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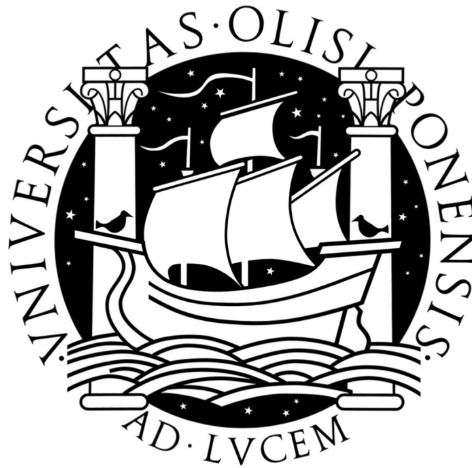

ROLE OF THE NEUROTROPHIC FACTOR RECEPTOR RET IN HAEMATOPOIESIS

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RESUMO

O sistema hematopoiético, que inclui as células do sistema imunitário, é altamente complexo e dinâmico, e como tal, está sujeito a uma regulação apertada. Assim, o estudo dos mecanismos moleculares responsáveis pelo desenvolvimento de células hematopoiéticas é essencial para compreender a forma como este sistema funciona de modo a manter o equilíbrio entre o número de células necessárias em homeostasia e uma rápida resposta em situações de desequilíbrio.

A tirosina cinase RET é o receptor para os factores neurotróficos da família do GDNF (glial cell line-derived neurotrophic factor) (GDNF family ligands – GFLs) e tem uma função crucial no desenvolvimento e manutenção do sistema nervoso, no desenvolvimento embrionário do rim e na espermatogénese. Curiosamente, a expressão de *Ret* já foi detectada em várias populações de células hematopoiéticas e em órgãos linfoides primários como o fígado fetal, a medula óssea e o timo. Para além disso, foi também demonstrado um papel crucial da sinalização por RET em células hematopoiéticas envolvidas na organogénese das placas de Peyer no intestino durante a vida embrionária. No entanto, apesar de a expressão de RET ter sido identificada em várias populações celulares, uma possível função de RET no desenvolvimento ou função de células hematopoiéticas não é ainda conhecida.

As células estaminais hematopoiéticas, pelas suas capacidades de autorrenovação e proliferação, são a base do programa de desenvolvimento e diferenciação que origina todas as células do sangue. Por este motivo, é crucial compreender os complexos mecanismos que regulam as células estaminais hematopoiéticas. Assim, no laboratório estamos particularmente interessados no papel de RET na função das células estaminais hematopoiéticas e no modo como a sinalização por RET pode ser usada de forma a modular a sua resposta em situações de desequilíbrio como a transplantação.

Uma vez que foi proposto um efeito do ligando de RET GDNF na sobrevivência de timócitos *in vitro*, estamos também interessados em compreender a função de RET no desenvolvimento de células T *in vivo*. O timo é responsável pela produção de todas as populações de células T, que são essenciais para respostas imunitárias eficientes. Como tal é indispensável perceber de que modo o microambiente tímico fornece uma complexa rede de sinais que levam ao desenvolvimento de células T a partir de precursores da medula óssea.

No laboratório descobrimos que a tirosina cinase RET tem uma função crítica na sobrevivência e função das células estaminais hematopoiéticas. Estas expressam a maquinaria de sinalização por RET, que inclui o receptor RET e os seus co-receptores, enquanto o microambiente onde as células estaminais hematopoéticas se encontram providencia os ligandos de RET necessários. Para além disso, a ablação de *Ret* leva à redução do número de células estaminais hematopoiéticas e ao recrutamento de células quiescentes para o estado proliferativo. Apesar de os progenitores hematopoiéticos deficientes em RET terem um potencial de diferenciação normal, apresentam uma fraca resposta ao stresse *in vivo* e um potencial de reconstituição reduzido. Importante, a sinalização de RET fornece factores de sobrevivência às células estaminais hematopoiéticas, por a jusante resultar na fosforilação da cinase p38/MAPK e na activação do factor de transcrição CREB. Em concordância, a sobre-expressão dos genes alvo a jusante de RET, *Bcl2* ou *Bcl2l1*, resgata *in vivo* a função hematopoiética de progenitores deficientes em *Ret*, aumentado o seu potencial te transplantação. Na verdade, a activação de RET aumenta a sobrevivência de células estaminais hematopoiéticas e a sua eficiência de transplantação *in vivo*, revelando novas possibilidades de intervenção em terapias de transplantação e expansão *ex vivo* de células estaminais hematopoiéticas. Assim, o nosso trabalho mostra que factores neurotróficos presentes no nicho das células estaminais hematopoiéticas regulam a sua função através do receptor RET.

Embora tenhamos confirmado que as moléculas envolvidas na sinalização de RET são expressas no timo, especialmente na população de timócitos negativa para os co-receptores CD4 e CD8 (DN), a remoção de *Ret* ou dos seus co-receptores *Gfra1* ou *Gfra2* não afecta a timopoiese fetal. Concordantemente, animais adultos com eliminação condicional de *Ret* em timócitos revelam que a capacidade de desenvolvimento de timócitos deficientes em *Ret é* semelhante à dos controlos selvagens. Do mesmo modo mutações que conferem um ganho de função no receptor RET não influenciam o desenvolvimento tímico. Assim, apesar de a sinalização por RET poder fornecer sinais de sobrevivência a timócitos, esta é dispensável para o desenvolvimento de células T *in vivo*, mesmo em condições de competição entre progenitores deficientes ou competentes em *Ret*.

No seu conjunto, o nosso trabalho demonstra que mecanismos moleculares geralmente atribuídos a tecidos específicos, podem ser mais amplamente utilizado por tipos de células não relacionadas, tais como células estaminais hematopoiéticas. Os nossos resultados também ilustram como uma mesma via de sinalização pode ser

regulada de forma a originar diferentes respostas celulares. Contrariamente a diversas populações de neurónios, que usam GFLs específicos dependendo do co-receptor que expressam, as HSCs expressam múltiplos coreceptores de RET respondem aos GFLs de forma redundante.

Existem cada vez mais evidências de que os sinais provenientes do sistema nervoso podem controlar hematopoiese, nomeadamente através da regulação da função dos osteoblastos e células estaminais mesenquimais presentes nos nichos das HSCs. Surpreendentemente, nós mostramos que os neurônios e as HSCs utilizam mecanismos comuns de regulação.. Deste modo, o nosso trabalho abre caminho a novos estudos na utilização factores neurotróficos em protocolos de expansão e transplantação de HSCs.

SUMMARY

Haematopoiesis is a developmental process that ensures the generation of all blood cell lineages throughout life. As a consequence, this is a highly complex and dynamic developmental cascade subject to tight regulatory mechanisms. Thus, the study of novel molecular signals is critical to further understand how haematopoiesis operates to ensure the balance between cell lineage commitment, cell homeostasis and efficient haematopoietic responses to insults and disturbances.

The tyrosine kinase RET is the receptor for the GDNF (glial cell line-derived neurotrophic factor) neurotrophic factor family (GDNF family ligands – GFLs). Productive RET signalling controls the development and maintenance of the enteric nervous system, kidneys and spermatogenesis. Interestingly, *Ret* expression was detected in haematopoietic cells and lymphoid organs and RET signalling was shown to regulate enteric lymphoid organogenesis. However the role of RET in haematopoiesis remains completely unexplored.

Haematopoietic stem cells (HSCs) are at the onset of the developmental cascade that generate all blood cells, thus we initially investigated the role of RET in HSC function and how modulation of RET signalling can be used to control HSC responses. Finally, we investigated the role of RET in late stages of haematopoietic cell precursor differentiation into the T cell lineage.

We found that the tyrosine kinase RET is critical to HSC survival and function. HSCs express RET signalling molecules and HSCs microenvironment provides RET ligands. Moreover *Ret* ablation leads to reduced HSC numbers and recruitment of quiescent cells into proliferation. Although RET null progenitors have normal differentiation potential, they exhibit impaired *in vivo* stress response and reconstitution potential. Remarkably RET downstream signalling results in p38/MAP kinase phosphorylation and CREB transcription factor activation, providing HSCs with critical surviving cues. In agreement, recue of *Ret* null progenitors was efficiently achieved *in vivo* by forcing the expression of RET downstream targets, *Bcl2* or *Bcl2l1*. Thus, RET activation improves HSC survival and *in vivo* transplantation efficiency, unveiling exciting new possibilities in transplantation and HSC *ex vivo* expansion. In addition, our work demonstrates that HSC use neurotrophic factors to regulate and maintain their fitness.

RET signalling molecules are also expressed in thymocytes, more specifically, we found their expression in CD4/CD8 double negative thymocytes (DN). Nevertheless, ablation of *Ret* or it co-receptors *Gfra1* and *Gfra2* had a minor impact in foetal thymopoiesis. In agreement, *Ret* conditional knockout mice had similar thymocyte development and fitness when compared to their WT counterparts. Thus, while RET signalling is critical to HSC function, it is dispensable for T cell development *in vivo*.

Altogether, our work show that molecular mechanisms usually assigned to specific tissues, can be more widely used by unrelated cell types, such as haematopoietic stem cells. Our findings also illustrate how a same signalling pathway can be regulated to originate different cell responses. Unlike several neuronal populations that use GFLs depending on the specific expressed co-receptor, HSCs express multiple RET co-receptors and respond to GFLs in a redundant fashion.

There is increasing evidence that nervous signals can control haematopoiesis, namely by regulating osteoblast and mesenchymal stem cell function in HSC niches. Herein, we show that neurons and HSC employ common regulatory mechanisms. Thus, our work paves the way to further studies employing neurotrophic factors in HSC expansion and transplantation protocols.

INTRODUCTION

The Haematopoietic system

The haematopoietic system, Ancient Greek for the system that makes blood, is composed by a wide range of molecular and cellular components. Haematopoietic cells are mainly found in lymphoid organs that promote haematopoietic cell interactions with themselves and with other systems in the body. The main functions of the haematopoietic system are: *i.* oxygen transportation, *ii.* blood coagulation, and *iii.* defence against foreigner antigens[1].

Since oxygen is a key element to energy production, this molecule is critically required for tissue development in embryonic life. In mammals, oxygen transportation is sustained by enucleated erythrocytes that contain haemoglobin, thus it is not surprising that these cells are among the first haematopoietic cells to be produced in the embryo. In mice they appear in the yolk sac's blood island (YS) at embryonic day 7.5 (E7.5) as primitive erythrocytes; however, the first enucleated definitive erythrocyte only appears by E12.5[2].

Blood coagulation prevents excessive blood loss from disrupted vessels, thus ensuring haemostasis. Quickly after injury, platelets aggregate locally, forming a plug at the site of injury, in an event called primary haemostasis[3]. Simultaneously, a myriad of coagulation factors in the blood plasma, react in a complex signalling cascade that result in the formation of fibrin strands that strengthen the platelet plug and make the scaffold for consecutive tissue remodelling and cicatrization[4].

Defence against potential pathogenic microorganisms and non-self antigens is provided by white blood cells, called leukocytes. Leukocytes are responsible for distinct immune responses in the body and encompass multiple immune cell populations. Immune responses are traditionally subdivided into innate or adaptive responses that cooperate for the elimination of infection or foreigner antigens. Innate immune responses are considerably faster than adaptive immune responses and provide non-specific inflammatory responses to pathogen and damage molecular patterns, thus they constitute an initial front-line of protection. The main players of innate immunity are myeloid cells, macrophages and granulocytes, which produce inflammatory molecules and can phagocyte pathogens. Importantly, an emergent family of innate lymphoid cells (ILC), which includes natural killer (NK) cells and ILC 2 and 3 have been recognized as main regulators of immune responses at mucosal

sites. In addition, NK cells can recognise and kill infected, transformed or altered cells, thus being very important for tumour surveillance[5-7].

The main feature of adaptive immune cells is their specificity to a given antigen. Adaptive responses can take some days to become efficient, requiring the engagement of antigen specific receptors in B and T lymphocytes, the B cell receptor (BCR) and T cell receptor (TCR), respectively. Remarkably, B and T cell responses generate potent memory cells that are long lived and react stronger and faster to previous encountered antigens. Thus, productive adaptive immune responses and generation of immunological memory are critical to efficient vaccination protocols.

Haematopoietic organs

Primary lymphoid organs

Haematopoiesis takes place in different tissues. Primary lymphoid organs ensure blood cell generation from haematopoietic stem cells and may allow differentiation of mature cells. Thus, their tissue microenvironment determines the development of haematopoietic progenitors into several blood cell lineages.

In mammals the first haematopoietic progenitors appear in the yolk sac's blood island (YS) and generate primitive erythrocytes, while in fish this process occurs in the intermediate cell mass[2,8,9]. Haematopoietic stem cells (HSCs) are generated at a later stage in the aorta-gonad-mesonephros region (AGM) and sequentially migrate to the foetal liver (FL) that becomes the main lymphoid organ of the embryo. Around birth, all liver haematopoiesis ceases, and the bone marrow becomes the main primary lymphoid organ[10,11]. In fish the kidney marrow and the spleen are the main primary lymphoid organs[12]. Some haematopoietic cell lineages require additional lymphoid organs to fully differentiate. As an example, the thymus provides the microenvironment for lymphoid progenitors that migrate from foetal liver and bone marrow to differentiate into mature T lymphocytes, and is therefore considered as a primary lymphoid organ[13-16]. In most mammals, B cell differentiation occurs in the bone marrow. However in sheep and cattle, the ileal Peyer's patch is the primary lymphoid organ for B cell development and in birds B cell development occurs in the bursa of Fabricius that gave the name to B cells (bursa-derived)[16-18].

Secondary lymphoid organs

Secondary lymphoid organs (SLOs), such as spleen and lymph nodes, are generated in embryonic life in strategic positions throughout the body and constitute specialized structures for cellular interactions. They are constituted by non-haematopoietic meschymal cells and antigen-presenting cells that organise the framework for T and B cell interactions. SLOs are interconnected by a network of lymphatic vessels maximizing, in this way, not only the chance of encounters between effectors cells and antigen-presenting cells loaded with foreigner antigens, but also to meet regulatory cells. This ensures a rapid, efficient and controlled immune response. Some secondary lymphoid organs were originated throughout evolution at mucosal

sites and constitute the first defence against pathogens. These include intestinal Peyer's patches, tonsils, and nasal (NALT) and bronchial-associated lymphoid tissues (BALT) in the airways[19-21].

Haematopoiesis

Every day there is a pressing need to replace aged or damaged blood cells due to homeostatic cell turnover, pathological conditions, illness or trauma. Haematopoiesis is the tightly orchestrated developmental process that is responsible for the continuous formation of all blood cells types that guarantees oxygen delivery, haemostasis and the ability to control microorganisms[22]. This process relies on the existence of rare long-lived and self renewing haematopoietic stem cells (HSCs), which by extensive proliferation accompanied by successive loss of multipotency, have the ability to differentiate into all blood cell lineages. In fact, their extraordinary features make them unique cell targets in the therapy of malignant and non malignant haematological problems[22-24].

Haematopoietic stem cell – from embryonic life to adulthood

In mice, the first haematopoietic activity is detected at embryonic day 7.5 (E7.5) in the yolk sac (YS) (Fig. 1A). These primitive haematopoietic progenitors form a cluster surrounded by endothelial cells that has been called 'blood island'. At this site, a first wave of nucleated primitive erythrocytes arises, followed by a second wave of erythrocyte and myeloid progenitors[2,9,25]. Nevertheless, the appearance and ontogeny of haematopoietic stem cells (HSCs) have been debatable matters in the last decades. Despite the existence of progenitors with multilineage potential in the YS, the emergence of true multipotent HSCs, with full multilineage reconstitution potential *in vivo*, is believed to first appear in the paraaortic splanchnopleura that later develops into the aorta-gonad-mesonephros region (AGM) around E8.5[11,26-28]. Strikingly, it was recently shown that the human AGM region harbours HSCs before they appear in the YS, reinforcing the concept of an AGM origin of HSC precursors and definitive HSCs[29]. Due to spatial and temporal proximity of blood and endothelial cell generation, and because both cells types have a mesodermal origin, a common embryonic progenitor, the haemangioblast, was first hypothesized and more recently proven to exist in different organisms[30-32]. In mice the haemangioblast was shown to be a tri-potent progenitor capable of generating haematopoietic, endothelial and vascular smooth muscle cells[33].

In addition to the YS and AGM regions, haematopoietic activity was also observed in other embryonic tissues in both mouse and humans, namely the vitelline and umbilical

arteries and the placenta. At E11, clusters of haematopoietic cells are found in vitelline and umbilical arteries and the frequencies of HSCs in these arteries were similar to those found in the AGM. Since haematopoietic activity is detected at these sites only after it begins in the AGM, it is unlikely that those HSC are locally generated *de novo*[34,35]. Interestingly, a significant number of definitive HSCs are found at E11 placentas, suggesting a role for HSC generation or expansion in placenta vasculature. In fact, HSC numbers expand until E13.5, being 15-fold higher than in the AGM. At E15.5 HSC numbers in the placenta start to decline, suggesting a displacement of HSCs to the foetal liver. Thus, the placenta is an important transitory haematopoietic niche responsible for the expansion, rather than generation, of definitive HSCs, before their expansion burst and differentiation in the foetal liver (FL) (Fig. 1B)[36-38].

After E12.5 the FL becomes the major haematopoietic organ in the mouse embryo (Fig. 1C). The colonization of the liver rudiment starts around E10-12 by AGM and YS progenitors. The first progenitors are YS myeloid cells and primitive erythrocytes. HSCs generated and expanded in the AGM and YS, and possibly in the placenta, also migrate to the FL[39,40]. Contrary to the adult bone marrow, the FL microenvironment provides the conditions for a massive HSC expansion thatexponentially increases their numbers until E15.5[41]. In addition, the FL provides the signals for complete differentiation of haematopoietic progenitors into different haematopoietic lineages, yet ensuring HSC maintenance[42]. From the FL, haematopoietic progenitors migrate to the foetal spleen (FS) and to the developing thymus anlagen and generate the first waves of T cells[43-46]. Just before birth, the FL haematopoietic activity diminishes; HSCs migrate and seed the bone marrow, which by then becomes the main lifelong primary lymphoid organ[47,48].

Circulating FL haematopoietic progenitors colonise the foetal splenic rudiment (FS) at E12.5, and splenic haematopoiesis is detected from day E14.5 until the first weeks after birth. In contrast to the FL, splenic HSCs do not expand significantly. Instead the FS environment drives differentiation of intermediate progenitor cells originated in the FL into erythrocytes, myeloid cells and lymphoid progenitors[44,49]. Nevertheless, removal of the neonatal spleen has no significant impact on bone marrow HSC numbers, thus suggesting that the foetal and neonatal spleen have an important but transitory role in maintaining blood cell numbers in newborns[50].

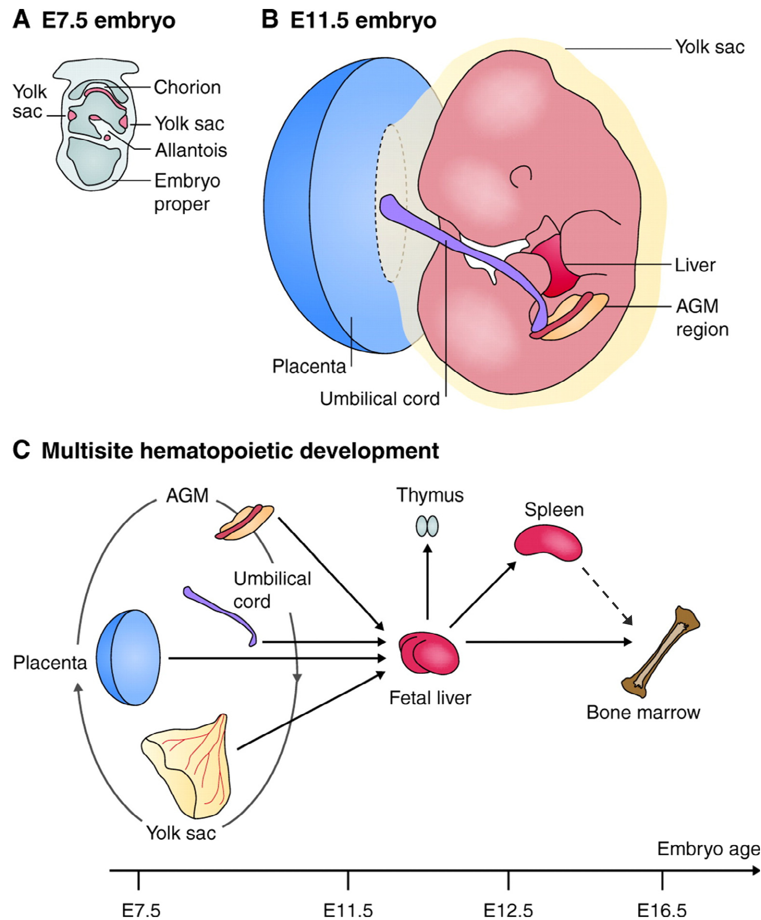

Figure 1. Embryonic haematopoietic development. **A.** schematic view of E7.5 embryo showing extraembryonic haematopoietic sites (pink). **B.** schematic view of E11.5 embryo showing tissues where embryonic haematopoietic stem cell (HSC) development occurs. **C.** model of multisite haematopoietic development. HSCs colonise the foetal liver. After expansion in the FL, haematopoietic progenitors and HSCs colonise the thymus, spleen and bone marrow. At birth, haematopoietic cells seed definitively in the bone marrow. Migration of progenitors from the spleen to the bone marrow has been also proposed (adapted from Medvinsky *et al* 2011[51]).

From day E16.5-17.5 HSCs leave the FL and are attracted to the bone marrow (BM), where they proliferate until three weeks after birth. In the following week, HSCs expansion stops and most HSCs enter in a dormant state, called quiescence[47,48]. These dramatic changes in cell cycle status between foetal and adult HSCs were proposed to prevent stem cell exhaustion resulting from massive proliferation and DNA damage that could lead to malignancy[52,53]. Thus, HSCs have a small contribution in the daily basis blood cell generation in adulthood, which relies mainly on the proliferation of multipotent and immature progenitors. However, HSC self-renewal and multipotent capacities are crucial to restore normal blood homeostasis in responses to stress conditions, such as inflammation and infections, toxic aggressions and transplantation. In fact it was shown that HSCs can reversibly switch from dormancy to an activated self-renewing status in homeostasis or under haematopoietic stress[54-61].

Throughout life HSC can also be found in the blood, although in very low percentages. The presence of HSCs in the blood was explained by their asymmetric division followed by migration of the daughter cells into circulation, but active division-independent egress of HSC was also demonstrated[62]. The biological function of these circulating HSCs has not been completely elucidated. One hypothesis is the search for vacant niches in distant bones; or, as shown by Massberg and colleagues, the differentiation into mature immune cells in peripheral organs[63].

In normal homeostatic conditions virtually all haematopoiesis is restricted to haematopoietic progenitors in the bone marrow. Nevertheless in pathological scenarios that cause severe depletion of haematopoietic cells, like anaemia or chronic infections, haematopoietic activity is detected outside the bone marrow[64,65]. This process, called extramedullary haematopoiesis, occurs in the adult spleen (Sp) and to a lesser extend in the adult liver. Extramedullary haematopoiesis is critically important in stress erythropoiesis that follows severe anaemia and transplantation in myeloablative conditions[66]. In fact, one of the first demonstrations of a clonogenic haematopoietic progenitors came from the work of Till and McCulloch demonstrating that upon transplantation of bone marrow cells into irradiated animals, some cells migrate to the spleen, proliferate and rapidly differentiate in order to protect the host. These highly proliferative multipotent progenitors were later called colony-forming unit-spleen (CFU-s)[67-69]. More recently true haematopoietic stem cells were observed in the Sp after mobilization, contacting sinusoidal endothelial cells, and the existence of a HSC niche in the spleen was also suggested[70]. In agreement, splenic stroma cells have been shown to support *in vitro* development of dendritic cells from BM and Sp haematopoietic precursors[71]. Albeit a crucial role for the spleen as a primary haematopoietic organ in normal healthy individuals is unlikely, the importance of spleen as an active site of haematopoiesis in pathological conditions, especially those that interfere with the normal haematopoietic activity in bones, needs to be carefully addressed.

Haematopoietic stem cell – characterization and function

Haematopoietic stem cells (HSC) are multipotent cells that have the ability to give rise to all mature haematopoietic cell types through a process of expansion and continuous loss of potential[72,73]. HSCs have the ability to self-renew their own pool of undifferentiated progenitors. Although the self-renewal capacity may occur by alternate

divisions, were in one round of division only stem cells are produced proceeded by a round of division that generate only committed cells; asymmetric cells divisions, where in each round of division one stem cell and one committed cells are generated, were found to be the dominant event[23,73-75]. Their joint ability to self-renew and differentiate into all mature blood cells has been taken in advantage by physicians in HSC transplantation protocols[60]. Indeed the capacity to fully reconstitute a recipient after transplantation became the major functional hallmark of HSCs. As such, long term repopulating assays, alone or in competition with cells bearing distinctive markers, are widely used to evaluate the haematopoietic activity of unknown progenitors[76-80]. Interestingly, HSCs differ from embryonic stem cells since their self-generation and differentiation capacity are not unlimited and serial transplantations can only succeed three of four times[74].

As previously discussed, the first demonstration of functional differentiation plasticity in haematopoietic progenitors derive from the work of Till, McCulloch and colleagues when transplanting BM cells into lethally irradiated mice. In these experiments some haematopoietic progenitors home to the spleen of irradiated recipients and form colonies that appear at day 8 or 12 after transplantation. Analysis of these colonies revealed that most of the cells present in a colony came from the same progenitor cell[67,68]. Although it was later shown that colony-forming unit-spleen (CFU-s) were not true HSCs, but myeloerythroid and multipotent progenitors, this work paved the way to the subsequent identification of HSCs, and established CFU-s_{d8} and CFU-s_{d12} as short term rescue assays of haematopoietic activity[72,81,82].

With the advent of fluorescence-activated cell sorting (FACS)[83] many different approaches were developed to purify pluripotent haematopoietic stem cells from multipotent progenitors. The first approaches took advantage of morphological properties of cells, such as size and granularity, by binding to fluorochrome conjugated wheat germ lectin, and by using cell penetrable dyes like rhodamine 123 (Rho) after density separation[84,85].

Since most immature haematopoietic stem cells have multidrug pumps from the ABC transporter family, using vital dyes, like Hoechst 33342, and displaying their fluorescence in two different wavelengths it is possible to identify a cell population with low fluorescence, designated side population (SP), with phenotypic and functional markers of HSCs[86-88]. Inside SP cells, $Rho^{neg/dull}$ cells are further enriched for HSCs[85,89]. However, the usage of vital dyes is not trivial and limits their application to

cell suspension samples. Finally, it is noteworthy that any event that interferes with ABC transporters kinetics, like activation and proliferation, will directly impact on SP[90].

In 1988, using fluorescent labelled antibodies, Weissman's lab described a bone marrow cell population that was Lin^{neg}Thy1^{lo}Sca1^{pos} and that contained virtually all haematopoietic stem cells[72]. However, Thy1 expression varies according to different mouse strains, which makes its use impractical for common strain that do not carry the haplotype Thy1.1, like the C57Bl/6 mice. In this regard, a continuous effort has been made to identify HSC specific markers.

Consecutive work by Ogawa and colleagues revealed that the tyrosine kinase receptor cKit (CD117) is expressed by many haematopoietic progenitor cells that lack the expression on lineage markers[91]. Haematopoietic activity was then described in the population of Lin^{neg}cKit^{pos}Sca1^{pos} (LSK) comprising approximately 0.05%-0.1% of BM and FL cells, making cKit and Sca1 the most commonly used markers to define the progenitor population that contains HSCs[92,93]. The tyrosine kinase cKit is the receptor for stem cell factor (SCF), also called mast cell growth factor or Kit ligand. Spontaneous mutations in either *Kit* or *Kitl locus* cause severe haematopoietic problems such as macrocytic anaemia[94-97]. , In conjunction with other cytokines and growth factors, like IL-3 and IL-6, Kit ligand is a potent HSC mitogen, and although its role in HSC self renewal is controversial, it is critic for their proliferation, survival, and possible adhesion through interaction of cKit with membrane bound Kit ligand present in the HSC microenvironment[93,98-101].

Sca1 was suggested to regulate HSC self-renewal, especially in haematopoietic stress, since Sca1 null mice have thrombocytopenia, decreased multipotent progenitors and CFU-s activity, and a competitive disadvantage in transplantation compared with WT HSCs. Sca1 was also shown to be downstream of IFNαR-STAT1 signalling mediating IFNα induced HSC proliferation. Similarly to other GPI-linked proteins, Sca1 was proposed to modulate signalling molecules in the lipid rafts. In fact, Sca1 was suggested to interact with cKit signalling, as Sca1 deficiency together with cKit partial loss of function ($Kit^{Wv/Wv}$) results in increased embryonic lethality and generation of severely anaemic foetus[102].

