **122**, 875–886 (2005).
224. Cho, P. F. *et al.* Cap-Dependent Translational Inhibition Establishes Two Opposing Morphogen Gradients in Drosophila Embryos. *Curr. Biol. CB* **16**, 2035–2041 (2006).
225. Cho, P. F. *et al.* A New Paradigm for Translational Control: Inhibition via 5'-3' mRNA Tethering by Bicoid and the eIF4E Cognate 4EHP. *Cell* **121**, 411–423 (2005).
226. Cao, Q., Padmanabhan, K. & Richter, J. D. Pumilio 2 controls translation by competing with eIF4E for 7-methyl guanosine cap recognition. *RNA* **16**, 221–227 (2010).
227. Friend, K. *et al.* A conserved PUF–Ago–eEF1A complex attenuates translation elongation. *Nat. Struct. Mol. Biol.* **19**, 176–183 (2012).
228. Nolde, M. J., Saka, N., Reinert, K. L. & Slack, F. J. The Caenorhabditis elegans pumilio homolog, puf-9, is required for the 3'UTR-mediated repression of the let-7 microRNA target gene, hbl-1. *Dev. Biol.* **305**, 551–563 (2007).
229. Leibovich, L., Mandel-Gutfreund, Y. & Yakhini, Z. A structural-based statistical approach suggests a cooperative activity of PUM1 and miR-410 in human 3'-untranslated regions. *Silence* **1**, 17 (2010).
230. Piqué, M., López, J. M., Foissac, S., Guigó, R. & Méndez, R. A combinatorial code for CPE-mediated translational control. *Cell* **132**, 434–448 (2008).
231. Kaye, J. A., Rose, N. C., Goldsworthy, B., Goga, A. & L'Etoile, N. D. A 3'UTR pumilio-binding element directs translational activation in olfactory sensory neurons. *Neuron* **61**, 57–70 (2009).
232. Saint-Georges, Y. *et al.* Yeast Mitochondrial Biogenesis: A Role for the PUF RNA-Binding Protein Puf3p in mRNA Localization. *PLoS ONE* **3**, e2293 (2008).
233. Gu, W., Deng, Y., Zenklusen, D. & Singer, R. H. A new yeast PUF family protein, Puf6p, represses ASH1 mRNA translation and is required for its localization. *Genes Dev.* **18**, 1452–1465 (2004).
234. Zipor, G. *et al.* Localization of mRNAs coding for peroxisomal proteins in the yeast, Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 19848–19853 (2009).
235. Vessey, J. P. *et al.* Mammalian Pumilio 2 regulates dendrite morphogenesis and synaptic function. *Proc. Natl. Acad. Sci.* **107**, 3222–3227 (2010).
236. Kershner, A. M. & Kimble, J. Genome-wide analysis of mRNA targets for Caenorhabditis elegans FBF, a conserved stem cell regulator. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 3936–3941 (2010).
237. Merritt, C. & Seydoux, G. The Puf RNA-binding proteins FBF-1 and FBF-2 inhibit the expression of synaptonemal complex proteins in germline stem cells. *Dev. Camb. Engl.* **137**, 1787–1798 (2010).
238. Lamont, L. B., Crittenden, S. L., Bernstein, D., Wickens, M. & Kimble, J. FBF-1 and FBF-2 regulate the size of the mitotic region in the C. elegans germline. *Dev. Cell* **7**, 697–707 (2004).
239. Chen, D. *et al.* Pumilio 1 suppresses multiple activators of p53 to safeguard spermatogenesis. *Curr. Biol. CB* **22**, 420–425 (2012).
240. Curtis, D. *et al.* A CCHC metal-binding domain in Nanos is essential for translational regulation. *EMBO J.* **16**, 834–843 (1997).
241. Curtis, D., Apfeld, J. & Lehmann, R. nanos is an evolutionarily conserved organizer of anterior-posterior polarity. *Dev. Camb. Engl.* **121**, 1899–1910 (1995).
242. Kraemer, B. *et al.* NANOS-3 and FBF proteins physically interact to control the sperm-oocyte switch in Caenorhabditis elegans. *Curr. Biol. CB* **9**, 1009–1018 (1999).
243. Sonoda, J. & Wharton, R. P. Recruitment of Nanos to hunchback mRNA by Pumilio. *Genes Dev.* **13**, 2704–2712 (1999).