Despite containing all haematopoietic activity, LSK cells are still heterogeneous and less than 10% were calculated to be HSCs. Humans HSC and progenitor cells were identified within a population expressing CD34 in the BM and blood, which can be further enriched in HSCs by selecting CD34^{pos}CD38^{neg} cells[103,104]. Interestingly, and in

contrast to humans, most immature HSCs in mice reside in the CD34^{neg}, and injection of a single LSK CD34^{neg} cell can reconstitute around 20% of the recipient mice[56]. Thus, together with the tyrosine kinase receptor Flt3, also called Flk2 or CD135, (fms-like tyrosine kinase receptor-3 or foetal liver kinase-2), expression of CD34 defines three categories of haematopoietic progenitors: LSK CD34^{neg}Flt3^{neg} cells are long-term reconstitution haematopoietic stem cells (LT-HSC) with self-renewal capacity, responsible for long term engraftment and reconstitution of an irradiated host; LSK CD34^{pos}Flt3^{neg} cells are short-term reconstitution haematopoietic stem cells (ST-HSC), which are active proliferating HSCs, with multipotency, limited self renewal capacity, generate CFU-s and rapidly restore myelopoiesis and erythropoiesis; and finally acquisition of Flt3 expression by LSK CD34^{pos}Flt3^{pos} cells, a tyrosine kinase receptor that gives proliferative and differentiation signals to many immature progenitors, characterises multipotent progenitors (MPP) with myeloid and lymphoid-biased potential, but with no self renewal capacity[99,105-107].

In contrast to human HSCs, mouse HSCs express CD38 within LSK cells in foetal and adult life. CD38 is a useful marker that correlates with HSC functions: LSKCD38^{neg} cells have most of the CFU-s but no long-term reconstituting cells (ST-LSK) and LSK CD38^{pos} cells contain virtually all long-term reconstituting stem cells (LT-LSK)[108]. Nevertheless, the usage of CD34 and CD38 as markers for HSCs needs to be carefully addressed since their expression varies in ontogeny and with cell activation. YS primitive haematopoietic progenitors and definitive FL and BM HSCs from young mice express CD34, a phenomenon also observed in mobilised or injury-activated HSCs[109,110]. Nevertheless, it is interesting to notice that YS primitive haematopoietic progenitors already express CD38[111]. Conversely, activated HSCs down regulate CD38 expression, acquiring a phenotype similar to human stem cells[112]. As such, mice mobilised HSCs appear CD34^{pos}CD38^{neg}. Interestingly, when homeostasis is achieved after transplant, the HSC phenotype reverts to CD34^{neg}CD38^{pos}, reflecting the active changes of HSCs under homeostasis and haematopoietic stress[54]. Together with the fact that multilineage repopulating activity was also found in human CD34^{neg} progenitor cells, including the generation of CD34^{pos} cells, these findings open the debate on whether to use CD34 and CD38 as markers for HSCs, highlighting the differences between homeostatic slow dividing and activated proliferating HSCs[112,113].

In addition to CD34 expression, many phenotypical and functional differences were revealed between foetal and adult HSCs. Mac1, or CD11b, a marker that identifies adult myeloid cells, is expressed by foetal haematopoietic progenitors, but its

expression is restricted to foetal haematopoietic stem cells[41]. The expression of CD45RB and CD93, recognised by the AA4.1 monoclonal antibody, as well as being Rho$^{pos/bright}$, also distinguishes foetal from adult stem cells[79,114]. Interestingly, FL HSCs have increased haematopoietic activity when compared to HSCs from adult BM, as revealed by their reconstitution potential in competitive repopulation assays with their BM counterparts[77-79].

Some of the markers commonly used to purify HSC populations, such as Thy1 and Sca1, were shown to be differentially expressed in mice strains carrying different haplotypes, thus limiting the comparison between assays using different models[115,116]. In 2005 Kiel and colleagues found that the SLAM family receptors (Signalling Lymphocyte Activation Molecule) were highly expressed in haematopoietic progenitors and could distinguish HSCs from committed progenitors. Therefore, most HSCs are CD150^{pos}CD48^{neg}CD244^{neg} (many times described only as CD150^{pos}CD48^{neg}), whereas MPP are CD150^{neg}CD48^{neg}CD244^{pos} and restricted progenitors are CD150^{neg}CD48^{pos} CD244^{neg} [70]. Using these markers, these authors achieved the same reconstitution capacity as with the LSKCD34^{neg} criteria (20%). When SLAM markers were applied within the LSK compartment, almost 50% of engraftment and long-term multilineage reconstitution of recipients could be obtained by transplanting a single LSK CD150^{pos}CD48^{neg} cell. Thus this marker combination is currently the best manner to define long-term HSCs. Importantly, the expression patterns of SLAM receptors is conserved among many mouse strains, characterises both adult and foetal HSCs, and is maintained in mobilised and extramedullary HSCs[70,117]. The purity of HSCs can be further enriched by excluding megakaryocyte progenitors with CD41, a marker for megakaryocyte lineage cells that is also expressed in primitive haematopoietic progenitors but is down regulated in definitive haematopoiesis[118-121].

Recent work has shed light into other HSC markers. CD201 expression, the endothelial protein C receptor (EPCR), alone or in combination with previously described markers, identifies a HSC population with long-term reconstitution activity and self-renewal potential in foetal, adult and cultured progenitors[122-124]. Long-term repopulating HSCs are also positive for endoglin or CD105, a TGFβ accessory receptor, and simple CD105^{pos}Sca1^{pos}Rho^{low} analysis defines a nearly homogeneous LT-HSC population[125,126].

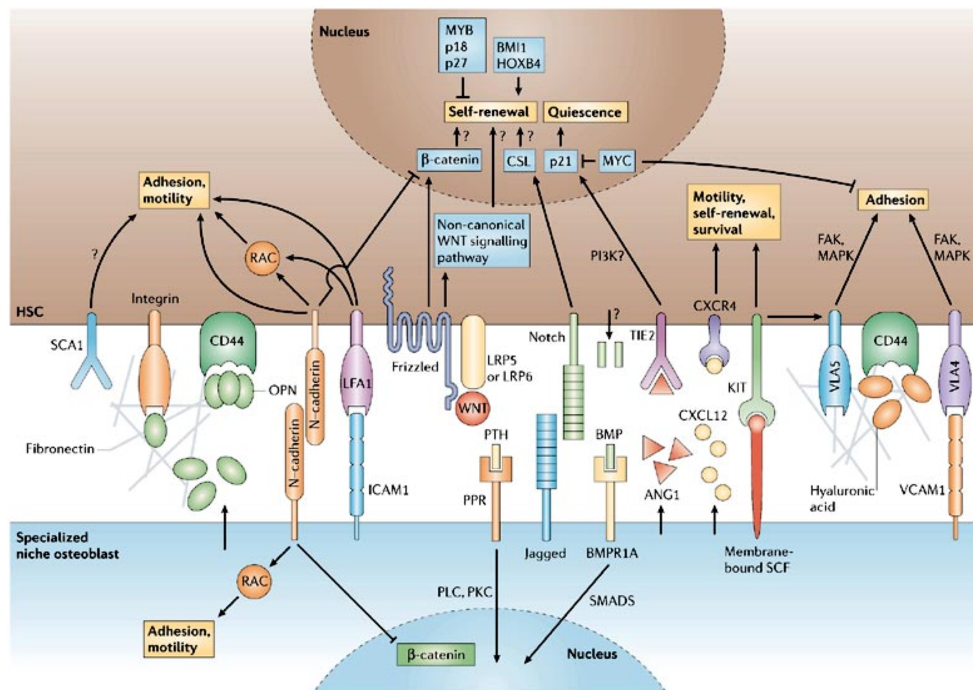

Figure 2. Critical signals for HSC maintenance. Schematic view of the molecular cascades in the HSC-niche. Ligand–receptor interactions and interactions between adhesion molecules are depicted. Contradictory evidence and indirect or unknown mechanisms are denoted by question mark (adapted from Wilson & Trumpp, 2006[127]).

In order to maintain their function, HSCs need to balance multiple signals from cytokines, chemokines and growth factors (Fig. 2)[127]. HSCs express the thrombopoietin receptor MPL. Its downstream signalling provides survival, proliferative and differentiation cues; more specifically it controls megakaryocyte differentiation[128-131]. Thrombopoietin also regulates maintenance of quiescent adult HSC[132,133]. Growth factors, such as insulin-like growth factor 2, were also shown to drive HSC expansion and both foetal and adult HSCs express insulin-like growth factor 2 receptor (IGF2R)[134]. In contrast, signals from angiopoetin-1 and its receptor the tyrosine kinase Tie2, also called TEK, are critical to maintain quiescence and HSC repopulating activity through cell division inhibition and improvement of HSC adhesion to the microenvironment[135,136]. The maintenance of the HSC pool, as well as the migration and retention of HSCs in the BM relies on CXCL12 interaction with CXCR4. CXCR4 antagonists mobilise human and murine HSCs from the BM into blood, a process that is currently used in the clinics to collect human haematopoietic progenitor cells[137-139]. Finally, HSCs also express numerous adhesion molecules such as N-cadherin, CD44 and various integrins, that collectively control HSC homing and maintenance in specific microenvironments[127,140].

While a significant proportion of foetal liver HSCs are in cycle, adult BM HSCs are mostly slow dividing quiescent cells[48]. Several models were proposed to explain HSC cell cycle regulation. The first model suggested a slow and continuous recruitment into cell cycle, where at any time point 75% of HSCs would be in G_0, but eventually all would divide on average every 57 days[141]. However, a hierarchical model recently postulated that the HSC population contains active and dormant HSCs (dHSCs). These dHSCs would divide on average 5 times per lifetime, and are detected as label-retaining cells (LRC) by pulsed labelling with BrdU or histone H2B-GFP fusion protein[54]. In addition, a 'dynamic repetition' model was also proposed, whereby long term reconstituting HSCs would be found both in cycling and quiescent populations. More specifically, this model proposes that HSCs have the capacity to repetitively change between cycling and quiescent states. Thus HSCs that for a given time are in cycle may enter quiescence and vice versa[142].

Haematopoietic stem cell – molecular regulation

To maintain haematopoiesis throughout life and the capacity to respond upon physiological demand, HSCs need to balance between non-dividing quiescent state, self-renewal and differentiation. As such, HSCs are subject to a tight regulation. Active inhibition of the cell cycle is crucial to maintain quiescence and avoid HSC exhaustion that results from persistent proliferation. Cyclin-dependent kinase inhibitor p21 (encoded in *Cdkn1a* gene), and its regulator, the zinc-finger repressor transcription factor GFI-1 (growth factor independent 1), are critical to restrict proliferation and maintain quiescence[143,144]. Although their ablation results initially in increased HSCs numbers and cycling HSCs, these cells have a defective stress response, impaired repopulation capacity in transplantation and are rapidly exhausted. Other cyclin-dependent kinase inhibitors, like p57 and p27, were also shown to be involved in HSC maintenance and quiescence[145-147].

Several other transcription factors were shown to regulate HSC homeostasis by controlling cell proliferation. The transcription factor EGR1 (early growth response 1), is highly expressed in LT-HSCs and controls both quiescence and retention in HSCs niches. Absence of EGR1 in *Egr1*$^{-/-}$ mice results in increased proliferation and mobilisation of HSCs to peripheral blood[148]. Interestingly, reduction in EGR1 also causes reduced p21 levels. The transcription factor EVI1 (ecotropic viral integration site 1), which was also shown to be over expressed in several myeloid malignancies, is

also critical to HSC self-renewal and long-term HSC activity[149,150]. Other transcription factors regulate HSC function by inhibiting cell differentiation. The inhibitor of DNA binding family member, ID1 regulate HSC fate by restraining myeloid commitment and differentiation[151].

The capacity of HSCs to self-renew is crucial when they need to rapidly expand, such as during foetal development and HSC stress responses. Several members of the HOX protein family, and their co-regulators, such as *Meis1* and *Pbx3*, are expressed by HSCs and are critical for HSC maintenance. Over expression of HOX proteins in haematopoietic progenitors results in their expansion, and although they have redundant functions, cumulative ablation of HOXA9, HOXB3 and HOXB4 cause severe haematopoietic defects[152,153]. Importantly, *Mixed Lineage Leukemia* gene (*Mll*), which is critical for primitive haematopoiesis in the YS and definitive foetal and adult haematopoiesis, is an activator of several *Hox* genes and drives expansion of haematopoietic progenitors[154-158].

In addition to the direct transcriptional regulation of HSC function, posttranscriptional mechanisms also take place in HSC regulation. The E3 ubiquitin ligase Fbw7 control HSC quiescence and differentiation by regulating the levels of MYC protein that is required for HSC proliferation[159,160]. Although sharing similar regulatory strategies, foetal and adult HSCs differ in their proliferation status, haematopoietic activity and differentiation potential. The balance between Lin28b transcriptional repressor and the let-7 family of micro RNAs (miRNAs) is responsible for the switch from foetal to adult HSC differentiation potential, in which expression of let-7 miRNAs is associated with adult HSC profile and expression of Lin28b, a let-7 miRNAs blocker, correlates with a foetal-like potential[161]. Interestingly, the *stem-cell leukaemia SCL/tal-1* is essential for HSC genesis in embryonic development, but it is dispensable for long-term repopulation activity and multipotency of adult HSCs[162-164]. Hedgehog signalling was also shown to be dispensable for adult HSC function, but it was suggested to regulate foetal HSC development[165,166].

Regulation of mitochondrial integrity, cell metabolism and control of oxidative stress play crucial role in HSC homeostasis. Activation of mTOR complex, a known nutrient sensing system, dramatically reduced haematopoiesis, promoting quiescent HSCs into rapid cycling and increasing mitochondrial biogenesis and reactive oxygen species (ROS) levels[167]. WNT signalling, which was shown to be critical to HSC maintenance, counteracts mTOR signalling, and concomitant activation of WNT and inhibition of mTOR increases the number of long-term HSCs *in vivo*[168,169]. The energy metabolism

regulator LKB1, the major kinase that phosphorilates AMP-activated protein kinase (AMPK) also regulate HSC quiescence, cell cycle and metabolic homeostasis, thus determining HSC survival[170-172]. Conditional ablation of *Lkb1* results in a dramatic decrease in energy content and increased mitochondria defects, causing rapid entry of haematopoietic progenitors into cell cycle and HSC exhaustion. Likewise, DNA repair machinery pathways and autophagy, critical in the protection against ROS and cellular damage, are essential for HSC function and maintenance, and absence of molecules involved in these pathways, such as *Paf* and *Atg7*, results in increased proliferation and HSC failure[173,174].

Several members of B-cell lymphoma 2 family (BCL2) have being implicated in HSC survival[175]. *myeloid cell leukaemia 1* (*Mcl1*), a BCL2 family member, is highly expressed in HSCs and was shown to be critical for HSC survival and other early haematopoietic progenitors[176]. Interestingly, known haematopoietic growth factors, particularly the Kit ligand SCF, were shown to upregulate *Mcl1* expression. Moreover, proteins involved in MCL1 stabilization and maintenance of mitochondrial integrity, such as heat shock protein 70 family members (HSC70) and guanosine nucleotide-binding protein GIMAP5, are also important regulators of HSC and progenitor cell survival[177]. Other BCL2 family member that bind to GIMAP5, BCLxL (encoded in the *Bcl2l1* gene) also play a role in HSC survival, as mice with BCLxL deficiency present massive cell death of several haematopoietic progenitors[178,179]. BCLxL was also shown to be involved in HSC[180]. Although the role of BCL2 in survival of HSCs is controversial, loss of BCL2 results in lymphocytopenia and its upregulation was shown to enhance hematopoietic differentiation from murine embryonic stem cells *in vitro*. Moreover, overexpression of *Bcl*2 increases HSC numbers and their repopulation potential[181-183]. Interestingly, it was shown that BCL2 causes upregulation of p27 cell cycling inhibitor, thus coupling HSC survival with cell cycle regulation. Importantly, some transcription factors shown to regulate HSC survival, maintenance and differentiation, such as the GA binding protein (GABP), were shown to target BCL2, BCLxL and MCL1[184]. In addition, calmodulin-dependent Protein Kinase IV (CaMKIV) regulates HSC maintenance controlling *Bcl2* expression downstream of the CREB transcription factor and CREB-binding protein (CBP)[185]. Thus, several anti-apoptotic factors regulate HSC survival, and their requirement may be influenced by particular physiological contexts.

Haematopoietic lineage development and commitment

Mature blood cells with diverse functions can be divided in three different lineages according to their ontogeny: erythroid, myeloid and lymphoid lineages. Haematopoietic stem cells and the more committed multipotent progenitors (MPP) generate all mature blood cells, but the mechanisms by which lineage restriction occurs are not obvious. The classical model of haematopoietic differentiation proposes that the first commitment of MPPs separates a common lymphoid progenitor (CLP) from myeloid progenitors (MP) that include a common myeloid progenitor (CMP) with myeloid-erythroid-megakaryocyte potential (Fig. 3). Therefore the CLP is a $Lin^{neg}IL\text{-}7R\alpha^{pos}$ $cKit^{low}Sca1^{low}$ population with rapid lymphoid (B, T and NK cells) reconstitution capacity but with no myeloerythroid capacity, while $Lin^{neg}IL\text{-}7R\alpha^{neg}$ $cKit^{pos}Sca1^{neg}$ MP are divided with CD34 and FcγR (CD16/32) in $Lin^{neg}IL\text{-}7R\alpha^{neg}cKit^{pos}Sca1^{neg}Fc\gamma R^{low}CD34^{pos}$ CMP, $Lin^{neg}IL\text{-}7R\alpha^{neg}cKit^{pos}Sca1^{neg}Fc\gamma R^{high}CD34^{pos}$ granulocyte-macrophage progenitors (GMP) and $Lin^{neg}IL\text{-}7R\alpha^{neg}cKit^{pos}Sca1^{neg}Fc\gamma R^{low}$ $CD34^{neg}$ megakaryocyte-erythrocyte progenitors (MEP)[186,187]. More recently, first in the foetal liver and later in the adult bone marrow, several studies have identified haematopoietic progenitors that do not fit the previous rigid model of haematopoietic development, such as the existence of $LSKCD34^{pos}Flt3^{high}$ lymphoid-primed multipotent progenitors lacking megakaryocytic and erythroid potential but with myelolymphoid potential, and progenitors that can generate macrophages, B and T cells (M/T, M/B and M/T/B progenitors). Thereby a revised or alternative model of haematopoietic commitment is a myeloid-biased model. In this model, the generation of erythroid-megakaryocyte and lymphoid progenitors within the MMPs are mutually exclusive, whereas the myeloid potential is promiscuous and kept longer, thus generating common myelolymphoid (CMLP) and common myeloerythroid (CMEP) progenitors[188-196].

The differentiation potential of intermediate progenitors has been analysed *in vivo* by transplantation into irradiated recipients and *in vitro* by identification of colony-forming cells (CFC) using semi-solid media supplemented with known cytokine cocktails. In these assays, progenitors with uni, bi and oligopotential were identified. Progenitors with restricted potential are colony-forming units-erythrocyte (CFU-E), and the more immature burst-forming unit-erythrocyte (BFU-E); colony-forming unit-megakaryocyte (CFU-Mk); colony-forming unit-granulocyte (CFU-G) and colony-forming unit-macrophage (CFU-M). Bipotential colonies are colony-forming unit-granulocyte/macrophage (CFU-GM). The more immature colony-forming units detected in these assays generate all myeloerythroid lineages and are called colony-

forming unit-granulocyte-erythrocyte-megakaryocyte-macrophage (CFU-GEMM). Although colony-forming unit-PreB cells (CFU-PreB) can be detected with very specific cytokine mixture in methylcellulose media, analysis of B and T potential normally require co-culture of haematopoietic progenitors with stromal cells or, especially for T cell development, complex tissue cultures such as foetal thymic organ cultures (FTOC)[197-199].

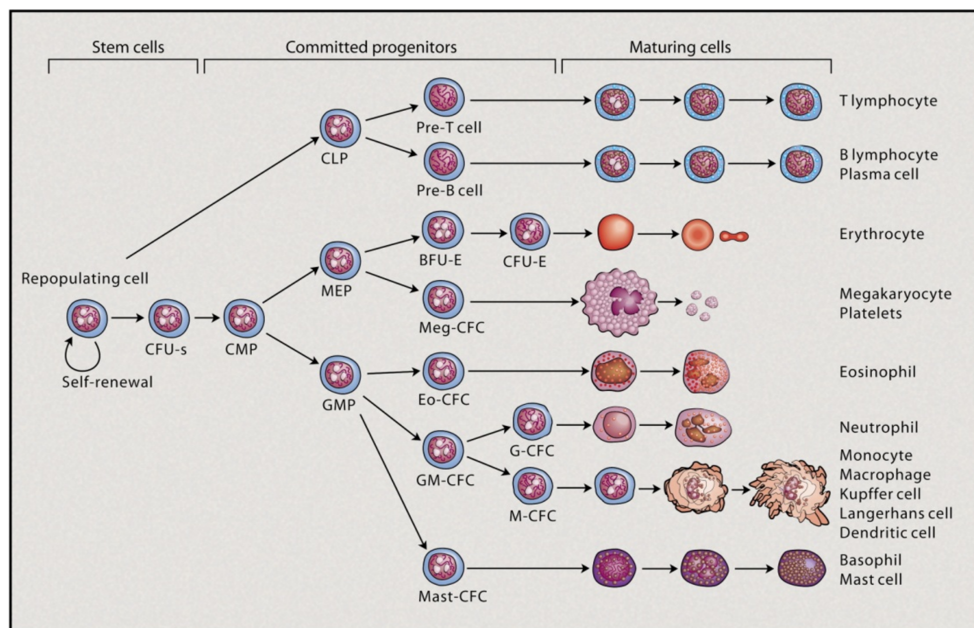

Figure 3. Lineage commitment of haematopoietic stem cells. Schematic view of a classical model of haematopoietic differentiation. Self-renewing HSCs give rise to multipotent progenitors that proliferate and differentiate into lineage-restricted progenitors. These generate mature blood cells. Alternatively, HSCs first restrict erythroid-megakaryocyte and lymphoid potentials by generating common myelolymphoid (CMLP) and common myeloerythroid (CMEP) progenitors, retaining myeloid potential longer in the restriction developmental program (adapted from Metcalf 2007[74]).

The erythroid call lineage is the first to appear in development, giving rise to erythrocytes that transport oxygen and megakaryocytes that fragment into platelets[1-4].

Haematopoietic cells that derive from the myeloid lineages are divided into two main groups according to their nucleus morphology. Mononuclear cells are composed by circulating monocytes and their progeny, dendritic cells and phagocytic macrophages that reside in SLOs and peripheral tissues. Polymorphonuclear cells, called granulocytes, include neutrophils, eosinophils, basophils and mast cells. Neutrophils are one of the first responding cells to local inflammation and are crucial to resolve bacterial infections; eosinophils are associated with responses to parasites and allergic reactions; basophils and mast cells are responsible for the induction of allergic inflammatory responses and have a role in ectoparasite infections[1,195,200,201].

The lymphoid lineage generates three main types of mature cells: innate lymphoid cells (ILCs); B cells and T cells. Nevertheless, some dendritic cell populations were also shown to originate from CLP[186,201]. Innate lymphoid cells are a heterogeneous family that includes cells capable of recognising and killing infected or malignant cells, driving inflammatory responses against pathogens but also implicated in autoimmunity and allergy. They are also crucial for SLOs formation in development[7]. B cells express on their surface a receptor known as the B cell receptor (BCR) and are the effector arm of humoral immunity. Upon BCR engagement with the cognate antigen, B cells can differentiate into plasma cells which produce large amounts of soluble antibodies that bind to the original antigen. B cells are also known to produce cytokines for other immune cells[202]. T cells, also express a unique receptor, the T cell receptor (TCR). The majority of T cells express a TCR composed of an α and a β chains (TCRαβ) and are divided into two main populations based on the expression of CD4 or CD8 co-receptors. CD8^{pos} T cells are cytotoxic cells capable of killing neoplastic or infected cells. CD4^{pos} T cells can have multiple cytokine production profiles and are specialised in providing help to other cells from the immune system in specific immune responses, such as the control of antibody production by B cells. A small population of CD4^{pos} cells is characterised by the expression of the transcription factor FOXP3. These FOXP3^{pos} regulatory cells control excessive immune activation, thereby preventing inflammatory diseases and autoimmunity[203,204]. The remaining T cells express a TCR composed of a γ and a δ chains (TCRγδ) and are important inflammatory cytokine producers that also bear cytotoxic activity[205].

Specific microenvironments – haematopoietic stem cell niches

To regulate the balance between self-renewal and differentiation, stem cells require a myriad of environmental signals. Stem cell niches can be defined as tri-dimensional microstructures in which their cellular and molecular components ensure stem cell maintenance. The concept of the stem cell niche was first proposed by Schofield in 1978[82]. Since then, many non haematopoietic cell types were described to support HSC function and were included the haematopoietic stem cell niche[127,206-208].

Bone-lining, spindle-shaped N-cadherin^{pos} osteoblasts (SNOs) were the first cells described to support HSC maintenance, and to form a HSC niche in the bone marrow, the endosteal niche[209,210]. This niche has a hypoxic state with low blood perfusion and is thought to provide HSCs with soluble molecules, such as angiopoetin 1 and

thrombopoietin, and membrane-bound signals that regulate quiescence of dormant HSCs[132,136,207]. Nevertheless, only 14% of HSCs were found to contact with endosteum, and many contact with sinusoidal vascular endothelium, leading to the concept of a perivascular endothelial niche[70]. Endothelial niches were also suggested to exist in extra-medullary haematopoiesis sites and to control HSC engraftment. This niche also supports activated HSCs, by controlling HSCs proliferation, differentiation and entry into circulation[70,207,211]. Importantly, the sinusoidal endothelium is also found near the endosteum, increasing the debate of HSCs niches as separate entities with different functionalities, or as a rather complex unifying entity with multiple players. Recently, several other cell types were shown to be part of the HSC niche. CXCL12-abundant reticular (CAR) cells, an immature progenitor cell that express large amounts of the chemokine CXCL12, are in contact with all HSCs and appear to be components of both the endosteal and perivascular niche. CAR cells also form a unique niche with cells that do not contact with the endosteum or sinusoidal endothelium, the reticular niche[137]. Nestin^{pos} mesenchymal stem cells (MSC), which have potential to self-renew and differentiate into mesenchymal lineages, also express genes involved in HSC maintenance and are in contact with at least 60% of the HSCs in the BM. Their depletion leads to a rapidly decrease of HSCs in BM, which is indicative of a common niche to these two stem cells, or a critical role of MSCs in controlling HSC niches [212]. Using flow cytometry the main cellular HSC niche components can be separated: endothelial cells are CD45^{neg}Lin^{neg}CD31^{pos}Sca1^{pos} cells; osteoblasts are CD45^{neg}Lin^{neg} CD31^{neg}Sca1^{neg}CD51^{pos} cells; MSCs are CD45^{neg}Lin^{neg}CD31^{neg}Sca1^{pos}CD51^{pos} cells and the remaining population of CD45^{neg}Lin^{neg}CD31^{neg}Sca1^{neg}CD51^{neg} cells contain CAR cells that can be selected based on the expression of PDGFRβ[206,213]. Some haematopoietic populations, such as macrophages, can also promote the retention of HSCs in their niches, and their conditional depletion results in HSC mobilization[214,215].

In recent years several studies indicated a crosstalk between the nervous and haematopoietic system in order to keep the balance between regular blood production in homeostasis and rapid immune responses in conditions of stress[216,217]. Interestingly, sympathetic nerve fibres in the BM are associated with cells of the HSC niche, such as MSCs and osteoblasts. These studies revealed that neurons regulate MSCs and osteoblast functions, thus indirectly regulating HSC maintenance. Moreover, glial cells unsheathing autonomic nerves were described to maintain HSC quiescence directly by activating TGF-β. Therefore cells from the nervous system, namely glial cells, are also components of the HSC niche[212,217-222].

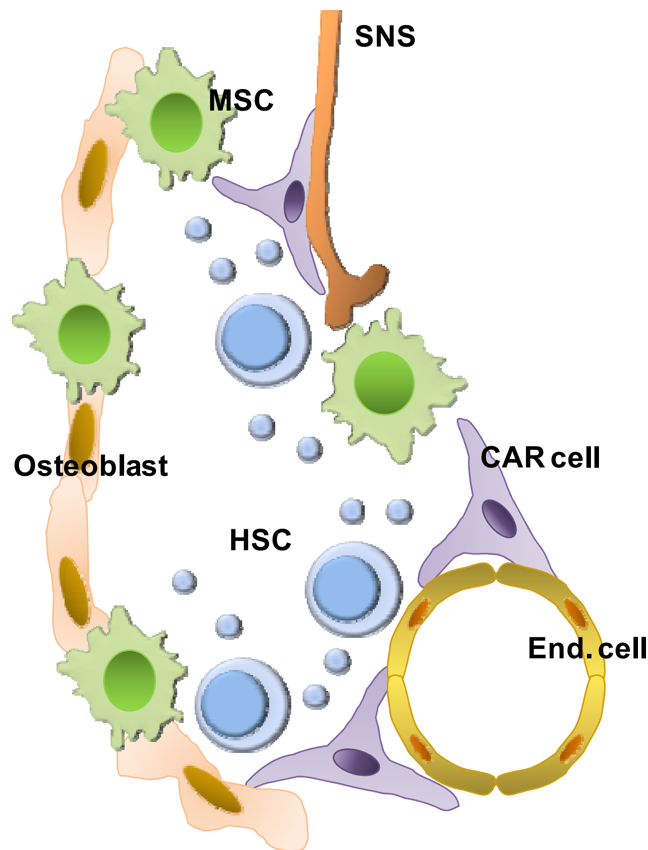

Figure 4. Bone marrow haematopoietic stem cell niches. Osteoblasts in the endosteum were proposed to be a niche that maintains dormant HSCs, whereas endothelial cells constitute a perivascular niche for activated HSCs. Mesenchymal stem cells (MSC), CXCL12-abundant reticular cells and cells form nervous system (SNS) were found to be involved in the previous niches or to form an alternative niche on their own. It is still unclear whether different niches are separate entities with distinct physiological functions; sites spatially separated but with overlapping functions; or part of a unique multicellular complex.

The increasing complexity of cellular players and functions of specific HSC microenvironments created a great debate around the identity and definition of the HSC niche(s) (Fig. 4). The endosteal and perivascular niches were described to favour HSC quiescence or proliferation, respectively, thus nurturing the concept that distinct HSC niches fulfil specific functions[61,207]. Nevertheless, other cell types can maintain dormancy or sustain proliferation of HSCs, such as MSCs and CAR cells, by sharing with osteoblasts and endothelial cells the capacity to produce important molecules for HSC maintenance, like SCF and CXCL12. This raises the possibility that spatially different HSC niches may co-exist in the bone marrow with seemingly overlapping functions. Another theoretical possibility is that instead of defined niches, a diffuse HSC maintenance zone would encompass the endosteum and perivascular cells. However, this hypothesis is unlikely since a diffuse maintenance zone would have difficulty in providing the contact dependent cell to cell interactions shown to be crucial

for HSC maintenance. Finally, it is tempting to speculate that only a discrete common unifying niche would gather the signals from both endosteum and endothelial cells[208].

Throughout embryonic development, the generation, expansion and differentiation of primitive haematopoietic progenitors is thought to depend on interactions between haematopoietic cells and a myelo-supportive stroma[8]. In the foetal liver, the cellular interactions that are established between HSCs and non-haematopoietic cells are not completely understood. Thus, HSC niches have not been formally defined in this context[8]. Nevertheless non-haematopoietic cells in the FL were claimed to support HSCs and some similarities with adult HSC niche players were described. Cells in the microvasculature of developing organs, such as YS, FL and placenta were suggested to form an active HSC niche. In the FL, slowly dividing HSCs with long-term reconstitution potential were found associated with perisinusoidal endothelial cells, and these cells were also shown to maintain reconstitution capacity of HSCs *in vitro*[36,124]. Foetal liver hepatocyte progenitors present in the HSC microenvironment were claimed to be a supportive cell type for HSCs by producing key molecules like IGF2 and CXCL12 and membrane-bound SCF[223]. The FL stroma, which consists of cells that have an epithelial-to-mesenchymal transition phenotype, are an immature cell type that shares some similarities with MSCs in the BM. FL stroma cells support the expansion and differentiation of HSC, immature progenitors and lineage committed cells, and were shown to amplify the repopulation ability of AGM-derived HSCs *in vitro*[224-226]. Other cell types, such as epithelial cells and macrophages, may also directly impact on HSC function and be part of a yet unidentified niche.

T cell development

The thymus is a bilobular organ located in the thoracic cavity near the heart. It has a compartmentalised structure, forming several microenvironments that support T cell development. The thymus is constituted by a blood vessel rich cortico-medullary junction, an outer cortex that finishes in the subcapsular zone, and an inner medulla[227]. Surprisingly, small cervical thymi , having a medulla-cortex structure and supporting T cell development, were found in mice[228].

As the thymus does not maintain self-renewing haematopoietic stem cells, the continuous generation of T cells depends on permanent waves of immigrant foetal liver or bone marrow-derived progenitors[13-15,229-231]. Nevertheless, recent reports found substantial self-renewing capacity of thymic progenitors, suggesting that continuous colonisation and survival of thymocytes is caused by competition and expulsion of resident cells by freshly incoming progenitors[232,233].

Early immature progenitors in the thymus

Despite several studies addressing T cell lineage commitment, there are still controversies on the nature of the haematopoietic progenitors that seed the thymus. Early thymic progenitors (ETPs) have some multilineage potential, since in addition to strong T cell potential, they can generate NK cells, myeloid cells and dendritic cells. Interestingly, different studies reported reduced B cell potential, suggesting that CLPs, which *in vitro* were shown to have T/B/ILC(NK) potential, migrate poorly to the thymus. Thus, other immature progenitors, such as lymphoid-primed multipontent progenitors (LMPPs) or M/T progenitors, must also colonise the thymus. It is only inside the thymic compartment that T cell lineage restriction occurs[43,234,235]. Nevertheless, genetic fate mapping studies with IL-7Rα reporter mice, showed different origins of T and myeloid cells, dissociating differentiation potential from *in vivo* developmental relevance[236].

Immature thymic progenitors lack expression of lineage-associated co-receptors that characterise mature T cells, CD4 and CD8, thereby being defined in the thymus as double-negative cells (DN). Using CD44 and CD25 markers, DN cells can be further divided into four progressive developmental stages, which are designated DN1 ($CD4^{neg}CD8^{neg}CD44^{pos}CD25^{neg}$), DN2 ($CD4^{neg}CD8^{neg}CD44^{pos}CD25^{pos}$), DN3 ($CD4^{neg}$ $CD8^{neg}CD44^{neg}CD25^{pos}$) and DN4 ($CD4^{neg}CD8^{neg}CD44^{neg}CD25^{neg}$). However, only a minority of $cKit^{pos}CD24^{neg}$ cells inside the DN1 population are thought to be colonising

cells with a foetal liver or bone marrow origin[235]. Seeding of the embryonic thymus by haematopoietic progenitors occurs around E13.5 and few thymocytes are beyond the DN stage until E16.5[237]. Full maturation of αβ T cells is residual before E19.5, but some unique γδ T cell populations are produced exclusively at these foetal stages[205,237].

Immature progenitors enter the thymic compartment in the cortico-medullary junction. Once in the thymus, DN1 and DN2 cells enter a coordinated migration through the thymic cortex toward the subcapsular zone. In this process, thymic progenitors proliferate extensively, progressively loose the potential to generate myeloid cells, NK cells and dendritic cells, and initiate TCR gene rearrangements[228,235]. This process is driven by several cytokines, chemokines and adhesion molecules in the thymus stromal cells. The commitment from immature progenitors to T cells is critically dependent on Notch signalling, especially before TCR signals[205,227,235,238]. By the DN3 stage, T cell commitment is complete; thymocytes stop proliferating and dramatically increase rearrangement of the TCR β, γ and δ genes, which will lead to β or γδ selection (Fig. 5).

T cell lineages – αβ and γδ fate choice

The transition to DN3 thymocytes marks an important step in T cell development. Thymocytes stop proliferating and start generating productive TCR products. Rearrangements in the *Tcrg*, *Trcb* and *Tcrd* locus will generate the first separation in the T cell lineage, as TCRγ and TCRδ chains heterodimerise generating γδ T cells – γδ-selection – whereas TCRβ associate with germline encoding pre-TCRα (pTα) to form pre-TCR complex – β-selection[239]. DN3 thymocytes can be divided into a DN3a subset, with low expression of CD27 that did not yet pass TCR selection, and $CD27^{high}$ DN3b cells, which are TCRγδ or β-selected cells[240]. Post-selection DN3 thymocytes receive multiple survival and proliferative signals form cortex stromal cells such as the activation of integrins and TCR/preTCR complex itself[227]. Although both TCRαβ and TCRγδ share the same CD3 signalling complex, the initial signalling from TCRγδ and pTα-TCRβ differ in their outcomes, initiating two different developmental programs. Indeed signalling-strength and TCR-Notch synergy models have been used to explain αβ (weak TCR signals and favoured by Notch signalling) or γδ (strong TCR signals) T cell lineage choice[205,241].

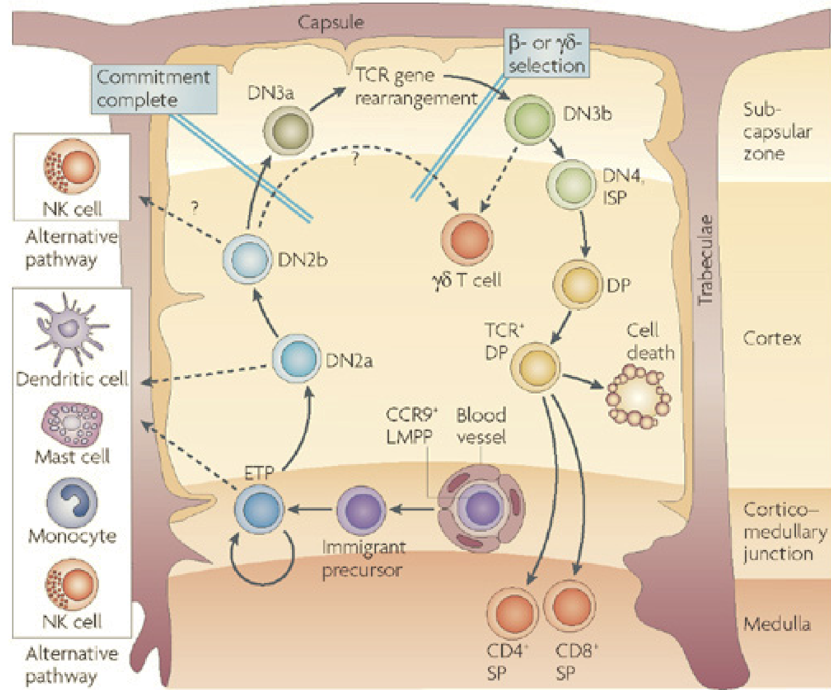

Figure 5. T cell developmental stages. Cross-section through an adult thymic lobule showing the migration path of haematopoietic precursors during T cell development. Immature progenitors enter the thymus through blood vessels near the cortico–medullary junction, and migrate to the outer rim of the thymic cortex, the subcapsular zone, finishing the T cell commitment. After TCR gene rearrangement TCRγδ^{pos} T cells are generated and TCRβ-selected cells start a reverse migration towards the thymic medulla, becoming CD4/CD8 double positive (DP) cells. This allows positive and negative selection to occur, generating mature CD4^{pos} and CD8^{pos} TCRαβ^{pos} T cells. Dashed arrows depict proposed developmental outcomes of immature progenitors and alternative generation pathways for γδ T cells (adapted from Rothenberg *et al* 2008[235]).

γδ-selected DN3 cells do not proceed to DN4 and DP stages characteristic of β-selected cells, but instead enrol a distinct developmental program. Contrary to β-selected cells, γδ-selected DN3 cells critically depend on IL-7 signalling but are less depended on Notch signalling. They also diverge in their proliferative kinetics and in the expression of activation markers such as CD5[240]. Differentiation of γδ T cells requires, among different signals, the transcription factor SOX13, *trans* conditioning lymphotoxin mediated signals by αβ DP cells and CD27 signals[242-244]. This result in the generation of fully functional γδ T cells that are imprinted already in the thymus with effector cytokine profiles as CD27^{pos} IFN-γ producers or CD27^{neg} IL-17-producing γδ T cells. Interestingly, the generation of tissues-specific γδ T cells subpopulations bearing monoclonal or oligoclonal TCR rearrangements are produced exclusively in the embryo[205,241,245].

From β-selection DN3 cells progress to DN4, which upregulate CD8, becoming first immature single positive (ISP or ImmCD8) and then CD4 positive, forming a double-positive population (DP), responsible for the generation of αβ T cells. DP thymocytes start a inward migration through the cortex from the subcapsullar zone towards the medulla[227]. In this DP state thymocytes stop cell division, possible waiting for the next developmental check point, avoiding uncontrolled growth of non-functional T cells.

αβ T cell populations – CD4 and CD8 T cells

DP cells are thymocytes that received survival and differentiation signals from pre-TCR complex after successful TCRβ-seletion. In this state they start and complete the *Tcra* rearrangement, being the first thymocytes to generate an αβ TCR[239]. As DP cells do not receive surviving signals from cytokines, αβ TCR signalling is critical for survival and maturation of DP cells, which die by neglect if their TCR is not capable of engaging peptide-loaded MHC class I or class II molecules – a process called positive selection[246-248].

Apart from positive selection, the specific TCR-MHC interactions dictate the critical divergence in the two main αβ T cell populations, $CD4^{pos}$ helper T cells and $CD8^{pos}$ cytotoxic T cells. DP cells with TCR restricted to MHC class I (MHC I) differentiate into CD8 T cells whereas signals through MHC class II (MHC II) restricted TCR results in the differentiation into CD4 T cells[249]. Consistent with this fact, differentiation of other αβ T cell populations from DP thymocytes, like NKT cells, also require specific interactions with MHC-like molecules, such as CD1d, which present glycolipid antigens[250].

Several models have been postulated to explain CD4/CD8 lineage choice and the generation of CD4 and CD8 single positive (SP) cells. The first classical models, stochastic and instructional models, couple lineage commitment and positive selection induced simultaneously by TCR signals. The stochastic model suggests that during positive selection DP cells randomly terminate expression of one of the co-receptors. Consequently, only thymocytes that express the appropriate co-receptor for their TCR will receive the required maturation and survival signals[203]. The instructional models postulate that the strength/duration of TCR signals in the positive selection directs DP cells to CD8SP or CD4SP, with weak/short interactions of TCRs with MHC I molecules engaging a CD8 fate, while strong/long MHC II-TCRs interactions drive CD4

differentiation[203]. More recently a kinetic model proposes that positive selection is dissociated from the CD4/CD8 choice. These processes would be sequential rather than simultaneous, and lineage choice would be determined by TCR signal duration balanced by cytokines from common cytokine receptor γ-chain (γ_c) family, particularly IL-7. According to this model, positive selecting TCR signals, derived either by MHC class I or MHC class II molecules, terminate *Cd8* gene transcription, which makes DP thymocytes acquiring a CD4^{pos}CD8^{low} phenotype. If TCR signals persist in the absence on CD8, IL-7 signalling is inhibited and thymocytes differentiate into CD4^{pos} T cells. If TCR signalling ceases, IL-7 signalling is induced and thymocytes differentiate into CD8^{pos} T cells – a process called co-receptor reversal. As co-receptor reversal occurs in cells that no longer receive TCR signals, γ_c cytokines such IL-7 are critical for their survival[203].

From the cortex, early SP cells leave for the last part of their journey in the medulla, where they receive the signals to become mature CD4 and CD8 T cells that can then be exported to peripheral tissues. At this point, potentially self-reactive thymocytes are eliminated from the thymocyte pool by a process called negative selection[248,251]. This occurs when thymocytes receive signals from a TCR that engages a peptide–MHC ligand with high affinity. Nevertheless, although less frequent, negative selection can also occur in the thymus cortex. Contrary to pro-survival signals received by low affinity TCR-MHC in positive selection, this strong TCR signals lead to activation of apoptotic pathways resulting in cell death . The elimination of thymocytes that recognise self antigens requires the expression and presentation of tissue-specific antigens in the thymus environment. This is accomplished by medullary thymic epithelial cells, as well as cross-presentation by dendritic cells. Moreover, it was proposed that negative selection also requires second co-stimulatory signals[251]. Thus, by deleting auto-reactive thymocytes, a largely self-tolerant peripheral T-cell repertoire is generated.

The relatively few auto-reactive T cells that evade thymic negative selection are actively controlled by tolerance mechanisms that depend on regulatory cells. Interestingly, the main regulatory cells are FOXP3^{pos} regulatory T cells (T_{reg}), which are generate in the thymus from cells that bear TCR with intermediate self-reactivity and affinity to peptide–MHC ligand[204].

After receiving final maturation signals from thymic stromal cells, newly generated T cell populations are exported from the thymus to secondary lymphoid organs and peripheral tissues where they will exert their functions.

RET tyrosine kinase receptor

Since its discovery, the tyrosine kinase receptor RET has been critically implied in spermatogenesis and embryonic development of kidneys, nervous system and enteric lymphoid organogenesis, controlling important biological processes such as growth and differentiation, cell survival, migration and chemotaxis[252-257].

The *Ret* locus, encoding the RET receptor, was identified as a proto-oncogene by Takahashi and colleagues in 1985, and encodes a tyrosine kinase receptor family member[258-260]. The name *Ret* stands for rearranged during transfection, as this gene was identified as rearranged gene segments within a NIH 3T3 fibroblast cell line that was transfected with human lymphoma DNA. The subsequent gene product encodes for a fusion protein with a dimerizing motif in the N-terminal region fused with the RET tyrosine kinase domain leading to kinase activation.

RET is composed of three main domains: an extracellular N-terminal domain that has four cadherin-like repeats with calcium binding sites and a cysteine rich region ; a transmembrane domain with two hydrophobic regions; and a cytoplasmatic C-terminal domain, with two tyrosine kinase subdomains[259-262]. In humans, the cytoplasmatic domain can have different isoforms that differ in their C-terminal amino acids: RET9; RET43 and RET51[263,264]. The most studied isoforms, the short isoform – RET9 – and the long isoform – RET51 – are conserved among vertebrates, including mice, and although sharing the same extracellular binding domain, they generate different signalling complexes, do not associate with each other, and control distinct biological processes. Indeed, only ablation of the short isoform cause similar phenotype in kidney and nervous system as the complete mutation of *Ret* and forced expression of *Ret9* can rescue *Ret* deficient mice[253,265-267].

The RET extracellular domain has the capacity to bind different RET ligands. RET functions as the receptor for the glial cell line-derived neurotrophic factor (GDNF) and its family ligands (GFLs), also called RET-ligands. In order to signal through RET, GFLs associate with GDNF family receptor-alpha (GFRαs) receptors[268,269]. Nevertheless, the RET extracellular domain has cadherin-like repeats with calcium binding sites, crucial for cadherins homophilic adhesions. Thus RET is also a distant member of the cadherin superfamily. This property raised the hypothesis that RET may have adhesion functions as a 'sensing' cadherin[262,270]. Despite the fact that a direct adhesion-derived signalling was not yet found, a recent report showed that the cleaved extracellular portion of RET, deprived of its kinase domain, can act as a

cadherin accessory protein stabilising N-cadherin, resulting in increased cell adhesion[271]. Interestingly, it was also shown that RET kinase receptors physically interact in their extracellular domain with protocadherins (Pcdhs) complexes, stabilising Pcdhs intracellular domains. In turn, Pcdhs stabilise RET activation by GFLs and delay RET degradation, specially the fast degrading RET51 isoform[272].

Increasing the complexity of its interactions and signalling axes, it was recently found in motor neurons that RET bound to ephrin-A, functions as a receptor driving reverse signalling in the ephrin-A:ephrin-A receptor (Eph) system. Moreover RET integrates both ephrin-As and GFL signals to achieve optimal neuronal function[273]. Interestingly RET was also shown to induce apoptosis by a ligand-independent mechanism, acting as a dependence receptor. In the absence of its cognate ligands, RET can be cleaved by proteases, like caspases, exposing an intracellular pro-apoptotic domain that drives apoptosis through p53 pathway[274-276]. Nevertheless GFL-RET signalling was described to have anti-apoptotic effects. Thus, at least in several cell lines *in vitro* and in somatotrophs in adenopituitary gland *in vivo*, RET acts as a switch receptor that balances survival and apoptosis based on GFL availability.

GDNF family ligands

The RET tyrosine kinase was identified as the signalling receptor for the neurotrophic factor GDNF, a distant member of TGF-β superfamily. However, GDNF does not bind directly to RET and instead requires the formation of heterodimeric complexes with the GDNF family receptor alpha (GFRα1), a glycosylphosphatidylinositol (GPI)-linked cell surface receptor[277-280]. Other GDNF family members (GFLs) were identified: neurturin (NRTN); artemin (ARTN) and persephin (PSPN), and their productive activation of RET was shown to depend on specific GPI-linked co-receptors, GFRα2, GFRα3 and GFRα4, respectively (Fig. 6a)[281-288]. Despite the GFL-GFRα-RET high specificity, some degree of crosstalk was found *in vitro* between GDNF, NRTN and ARTN and the co-receptors GFRα1, GFRα2 and GFRα3[285-288].

The original model for the initiation of GFL signalling complexes indicates that a GFL homodimer binds to GFRα co-receptors, sequentially the GFL-GFRα complex brings together two RET molecules, thereby inducing RET homodimerization and tyrosine autophosphorylation, which then allows the assembly of intracellular signalling

components (Fig. 6b). Nevertheless, weaker interactions between RET and GFRαs can also occurs in the absence of GFLs[268,269]. GFRα co-receptors are GPI-linked membrane bound proteins that locate to lipid rafts, where they recruit RET for efficient downstream signalling – signalling in *cis*[289,290]. Nevertheless, soluble forms of co-receptors originated from enzyme cleavage or alternative splicing, are produced by many cell types and are capable of activate RET with delayed kinetics – *trans* signalling[284,291,292]. Activation of RET in *trans* is slower, but more sustained than in *cis* activation, and may first occur outside lipid rafts. Ligand-activated RET associate with different adaptor proteins depending on its localization outside or within the lipid rafts, which in turn influences the intracellular signalling pathways activated by GFLs[268,269]. *In vitro* studies in neurons suggested that GFRα signalling in *cis* and *trans* may cooperate in the same cells for optimal effects of GFLs signals[291,293]. Nevertheless, *trans* signalling in neurons has a reduced physiological relevance *in vivo*, since mice that express GFRα1 in all RET^{pos} cells but lack GFRα1 in other cells (*Gfra1*$^{-/-}$.*Ret*$^{+/Gfra1}$ mice), thus enabling only high affinity *cis* signalling, have a normal phenotype with no developmental nervous problems even in processes where *trans* signaling has been shown *in vitro*[294]. Strikingly, haematopoietic cells in the gut that express RET but lack GFRa co-receptors rely on GFRas provides by neighbour cells and therefore employ RET signalling *in trans*[256,295].

All GFLs are thought to activate the same downstream signalling pathways, since the kinetics of binding and RET activation in the four key tyrosine residues (Y905, Y1015, Y1062, and Y1096 exclusively in the RET51 long isoform) is similar for all GFLs-GFRαs pairs[293]. Nevertheless, a selective recruitment of signalling partners by GFL-GFRα complexes cannot be excluded since the RET intracellular domain has 1 serine residue and 16 or 18 tyrosine residues in the short and long RET isoforms, respectively. Moreover, even the same phophorylated residues can assembly different adaptors that produce distinct biological responses. Importantly, the fact that each GFRα has a unique developmentally regulated tissue specific expression pattern indicates that despite the detected *in vitro* crosstalk, each GFL-GFRα pair has non-redundant roles *in vivo*, which is confirmed by the very different outcomes from specific ablation of each GFL or GFRα[268,269].

Recently, two mechanisms of RET-independent GFL signalling were discovered for GDNF and its specific co-receptor GFRα1. In RET-deficient neurons that express GFRα1, GDNF triggers induction of Fos, Src family kinases (SFKs) activation and phosphorylation of phospholipase C-γ (PLC-γ), mitogen-activated protein kinases

(MAPK) ERK1/ERK2 and cAMP response element binding protein (CREB). Extracellular GPI-linked GFRα1 is not likely to direct interact with cytoplasmatic membrane-bound SFKs, and instead were proposed to co-localise in lipid rafts, where they would require an additional transmembrane glycoprotein or linker[289,296]. Consequently, active SFKs can activate other tyrosine kinase receptors, as it was shown for the indirect phophorylation of MET receptor by GDNF signalling[297]. The neural cell adhesion molecule (NCAM) was also shown to mediate RET-independent GFL signalling. In the presence of GFRα1, GDNF can bind to NCAM and activate cytoplasmatic kinases in Schwann cells and neurons to stimulate cell migration and axonal growth. In the absence of GDNF, GFRα1-NCAM interactions are responsible for inhibiting NCAM-mediated cell adhesion[298]. Nevertheless, RET-independent GDNF/GFRα1 signalling is dispensable for normal neuronal development and may play only a minor role *in vivo*[294].

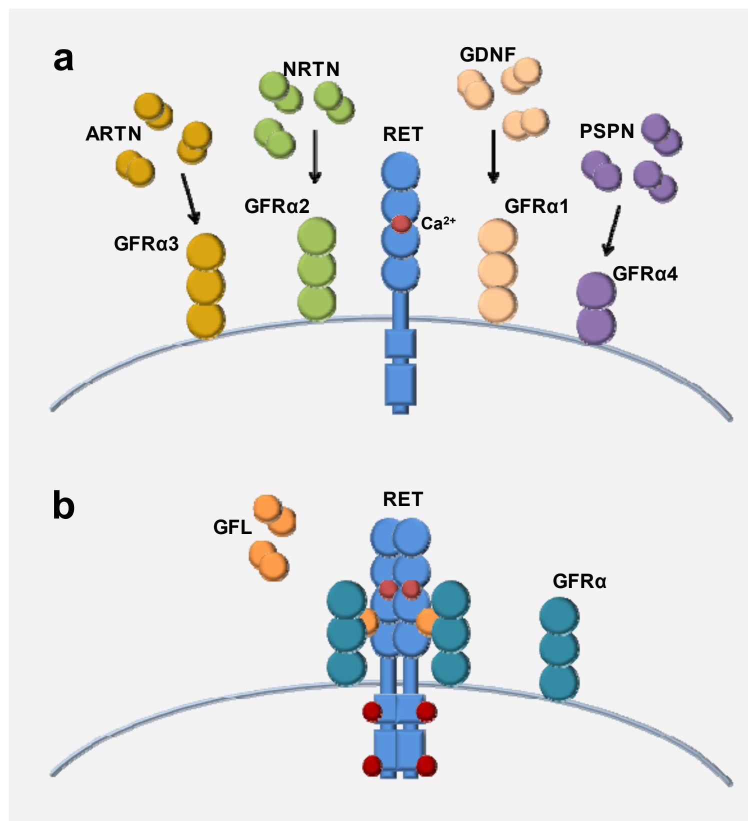

Figure 6. GDNF family ligands (GFLs) and signalling through RET. a. homodimeric GDNF family ligands (GFLs) first bind to specific GDNF family receptor-α. Arrows indicate the specific ligand-receptor interactions *in vivo*: GDNF binds to GFRα1; NRTN to GFRα2; ARTN to GFRα3 and PSPN to GFRα4. Binding of calcium ions (Ca^{2+}) to RET cadherin-like domains is required for RET activation. **b.** Association of GFL-GFRα complexes with RET extracellular domain triggers RET homodimerization and autophosphorylation of tyrosine residues in the kinase domain, driving its intracellular signalling[268,269].

RET signalling axes

RET activates several signalling cascades such as Ras/ERK; PI3K/Akt; PLC-γ; JAK/STAT; JNK, p38MAPK and ERK5. Activated RET has multiple phophorylated tyrosine residues that have different functions. Phophorylation of Y905 stabilises the kinase conformation and facilitates the autophosphorylation of other C-terminal tyrosine residues. This residue is also a docking site for Grb7/10 adaptors[299]. Y752 and Y928 were described to be a docking site and activate STAT3[300]. Phosphorylation of Y981 results in binding and activation of SFKs that have been implied in survival and migration processes[301]. PLC-γ interacts with RET in the Y1015, and deficiencies in Y1015 result in defective kidney development[302,303]. Activation of PI3K/Akt pathway can be achieved by phosphorylation of Y1096, one of the two tyrosine residues that are only present in the RET51 isoform[304]. Apart from tyrosine phosphorylation, serine phosphorylation in S697 residue is also critical for activation of JNK pathway and the lack of S697 results in enteric nervous system defects and impairs lamellipodia formation[305,306].

Y1062 is possibly the most important residue in RET kinase domain, since it acts as a multidocking binding site for many adaptor and effector proteins. Phosphorylation of Y1062 is the most studied RET activation site and results in the activation of Ras/ERK; PI3K/Akt; JNK, p38MAPK and ERK5 intracellular signalling pathways. This leads to activation of important transcription factors responsible for the pleiotropic effects of RET signalling such cAMP response element binding protein (CREB) and NF-κB[304,307]. Activation of these signalling pathways results in survival, proliferation and differentiation programs downstream of RET, which are also implicated in neoplastic diseases associated with increased or constitutive RET function[268,269,308]. Surprisingly, in addition to its role in cell development and maintenance, RET was also shown *in vitro* to have a role in stress response pathways, driving the expression of heat shock protein (Hsp) family members[309].

Deregulation of RET signalling

Ret mutations that result in deregulated RET signalling are responsible for several human pathologies. Hirschprung's disease (HSCR) is a multigenic disorder of neural crest development characterised by reduction or absence of autonomic neuronal ganglia in the colon – colonic aganglionosis – which results in a life-threatening

condition of severe constipation and intestinal obstruction in neonates and megacolon in children and adults. RET loss-of-function dominant mutations, both in the tyrosine kinase domain and in the extracellular domain, were implicated in the majority of familial HSCR cases[310,311]. These mutations result in the unresponsiveness to GDNF and impairment of neuronal migration. In mice, a dominant negative mutation in *Ret* (*Ret*$^{+/DN}$) causes intestinal hypoganglionosis and aganglionosis in the distal intestine similar to HSCR disease in humans[254]. Accordingly, mice homozygous for a null mutation of *Ret* (*Ret*$^{-/-}$) are completely deprived of enteric neurons throughout the digestive tract, and also show renal agenesis, confirming RET requirement for enteric neurogenesis and kidney organogenesis[253].