244. Nakahata, S. *et al.* Biochemical identification of *Xenopus* Pumilio as a sequence-specific cyclin B1 mRNA-binding protein that physically interacts with a Nanos homolog, Xcat-2, and a cytoplasmic polyadenylation element-binding protein. *J. Biol. Chem.* **276**, 20945–20953 (2001).
245. Ginter-Matuszewska, B. *et al.* NANOS1 and PUMILIO2 bind microRNA biogenesis factor GEMIN3, within chromatoid body in human germ cells. *Histochem. Cell Biol.* **136**, 279–287 (2011).
246. Richter, J. D. & Lasko, P. Translational control in oocyte development. *Cold Spring Harb. Perspect. Biol.* **3**, a002758 (2011).
247. Ortiz-Zapater, E. *et al.* Key contribution of CPEB4-mediated translational control to cancer progression. *Nat. Med.* **18**, 83–90 (2012).
248. Standart, N. & Minshall, N. Translational control in early development: CPEB, P-bodies and germinal granules. *Biochem. Soc. Trans.* **36**, 671–676 (2008).
249. Luitjens, C., Gallegos, M., Kraemer, B., Kimble, J. & Wickens, M. CPEB proteins control two key steps in spermatogenesis in *C. elegans*. *Genes Dev.* **14**, 2596–2609 (2000).
250. Sonoda, J. & Wharton, R. P. *Drosophila* Brain Tumor is a translational repressor. *Genes Dev.* **15**, 762–773 (2001).
251. Neumüller, R. A. *et al.* Mei-P26 regulates microRNAs and cell growth in the *Drosophila* ovarian stem cell lineage. *Nature* **454**, 241–245 (2008).
252. Frank, D. J. & Roth, M. B. *ncl-1* is required for the regulation of cell size and ribosomal RNA synthesis in *Caenorhabditis elegans*. *J. Cell Biol.* **140**, 1321–1329 (1998).
253. Schwamborn, J. C., Berezikov, E. & Knoblich, J. A. The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. *Cell* **136**, 913–925 (2009).
254. Loedige, I., Gaidatzis, D., Sack, R., Meister, G. & Filipowicz, W. The mammalian TRIM-NHL protein TRIM71/LIN-41 is a repressor of mRNA function. *Nucleic Acids Res.* **41**, 518–532 (2012).
255. Harris, R. E., Pargett, M., Sutcliffe, C., Umulis, D. & Ashe, H. L. Brat Promotes Stem Cell Differentiation via Control of a Bistable Switch that Restricts BMP Signaling. *Dev. Cell* **20**, 72–83 (2011).
256. Fiore, R. *et al.* Mef2-mediated transcription of the miR379–410 cluster regulates activity-dependent dendritogenesis by fine-tuning Pumilio2 protein levels. *EMBO J.* **28**, 697–710 (2009).
257. Lin, H. & Spradling, A. C. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Dev. Camb. Engl.* **124**, 2463–2476 (1997).
258. Forbes, A. & Lehmann, R. Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. *Dev. Camb. Engl.* **125**, 679–690 (1998).
259. Souza, G. M., da Silva, A. M. & Kuspa, A. Starvation promotes Dictyostelium development by relieving PufA inhibition of PKA translation through the YakA kinase pathway. *Dev. Camb. Engl.* **126**, 3263–3274 (1999).
260. Kennedy, B. K. *et al.* Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in *S. cerevisiae*. *Cell* **89**, 381–391 (1997).
261. Siemen, H., Colas, D., Heller, H. C., Brüstle, O. & Reijo Pera, R. A. Pumilio-2 Function in the Mouse Nervous System. *PLoS ONE* **6**, e25932 (2011).
262. Tordai, A. *et al.* Evaluation of biological pathways involved in chemotherapy response in breast cancer. *Breast Cancer Res.* **10**, R37 (2008).
263. Olsson, A. Y. *et al.* Role of E2F3 expression in modulating cellular proliferation rate in human bladder and prostate cancer cells. *Oncogene* **26**, 1028–1037 (2007).
264. Cooper, C. S. *et al.* Nuclear overexpression of the E2F3 transcription factor in human lung cancer. *Lung Cancer Amst. Neth.* **54**, 155–162 (2006).