Over expression, gain-of-function mutations and production of aberrant forms of RET are associated with oncogenesis[312]. Somatic chromosomal rearrangements involving the *Ret* gene were found to cause papillary thyroid carcinoma (PTC), one of the most common types of thyroid malignancies[312,313]. These rearrangements lead to generation of chimeric oncogenes, designated as RET/PTC, as a result of fusion of the RET tyrosine kinase domain with the 5′-terminal regions of heterologous genes[314,315]. Similarly, translocation of the *Ret* kinase domain with genes encoding transmenbrane receptors like *Bcr* and *Fgfr1* were also found to contribute to chronic myelomonocytic leukaemia[316]. The generated oncogenes can cause malignancy by multiple mechanisms: substitution of *Ret* regulatory elements by the one of the 5′-terminal gene results in ectopic *Ret* expression in cells where it is normally silent; or constitutive activation of RET kinase domain in the generated oncogenes, since many of the fusion partner genes have dimerizing domains. Finally, if the 5′-terminal gene has regulatory or anti-tumour roles, its normal function will be disrupted as it occurs with the RET/PTC chimeric oncogene[269].

Familial cancer syndrome of multiple endocrine neoplasia type 2 (MEN 2) is also caused by germline activating point mutations of *Ret* that are inherited in an autosomal dominant manner. MEN 2 can be divided into three variants, with different genetic alterations and clinical outcome: MEN 2A, MEN 2B, and familial medullary thyroid carcinoma (FMTC) [312,314]. Medullary thyroid carcinoma (MTC), which is a malignant tumour arising from calcitonin-secreting parafollicular C cells, is the central feature of MEN 2 syndromes. In addition, MEN 2A is also characterised by pheochromocytoma and parathyroid hyperplasia or adenoma, although rare manifestations can include cutaneous lichen amyloidosis, a pruritic cutaneous lesion, and intestinal aganglionosis as in HSCR. FMTC is the least aggressive of the MEN 2 subtypes and consist on

MTC, with rare cases of associated HSCR[317-319]. The mutations that cause MEN 2A and FMTC are similar, and in most cases cause a substitution of a cysteine residue, particularly in the extracellular domain, but rarely additional cysteine residues can be generated by base pair duplication. The generation of an unpaired cysteine forms an activating intermolecular bridge that result in ligand-independent dimerization and constitutive activation of RET. Moreover, some mutations can reduce cell surface localization[269]. MEN 2B is the most aggressive of the MEN 2 variants and is characterized by early MTC onset associated with pheochromocytoma, and less frequently with developmental abnormalities as intestinal and mucosal ganglioneuromatosis, ocular and skeletal abnormalities[317]. Approximately 95% of the MEN 2B mutations are highly specific methionine to threonine substitutions in the position 918. MEN 2B mutations also cause constitutive activation of RET, but were proposed to alter the quality of RET intracellular signals by modifying substrate specificity. Contrary to MEN 2A, MEN 2B ligand-independent activation of the kinase occurs without causing a constitutive dimerization of RET molecules. Furthermore RET ligands can induce dimerization or RET MEN 2B molecules, further enhancing kinase activity, which can explain the aggressiveness of MEN 2B syndrome[269]. Finally, several mice models with altered RET residues present many developmental malformations and oncogenic transformations that mirror MEN 2 syndromes, although less aggressive than in humans[269,320].

Role of RET in the haematopoietic system

RET signalling functions have been intensively explored in nervous system development and maintenance, but it has also been shown that RET controls critical events in other systems, such as kidney development and spermatogonia differentiation. Increasing evidence has highlighted striking similarities between the nervous and the immune system, such as complex membrane synapse and establishment of memory cells[216,217]. Interestingly, receptors for neurotransmitters were found in human HSCs, fostering the concept of active crosstalk between nervous and haematopoietic cells and unveiling putative common molecular pathways used by these two systems[221].

The tyrosine kinase receptor RET belongs to the same family as cKit, Tie2 and Flt3 receptors. These receptors are critical for proliferation, differentiation, migration and survival of several haematopoietic progenitor cells. During embryonic development,

RET is expressed by haematopoietic lymphoid tissue initiator cells (LTin) and is essential for Peyer's patch organogenesis[256]. These LTin cells are attracted by GFLs and RET signalling cause changes in motility of LTin cells, resulting in adhesion-dependent cell arrest that induces maturation of non-haematopoietic stromal cells , thus initiating the priming events that lead to the formation of Peyer's patches[295]. Interestingly, this is the only process where RET signalling *in trans* has a physiological relevance *in vivo,* since haematopoietic cells in the gut express RET but lack GFRa co-receptors and rely on GFRas provided by neighbouring cells[256,295].

In the embryo, expression of *Ret* was observed in the developing liver and was suggested to be restricted to cells from haematopoietic origin, since the adult liver do not express *Ret*[321]. Sequentially, *Ret* expression was also shown in the thymus, spleen and lymph nodes of young and adult rats, as well as in bone marrow haematopoietic cells[322,323]. In the bone marrow, stromal cells were also shown to express RET signalling partners, *Gfra1*, *Gfra2*, *Gdnf* and *Nrtn*, strongly suggesting a broader role of RET in the haematopoietic system[323,324]. Although with unknown functions, expression of *Ret* and *Gfra* co-receptors was already described in many human haematopoietic cells populations, from CD34^{pos} haematopoietic progenitors, to mature monocytes, T cells and B cells[324-326].

As a proto-oncogene, RET activation can lead to malignancies, like in the cases of multiple endocrine neoplasias type 2. Moreover *Ret* expression was detected in multiple leukaemia/lymphoma cell lines and in primary haematopoietic malignancies, uncovering a possible impact of deregulated RET signalling in the establishment of haematological cancers[316,323,324,327-329]. Interestingly forced activation of RET in immature Lin^{neg} progenitors was found to drive differentiation to monocyte/macrophage lineage that ultimately lead to leukaemia, uncovering a possible function of RET in myeloid cell differentation[316].

Previous studies have indicated RET expression in diverse steps of lymphocyte differentiation. *Ret* expression was shown to be tightly regulated in B cell development. It is already detected in the most immature B cell progenitor, pre-Pro-B cell, and increase until the late Pro-B/large Pre-B stage. Thereafter its expression decreases until the mature B cell stage in the BM. Interestingly, this tightly regulated reduction of *Ret* expression was proposed to depend on productive immunoglobulin rearrangements of both heavy (*Igμ*) and light (*Igκ*) chains, as Pro-B cells generated in recombination-deficient mice, SCID and *Rag1*$^{-/-}$, have a 3-fold induction of *Ret* expression compared to wild-type mice, a phenotype rescued if SCID and *Rag1*$^{-/-}$ had *μ*

and κ transgenes[330]. Strikingly, forced expression of constitutively activated *Ret* oncogene under the enhancer of immunoglobulin heavy chain (Eμ-Ret mice), active in Pro-B cells, results in increased proliferation of foetal B-cell progenitors, expansion of immature progenitors in the BM, impairment in the elimination of late Pro-B cells with non-productive immunoglobulin rearrangements and cell cycle and apoptosis disturbances, which give rise to B cell precursor leukaemia[328,329]. Notwithstanding, whether RET signalling plays a role in normal B cell development is still unclear.

Expression of *Ret*, *Gfra1* and *Gdnf* was also detected in the foetal and adult thymus. *Gfra1* and *Gdnf* expression were found respectively in thymocytes and thymic stromal cells, suggesting an involvement of GFL signalling in T cell development[331]. E14.5 foetal thymocytes strongly express *Gfra1*, but this gene expression is thereafter reduced in adulthood, remaining only in the double negative (DN) stage of T cell development. Furthermore, GNDF was proposed to provide thymocyte growth and survival without affecting cell differentiation. Nevertheless, it is still unclear whether RET signals are required for proper thymopoiesis *in vivo*.

Taken together, numerous observations have suggested a possible broad role of RET in haematopoietic cell biology, but the precise consequences and molecular mechanisms of these putative functions are poorly understood.

AIMS OF THIS THESIS

For proper haematopoiesis, HSCs need to regulate the delicate balance between maintenance, self-renewal and differentiation. To achieve that HSCs require multiple soluble and contact-dependent signals provided by the HSC microenvironments[127]. Previous studies have demonstrated a key role of several tyrosine kinase receptors in controlling different haematopoietic stem cell functions. Tyrosine kinase receptors respond to many different cytokines and growth factors, such SCF and angiopoetin, which in HSCs control processes of survival, self-renewal, quiescence proliferation and differentiation. Interestingly, it was shown that RET signals are crucial for enteric lymphoid organogenesis[256]. Moreover, expression of the tyrosine kinase receptor RET and its neurotrophic factor ligands, was previously described in primary lymphoid organs and immune cells, and a function on thymocyte survival and maturation was also suggested[321-331]. Nevertheless, the role of RET in HSC function and thymocyte development, *in vivo* remains totally unexplored.

In this thesis, we aimed at determining the role of RET in HSC function and thymopoiesis. By analysing loss and gain of function mouse models of *Ret* we characterized the impact of RET in HSC function in normal haematopoietic development and stressful conditions[253,320]. We then aimed to understand how manipulation of RET signalling influences transplantation protocols in order to favour critical HSC responses.

Despite an increased survival of GDNF-treated thymocytes, the physiological relevance of GFL signalling in T cell development *in vivo* remains elusive[331]. Therefore, we aim to dissect the role of RET signalling in foetal and adult T cell differentiation and understand the differential contribution of GFLs and GFRα co-receptors in this process.

Similarities between the haematopoietic system and the nervous systems were recently highlighted, as cells from both systems share common features[216]. With our work we aim to understand how the neurotrophic receptor RET is used by haematopoietic cells in order to orchestrate their function, thus shedding light into unexpected regulatory mechanisms used to control haematopoiesis.

THE NEUROTROPHIC FACTOR RECEPTOR RET DRIVES HAEMATOPOIETIC STEM CELL SURVIVAL AND FUNCTION

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Abstract

Haematopoiesis is a developmental cascade that generates all blood cell lineages in health and disease. This process relies on quiescent Haematopoietic Stem Cells (HSCs) capable to differentiate, to self renew and to expand upon physiological demand[61]. However, the mechanisms regulating HSC homeostasis and function remain largely unknown. Here we show that the neurotrophic factor receptor RET is critical to HSC survival and function. We found that RET signalling molecules are expressed by HSCs and *Ret* ablation leads to reduced HSC numbers. Despite normal differentiation potential, RET null progenitors exhibit loss of *in vivo* stress response and reconstitution potential, being rapidly exhausted. Strikingly, RET signals provide HSCs with critical *Bcl2* and *Bcl2l1* surviving cues, downstream of p38/MAP kinase and CREB activation. Accordingly, enforced expression of RET down-stream targets, *Bcl2* or *Bcl2l1*, is sufficient to restore the activity of *Ret* null progenitors *in vivo*. Remarkably, activation of RET results in improved HSC survival and *in vivo* transplantation efficiency, thus opening new horizons to the usage of RET agonist in HSC expansion and transplantation protocols. Finally, our work shows that RET is an essential cell-autonomous regulator of HSCs function, revealing neurotrophic factors as novel components of the HSC microenvironment.

Methods

Mice: C57BL/6J (CD45.2 and CD45.1), *Rag1*$^{-/-}$ (CD45.2 and CD45.1)[332], *Vav1*-iCre[333], *Gfra1*$^{-/-}$ [334], *Gfra2*$^{-/-}$ [335], *Gfra3*$^{-/-}$ [336], *Ret*^{MEN2B} [320] and *Ret*$^{-/-}$ [253] were on a C57BL/6J genetic background. All mice strains were bred and maintained at IMM animal facility. Animal procedures were performed in accordance to national and institutional guidelines.

Generation of *Ret* conditional knockout mice: To generate mice harbouring a conditional *Ret* knock-out allele we engineered a targeting construct that firstly, included the introduction of a floxed 2.1 kb, Neomycin resistance (Neo^r) cassette under the control of the phosphoglycerate kinase-1 (PGK) promoter and a polyA tail (pA). This cassette (PGK-NEO^r-pA) was inserted approximately 4.5 kb upstream at the Xho I site of the pBluescript KS (pBS KS) vector that carried approximately 13 kb of the 5' end of mouse *Ret* genomic locus flanking exon 1. The second modification included an insertion of a loxP ~ 2.5 kb downstream of exon 1, at the Hind III site in the intron between exons 1 and 2 of the mouse *Ret* locus. Finally, a viral thymidine kinase cassette (~3 kb) under the control of the PGK promoter (PGK-TK-pA) was inserted at the Hind III site ~5kb downstream of the inserted LoxP site. To obtain homologous recombination, this targeting construct was linearised by Xho I, purified by gel elution and extraction using the Qiaquick gel extraction kit (Qiagen), prior to electroporation into 129SvJ-derived R1 ES cells grown on mouse embryonic fibroblast (MEF) feeder layers. Following double selection with 300μg/ml Geneticin (G418, Invitrogen) and 2μM Gancyclovir (Sigma), positive clones were identified by Southern blotting. Genomic DNA was digested with Hind III restriction enzymes and a 5' external probe of 500bp (see figure) was used to screen for positive clones. With the Hind III digest the WT and mutant alleles showed a band size of 16.5 kb and 6 kb respectively. Positive animals were subsequently crossed with transgenic mice expressing *Vav1*-iCre[333] in order to delete the PGK-NEO^r-pA cassette. This recombination resulted in generating the floxed Ret mice wherein the two remaining LoxP sites were found flanking the first exon of the *Ret* locus, or the complete deletion of the first exon. These mice are further designated as *Ret* floxed (*Ret*^{fl}) and *Ret* null (*Ret*^{null}). Mice were further screened by PCR. Primer sequences were: P1: AAG CTC CCT CCT ACC GTG CT; P2: TGG GAT GAA CTC TGC CCA TT; P3: TGC TGC TCC ATA CAG ACA CA; P4: TAC ATG CTG TCT GCT CTC AG.

Colony-Forming Units assays and homing capacity: $5x10^3$ E14.5 Lin^{neg}cKit^{pos} cells MACS purified (Miltenyi Biotec) from WT, *Ret*$^{-/-}$ or *Ret*^{MEN2B} were cultured in

M3434 (Stem Cell Technologies) and scored at day 8 to 10 by flow cytometry and microscope analysis. $3x10^4$ E14.5 $Lin^{neg}cKit^{pos}$ cells were MACS (Miltenyi Biotec) purified from WT, *Ret*$^{-/-}$ or *Ret*^{MEN2B} and were injected into lethally irradiated mice (9Gy) and CFU-s scored at day 8 or 12 by flow cytometry and microscope analysis. Homing assays were done using E14.5 $Lin^{neg}cKit^{pos}$ cells labelled with CMTMR, injected into lethally irradiated mice. Flow cytometry analysis was performed 20h post-injection.

Transplantation experiments: For reconstitution experiments with foetal liver, $1x10^5$ E14.5 $Lin^{neg}cKit^{pos}$ cells MACS purified from WT, *Ret*$^{-/-}$ or *Ret*^{MEN2B} were injected alone or in direct competition (1:1 ratio) into lethally irradiated *Rag1*$^{-/-}$ CD45.1 mice. For the 3:1 ratio $1.5x10^5$ WT CD45.1/CD45.2 cells were co-injected with $0.5x10^5$ *Ret*$^{-/-}$ CD45.2 cells; for the 1:3 ratio $0.5x10^5$ WT CD45.1/CD45.2 cells were co-injected with $1.5x10^5$ *Ret*$^{-/-}$ CD45.2 cells. For secondary reconstitution experiments with bone marrow $2.5x10^5$ cells from each genotype were FACS sorted from primary recipients and injected in direct competition into lethally irradiated *Rag1*$^{-/-}$ CD45.1 mice.

5-FU treatment: *Vav1*-iCre^{pos}.*Ret*$^{null/fl}$ and littermate controls were injected weekly with 150 µg of 5-FU per g of animal.

Rescue of *in vivo* transplantation: E14.5 $Lin^{neg}cKit^{pos}$ WT or *Ret*$^{-/-}$ cells were transduced overnight with pMig.IRES-GFP retroviral vector containing *Ret9*, *Bcl2* or *Bcl2l1* and GFP^{pos} cells injected into lethally irradiated mice. 6 to 8 weeks later transduced BM $Lin^{neg}CD45.2^{pos}GFP^{pos}$ were purified by flow cytometry and 10^5 cells were co-injected with a radio-protective dose 10^5 CD45.1 BM cells into lethally irradiated recipients.

Flow cytometry: Embryonic foetal livers were micro-dissected and homogenized in 70µm cell strainers. Bone marrow cells were either collected by flushing or crushing bones. Cell suspensions were stained with: anti-CD117 (cKit) (2B8), anti-Ly-6A/E (Sca-1) (D7), anti-CD16/32 (FcRγII/III) (93), anti-CD3 (eBio500A2), anti-CD150 (mShad150), anti-CD48 (HM48-1), anti-CD19 (eBio1D3), anti-CD11b (M1/70), anti-Ly-6G (Gr-1) (RB6-8C5), anti-Ly79 (TER119), anti-NK1.1 (PK136), anti-CD11c (N418), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD54 (ICAM-1) (YN1/1.7.4), anti-CD34 (RAM34), anti-CD51 (RMV-7) and anti-CD41 (eBioMWReg30) from eBioscience; anti-CD38 (90), anti-CD3 (145-2C11), anti-CD34 (HM34) and anti-CD31 (390) from BioLegend; anti-Ly6C (HK1.4) from Abcam, Annexin V from BD Pharmingen. Lineage cocktail include anti-CD3, anti-CD19, anti-Ly-6G, anti-Ly6C, anti-Ly79, anti-NK1.1, anti-CD11c for embryonic foetal livers plus anti-CD11b for adult bone marrow cells. Human

cord blood was enriched in $CD34^{pos}$ cells using CD34 MicroBead Kit (Miltenyi Biotec) after Histopaque separation (Sigma) and stained with anti-human CD34 (AC136) (Miltenyi Biotec) and anti-human CD38 (HIT2) (eBioscience). Samples were sorted on a FACSAria I or FACSAria III and analysed on a FACSCanto or LSRFortessa (BD). Flow cytometry data was analysed with FlowJo 8.8.7 software (Tree Star).

Cell cycle analysis and intracellular staining: Animals were injected 12 hours before analysis with 66.6 µg of BrdU per g of animal. Intracellular stainings were done using BrdU Flow Kit and anti-BrdU (3D4), 7AAD, anti-Ki-67 (B56), anti-S6 (pS235/pS236) (N7-548) and anti-Akt (pT308) (JI-223.371) from BD Pharmingen, anti-human RET (132507) from R&D Systems, anti-PIP_3 (Z-P345) from Echelon Biosciences, anti-CREB (pS133) (87G3), anti-p38 (pT180/Y182) (28B10), anti-Akt (pS473) (D9E) and anti-ERK1/2 (pT202/pY204) (D13.14.4E) from Cell Signaling Technology.

in vitro **culture of haematopoietic progenitors:** 10^6 E14.5 WT $Lin^{neg}cKit^{pos}$ cells were cultured in DMEM and starved for 2 hours. To test CREB phosphorilation upon GFL stimulation $Lin^{neg}cKit^{pos}$ cells were stimulated 1 hour with 500ng/ml of each GFL and co-receptor. LSK cells were purified by flow cytometry and stimulated overnight with GFL/GFRα combinations in order to determine *Bcl2 and Bcl2l1* expression levels. For inhibition experiments cells were incubated 2hours prior GFLs stimulation, to test CREB phosphorilation, or during overnight stimulation with GFLs, to determine *Bcl2 and Bcl2l1* expression levels, with SB 202190 (10 µM) and PD98,059 (40 µM) from Sigma-Aldrich or Akt1/2, Akt InhibitorVIII (58 nM) and CBP-CREB Interaction Inhibitor (2.5 µM) from Calbiochem. $Lin^{neg}cKit^{pos}$ cells were stimulated with GFL/GFRα for 120 hours and $2.5x10^5$ $CD45.2^{pos}Lin^{neg}cKit^{pos}$ cells were sequentially analysed by flow cytometry and transplanted into lethally irradiated hosts with a radio-protective dose $2.5x10^5$ CD45.1 BM cells. To detect Annexin V, $4x10^4$ E14.5 WT or $Ret^{-/-}$ $Lin^{neg}ckit^{pos}$ cells per well were cultured overnight in DMEM alone or with GFL/GFRα. When analysed by flow cytometry haematopoietic progenitors were further stained with an LSK antibody cocktail.

Real-time PCR analysis: RNA was extracted from cell suspension using RNeasy Mini Kit or RNeasy Micro Kit (Qiagen). Real-time PCR for *Ret*, *Gfra1*, *Gfra2* and *Gfra3* were done as previously described [256,337]. *Hprt1* was used as housekeeping gene. For TaqMan assays (Applied Biosystems) RNA was retro-transcribed using High Capacity RNA-to-cDNA Kit (Applied Biosystems), followed by a pre-amplification PCR using TaqMan PreAmp Master Mix (Applied Biosystems). TaqMan Gene Expression Master

Mix (Applied Biosystems) was used in real-time PCR. TaqMan Gene Expression Assays bought from Applied Biosystems were the following: Gapdh Mm99999915_g1; *Hprt1* Mm00446968_m1; *Gusb* Mm00446953_m1; *Mpl* Mm00440310_m1; *Mcl1* Mm00725832_s1; *Meis1* Mm00487664_m1; *Angpt1* Mm00456503_m1; *Eya1* Mm00438796_m1; *Eya2* Mm00802562_m1; *Egr1* Mm00656724_m1; *Tek* Mm00443243_m1; *Slamf1* Mm00443316_m1; *Lef1* Mm00550265_m1; *Thy1* Mm00493681_m1; *Mllt3* Mm00466169_m1; *Hoxa5* Mm00439362_m1; *Hoxa9* Mm00439364_m1; *Hoxc4* Mm00442838_m1; *Pbx3* Mm00479413_m1; *Ndn* Mm02524479_s1; *Evi1* Mm00514814_m1; *Mll1* Mm01179213_g1; *Hlf* Mm00723157_m1; *Cxcr4* Mm01292123_m1; *Sm*o Mm01162710_m1; *Igf2r* Mm00439576_m1; *Cdkn1a* Mm00432448_m1; *Notch1* Mm00435249_m1; *Kitl* Mm00442972_m1; *Thpo* Mm00437040_m1; *Bcl2l1* Mm00437783_m1; *Bcl2* Mm00477631_m1; *Pspn* Mm00436009_g1; *Artn* Mm00507845_m1; *Nrtn* Mm03024002_m1; *Gdnf* Mm00599849_m1; *Ret* Mm00436304_m1. For HSC signature gene arrays, gene expression levels were normalized to *Gapdh*, *Hprt1* and *Gusb*. For *Bcl2* / *Bcl2l1* expression after HSC stimulation and *Ret* expression levels after *in vivo* transfer gene expression levels were normalized to *Gapdh* and *Hprt1.*

Statistics: Statistical analysis was done using Microsoft Excel. Variance was analyzed using F-test. Student's *t*-test was performed on homocedastic populations and student's *t*-test with Welch correction was applied on samples with different variances. Kaplan-Meier survival curves were analyzed using a log-rank test.

Results

Haematopoiesis starts during embryonic life, mainly in the Foetal Liver (FL), and is maintained throughout adulthood in the Bone Marrow (BM). Although HSCs are mostly quiescent in adults, they become proliferative upon physiological demand. Interestingly, autonomic nerves have been recently shown to actively participate in HSC niches raising the hypothesis that neurotrophic factors may regulate HSC function[220]. The neuronal growth factor family includes the glial cell-line derived neurotrophic factor (GDNF) ligands (GFLs), which signal through the RET tyrosine kinase receptor and act mainly in the autonomous nervous system, kidney and mature lymphoid cells[256,268,325,331].

To explore the role of GFLs in HSC biology we initially determined the expression of their canonical receptor RET in embryonic day 14.5 (E14.5) FL Lin^{neg}Sca1^{pos}cKit^{pos} (LSK) cells, a population highly enriched in HSCs. When compared to myeloid progenitors (Lin^{neg}Sca1^{neg}cKit^{pos}) (MP), LSKs expressed high levels of *Ret* and its co-receptors *Gfra1*, *Gfra2*, and *Gfra3*; a finding also confirmed in BM LSKs and human CD34^{pos}CD38^{neg} cord blood progenitors (Fig. 1a; Supplementary Fig. 1). Strikingly, *Ret* expression in FL was restricted to Lin^{neg}Sca1^{pos}cKit^{pos}CD150^{pos}CD48^{neg} haematopoietic stem cells (HSC), while multipontent progenitors (Lin^{neg}Sca1^{pos}cKit^{pos}CD150^{neg}CD48^{pos} (MPPs)) expressed this gene poorly (Fig. 1b)[70,117]. Interestingly, FL mesenchymal cells and BM osteoblasts, which were described as important players in HSC microenvironment, co-expressed the neurotrophic RET ligands *Gdnf*, *Nrtn* and *Artn*, further suggesting an unexpected involvement of RET signalling in HSCs (Fig. 1c; Supplementary Fig. 2)[61].

To dissect this hypothesis we analysed mice with a null mutation of *Ret*[253]. E14.5 Ret deficient progenitors were generated in similar proportions to their WT littermate controls (Fig. 1d). However, LSK numbers and total foetal liver cellularity were strongly reduced in *Ret*$^{-/-}$ embryos (Fig. 1e). Despite their reduced cell number, the differentiation potential of *Ret*$^{-/-}$ LSKs was intact as revealed by normal numbers of methylcellulose colony-forming units (CFU) (Fig. 1f). Further dissection of LSK cells into short-term (ST-LSK) and long-term LSKs (LT-LSK) by CD38 expression[108], revealed a preferential reduction of *Ret* deficient LT-LSKs (Supplementary Fig. 3a). This abnormal LSK population structure correlated with an altered cell cycle status of *Ret*$^{-/-}$ LSK. Thus, despite similar G1/S/G2+M fractions (Supplementary Fig. 3b), and turnover rate determined by BrdU incorporation (Fig. 1g), quiescent Ki-67^{neg} G0 phase

LSKs were consistently reduced in *Ret*$^{-/-}$ animals (Fig. 1g-h). Accordingly, *Ret* deficiency resulted in decreased HSC numbers (Fig. 1i).

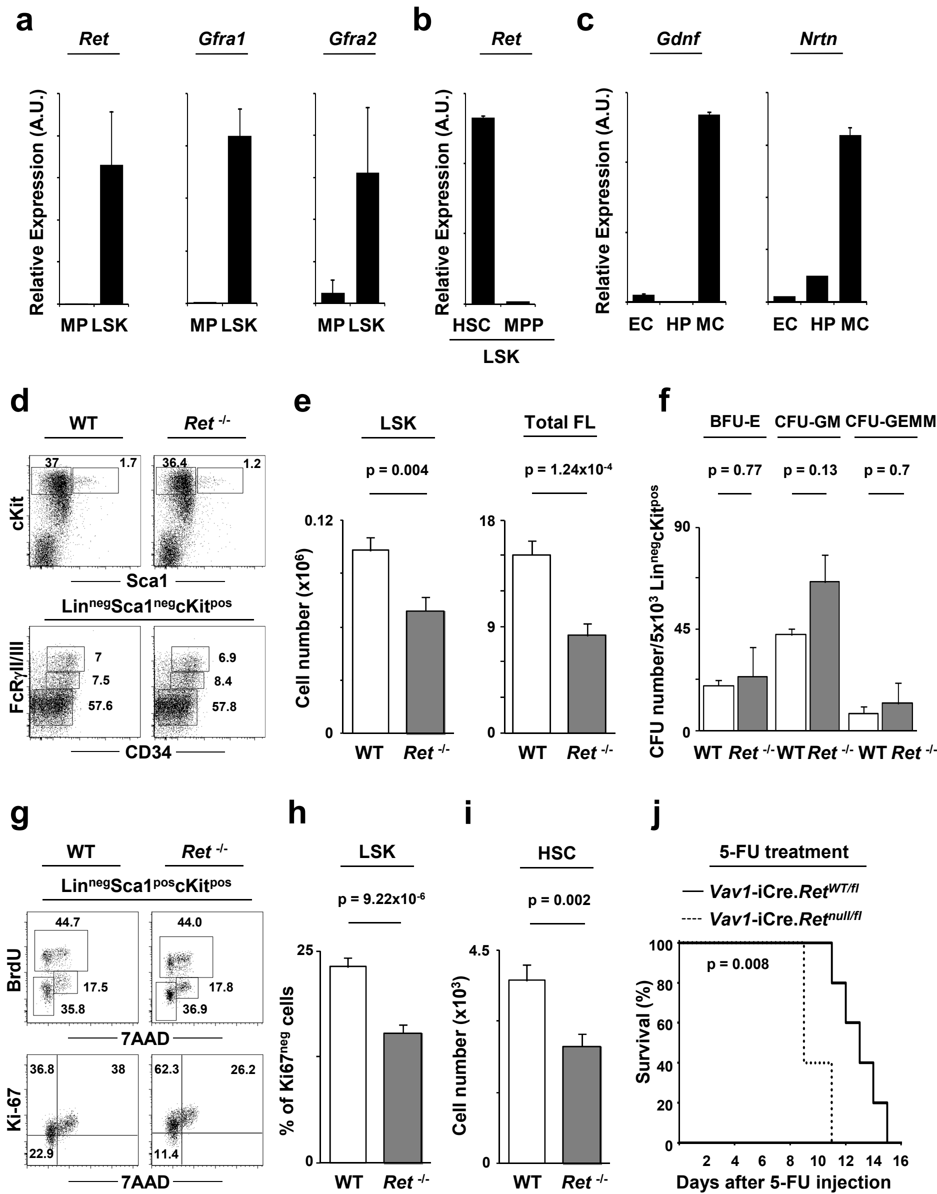

Figure 1. *Ret* deficiency leads to reduced HSCs. a., b. FL E14.5 MP and LSK, HSCs and MPPs were analysed by quantitative RT-PCR. **c.** FL E14.5 TER119^{neg}CD45^{neg}CD31^{pos} endothelial cells (EC), TER119^{neg}CD45^{neg}CD31^{neg}cKit^{pos}ICAM-1^{neg} hepatocyte progenitor cells (HP) and TER119^{neg}CD45^{neg} CD31^{neg}cKit^{neg}ICAM-1^{pos} mesenchymal cells (MC) were analysed by quantitative RT-PCR. **d.** Flow cytometry analysis of E14.5 *Ret*$^{-/-}$ *and* WT littermate control LSKs (top) and MPs (bottom). **e.** Number of LSKs and total FL cells. WT n=18; *Ret*$^{-/-}$ n=17. **f.** Day 8 CFU colony numbers. WT n=3; *Ret*$^{-/-}$ n=3. **g.** BrdU and Ki-67 in E14.5 LSK cells. **h.** Percentage of Ki-67^{neg} LSKs (G0 cells). WT n=12; *Ret*$^{-/-}$ n=14. **i.** Number of E14.5 HSCs. WT n=20; *Ret*$^{-/-}$ n=18. **j.** Survival of VaviCre.*Ret*$^{null/fl}$ and littermate controls after 5-FU treatment. *P* value for log-rank test is indicated. *Vav1* iCre.*Ret*$^{WT/fl}$ n=5; *Vav1* iCre.*Ret*$^{null/fl}$ n=5. Two tailed *t*-test *P* values are indicated. Error bars show s.e..

To test whether RET also affects adult HSCs we generated *Ret*$^{fl/fl}$ mice that were bred to *Vav1*-iCre mice (Supplementary Fig. 4)[333]. Strikingly, despite low levels of *Ret* expression in adult HSCs, *Ret* conditional ablation led to reduced quiescent HSCs numbers (Supplementary Fig. 1, 4). These data suggested that RET may regulate HSC responses to physiological demands. In agreement, serial treatments with 5-fluorouracil (5-FU) revealed that *Vav1*-iCre^{pos}.*Ret*$^{null/fl}$ mice promptly died upon 5-FU treatment when compared to their *Vav1*-iCre^{pos}.*Ret*$^{wt/fl}$ littermate controls (Fig. 1j). Taken together, these results indicate that RET is required to the maintenance of a normal pool of quiescent HSCs and for haematopoietic stress responses.

In the past, reduction of quiescent HSCs was correlated to impaired haematopoietic activity on a per cell basis[143,338]. Accordingly, *Ret*$^{-/-}$ progenitors exhibited reduced CFU-s potential upon transplantation into lethally irradiated hosts (Fig. 2a). In order to assess whether *Ret* is required for long-term HSC transplantation we performed in vivo repopulation assays. Strikingly, despite similar BM and Spleen colonising capacity (Fig. 2b), *Ret* deficient progenitors failed to rescue lethally irradiated mice, resulting in 100% host lethality (Fig. 2c). Since loss of reconstitution potential makes it impossible to evaluate the fate of *Ret* null progenitors, we performed competitive transplantation assays. Thus, foetal *Ret*$^{-/-}$ progenitors were co-transplanted with equal numbers of WT littermate progenitors that also provide supportive haematopoiesis and ensure host survival (Fig. 2d). Analysis of recipient mice revealed that *Ret* deficient progenitors lost their transplantation fitness across all blood cell lineages (Fig. 2e-f), a finding also confirmed when different donor cell ratios were transplanted (Supplementary Fig. 5). These data suggested a role of RET in HSCs. Accordingly, BM analysis 4 months after transplantation showed minute frequencies of *Ret* deficient LSKs (Fig. 2g). Sequentially we performed highly sensitive secondary competitive transplantation assays with the same number of WT and *Ret*$^{-/-}$ BM cells isolated from primary recipients (Fig. 2d). We found minute frequencies of *Ret*$^{-/-}$ cells in blood (Fig. 2h-i), a defect already established in BM LSKs (Fig. 2j). Altogether, these findings demonstrate that RET is critically required for foetal and adult LSK function and transplantation activity, a finding also supported by *Ret* up-regulation upon LSK transplantation (Supplementary Fig. 5c).

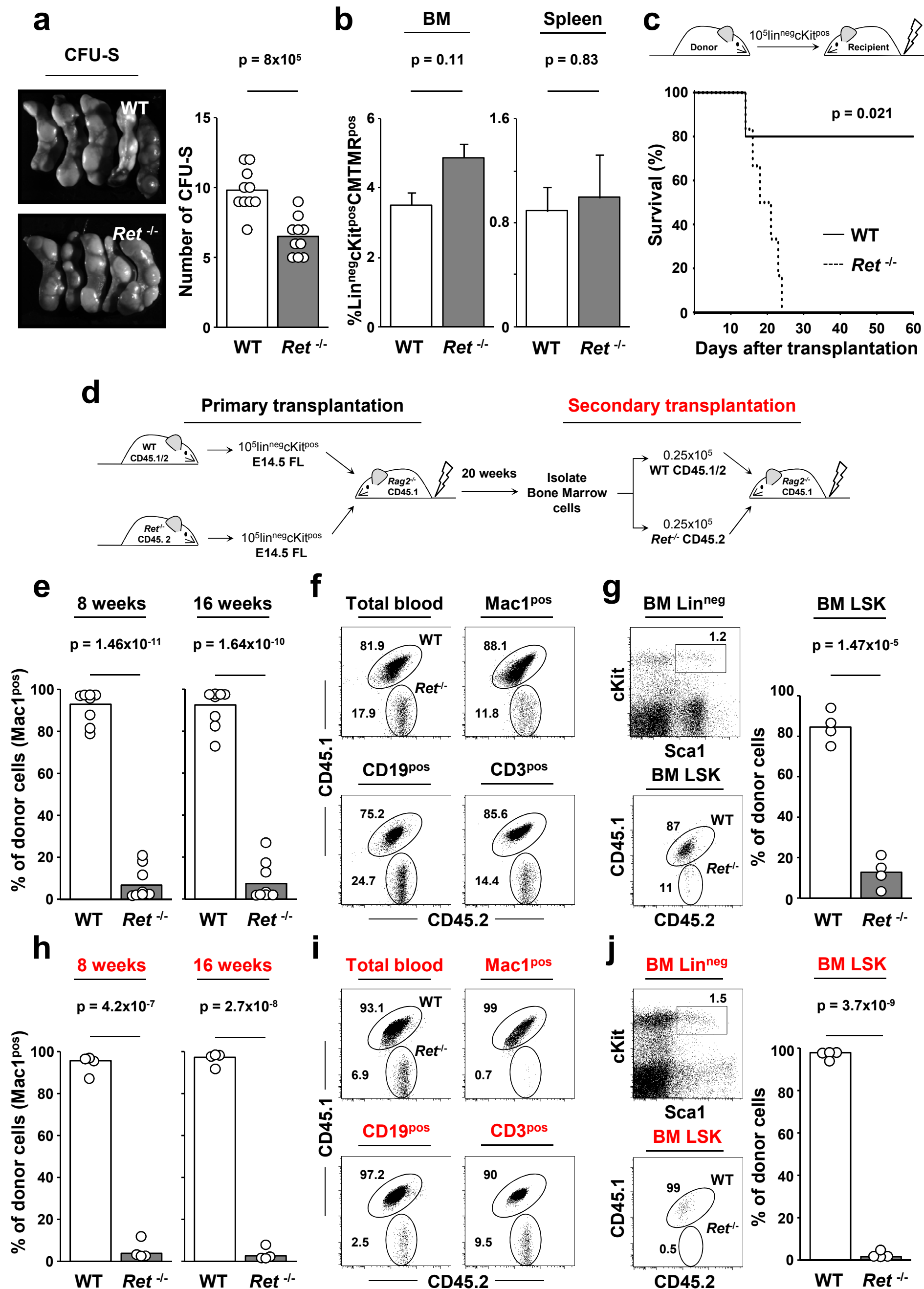

Figure 2. ***Ret***$^{-/-}$ **LSKs have poor in vivo reconstitution potential.** **a.** Day 12 CFU-s. WT n=10; *Ret*$^{-/-}$ n=10. **b.** Percentage of Lin^{neg}cKit^{pos}CMTMR^{pos} cells in BM and spleen 20h post-injection. WT n=3; *Ret*$^{-/-}$ n=3. **c.** Survival upon transplantation. *P* value for log-rank test is indicated. WT n=5; *Ret*$^{-/-}$ n=6. **d.** Scheme of Primary and Secondary (red) transplantation. **e.** Percentage of Mac1^{pos} cells after transplantation. WT n=8; *Ret*$^{-/-}$ n=8. **f.** Blood cell lineages 16 weeks post-transplantation. **g.** BM Lin^{neg} and LSK cells at 16 weeks and percentage of BM LSK cells. WT n=4; *Ret*$^{-/-}$ n=4. **h.** Percentage of Mac1^{pos} cells post-secondary transplantation. WT n=4; *Ret*$^{-/-}$ n=4. **i.** Blood cell lineages 16 weeks post-secondary transplantation. **j.** BM Lin^{neg} and LSK cells at 16 weeks and percentage of BM LSK cells. WT n=4; *Ret*$^{-/-}$ n=4. Two tailed *t*-test *P* values are indicated. Error bars show s.e..

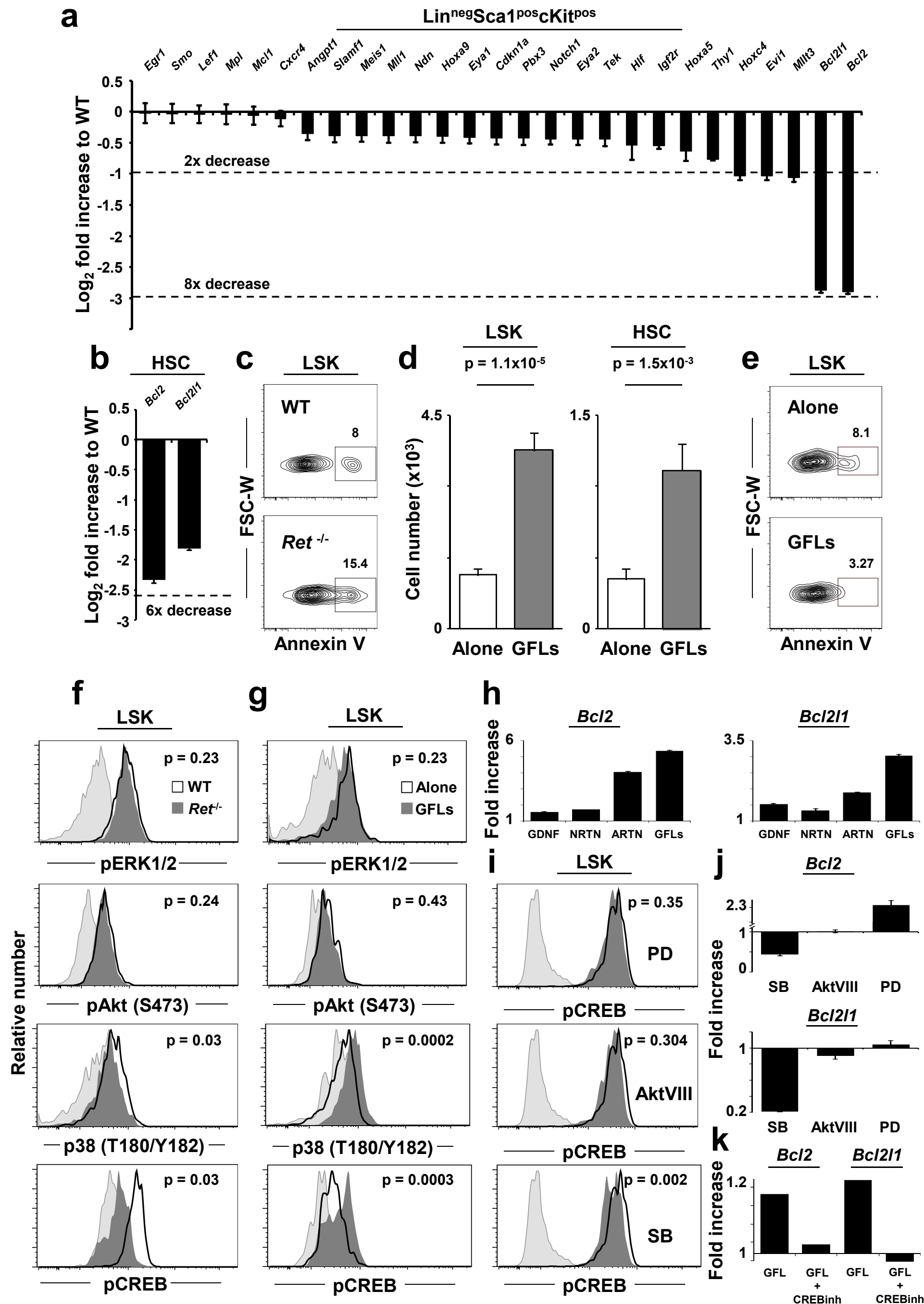

Figure 3. RET induces *Bcl2*/*Bcl2l1* downstream of p38/MAP and CREB activation. **a., b.** Quantitative RT-PCR for FL E14.5 *Ret*$^{-/-}$ and WT LSKs and HSCs. n=3. **c.** AnnexinV^{pos} cells in cultured E14.5 LSK cells. WT n=7; *Ret*$^{-/-}$ n=4. **d.** Number of recovered LSKs and HSCs treated with GFLs for 4 days. n=12. **e.** AnnexinV^{pos} cells in cultured LSK cells. Alone n=9; GFLs n=9. **f.** Flow cytometry of E14.5 *Ret*$^{-/-}$ and WT littermate control LSKs. WT n>8; *Ret*$^{-/-}$ n>7. **g.** Flow cytometry analysis of LSK cells in the absence or presence of GFLs for 1h. n>6. **h.** *Bcl2* and *Bcl2l1* expression upon GFL treatment. **i.** Flow cytometry analysis of LSKs cultured with GFLs (black line) or GFLs and the inhibitors SB 202190 (SB); PD98,059 (PD);or Akt1/2, Akt Inhibitor VIII (AktVIII) (solid grey). n>5. **j.** *Bcl2* and *Bcl2l1* expression upon GFL treatment and different inhibitors. **k.** *Bcl2* and *Bcl2l1* expression relative to untreated LSKs, upon GFL treatment or GFL+CBP-CREB interaction inhibitor (CREBinh). Two tailed *t*-test *P* values are indicated. Error bars show s.e.. Light grey: isotype control.

The marked deficiencies of RET null HSCs led us to investigated putative changes at the molecular level. Previous reports have identified a gene signature, associated with long term HSC activity[151,159,193,339]. Strikingly, while most of those genes were not significantly modified, *Bcl2* and *Bcl2l1* were heavily reduced in *Ret* deficient LSKs and HSCs (Fig. 3a-b; Supplementary Fig. 6a). The marked reduction of *Bcl2* and *Bcl2l1* anti-apoptotic genes suggested that RET could provide HSCs with critical survival signals. Accordingly, *Ret* null LSKs were highly susceptible to apoptosis, and GFLs efficiently increased LSK and HSC survival in culture conditions (Fig. 3c-e; Supplementary Fig. 6b-c).

RET activation in neurons was shown to lead to ERK1/2, PI3K/Akt and p38/MAP kinase activation[268], while phosphorylation of the transcription factor CREB can induce *Bcl2* gene family expression[340,341]. Analysis of p38/MAP kinase and CREB in $Ret^{-/-}$ LSKs revealed that these molecules were consistently hypo-phosphorylated, while ERK1/2 and PI3K/Akt activation was seemingly unperturbed when compared to their WT counterparts (Fig. 3f; Supplementary Fig. 6d). Accordingly, GFL induced RET activation led to rapid p38/MAP kinase and CREB phosphorylation and increased *Bcl2*/*Bcl2l1* expression by LSKs, while ERK1/2, PI3K/Akt phophorylation was stable (Fig. 3g-h; Supplementary Fig. 6e). Importantly, inhibition of p38/MAP kinase upon GFL activation led to impaired CREB phosphorylation and *Bcl2*/*Bcl2l1* expression while inhibition of ERK1/2 and PI3K/Akt had no significant impact on these molecules (Fig. 3i-j). Finally, inhibition of CREB upon GFL activation resulted in decreased *Bcl2*/*Bcl2l1* levels (Fig. 3k). Altogether, these data demonstrate that RET deficient progenitors express reduced *Bcl2* and *Bcl2l1*, downstream of impaired p38/MAP kinase and CREB activation.

The aberrant molecular signature of *Ret* deficient HSCs, suggested that the minute levels of *Bcl2* and *Bcl2l1* were responsible for the observed LSK unfitness. Using retro-viral transductions we found that *Bcl2* and *Bcl2l1* expression levels were quickly restored in $Ret^{-/-}$ LSKs transduced with WT *Ret*, while other signature genes were unperturbed by this immediate rescue of RET function (Fig. 4a). Thus, in order to test whether $Ret^{-/-}$ progenitor fitness could be restored by enforced expression of *Ret* we performed competitive transplantation assays with *Ret* deficient progenitors transduced with pMig.*Ret9*.IRES.GFP or pMig.Empty.IRES.GFP retro-virus together with a competitive/radio-protective dose of CD45.1 BM (Supplementary Fig. 7a). Restoration of RET expression fully rescued $Ret^{-/-}$ progenitors transplantation (Fig. 4b-d).

Strikingly, enforced expression of RET down-stream targets, *Bcl2* or *Bcl2l1*, was sufficient to recover the engraftment of *Ret* null LSKs (Fig. 4b-d).

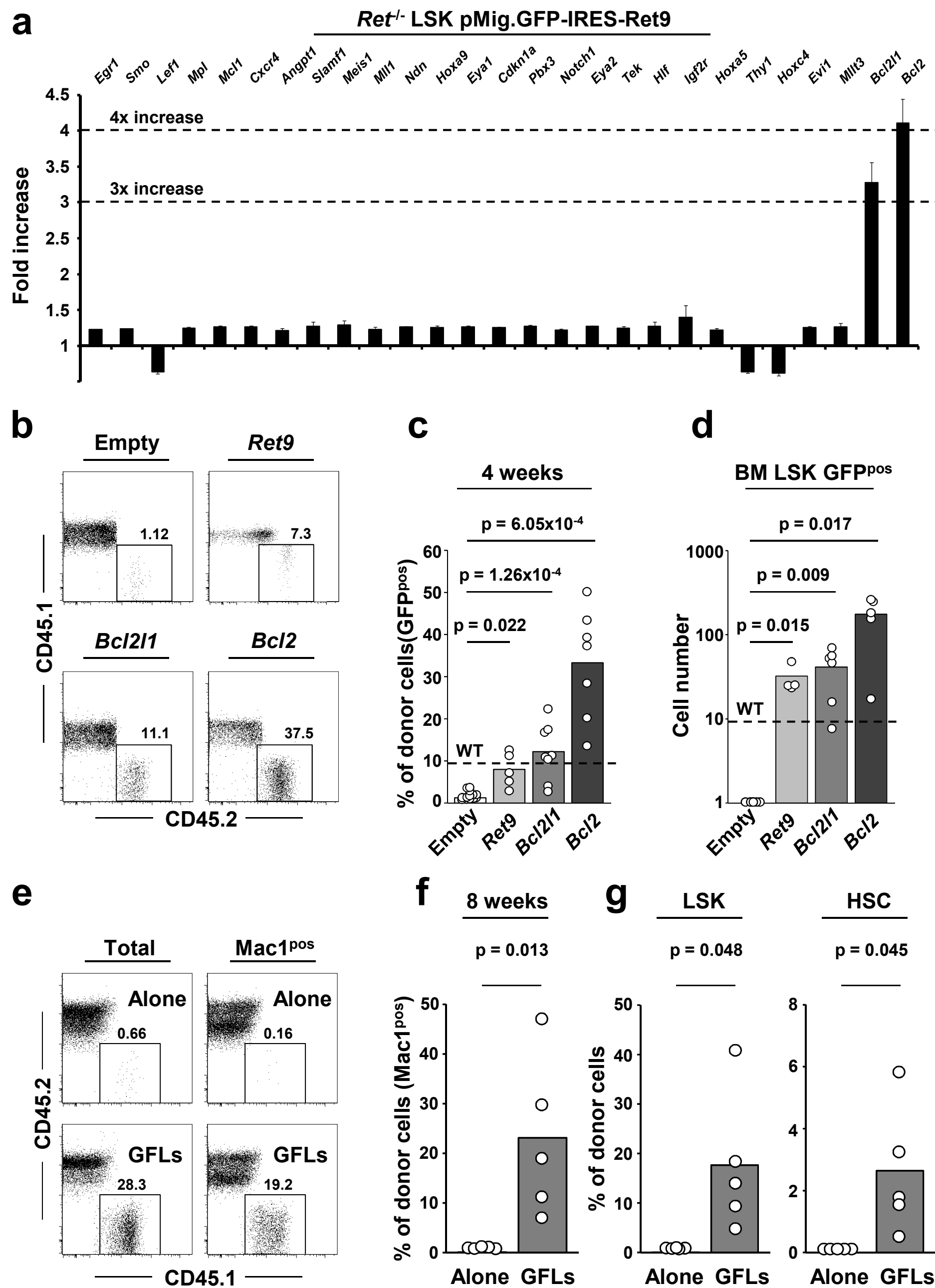

Figure 4. Triggering of RET signalling improves transplantation activity. a. Quantitative RT-PCR. Fold increase ratio between *Ret*$^{-/-}$ pMig.GFP-IRES-*Ret9* and empty vector. n=3. **b.** Flow cytometry analysis of *Ret*$^{-/-}$ blood cells at 4 weeks upon transduced cell transplantation. **c.** Percentage of CD45.2^{pos}GFP^{pos} in the blood at 4 weeks post transplantation with *Ret*$^{-/-}$ progenitors transduced with pMig.GFP-IRES-Empty (Empty), pMig.GFP-IRES-*Ret9* (*Ret9*), pMig.GFP-IRES-*Bcl2l1* (*Bcl2l1*) or pMig.GFP-IRES-*Bcl2* (*Bcl2*). Empty n=13; *Ret9* n=5; *Bcl2l1* n=8; *Bcl2* n=7. **d.** Number of BM LSK cells transduced as indicated in Fig.4b. *Ret*$^{-/-}$ Empty n=13; *Ret9* n=4; *Bcl2l1* n=6; *Bcl2* n=5. **e.** Flow cytometry analysis of blood cells at 8 weeks upon transplantation. **f.** Percentage of CD45.2^{pos} donor cells in Mac1^{pos} cells from blood. Alone n=5; GFLs n=5. **g.** Percentage of BM CD45.2^{pos} donor LSK and HSC cells, 12 weeks upon transplantation. Alone n=5; GFLs n=5. Two tailed *t*-test *P* values are indicated.

Altogether, these data suggest that RET signals might be used to improve blood cell transplantation. To directly test this hypothesis, initially we used *Ret*^{MEN2B} mice, which have improved ligand-dependent RET activation[320]. Early haematopoietic progenitors in these animals exhibited a remarkable increased CFU-s activity and reconstitution potential. However, no difference was observed when comparing embryonic LSK numbers and downstream haematopoietic progenitors between *Ret*^{MEN2B} and wild type mice (Supplementary Fig. 7b-f). Hence, positive modulation of RET-signalling was clearly beneficial in transplantation without compromising steady state haematopoiesis. Further evidence that RET signalling axes promotes HSC *in vivo* fitness was provided by transplantation of GFL pre-treated progenitors. E14.5 LSKs were treated with GFLs or medium alone and were transplanted with competitor CD45.1 BM (Supplementary Fig. 7g). While untreated progenitors engrafted poorly in these conditions; GFL treated progenitors had strikingly increased transplantation fitness and HSC engraftment (Fig. 4e-g).

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Discussion

Our results reveal that RET signalling is a crucial novel pathway regulating foetal and adult HSC activity by providing critical surviving signals through BCL2 family members. Although no appreciable HSC survival deficiencies were reported in *Bcl2* deficient mice, it is possible that haematopoietic stress conditions could reveal such deficits[181]. Alternatively, *Bcl2* and *Bcl2l1* may have redundant roles in HSCs, an idea supported by our data demonstrating that *Bcl2l1* or *Bcl2* are independently sufficient to fully rescue *Ret* deficient HSC function (Fig. 4b-d).

Haematopoietic progenitors express multiple RET co-receptors and actively respond to their respective ligands (Fig. 1a; Fig. 3g-h; Supplementary Fig. 6). We hypothesise that contrary to nervous cells, HSCs might use GFLs in a versatile and redundant manner since analysis of RET co-receptors single knockouts revealed normal LSK numbers (Supplementary Fig. 8)[334-336]. In agreement with this concept, it was recently shown that Lymphoid Tissue initiator cells can respond redundantly to all RET ligands[295].

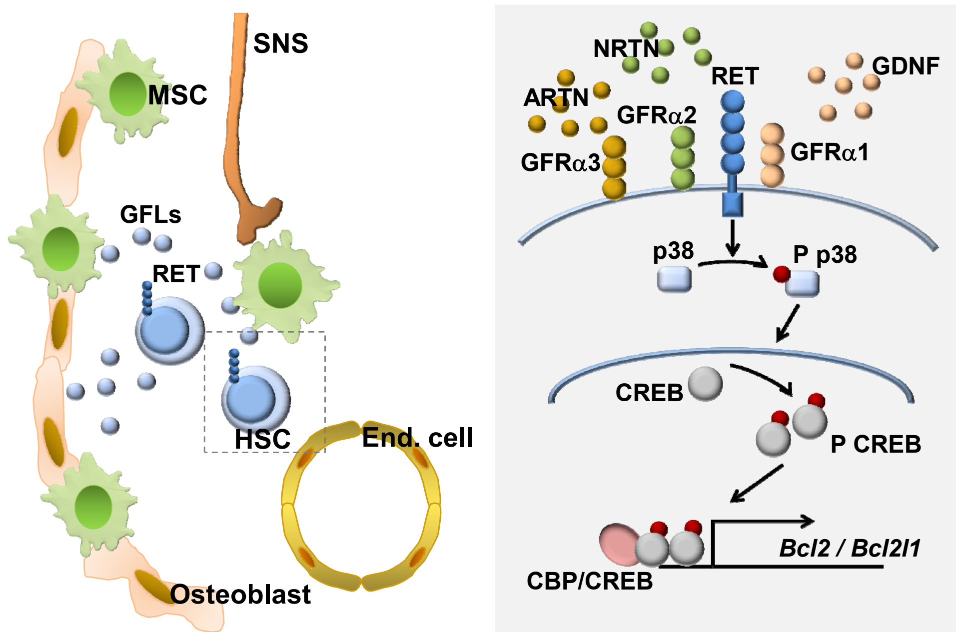

Figure 5. Neuronal growth factors regulate HSC response to physiological demand. The neurotrophic factors GDNF, NRTN and ARTN are produced by cells in the HSC microenvironment and act directly on HSCs by binding to RET/GFRa heterodimers. Highlighted area: RET stimulation results p38/MAP kinase and CREB activation leading to *Bcl2* and *Bcl2l1* expression. Thus, RET signal provide HSCs with critical survival signals.

Our data indicate that absence of neurotrophic factor cues leads to impaired HSC survival and possible recruitment of quiescent LT-HSCs resulting in a pronounced deficiency of HSC transplantation *in vivo*. Accordingly, activation of RET results in increased *Bcl2/Bcl2l1* expression, improved HSC survival and *in vivo* transplantation efficiency. Thus, we propose that RET controls HSC responses to physiological proliferative demands (Fig. 5). These findings and the expression of RET in human $CD34^{pos}CD38^{neg}$ cord blood progenitors open new horizons for testing the usage of GFLs in human haematopoietic stem cell expansion and transplantation therapy.

Previous work has revealed that nervous cells can modulate HSC function indirectly[212,218,220,221]. However, our findings reveal that HSCs are direct targets of neurotrophic factors, suggesting that haematopoietic and neuronal stem cells require similar survival signals. Whether HSCs can control autonomic nervous functions through neurotrophic factor consumption remains an elusive aspect. However, the presence of neurotrophic factors in the HSC environment and their critical role in HSC function pave the way to further studies deciphering whether haematopoietic stem cells regulate neural functions.