265. Feber, A. *et al.* Amplification and overexpression of E2F3 in human bladder cancer. *Oncogene* **23**, 1627–1630 (2004).
266. Oeggerli, M. *et al.* E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer. *Oncogene* **23**, 5616–5623 (2004).
267. Hurst, C. D., Tomlinson, D. C., Williams, S. V., Platt, F. M. & Knowles, M. A. Inactivation of the Rb pathway and overexpression of both isoforms of E2F3 are obligate events in bladder tumours with 6p22 amplification. *Oncogene* **27**, 2716–2727 (2008).
268. Dyrskjöt, L. *et al.* Genomic profiling of microRNAs in bladder cancer: miR-129 is associated with poor outcome and promotes cell death in vitro. *Cancer Res.* **69**, 4851–4860 (2009).
269. Miles, W. O., Tschöp, K., Herr, A., Ji, J.-Y. & Dyson, N. J. Pumilio facilitates miRNA regulation of the E2F3 oncogene. *Genes Dev.* **26**, 356–368 (2012).
270. Spassov, D. S. & Jurecic, R. Mouse Pum1 and Pum2 genes, members of the Pumilio family of RNA-binding proteins, show differential expression in fetal and adult hematopoietic stem cells and progenitors. *Blood Cells. Mol. Dis.* **30**, 55–69 (2003).
271. Jordan, C. T. *et al.* Long-term repopulating abilities of enriched fetal liver stem cells measured by competitive repopulation. *Exp. Hematol.* **23**, 1011–1015 (1995).
272. Phillips, R. L. *et al.* The genetic program of hematopoietic stem cells. *Science* **288**, 1635–1640 (2000).
273. Kim, M., Cooper, D. D., Hayes, S. F. & Spangrude, G. J. Rhodamine-123 staining in hematopoietic stem cells of young mice indicates mitochondrial activation rather than dye efflux. *Blood* **91**, 4106–4117 (1998).
274. Rebel, V. I., Dragowska, W., Eaves, C. J., Humphries, R. K. & Lansdorp, P. M. Amplification of Sca-1+ Lin- WGA+ cells in serum-free cultures containing steel factor, interleukin-6, and erythropoietin with maintenance of cells with long-term in vivo reconstituting potential. *Blood* **83**, 128–136 (1994).
275. Spangrude, G. J. & Scollay, R. A simplified method for enrichment of mouse hematopoietic stem cells. *Exp. Hematol.* **18**, 920–926 (1990).
276. Jurecic, R., Van, N. T. & Belmont, J. W. Enrichment and functional characterization of Sca-1+WGA+, Lin-WGA+, Lin-Sca-1+, and Lin-Sca-1+WGA+ bone marrow cells from mice with an Ly-6a haplotype. *Blood* **82**, 2673–2683 (1993).
277. Li, C. L. & Johnson, G. R. Murine hematopoietic stem and progenitor cells: I. Enrichment and biologic characterization. *Blood* **85**, 1472–1479 (1995).
278. Lee, M.-H. *et al.* Conserved regulation of MAP kinase expression by PUF RNA-binding proteins. *PLoS Genet.* **3**, e233 (2007).
279. Vessey, J. P. *et al.* An Asymmetrically Localized Staufen2-Dependent RNA Complex Regulates Maintenance of Mammalian Neural Stem Cells. *Cell Stem Cell* **11**, 517–528 (2012).
280. Shigunov, P. *et al.* PUMILIO-2 is involved in the positive regulation of cellular proliferation in human adipose-derived stem cells. *Stem Cells Dev.* **21**, 217–227 (2012).
281. Urano, J., Fox, M. S. & Reijo Pera, R. A. Interaction of the conserved meiotic regulators, BOULE (BOL) and PUMILIO-2 (PUM2). *Mol. Reprod. Dev.* **71**, 290–298 (2005).
282. Xu, E. Y., Chang, R., Salmon, N. A. & Reijo Pera, R. A. A gene trap mutation of a murine homolog of the Drosophila stem cell factor Pumilio results in smaller testes but does not affect litter size or fertility. *Mol. Reprod. Dev.* **74**, 912–921 (2007).
283. Frosk, P. *et al.* Limb-girdle muscular dystrophy type 2H associated with mutation in TRIM32, a putative E3-ubiquitin-ligase gene. *Am. J. Hum. Genet.* **70**, 663–672 (2002).
284. Nicklas, S. *et al.* TRIM32 regulates skeletal muscle stem cell differentiation and is necessary for normal adult muscle regeneration. *PloS One* **7**, e30445 (2012).
285. Sada, A., Suzuki, A., Suzuki, H. & Saga, Y. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. *Science* **325**, 1394–1398 (2009).