Supplementary Figures

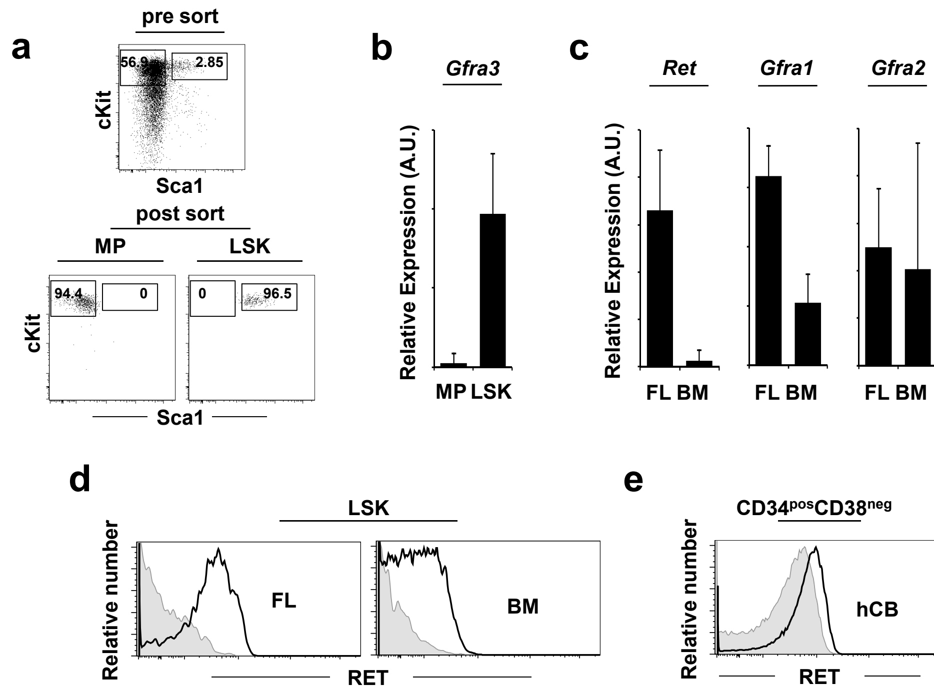

Figure S1. *Ret* expression in haematopoietic progenitors. a. Purification strategy of $Lin^{neg}Sca1^{neg}cKit^{pos}$ myeloid progenitors (MP) and $Lin^{neg}Sca1^{pos}cKit^{pos}$ (LSK) cells from E14.5 FL by flow cytometry. Top: pre sort gated on Lin^{neg} cells. Bottom: purity of $Lin^{neg}Sca1^{neg}cKit^{pos}$ myeloid progenitors (left) and $Lin^{neg}Sca1^{pos}cKit^{pos}$ cells (right) after sorting. **b.** FL E14.5 MP and LSK were analysed by quantitative RT-PCR. **c.** LSK cells from E14.5 FL and adult BM were analysed by quantitative RT-PCR. **d.** Flow cytomery analysis of E14.5 FL and adult BM LSK cells. **e.** Flow cytomery analysis of $CD34^{pos}CD38^{neg}$ cells from human cord blood (hCB). Light grey: isotype control.

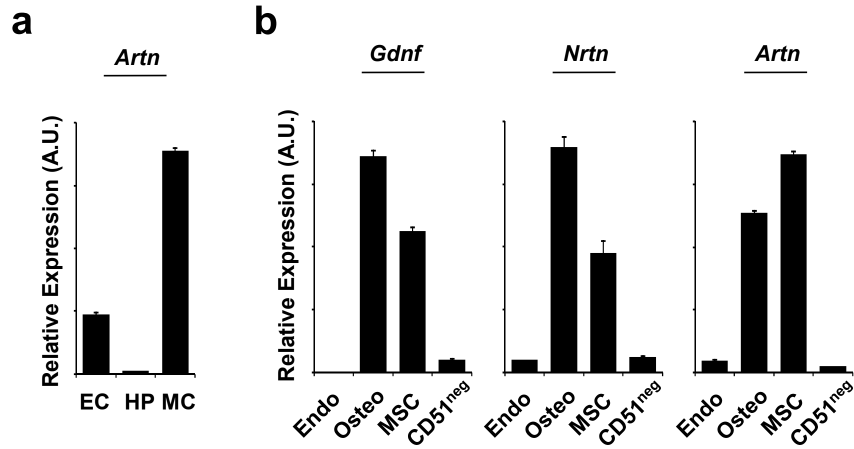

Figure S2. ***Ret*** **ligands are expressed in foetal and adult haematopoietic stem cell environment.** **a.** E14.5 FL TER119^{neg}CD45^{neg}CD31^{pos} endothelial cells (EC), TER119^{neg}CD45^{neg}CD31^{neg}cKit^{pos}ICAM-1^{neg} hepatocyte progenitor cells (HP) and TER119^{neg}CD45^{neg}CD31^{neg}cKit^{neg}ICAM-1^{pos} mesenchymal cells (MC) were analysed by quantitative RT-PCR. **b.** BM TER119^{neg}CD45^{neg}CD31^{pos}Sca1^{pos} endothelial cells (Endo), TER119^{neg}CD45^{neg}CD31^{neg}Sca1^{neg}CD51^{pos} osteoblasts (Osteo), TER119^{neg}CD45^{neg}CD31^{neg} Sca1^{pos}CD51^{pos} mesenchymal stem cells (MSC) and the remaining TER119^{neg}CD45^{neg}CD31^{neg} Sca1^{neg}CD51^{neg} (CD51^{neg}) cells were analysed by quantitative RT-PCR. Results are representative of three independent experiments.

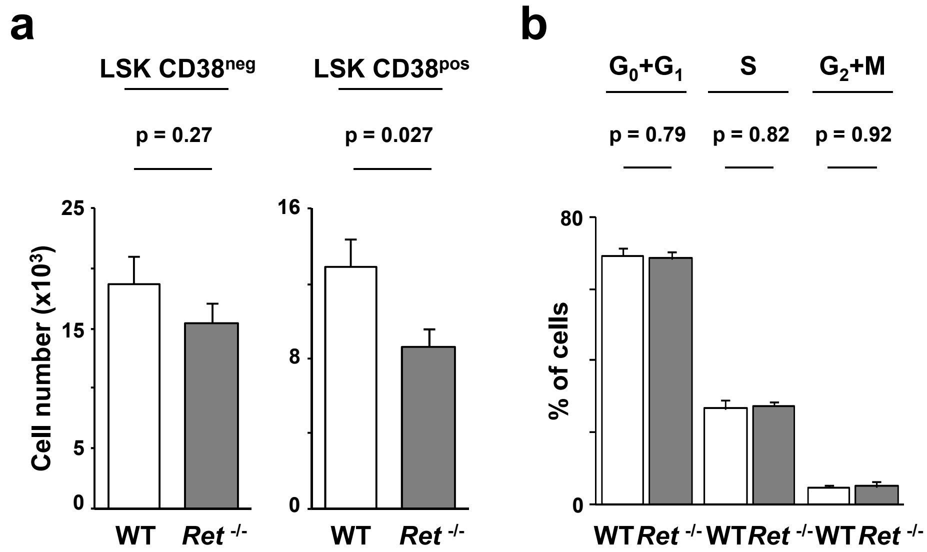

Figure S3. Long-term reconstituting LSK cell numbers are affected by *Ret* deficiency. a. Number of E14.5 ST-LSK ($LSKCD38^{neg}$) and LT-LSK ($LSKCD38^{pos}$) cells. WT n=12; $Ret^{-/-}$ n=11. **b.** Percentage of LSK cells in G0+G1, S or G2+M cell cycle phases according to DNA content (7AAD). WT n=7; $Ret^{-/-}$ n=6. Two tailed *t*-test *P* value is indicated. Error bars show s.e..

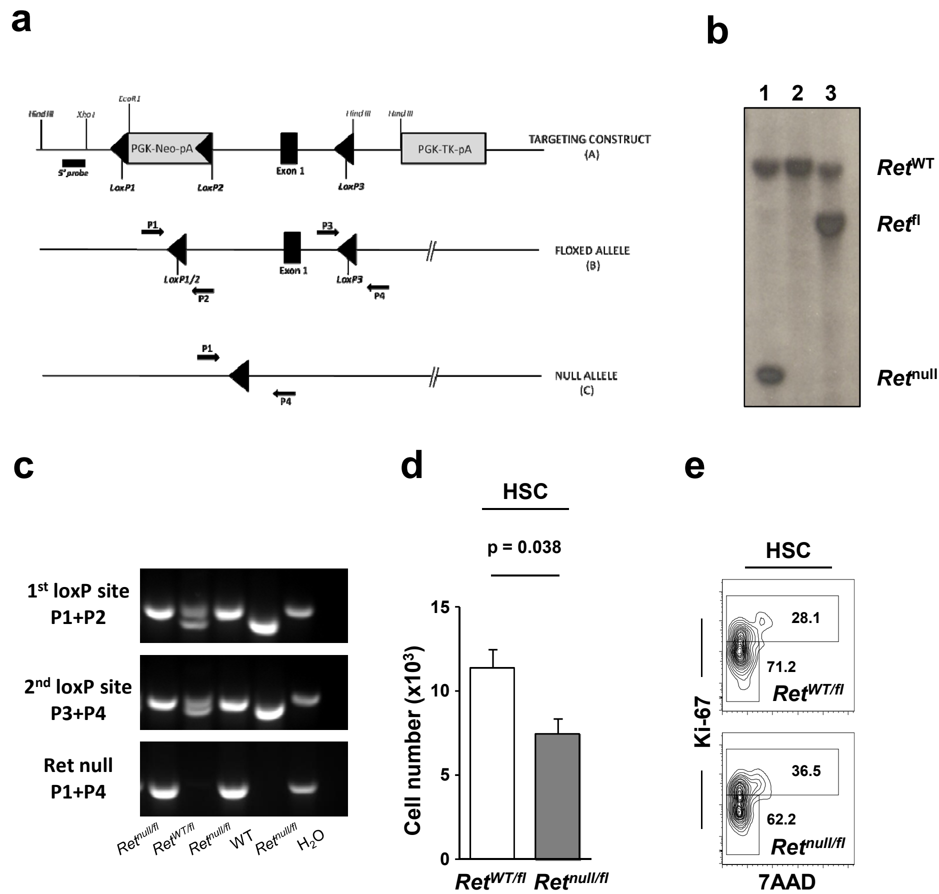

Figure S4. Generation of *Ret* conditional knockout mice and analysis of BM haematopoietic stem cells. a. (A) The floxed Neomycin cassette was inserted ~4.5 kb upstream of exon 1 of mouse Ret locus, a third loxP (LoxP3) was introduced downstream of exon 1 and ~ 5 kb downstream the PGK-TK-pA cassette was inserted to aid negative selection. Targeted events were identified by Southern analysis of either Hind III digests of genomic DNA using the 5' external probe. (B) The floxed allele was identified by PCR and the primers P1/P2 were used to identify the loxP that remained after excision of the Neomycin cassette (PGK-Neo-PA), while the loxP3 was identified using primers P3/P4. The primer sequences are in the methods section. (C) To screen for the null allele, primers P1 and P4 were used. **b.** Southern-blot using HindIII digest and the 5' external probe. **c.** Genotyping results from a litter of mice obtained from a $cRet131^{WT/null}$ *x* $cRet131^{fl/fl}$ breeding. In the loxP sites PCRs, upper band corresponds to the sequence with the loxP site and the lower band to the WT sequence. **d.** Numbers of BM HSCs. *Vav1*-iCre.$Ret^{WT/fl}$ ($Ret^{WT/fl}$) n=5; *Vav1*-iCre.$Ret^{null/fl}$ ($Ret^{null/fl}$) n=4. **e.** Ki-67 in HSCs. *Vav1*-iCre.$Ret^{WT/fl}$ =68.85 ± 1.43; *Vav1*-iCre.$Ret^{null/fl}$ =60.09 ± 1.59 (mean ± s.e.); *t*-test *P*=0.003. *Vav1*-iCre.$Ret^{nulll/fl}$ n=10; *Vav1*-iCre.$Ret^{null/fl}$ n=7. Two tailed *t*-test *P* value is indicated. Error bars show s.e..

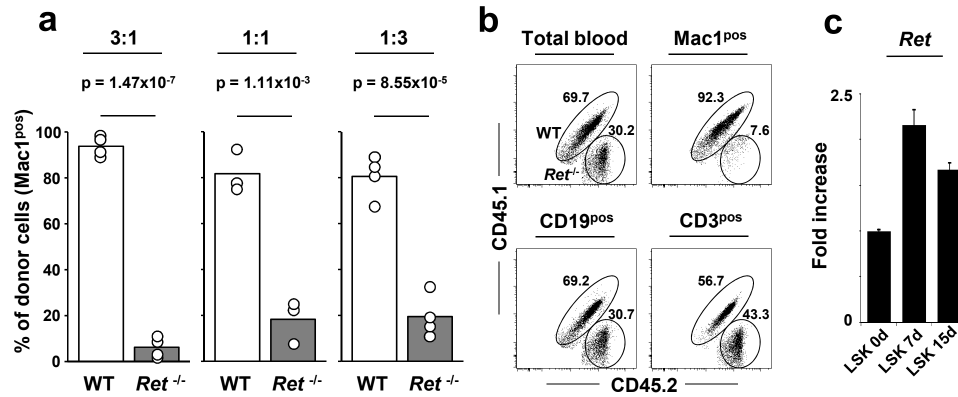

Figure S5. *Ret* deficient LSKs have reduced fitness at different transplantation ratios and *Ret* expression increases after LSK transplantation. a. Competitive transplantation assay was performed with $Lin^{neg}cKit^{pos}$ from E14.5 $Ret^{-/-}$ CD45.2 and WT CD45.1/CD45.2 in different proportions. Percentage of $Mac1^{pos}$ cells post-secondary transplantation. Left: co-injected $1.5x10^{5}$ WT CD45.1/CD45.2 and $0.5x10^{5}$ $Ret^{-/-}$ CD45.2 (3:1). Middle: co-injected $1x10^{5}$ WT CD45.1/CD45.2 and $1x10^{5}$ $Ret^{-/-}$ CD45.2 (1:1). Right: co-injected $0.5x10^{5}$ WT CD45.1/CD45.2 and $1.5x10^{5}$ $Ret^{-/-}$ CD45.2 (1:3). 3:1 n=4; 1:1 n=3; 1:3 n=4. **b.** Flow cytometry analysis of blood cells from 1:3 recipients at 8 weeks. **c.** E14.5 FL LSK cells were transplanted to lethally irradiated recipients. Transplanted LSK cells were purified at 7 and 15 days after transplant and analysed by quantitative RT-PCR. Two tailed *t*-test *P* values are indicated.

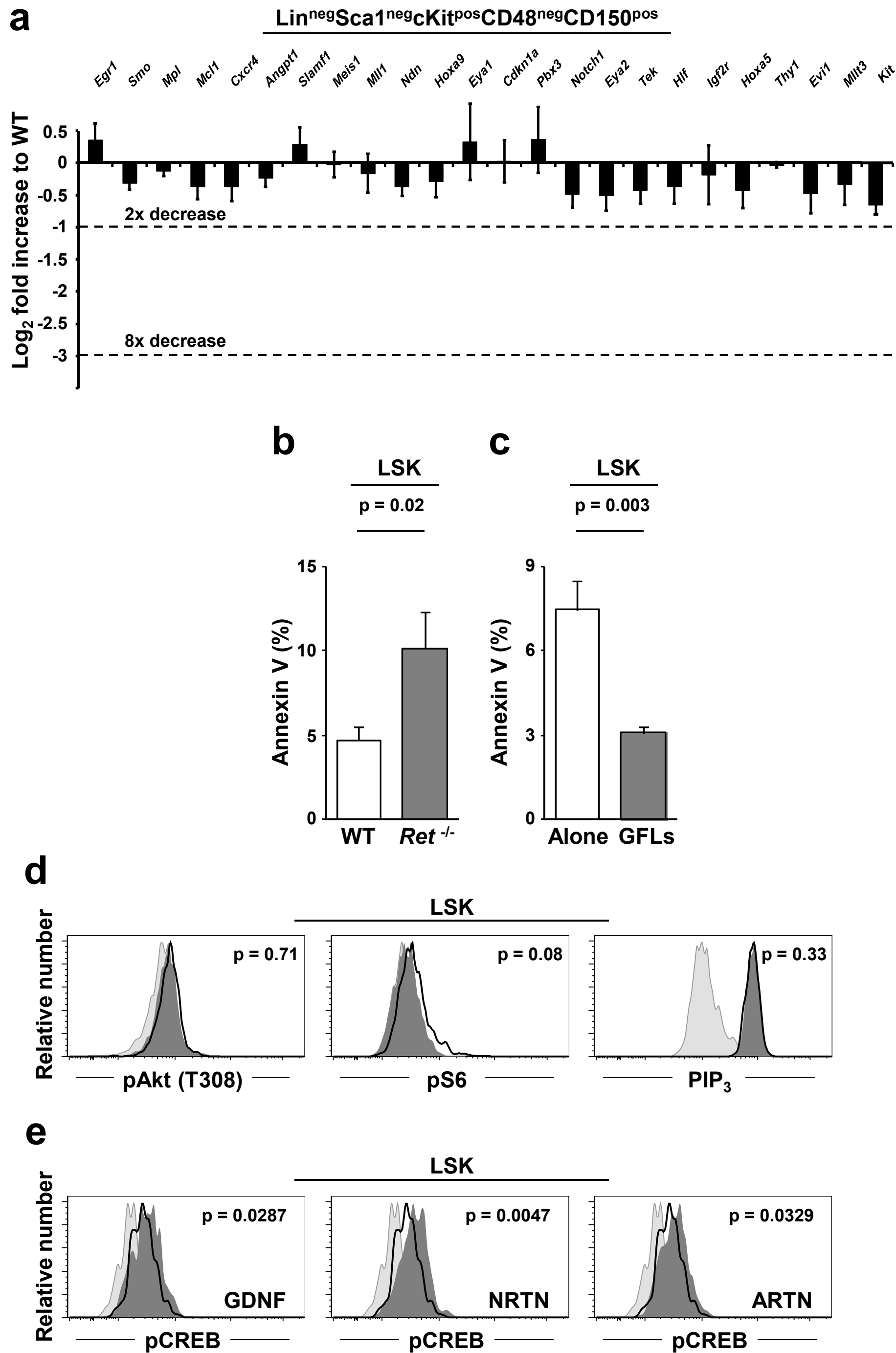

Figure S6. RET signalling results in increased survival and CREB phosphorylation. a. Quantitative RT-PCR for FL E14.5 *Ret*$^{-/-}$ and WT HSCs. n=3. **b.** Percentage of AnnexinV^{pos} LSK cells after culture. WT n=7, *Ret*$^{-/-}$ n=4. **c.** Percentage of AnnexinV^{pos} LSK cells after culture. Alone n=7, GFLs n=4. **d.** Flow cytometry analysis E14.5 *Ret*$^{-/-}$ or WT littermate control LSK cells. WT n=6; *Ret*$^{-/-}$ n=6. **e.** Flow cytometry analysis of LSK cells in absence or presence of GDNF, NRTN or ARTN for 1h. n = 6. Two tailed *t*-test *P* value is indicated. Error bars show s.e.. Light grey: isotype control.

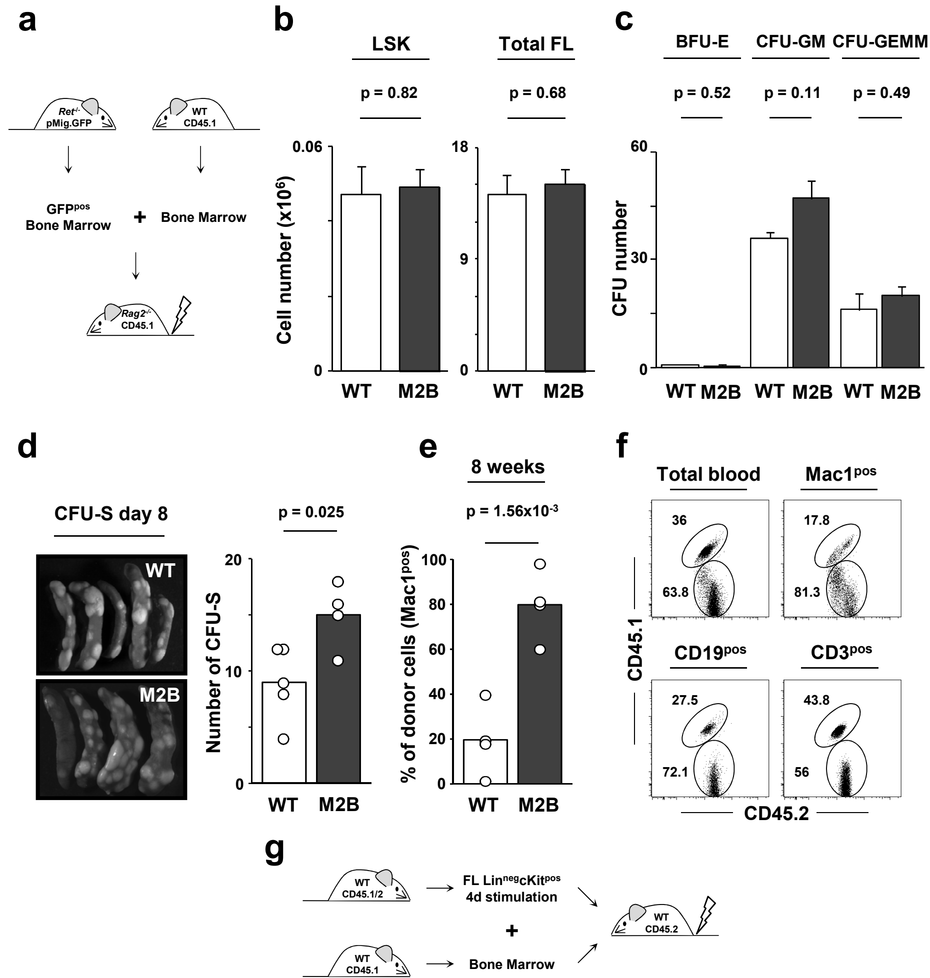

Figure S7. Analysis of *Ret*^{MEN2B} LSK differentiation and transplantation potential. a. Scheme of competitive transplantation rescue of *Ret* deficient progenitors. Relative to Fig.4b-d. **b.** Number of LSK and total FL cells. WT n=10; *Ret*^{MEN2B} n=15. **c.** Day 8 CFU colony numbers. WT n=3; *Ret*^{MEN2B} n=3. **d.** Day 8 CFU-s. WT n=5; *Ret*^{MEN2B} n=4. **e.** Competitive transplantation assay with *Ret*^{MEN2B} CD45.2 and WT CD45.1/2. Percentage of Mac1^{pos} cells post-secondary transplantation. n=4. **f.** Blood cell lineages from *Ret*^{MEN2B} CD45.2 and WT CD45.1/2 origin 8 weeks post-transplantation. **g.** Scheme of competitive transplantation with GFLs treatment. Relative to Fig.4e-g. Two tailed *t*-test *P* value is indicated. Error bars show s.e..

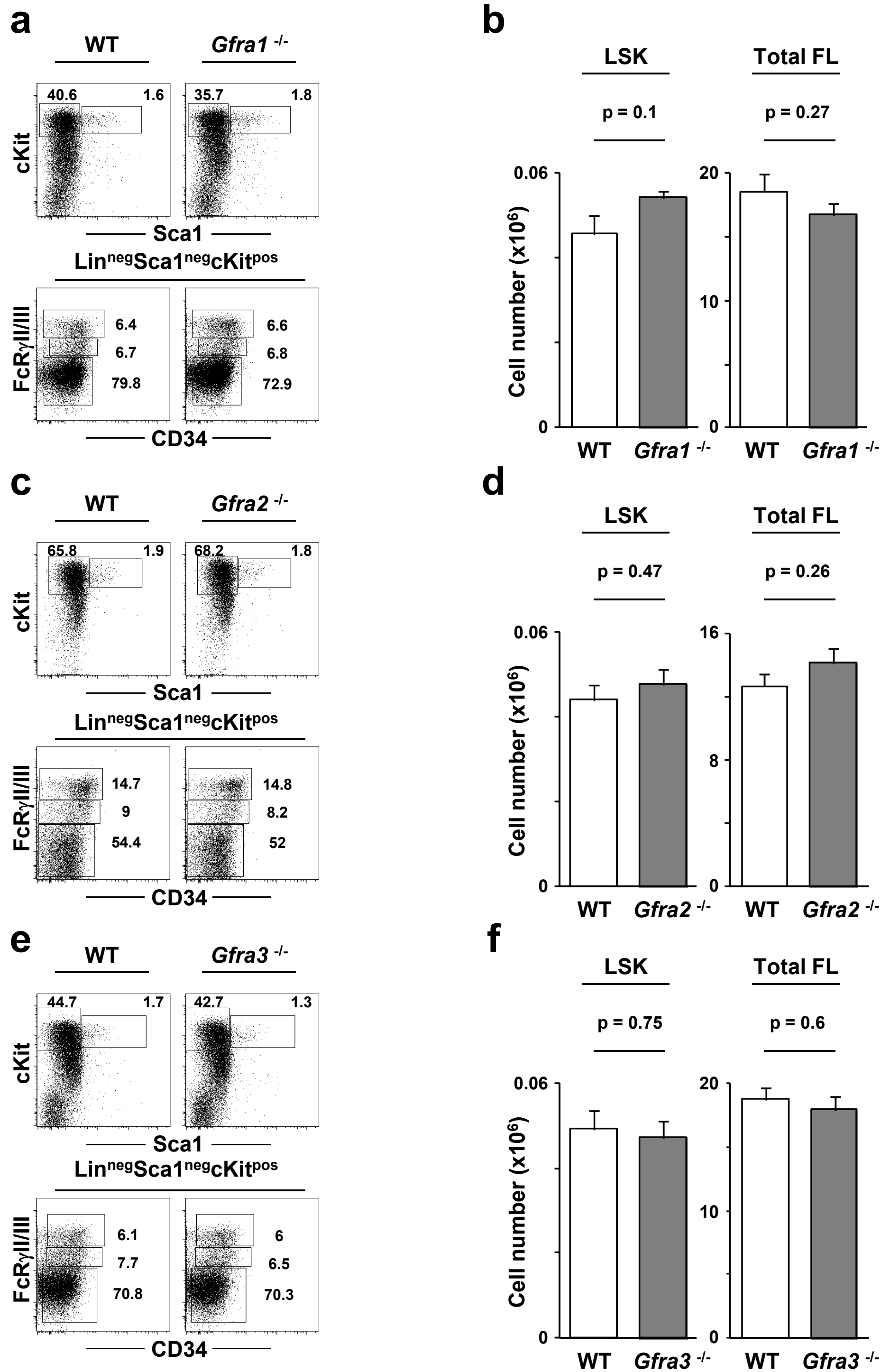

Figure S8. *Gfra* deficient embryos have normal LSK cell numbers. **a.** Flow cytometry analysis of E14.5 *Gfra1*$^{-/-}$ and WT littermate control LSKs (top) and MPs (bottom). **b.** Number of LSKs and total FL cells. WT n=9; *Gfra1*$^{-/-}$ n=10. **c.** Flow cytometry analysis of E14.5 *Gfra2*$^{-/-}$ and WT littermate control LSKs (top) and MPs (bottom). **d.** Number of LSKs and total FL cells. WT n=12; *Gfra2*$^{-/-}$ n=11. **e.** Flow cytometry analysis of E14.5 *Gfra3*$^{-/-}$ and WT littermate control LSKs (top) and MPs (bottom). **f.** Number of LSKs and total FL cells. WT n=11; *Gfra3*$^{-/-}$ n=20. Two tailed *t*-test *P* value is indicated. Error bars show s.e..

RET/GFRα SIGNALS ARE DISPENSABLE FOR THYMIC T CELL DEVELOPMENT *IN VIVO*

Afonso R. M. Almeida, Sílvia Arroz-Madeira, Diogo Fonseca-Pereira, Hélder Ribeiro, Reena Lasrado, Vassilis Pachnis and Henrique Veiga-Fernandes

Abstract

Identification of thymocyte regulators is a central issue in T cell biology. Interestingly, growing evidence indicates that common key molecules control neuronal and immune cell functions. The neurotrophic factor receptor RET mediates critical functions in foetal hematopoietic subsets, thus raising the possibility that RET-related molecules may also control T cell development. We show that *Ret*, *Gfra1* and *Gfra2* are abundantly expressed by foetal and adult immature DN thymocytes. Despite the developmentally regulated expression of these genes, analysis of foetal thymi from *Gfra1*, *Gfra2* or *Ret* deficient embryos revealed that these molecules are dispensable for foetal T cell development. Furthermore, analysis of RET gain of function and Ret conditional knockout mice showed that RET is also unnecessary for adult thymopoiesis. Finally, competitive thymic reconstitution assays indicated that *Ret* deficient thymocytes maintained their differentiation fitness even in stringent developmental conditions. Thus, our data demonstrate that RET/GFRα signals are dispensable for thymic T cell development in vivo, indicating that pharmacological targeting of RET signalling in tumours is not likely to result in T cell production failure.