286. Auvray, C. *et al.* HOXC4 homeoprotein efficiently expands human hematopoietic stem cells and triggers similar molecular alterations as HOXB4. *Haematologica* **97**, 168–178 (2012).
287. Haddad, R. *et al.* The HOXB4 homeoprotein differentially promotes ex vivo expansion of early human lymphoid progenitors. *Stem Cells Dayt. Ohio* **26**, 312–322 (2008).
288. Campisi, J. Aging, cellular senescence, and cancer. *Annu. Rev. Physiol.* **75**, 685–705 (2013).
289. Nijnik, A. *et al.* DNA repair is limiting for haematopoietic stem cells during ageing. *Nature* **447**, 686–690 (2007).
290. Rossi, D. J. *et al.* Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* **447**, 725–729 (2007).
291. Kitajima, K., Minehata, K., Sakimura, K., Nakano, T. & Hara, T. In vitro generation of HSC-like cells from murine ESCs/iPSCs by enforced expression of LIM-homeobox transcription factor Lhx2. *Blood* **117**, 3748–3758 (2011).
292. Xu, Y. *et al.* LH-2: a LIM/homeodomain gene expressed in developing lymphocytes and neural cells. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 227–231 (1993).
293. Pinto do O, P., Richter, K. & Carlsson, L. Hematopoietic progenitor/stem cells immortalized by Lhx2 generate functional hematopoietic cells in vivo. *Blood* **99**, 3939–3946 (2002).
294. Wilson, N. K. *et al.* Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. *Cell Stem Cell* **7**, 532–544 (2010).
295. Sirven, A. *et al.* Enhanced transgene expression in cord blood CD34(+)-derived hematopoietic cells, including developing T cells and NOD/SCID mouse repopulating cells, following transduction with modified trip lentiviral vectors. *Mol. Ther. J. Am. Soc. Gene Ther.* **3**, 438–448 (2001).
296. Zennou, V. *et al.* HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101**, 173–185 (2000).
297. Zand, M. S. *et al.* Apoptosis and complement-mediated lysis of myeloma cells by polyclonal rabbit antithymocyte globulin. *Blood* **107**, 2895–2903 (2006).
298. Francischini, C. W. & Quaggio, R. B. Molecular characterization of Arabidopsis thaliana PUF proteins--binding specificity and target candidates. *FEBS J.* **276**, 5456–5470 (2009).
299. Krosl, J. *et al.* In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. *Nat. Med.* **9**, 1428–1432 (2003).
300. Rossi, L. *et al.* Less is more: unveiling the functional core of hematopoietic stem cells through knockout mice. *Cell Stem Cell* **11**, 302–317 (2012).
301. Yoshihara, H. *et al.* Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell* **1**, 685–697 (2007).
302. Brun, A. C. M. *et al.* Hoxb4-deficient mice undergo normal hematopoietic development but exhibit a mild proliferation defect in hematopoietic stem cells. *Blood* **103**, 4126–4133 (2004).
303. Kayali, F., Montie, H. L., Rafols, J. A. & DeGracia, D. J. Prolonged translation arrest in reperfused hippocampal cornu Ammonis 1 is mediated by stress granules. *Neuroscience* **134**, 1223–1245 (2005).
304. Nover, L., Scharf, K. D. & Neumann, D. Cytoplasmic heat shock granules are formed from precursor particles and are associated with a specific set of mRNAs. *Mol. Cell. Biol.* **9**, 1298–1308 (1989).
305. Meacham, C. E. & Morrison, S. J. Tumour heterogeneity and cancer cell plasticity. *Nature* **501**, 328–337 (2013).







## RESUME

Les propriétés centrales des cellules souches sont la **pluripotence** et la capacité **d'auto-renouvellement**. Les **cellules souches hématopoïétiques** (CSHs) sont dotées de ces caractéristiques qui leur permettent de générer toutes les cellules du compartiment hématopoïétique, tout en maintenant en parallèle leur compartiment. Nous menons des approches visant à amplifier *ex vivo* les CSHs en les activant par HOXB4 exogène (CSHs humaines) ou via la signalisation Notch/DLL-4 (CSHs murines). Or deux analyses transcriptomiques indépendantes de ces deux modes d'activation ont de manière étonnante convergé sur une augmentation de l'expression de deux gènes jamais identifiés auparavant comme étant impliqués dans le maintien des CSHs : **Pumilio1** (Pum1) et **Pumilio2** (Pum2). Pum1 et Pum2 sont des **régulateurs post-transcriptionnels** appartenant à la famille Pumilio-FBF (PUF) des protéines liant l'ARN. Bien qu'il ait été établi que le rôle princeps de ces protéines PUF est de soutenir la prolifération des cellules souches chez les Invertébrés, jusqu'à présent on ne sait rien du rôle de Pum1 et Pum2 dans les CSH humaines et murines.

Pour toutes ces raisons, nous avons étudié le rôle et les mécanismes d'action de Pum1 et Pum2 dans les CSH murines et humaines en utilisant l'interférence ARN (ARNi). L'invalidation de Pum1 ou de Pum2 dans les CSHs murines conduit à une réduction de l'expansion et du potentiel clonogénique *ex vivo*, associée à une apoptose accrue et l'arrêt du cycle cellulaire en phase G0/G1. L'invalidation concomitante de Pum1 et Pum2 majore ces effets ce qui suggère un effet coopératif entre les deux protéines. L'expansion et le potentiel clonogénique des CSH invalidées pour Pum1 sont restaurés suite à l'expression forcée de Pum1 (insensible au shRNA utilisé), validant ainsi la spécificité de nos shRNAs. Par contre la surexpression de Pum1 dans les CSHs invalidées pour Pum2 ne restaure pas leurs fonctions, soulignant le rôle non redondant de chaque protéine. En outre, lorsque les CSHs invalidées pour Pum1 ou Pum2 sont inoculées à des souris irradiées létalement de suivre le potentiel hématopoïétique à long terme, seules quelques rares cellules de la moelle osseuse issues des CSH KD pour Pum1 ou Pum2 sont mises en évidence après 4 mois de reconstitution, contrairement aux CSH contrôles. Des résultats identiques ont été obtenus en invalidant Pum1 ou Pum2 dans les CSH humaines.

**En conclusion**, nos résultats démontrent **l'implication des facteurs Pumilio dans le maintien du potentiel souche**, l'expansion et la survie des CSHs murines et humaines. L'identification des facteurs Pumilio et de leurs cibles comme nouveaux régulateurs des CSHs permettra d'envisager de nouveaux outils en vue de perspectives thérapeutiques.

## ABSTRACT

The central properties of stem cells are the pluripotency and the capacity of self-renewal. Hematopoietic stem cells (HSCs) possess such common features that allows them to generate all the cells of the hematopoietic compartments, maintaining in the same time the HSC pool. We develop approaches focused on *ex vivo* HSC expansion through activation by exogenous HOXB4 (human HSCs) or Notch/Dll-4 ligand (murine HSCs). Two independent transcriptomic analyses surprisingly converged toward an increased expression of two genes never identified so far as crucial for HSC functions: **Pumilio1** (Pum1) and **Pumilio2** (Pum2). Pum1 and Pum2 are posttranscriptional regulators belonging to the Pumilio-FBF (PUF) family of RNA-binding proteins. Although it was established that the primordial role of PUF proteins is to sustain mitotic proliferation of stem cells in Invertebrates, so far nothing is known about the role of Pum1 and Pum2 in human and murine HSCs.

For these reasons, we have investigated the roles and mechanisms of action of Pum1 and Pum2 in murine and human HSCs through shRNA strategy. Pum1 and Pum2 knockdown (KD) in murine HSCs led to a decreased HSC expansion and clonogenic potential *ex vivo*, associated with an increased apoptosis and a cell cycle arrest in G0/G1 phase. KD of both Pum1 and Pum2 enhanced these effects, suggesting a cooperative effect. Expansion and clonogenic potential of KD Pum1 HSCs were rescued by enforced expression of Pum1 (insensitive to our shRNA), thus validating the specificity of our shRNA. Enforced expression of Pum1 could not rescue the functions of Pum2 KD HSCs, highlighting the non-redundant role of these proteins. Furthermore, when Pum1 or Pum2 KD HSCs were inoculated into lethally irradiated mice to follow the long-term hematopoietic potential, only rare bone marrow cells derived from Pum1 and Pum2 KD HSCs were evidenced after 4 months, contrary to control HSCs. Identical results were obtained with human Pum1 or Pum2 KD HSCs.

In conclusion, our results demonstrate the involvement of Pumilio factors in stemness maintenance, expansion and survival of murine and human HSCs. Identification of Pumilio factors and their targets as new regulators of HSCs expansion will allow consider them as new tools for therapeutic perspectives.