Methods

Mice: C57Bl/6J (CD45.2, CD45.1 and CD45.1CD45.2), *Rag1*$^{-/-}$ (CD45.2 and CD45.1)[332], *hCd2*-iCre[333], *Gfra1*$^{-/-}$ [334], *Gfra2*$^{-/-}$ [335], and *Ret*$^{-/-}$ [253], all in C57Bl/6J background, were bred and maintained at the IMM animal facility. Generation of as *Ret* floxed (*Ret*^{fl}) and *Ret* null (*Ret*^{null}) mice was already described in this thesis. All animal procedures were performed in accordance to national and institutional guidelines.

Flow Cytometry: Embryonic thymi were micro-dissected and either homogenized in 70µM cell strainers or digested with Accutase medium (PAA Laboratories, Austria), 30' at 37º. Adult thymi were homogenized in 70µm cell strainers. Single cell suspensions were stained with the following antibodies from ebioscience, Biolegend, or BD: anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-CD25 (7D4), anti-CD45 (30-F11), anti-CD45.1 (A20), antiCD45.2 (104), anti-γδ TCR (GL3), anti-CD117 (2B8), anti-Sca1 (D7), Lineage (Lin) cocktail (anti-CD19 (eBio1D3), anti-CD11b (M1/70), anti-Gr.1 (RB6-8C5), anti-Ly79 (Ter119) and anti-NK1.1 (PK136)). Antibodies were coupled to FITC, PE, PerCP, PerCP-Cy5, PE-Cy7, APC, APC-Cy7, Pacific Blue, Brilliant Violet 421 and Horizon V500 fluorochromes or to biotin. Secondary incubation with fluorochrome binding streptavidin was performed when biotin coupled antibodies were used. Anti hRET was performed with antibody from R&D (132507) and respective anti-mouse IgG1 isotype control. Flow cytometry analysis was performed on a LSR Fortessa (BD) and data was analyzed with FlowJo 8.8.7 software (Tree Star). Cell-sorting was performed on a FACSAria I or FACSAria III (BD), and purity of obtained samples was >97%. CD45^{pos} and CD45^{neg} populations were sorted from the same samples.

Real-time PCR analysis: RNA was extracted from sorted cell suspensions using RNeasy Micro Kit (Qiagen). Real-time PCR for Gfra1 and Gfra2 were done as previously described[256,337]. *Hprt1* was used as housekeeping gene. For TaqMan assays (Applied Biosystems) RNA was retro-transcribed using High Capacity RNA-to-cDNA Kit (Applied Biosystems), followed by a pre-amplification PCR using TaqMan PreAmp Master Mix (Applied Biosystems). TaqMan Gene Expression Master Mix (Applied Biosystems) was used in real-time quantitative PCR. TaqMan Gene Expression Assays bought from Applied Biosystems were the following: *Gapdh* Mm99999915_g1; *Hprt1* Mm00446968_m1; *Nrtn* Mm03024002_m1; *Gdnf* Mm00599849_m1; *Ret* Mm00436304_m1.

Competitive reconstitution chimeras: Foetal livers from C57Bl/6 (CD45.1/CD45.2), *hCd2*-iCre.*Ret*$^{null/fl}$ (CD45.2, conditional knockouts) or *hCd2*-iCre^{-} .*Ret*$^{WT/fl}$ (CD45.2, controls) donors were made into single cell suspensions and enriched for precursors by staining with anti-CD117-APC followed by magnetic cell sorting with anti-APC microbeads (Miltenyi Biotec). Cells were then mixed in a 1:1 ratio (50% CD45.1/CD45.2 and 50% CD45.2) and injected intra venous into irradiated (9Gy) *Rag1*$^{-/-}$ (CD451) hosts (4x10^6 progenitor cells/host). Chimeras were analyzed 8 weeks after reconstitution.

Statistics: Statistical analysis was done using Prism. Variance was analyzed using F-test. Student's *t*-test was performed on homocedastic populations and student's *t*-test with Welch correction was applied on samples with different variances.

Results

T cell development occurs mainly in the thymus[227]. However, by the time T cell precursors reach this primary lymphoid organ, they are not fully committed, and only later receive the cues that engage them on a T cell fate[205,227,342]. Recently it came to evidence that nervous system and immune systems share several regulatory mechanisms and signalling pathways[216]. RET signalling axes are critical to the neuronal system and kidney, but recent evidence indicates that RET signals are also key to intestinal lymphoid organ development[256,268,295]. Interestingly, it was shown that RET is expressed by mature lymphocytes and GDNF promotes DN thymocytes survival *in vitro*; thus, raising the exciting possibility that RET signalling may control thymocyte development *in vivo*[325,331].

Initially we investigated the expression of *Ret* and its co-receptors in E15.5 thymocyte subsets by RT-PCR. Although most E15.5 thymocytes are at the DN stage[237], due to minute cell numbers available at this developmental stage we sorted DN1+DN2 (pooling $CD4^{neg}CD8^{neg}CD3^{neg}CD44^{pos}CD25^{neg}$ and $CD4^{neg}CD8^{neg}CD3^{neg}$ $CD44^{pos}CD25^{pos}$ cells) and DN3+DN4 thymocytes ($CD4^{neg}CD8^{neg}CD3^{neg}CD44^{neg}$ $CD25^{pos}$ and $CD4^{neg}CD8^{neg}CD3^{neg}CD44^{neg}CD25^{neg}$) by flow cytometry. We found that while *Ret*, *Gfra1* and *Gfra2* were expressed in the foetal thymus, *Gfra3* and *Gfra4* were absent (Fig. 1a). Sequentially, quantitative RT-PCR analysis confirmed expression of *Ret* and *Gfra1* in thymocytes at all DN developmental stages, a finding also confirmed at the protein level for RET (Fig. 1b-c). In contrast, *Gfra2* was present in DN1+DN2 but absent from later DN stages (Fig. 1b). We then evaluated the expression of the RET-ligands *Gdnf* and *Nrtn* in the thymic environment. We found that the main source of these transcripts were $CD45^{neg}$ cells (Fig. 1d), while hematopoietic ($CD45^{pos}$) DN thymocytes only expressed minute levels of *Gdnf* and *Nrtn* (Fig. 1d-e). Thus, we confirmed that the molecules required for active RET signalling are expressed in the embryonic thymus, suggesting a role for these neurotrophic factor signalling axes in the early stages of foetal thymocyte development.

In order to determine whether RET mediated signals are required for foetal thymocyte development, we analyzed E18.5 thymus from $Ret^{-/-}$, $Gfra1^{-/-}$ or $Gfra2^{-/-}$ animals, thus including in our analysis DN thymocytes and emergent immCD8, DP and γδ TCR thymocytes[253,334,335].

Since expression of *Ret*, *Gfra1* and *Gfra2* is higher in early DN thymocytes (DN1 and DN2) (Fig. 1b), we initially evaluated these differentiation stages in *Ret*, *Gfra1* or

Gfra2 deficient embryos. We found that both the percentage and cell number of DN1-4 subsets were similar between *Ret*, *Gfra1* or *Gfra2* deficient embryos and their respective WT littermate controls (Fig.2a; Supplementary Fig. 1). Similarly, we found that total DN and ImmCD8 were equally represented in mutant embryos and their WT controls (Fig. 2b; Supplementary Fig. 1).

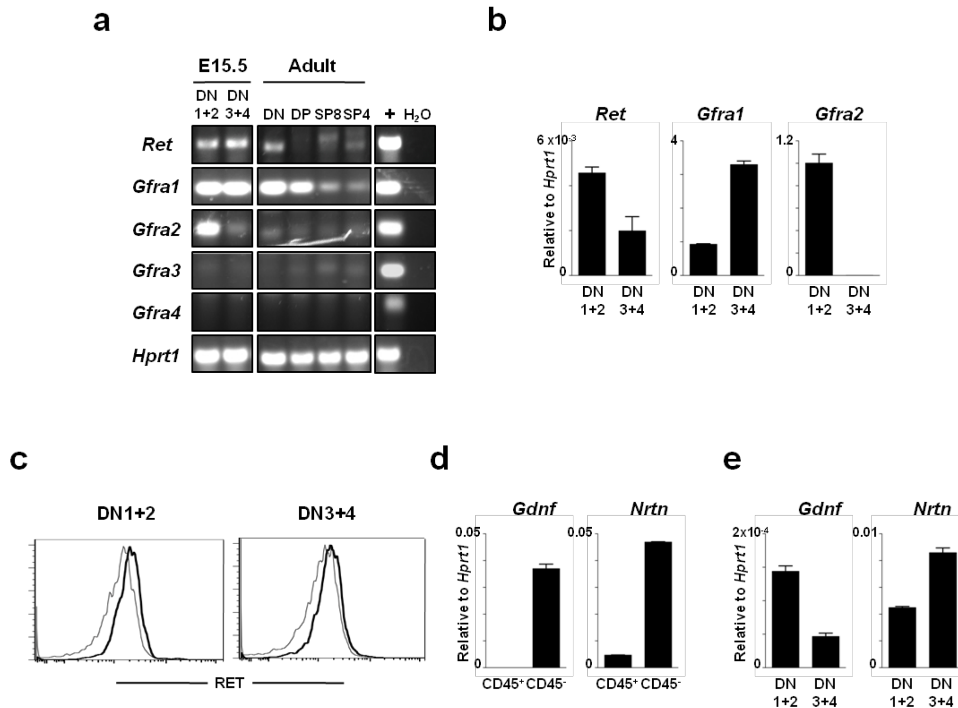

Figure 1. RET signalling molecules are express in embryonic thymus. a. E15.5 DN1-2 and DN3-4 and adult (8 weeks old) DN, DP, single-positive CD8 (SP8) and single-positive CD4 (SP4) thymocytes were analysed by RT-PCR. **b.** E15.5 DN1-2 and DN3-4 thymocytes were analysed by quantitative RT-PCR. **c.** RET expression in DN1-2 and DN3-4 thymocytes was determined by flow cytometry. RET: black bold line; Isotype control: grey line. **d.** Thymic E15.5 $CD45^{pos}$ or $CD45^{neg}$ cells were analysed by quantitative RT-PCR. **e.** DN1-2 and DN3-4 thymocytes were analysed by quantitative RT-PCR. Results from three independent measurements are represented. Error bars show s.e..

Sequentially, we analyzed later stages of the αβ TCR lineage development. Absolute numbers of DP thymocytes from $Ret^{-/-}$, $Gfra1^{-/-}$ or $Gfra2^{-/-}$ embryos were identical to WT littermate controls (Fig. 2b; Supplementary Fig. 1). Similarly, the fraction and absolute numbers of γδ TCR thymocytes, which are the majority of $CD3^{+}$ cells at E18.5, were unperturbed in *Ret*, *Gfra1* or *Gfra2* deficient animals (Fig. 2c; Supplementary Fig. 1)[237]. Consequently, absolute numbers of total thymocytes from *Ret*, *Gfra1* or *Gfra2* deficient embryos were similar to their WT littermate controls (Fig. 2d). Thus, we conclude that signals mediated by RET or by its co-receptors GFRα1 or GFRα2 are not required for foetal thymocyte development *in vivo*.

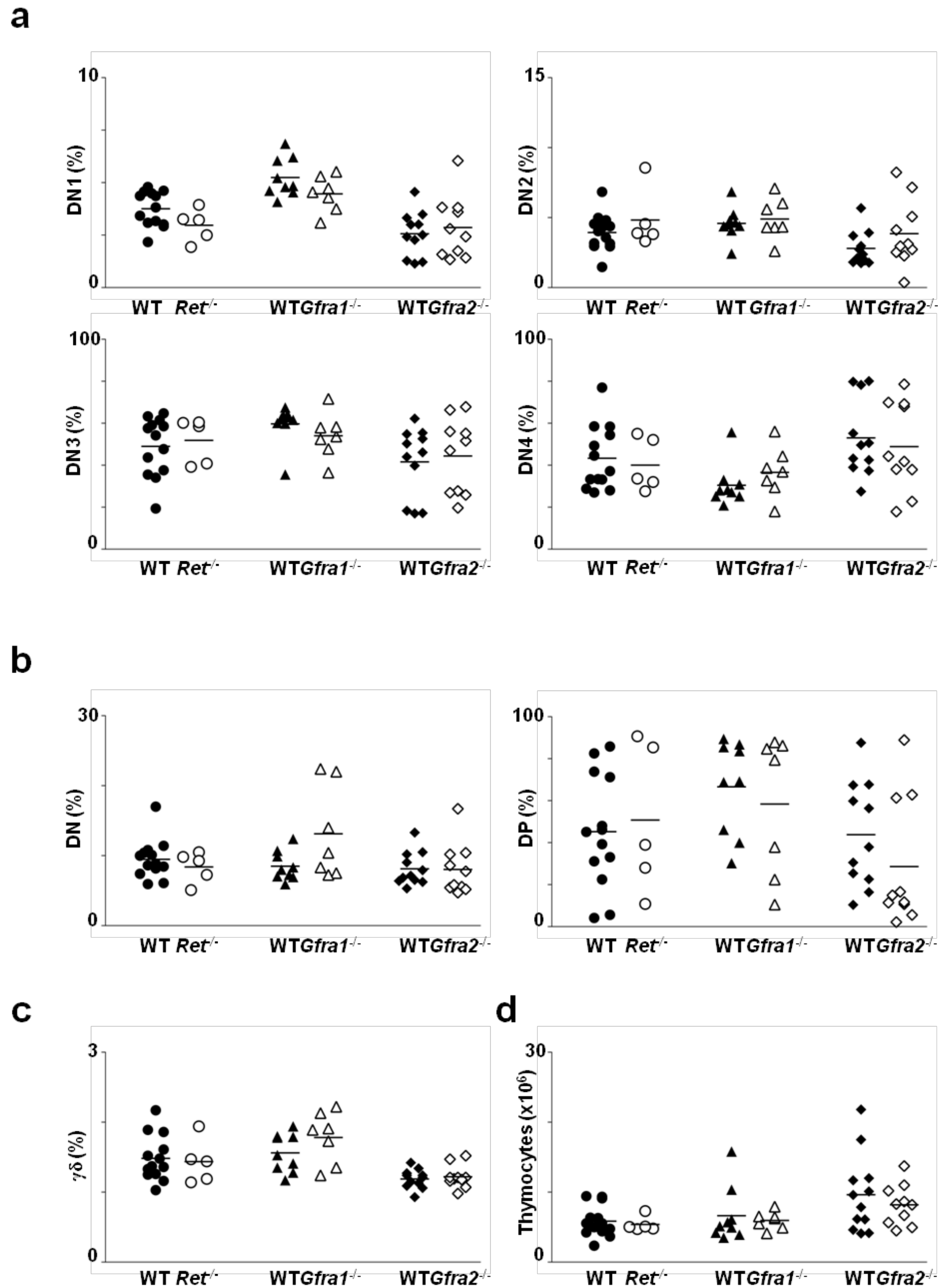

Figure 2. RET or co-receptor signals are not required in embryonic thymic development. E18.5 thymocytes were analyzed by flow cytometry. Null mice: open symbols; WT littermate controls: full symbols; Mean value: dash line. **a.** Percentages of DN thymocyte populations gated on $CD45^{pos}Lin^{neg}CD3^{neg}CD4^{neg}CD8^{neg}$ in E18.5 *Ret*$^{-/-}$, *Gfra1*$^{-/-}$ or *Gfra2*$^{-/-}$ thymocytes and respective WT littermate controls. n>5. **b.** Percentage of DN and DP thymocytes gated on $CD45^{pos}Lin^{neg}\gamma\delta TCR^{neg}$ in E18.5 *Ret*$^{-/-}$, *Gfra1*$^{-/-}$ or *Gfra2*$^{-/-}$ thymocytes and respective WT littermate controls. n>5. **c.** Percentage of γδ TCR expressing thymocytes in E18.5 *Ret*$^{-/-}$, *Gfra1*$^{-/-}$ or *Gfra2*$^{-/-}$ thymocytes and respective WT littermate controls. n>5. **d.** Total number of thymocytes in E18.5 *Ret*$^{-/-}$, *Gfra1*$^{-/-}$ or *Gfra2*$^{-/-}$ thymocytes and respective WT littermate controls. n>5. Two-tailed student's *t*-test analysis was performed between knockouts and respective WT littermate controls. No statistically significant differences were found.

The thymic environment supports T cell development in embryonic and adult life. Nevertheless, T cell development in the fetus and adult thymus employs differential pathways, leading to different viability, proliferation and lineage commitment[237]. Thus, we investigated whether *Ret* related genes maintain their expression through adult thymopoiesis.

DN (CD4^{neg}CD8^{neg}CD3^{neg}), DP, single-positive CD4^{pos} T cells (SPCD4) and single positive CD8^{pos} T cells (SPCD8) were FACS sorted and analyzed by quantitative RT-PCR analysis. RT-PCR analysis revealed that similarly to the foetal thymus only *Ret* and its co-receptors *Gfra1* and *Gfra2* were expressed in the adult thymus (Fig. 1a). Quantitative RT-PCR confirmed that *Ret*, *Gfra1* and *Gfra2* expression was mainly expressed by DN thymocytes, although low levels of *Gfra1* and *Gfra2* expression were also expressed by DP thymocytes, a finding also confirmed at the protein level for RET (Fig. 3a-b). Sequentially, we evaluated the expression of the RET-ligands *Gdnf* and *Nrtn* in the adult thymus. While *Gdnf* expression was mostly found on CD45^{neg} cells, *Nrtn* was expressed both by CD45^{neg} and CD45^{pos} DN and DP thymocytes (Fig. 3c).

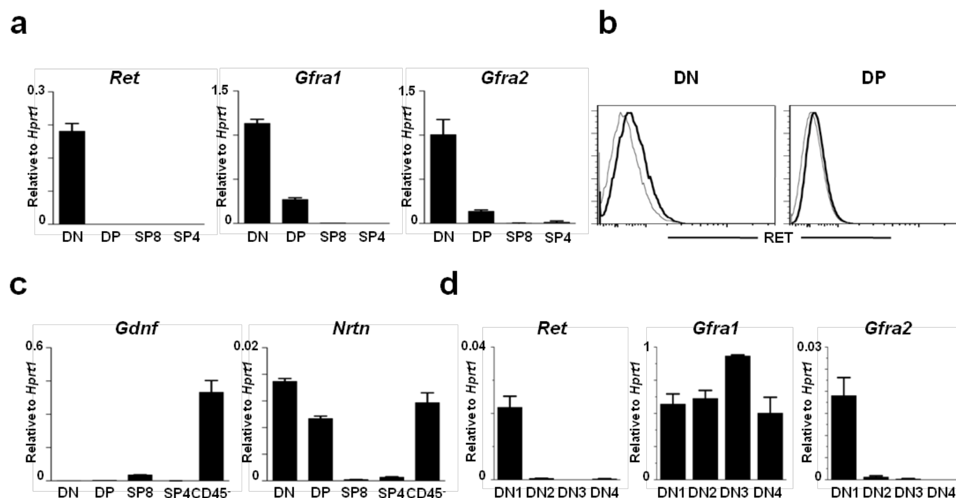

Figure 3. *Ret* and co-receptors are expressed in adult thymocytes. **a.** DN, DP, SPCD8^{pos} and SPCD4^{po} thymocytes were analysed by quantitative RT-PCR. **b.** RET expression in DN and DP thymocytes was determined by flow cytometry. RET: black bold line; Isotype control: grey line. **c.** DN, DP, SPCD8^{pos} and SPCD4^{pos} thymocytes and thymic CD45^{neg} cells were analysed by quantitative RT-PCR. **d.** DN1, DN2, DN3 and DN4 thymocytes were analysed by quantitative RT-PCR. Error bars show s.e.. Results from three independent measurements are represented.

Dissection of DN cells into DN1-DN4 subsets further revealed that DN1 thymocytes were the only DN subset that co-expressed appreciable levels of *Ret*, *Gfra1* and *Gfra2*, while all other DN subsets expressed *Gfra1* but only minute levels of *Ret* (Fig. 3d). Thus, we conclude that the expression of RET signalling partners in adult thymocytes

mirrors to large extend the expression patterns of foetal thymocytes, ie, *Ret*, *Gfra1* and *Gfra2* are most abundant in the earliest stages of T cell development, while *Gdnf* and *Nrtn* are mainly produced by non-hematopoietic thymic cells.

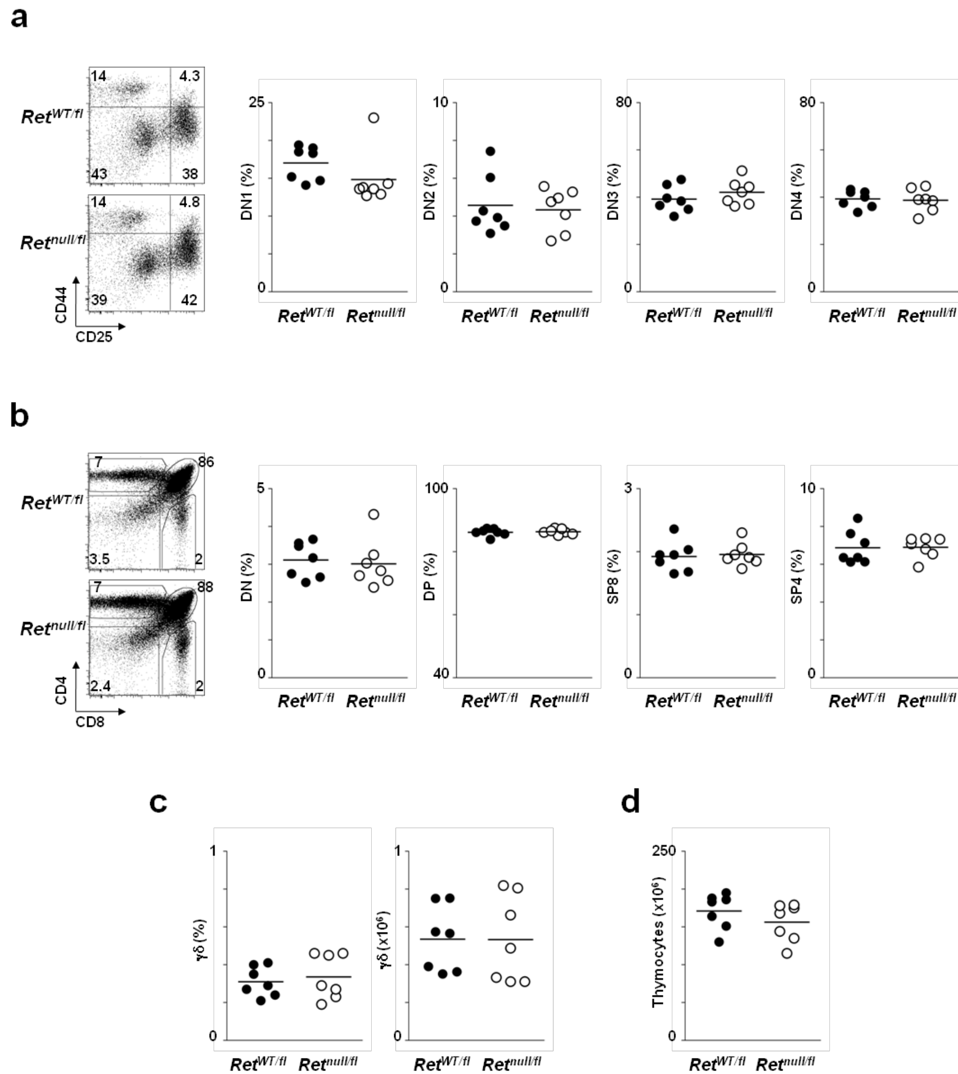

Figure 4. RET-mediated signals are dispensable in normal T cell development. 8 week old *Ret* conditional knockout *hCd2*-iCre.$Ret^{null/fl}$ ($Ret^{null/fl}$) and control *hCd2*-iCre^{-}.$Ret^{WT/fl}$ ($Ret^{WT/fl}$) mice were analyzed by flow cytometry. Percentages are indicated. Mean value: dash line. **a.** Left: Flow cytometry analysis of $CD4^{neg}CD8^{neg}CD3^{neg}$ thymocytes. Right: Percentage of DN1-DN4 in $Ret^{null/fl}$ and control $Ret^{WT/fl}$ mice. $Ret^{WT/fl}$ n=7; $Ret^{null/fl}$ n=7. **b.** Left: Flow cytometry analysis of CD4 versus CD8 expression profile. Right: Percentage of DN, DP, SP4 and SP4 in $Ret^{null/fl}$ and control mice. $Ret^{WT/fl}$ n=7; $Ret^{null/fl}$ n=7. **c.** Proportion and total numbers of γδ TCR expressing thymocytes in $Ret^{null/fl}$ and control mice. $Ret^{WT/fl}$ n=7; $Ret^{null/fl}$ n=7. **d.** Total thymocyte numbers. $Ret^{WT/fl}$ n=7; $Ret^{null/fl}$ n=7. Two-tailed student's *t*-test analysis was performed between mutant and control mice. No statistically significant differences were found.

$Ret^{-/-}$ animals die perinatally due to kidney failure, hindering analysis of adult T cell development[253]. Thus, in order to determine the role of RET signalling in adult

thymopoiesis, we developed a *Ret* conditional knockout model (*Ret*$^{fl/fl}$) that allows a lineage targeted strategy for *Ret* ablation. These mice were bred to human *hCd2*-iCre animals that ensure Cre activity from DN1 stage onwards (Supplementary Fig. 2)[333]. Analysis of the offspring of this breeding at 8 weeks of age showed that despite a marginal reduction in DN1 thymocyte numbers in *hCd2*-iCre.*Ret*$^{null/fl}$ animals, the subsequent DN stages were similarly represented in *hCd2*-iCre.*Ret*$^{null/fl}$ and *hCd2*-iCre^{-}.*Ret*$^{WT/fl}$ mice (Fig. 4a; Supplementary Fig. 3). Analysis of DN to SP αβ T cell development showed similar fractions and absolute numbers of thymocytes within each subset (DN, DP, SP4 and SP8) in both *hCd2*-iCre.*Ret*$^{null/fl}$ and *hCd2*-iCre^{-}.*Ret*$^{WT/fl}$ mice (Fig. 4b; Supplementary Fig. 3). Finally, the fraction and absolute numbers of thymic γδ TCR expressing T cells and total thymocyte numbers were not affected by *Ret* deletion (Fig. 4c-d). Thus, altogether our data indicate that RET-mediated signals are dispensable for foetal and adult thymic T cell development *in vivo*.

Over expression and expression of gain-of-function forms of RET are characteristic of proliferative cell phenotypes and oncogenic diseases[269]. Thus, in order to elucidate whether improved RET signals could affect thymopoiesis, we used a genetic model that drives a constitutively activated form of RET in *Ret* expressing cells (*Ret*^{MEN2B})[320]. These mice harbour a single point mutation (Met919Thr) introduced into the endogenous *Ret* gene locus, thus resulting in improved ligand-dependent RET activation[320].

Analysis of *Ret*$^{MEN2B/MEN2B}$ and their WT littermate controls at 8 weeks of age revealed that DN (DN1-DN4) to SP mature αβ T cell development had similar fractions and absolute numbers (Fig. 5a-c; Supplementary Fig. 4). Consequently, total thymocyte numbers were not affected by the *Ret*^{MEN2B} gain-of-function mutation (Fig. 5d), demonstrating that this *Ret* gain-of-function mutation does not affect thymopoiesis.

Ret signalling has been implicated in the survival of a variety of peripheral and central neural cells, including motor, sensory and autonomic neurons[268]. Furthermore, it was shown that the RET ligand GDNF could promote thymocyte survival in foetal thymus organ culture (FTOC) systems[331]. Thus, it is plausible that subtle effects of RET signalling on thymocyte development might be masked in *Ret* conditional knockouts by compensatory mechanisms at later stages of thymocyte differentiation.

In order to specifically address that hypothesis, we performed sensitive competitive thymic repopulation assays to assess whether T cell progenitors lacking RET are able to compete with RET competent T cell progenitors. Bone marrow cells from *hCd2*-

iCre.*Ret*$^{null/fl}$ or *hCd2*-iCre^{-}.*Ret*$^{WT/fl}$ (CD45.2) were transplanted into lethally irradiated *Rag1*$^{-/-}$ (CD45.1) recipients with C57Bl6 (CD45.1/2) competitor bone marrow cells in a 1:1 ratio (Fig. 6a). 8 weeks after transplantation *Ret*-deficient (*hCd2*-iCre.*Ret*$^{null/fl}$) and *Ret*-competent (*hCd2*-iCre^{-}.*Ret*$^{WT/fl}$) thymocytes had similar fitness when compared to the third part competitor thymocytes (C57Bl6 (CD45.1/2) across all analyzed stages of T cell development (DN1 to mature SP) (Fig. 6b-c). Thus, we conclude that RET signalling is dispensable for thymocyte competitive fitness and thymic reconstitution.

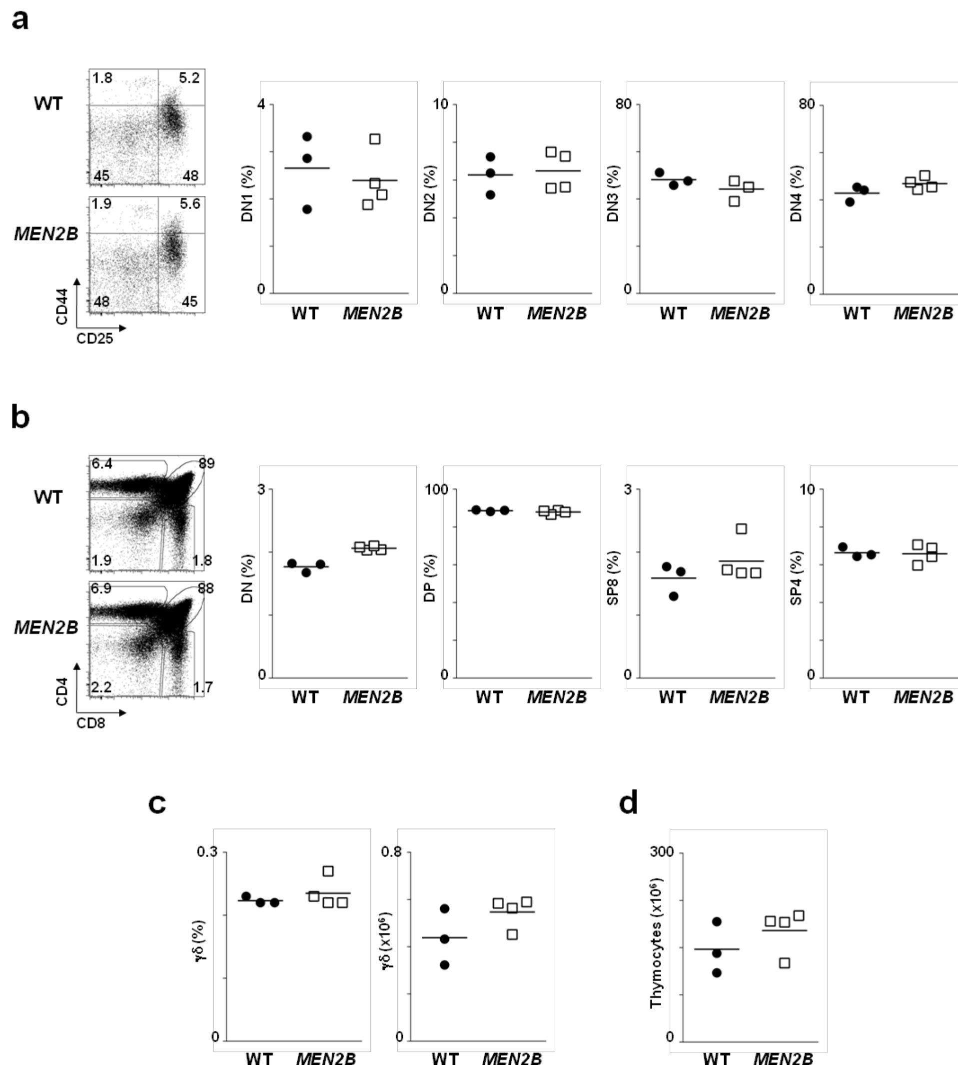

Figure 5. *Ret* gain-of-function mutations do not affect thymopoiesis. 8 week old *Ret*$^{MEN2B/MEN2B}$ (*MEN2B*) and their WT littermate controls were analyzed by flow cytometry. Percentages are indicated. Mean value: dash line. **a.** Left: Flow cytometry analysis of CD4^{neg}CD8^{neg}CD3^{neg} thymocytes. Right: Percentage of DN1-DN4 in *MEN2B* and WT control mice. WT n=3; *MEN2B* n=4. **b.** Left: Flow cytometry analysis of CD4 versus CD8 expression profile. Right: Percentage of DN, DP, SP4 and SP4 in in *MEN2B* and WT control mice. WT n=3; *MEN2B* n=4. **c.** Proportion and total numbers of γδ TCR expressing thymocytes in *MEN2B* and WT controls. WT n=3; *MEN2B* n=4. **d.** Total thymocyte numbers. WT n=3; *MEN2B* n=4. Two-tailed student's *t*-test analysis was performed between knockouts and WT controls. No statistically significant differences were found.

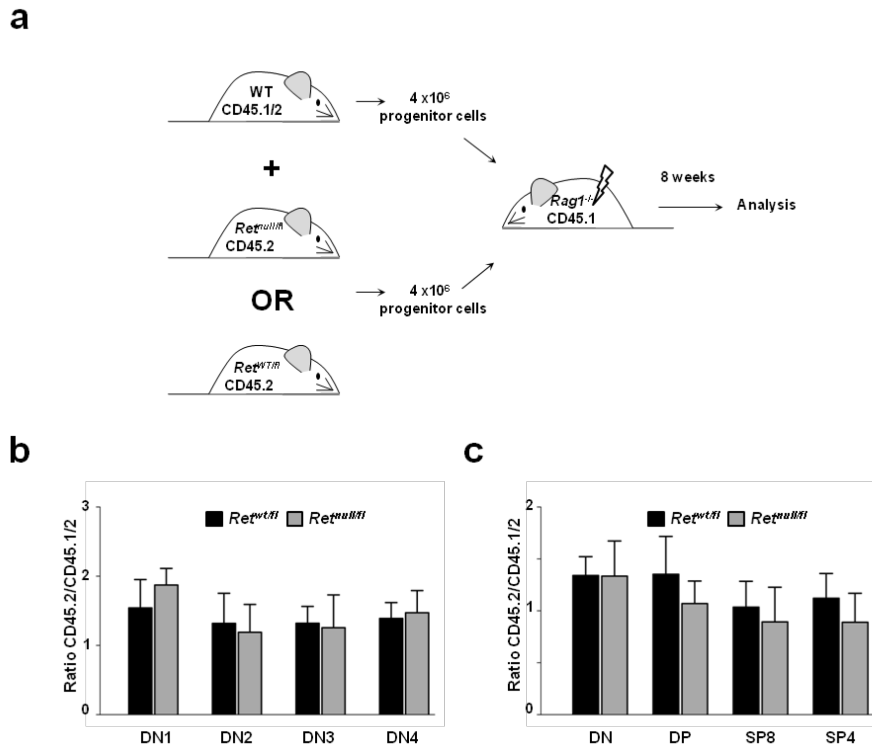

Figure 6. RET signalling is dispensable for thymic reconstitution and thymocyte fitness. a. Sheme of competitive transplantation. *hCd2*-iCre.*Ret*$^{null/fl}$ or *hCd2*-iCre^{-}.*Ret*$^{WT/fl}$ control progenitors (CD45.2) were transplanted together with competitor precursors (CD45.1/2) into lethally irradiated *Rag1*$^{-/-}$ (CD45.1). **b. c.** Ratio between *hCd2*-iCre.*Ret*$^{null/fl}$ (*Ret*$^{null/fl}$ – grey bars) or *hCd2*-iCre^{-}.*Ret*$^{WT/fl}$ (*Ret*$^{WT/fl}$ – black bars) and the third part WT competitor through thymic T cell development, 8 weeks after transplantation. *Ret*$^{WT/fl}$ n=4; *Ret*$^{null/fl}$ n=4. Error bars show s.d. Two-tailed student *t*-tests were performed. No significant differences were found.

Discussion

Our data indicate that the neuroregulatory genes *Ret*, *Gfra1* and *Gfra2* are expressed in discrete DN thymocytes, while Gfra3 and Gfra4 transcripts were absent in thymocytes. Interestingly, the RET ligands *Gdnf* and *Nrtn* are predominantly produced by non-hematopoietic thymic cells. These gene expression patterns raised the exciting possibility that RET signalling axes could control T cell development, adding to the growing body of evidence that the nervous and immune systems share similar key molecular signals[256,295,323-325,331]. In line with this hypothesis, it was previously shown that GDNF could promote survival and maturation of thymocytes *in vitro*[331].

In order to test whether RET signalling axes control T cell development, we analyzed the thymus of genetically mutant embryos for *Ret*, *Gfra1* or Gfra2 at E18.5[253,334,335]. Despite expression of Ret in foetal thymocytes, *Ret* deficient embryos showed normal DN, immCD8 DP and γδ T cell development. These findings were also consistent with normal T cell development in null mice for the RET co-receptors that provide specificity to the neurotrophic factors GDNF and NRTN, respectively *Gfra1*$^{-/-}$ and *Gfra2*$^{-/-}$ mice. Thus, we conclude that RET and its signalling partners GFRα1 and GFRα2, are dispensable for foetal T cell development.

Since T cell development in adulthood employs additional molecular mechanisms to foetal thymopoiesis, we investigated whether *Ret* related genes controlled adult T cell development. Our data indicate co-expression of *Ret*, *Gfra1* and *Gfra2* in the early DN1 stage, and production of *Gdnf* and *Nrtn* in the adult thymic microenvironment. Both foetal and adult immature thymocytes co-express *Ret*, *Gfra1* and *Gfra2*. These data are in line with a previous report indicating expression of these genes in the foetal thymus by *in situ* hybridization[343]. However, DP thymocytes expressed the RET co-receptors *Gfra1* and *Gfra2*, despite absence of *Ret* expression, and similarly, adult DN2-4 expressed *Gfra1* but lacked significant *Ret* expression. This observation is in line with previous reports showing that GFRαs are more abundantly expressed than RET, which suggests that GFRαs may modulate RET signalling in a non-cell-autonomous manner (signalling *in trans*)[291,344-346]. Accordingly, we have recently shown that Lymphoid Tissue initiator cells use unconventional RET signalling in which receptor activation is provided by soluble ligand and co-receptors *in trans* secreted from nearby cells[295]. Thus, it is possible that RET negative, GFRα positive thymocytes may modulate the activity of neighbouring non-hematopoietic RET expressing cells.

Analysis of adult conditional *Ret* mutant mice ($Ret^{fl/fl}$) bred to *hCd2*-Cre mice revealed a significant, but small impact on the absolute numbers of DN1 thymocytes. However, this reduction was not translated into consecutive developmental stages, indicating that RET mediated signals are dispensable to T cell production *in vivo*. The co-expression of *Ret/Gfra1* in DN1 and the decreased number of DN1 cells in $Ret^{fl/fl}$ mice are consistent with a previous report indicating that GDNF promotes thymocyte survival in foetal thymic organ cultures (FTOCs)[331]. Nevertheless, our data does not support a major role for this signalling axis in vivo, since *Ret* mutant thymocytes develop normally and positive modulation of RET-signalling (Ret^{MEN2B}) was not beneficial for T cell development. Thus, the contribution of RET signalling to T cell development in vivo appears to be insignificant. Moreover, while FTOCs reproduce several aspects of T cell development[199], they fail to mimic the exact events in T cell development[15,347], and therefore these different methodologies may also contribute to the observed discrepancies.

GDNF/GFRα1 have been shown to activate the transmembrane receptor RET and the neural cell adhesion molecule (NCAM) in neurons[278,298]. Thus, although activation of a putative NCAM analogue by GDNF cannot be fully discarded in thymocytes, this is unlikely to have a significant physiological relevance since NCAM downstream signalling requires GFRα1 and $Gfra1^{-/-}$ embryos displayed normal thymopoiesis[298].

In order to overcome possible viability/proliferative compensatory mechanisms that may arise through T cell development, we performed sensitive competitive reconstitution assays in vivo with *Ret* deficient (*hCd2*-iCre.$Ret^{null/fl}$) and *Ret* competent (*hCd2*-iCre^{-}.$Ret^{WT/fl}$) thymocytes. Our data demonstrate that even in a very sensitive competitive setting the fitness of *Ret* deficient T cell precursors is intact.

Finally, our findings indicate that pharmacological inhibition of the RET pathway in severe pathologies, such as medullary thyroid cancer, should not be confronted with undesirable T cell production failure[348,349].

In summary, our data demonstrate that RET signalling is dispensable to foetal and adult T cell development in vivo. Nevertheless, RET and its signalling partners are also expressed by mature T cells[325], thus, lineage targeted strategies will be critical to elucidate the contribution of RET signals to T cell function.

Supplementary Figures

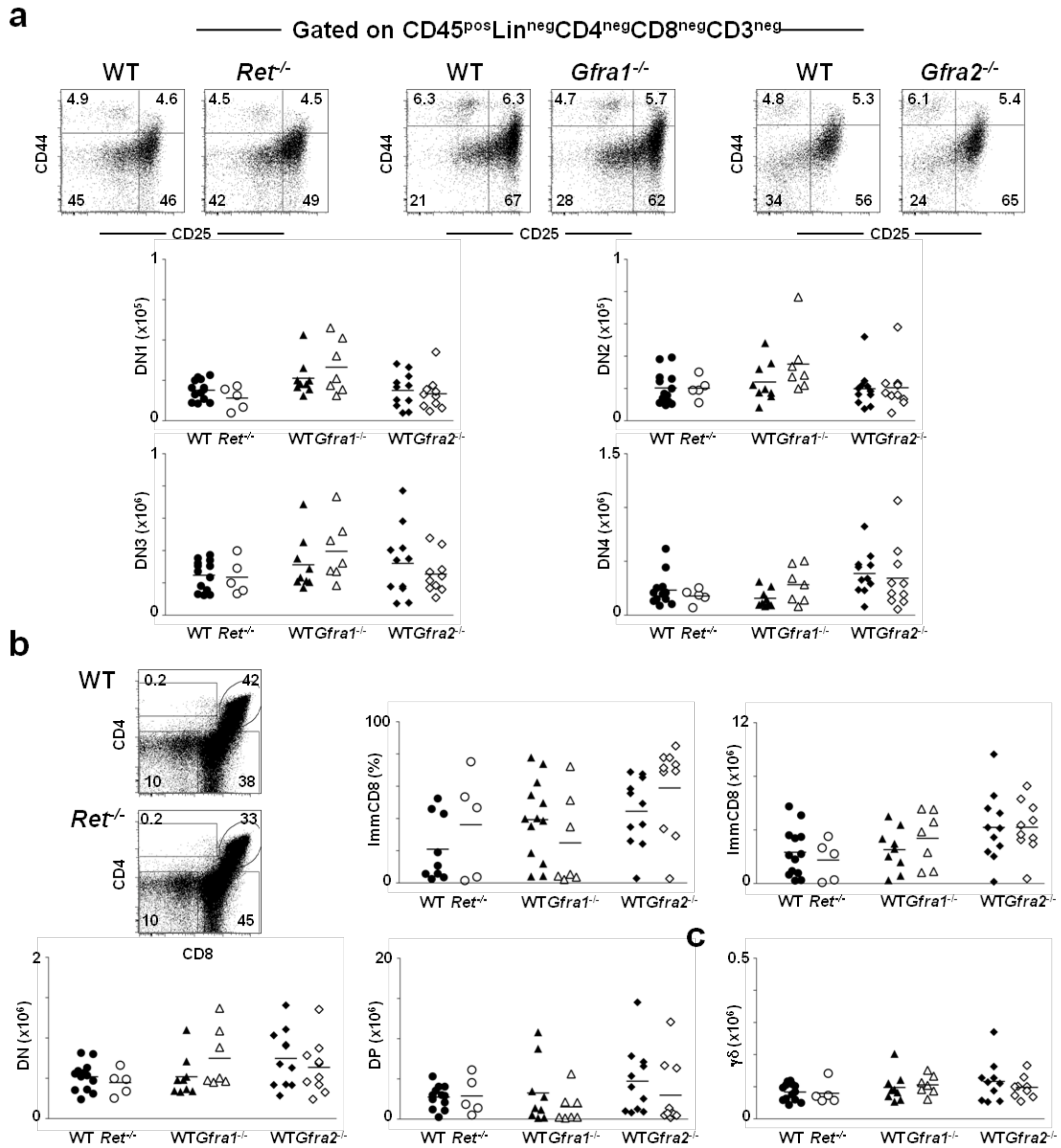

Figure S1. *Ret* and co-receptor deficiency does not affect embryonic thymic development. E18.5 thymocytes were analyzed by flow cytometry. Null mice: open symbols; WT littermate controls: full symbols; Mean value: dash line. **a.** Top: CD44 and CD25 expression profiles within the CD45^{pos}Lin^{neg}CD3^{neg}DN compartment for *Ret*$^{-/-}$, *Gfra1*$^{-/-}$, *Gfra2*$^{-/-}$ and respective WT littermate controls. Bottom: Numbers of DN1-DN4 in *Ret*, *Gfra1*and *Gfra2* deficient mice. n>5. **b.** Flow cytometry analysis of CD4 and CD8 expression profiles within the CD45^{pos}Lin^{neg}γδTCR^{neg} compartment from *Ret*$^{-/-}$and WT littermate controls. Note that within SPCD4 and SPCD8 gates >90% of cells were CD3^{neg} and are thus immature thymocytes. Percentage and number of immature CD8^{pos} thymocytes and total number of DN and DP thymocytes in *Ret*, *Gfra1*and *Gfra2* deficient mice. n>5. **c.** total number of γδ TCR^{pos} thymocytes in *Ret*, *Gfra1*and *Gfra2* deficient mice. Two-tailed student's *t*-test analysis was performed between knockouts and respective WT littermate controls. No statistically significant differences were found.

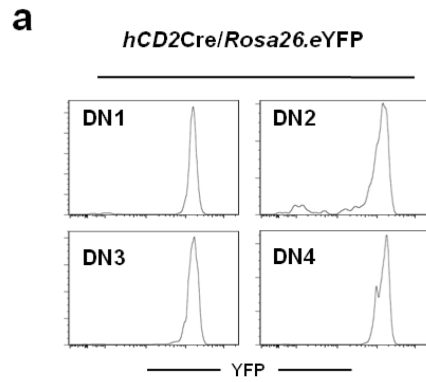

Figure S2. *hCd2*-iCre is active in thymocytes from DN1 developmental stage. In order to evaluate the activity of Cre recombinase driven by *hCd2*, we bred *hCd2*-iCre-expressing animals to *Rosa26eYFP* animals. Histograms show flow cytometry analysis of eYFP expression in DN1 to DN4 thymocytes.

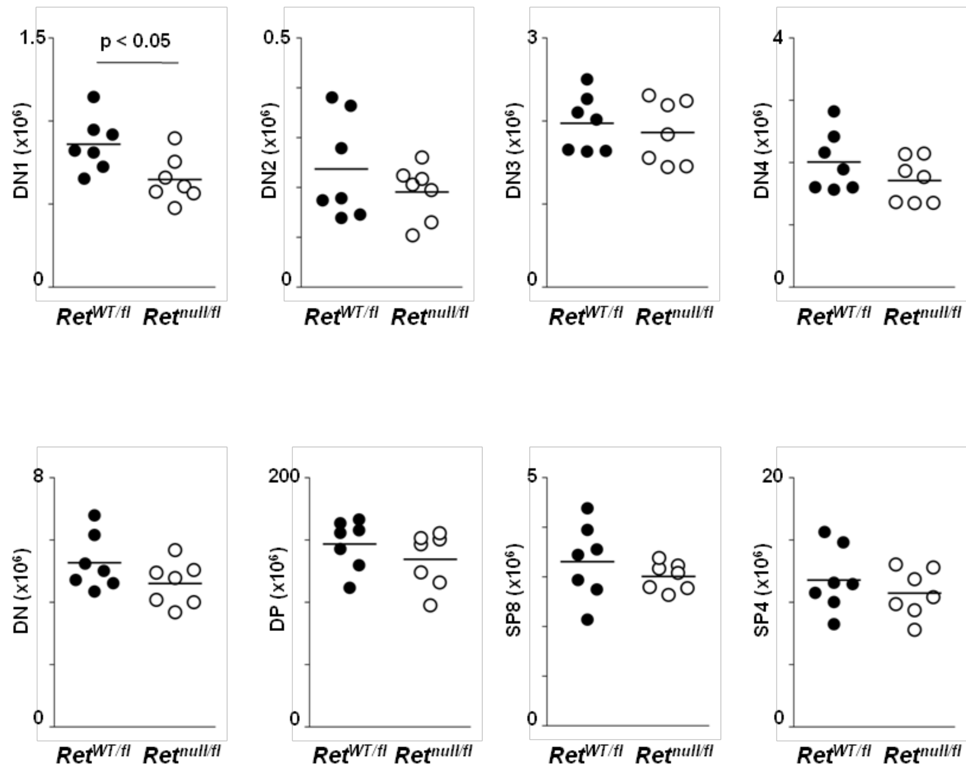

Figure S3. Conditional ablation of *Ret* does not impact thymocyte numbers. 8 week old *Ret* conditional knockout *hCd2*-iCre.*Ret*$^{null/fl}$ (*Ret*$^{null/fl}$) and control *hCd2*-iCre^{-}.*Ret*$^{WT/fl}$ (*Ret*$^{WT/fl}$) mice were analyzed by flow cytometry. Mean value: dash line. Top: numbers of DN1-DN4 subpopulations. Bottom: Number of total DN, DP, SP8, SP4 populations. *Ret*$^{WT/fl}$ n=7; *Ret*$^{null/fl}$ n=7. Two-tailed student's *t*-test analysis was performed between knockouts and respective WT littermate controls.

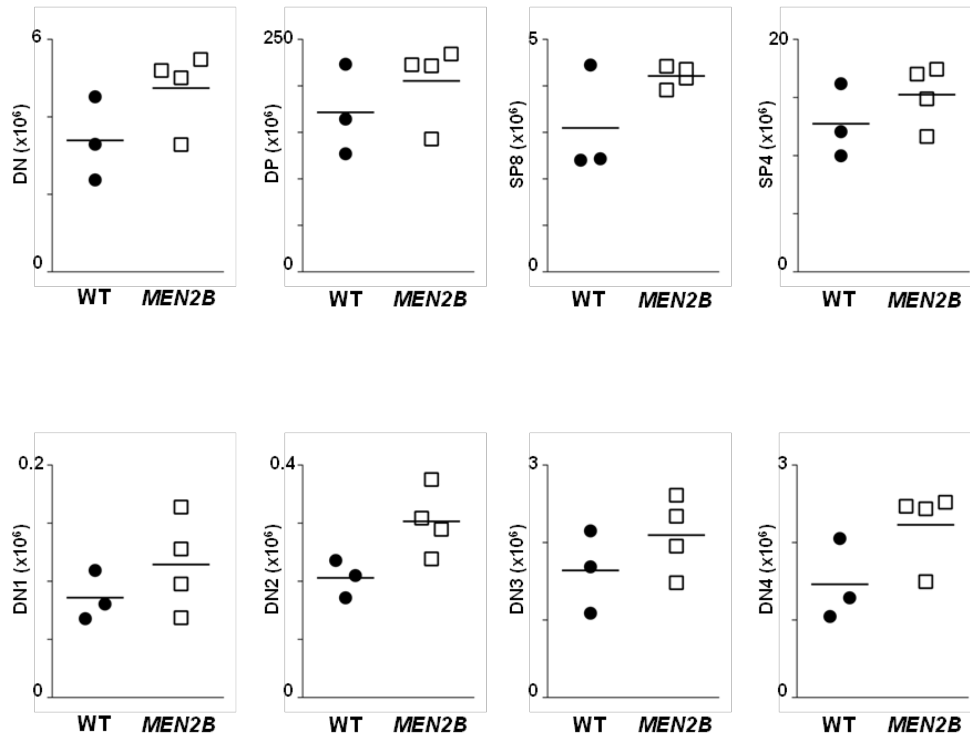

Figure S4. *Ret*^{MEN2B} gain-of-function mutation does not affect adult thymic development. 8 week old *Ret*$^{MEN2B/MEN2B}$ (*MEN2B*) and their WT littermate controls were analyzed by flow cytometry. Mean value: dash line. Top: numbers of DN1-DN4 subpopulations. Bottom: Number of total DN, DP, SP8, SP4 populations. WT n=7; *MEN2B* n=7. Two-tailed student's *t*-test analysis was performed between knockouts and respective WT littermate controls. No statistically significant differences were found.

GENERAL DISCUSSION

Reciprocal interactions between particular cell types and their neighbour cells are the driving force of tissue formation in ontogeny. Haematopoietic cells interact with non-haematopoietic stroma cells forming particular microenvironments, or niches. Haematopoietic stem cell niches provide the specific cues that regulate HSC function. These can be direct cell to cell interactions using membrane-bound receptors or soluble signalling molecules such as cytokines and growth factors. Tyrosine kinase receptors encompass receptors for soluble molecules as well as membrane-bound ligands. In these thesis we analysed the role of RET tyrosine kinase receptor in haematopoietic cell development. Our data revealed that RET is crucial for haematopoietic stem cell (HSC) survival, but unnecessary to T cell development.

Initially we confirmed that *Ret* and it co-receptors are expressed in haematopoietic cells in primary lymphoid organs and RET ligands are produced by stromal cells[321-326]. Surprisingly, we found that compared with more committed progenitors, HSCs express higher levels of RET signalling molecules and importantly, cells in the HSC microenvironment, such as osteoblasts and mesenchymal stem cells in the bone marrow niches, produce RET ligands. The production of soluble GFLs by neighbour cells in HSCs microenvironments is critical for their possible function in HSC biology as GFLs were described to bind to heparan sulphates in the extracellular matrix, thus preventing their diffusion and increasing local concentration[350].

In agreement, ablation of *Ret* resulted in reduced foetal and adult HSC numbers. *Ret*$^{-/-}$ HSCs were inefficient in reconstituting the haematopoietic system, which reveals the importance of RET signalling in HSC function. As *Ret* deficiency results in several problems in the nervous system and kidney development, systemic embryonic defects could contribute to the differences between *Ret*$^{-/-}$ and conditional *Ret* deficient embryos[253]. However, our data does not support this hypothesis. First, analysis of *Gfra1*$^{-/-}$ deficient animals, which phenocopy the defects of *Ret*$^{-/-}$ embryos in kidney and nervous system development and also die perinatally, show normal haematopoiesis, which indicates that intrinsic haematopoietic defects are present in *Ret* deficient animals[334]. Second, adoptive transfer the same number of RET deficient cells and WT competitor cells showed that *Ret*$^{-/-}$ haematopoietic progenitors have impaired haematopoietic activity. Whereas these could be explained in the primary transfers by deficiencies in the microenvironment in which HSCs where generated, secondary transplantation clearly shows cell autonomous requirement of RET by HSCs, since WT

and *Ret*$^{-/-}$ cells where maintained in the same animals. Third, conditional deletion of *Ret* in the haematopoietic system in *Vav1*-iCre^{pos}.*Ret*$^{null/fl}$ mice, which maintain a normal RET proficient animal; also present a significant reduction in HSCs.

Our data further suggest that RET signalling is particularly important in situations of proliferative responses, like foetal development and transplantation, as *Ret* is upregulated in haematopoietic progenitors after transplantation. In accordance, although *Ret* deficient animals in adulthood live with no noticeable problems, their stress response after chemical aggression is defective.

RET signalling has distinct outcomes depending on the cell types in which it operates. Nevertheless, it is reasonable to claim that providing migratory/chemotaxis and survival cues are two of its main functions[255-257,295]. Contrary to what was observed in lymphoid tissue initiator cells (LTin), where GFLs act by changing the motility behaviour of LTin cells, inducing adhesion-dependent motility arrest, we could not detect any effect of RET in the migration and homing of HSCs to lymphoid organs[295]. Strikingly our results show that RET provides anti-apoptotic signals to HSCs by cAMP response element-binding protein (CREB) dependent transcription of anti-apoptotic B-cell CLL/lymphoma 2 (BCL2) family members, BCL2 and BCLxL, and that restoration of these downstream surviving signals is required and sufficient to recover HSC fitness in the absence of RET. In fact, CREB dependent BCL2 expression was shown to be crucial for NGF-dependent survival of sympathetic neurons and for HSC maintenance downstream CaM kinase IV[185,351]. Moreover, overexpression of BCL2 increases the number, quiescence and repopulation capacity of haematopoietic progenitors[183].

Several canonical signalling pathways, such as mitogen-activated protein (MAP) kinases p42/p44 (ERK1/2), p38 MAP kinase and PI3K/Akt pathways, which were shown to act downstream of RET could be responsible for CREB phosphorylation and consequent *Bcl2* and *Bcl2l1* expression. In neuronal cell lines, CREB phosphorylation upon RET activation was shown to critically depend on ERK1/2 signalling pathway; however CREB activation downstream of several tyrosine receptor kinases depend both of ERK1/2 and p38 signalling axes[307,352,353]. Indeed our results indicate that in HSCs activation of p38 MAP kinase by RET signalling is critical for CREB phosphorylation and anti-apoptotic gene expression. Together these results show that haematopoietic and neuronal cells may use similar molecular mechanisms to obtain survival and maintenance signals, although the internal wiring of these signals may differ. Moreover, these data suggest a possible co-regulation in shared microenvironments, such as the bone marrow niches.

In our work we also observed that *Ret*$^{-/-}$ progenitors have a significant altered cell cycle status measured by Ki-67 expression. Compared with WT controls, *Ret* deficient animals have a higher proportion of cells in cycle (Ki-67^{pos}), and consequentially reduced number of quiescent cells, which was previously shown to result in HSC exhaustion and loss of haematopoietic activity[338]. Nevertheless, both in LSK cells and HSCs, the genetic signature of *Ret*$^{-/-}$ haematopoietic progenitors was not altered. Expression of genes involved in HSC expansion and proliferation such as HOX proteins and their co-regulators, *Meis1* and *Pbx3*, as well as relevant molecules for HSC quiescence and self-renewal, like *Evi1*, *Tek* and its ligand *Angpt1*, was similar between WT and *Ret*$^{-/-}$ progenitors[135,136,149,152,153,157]. Similarly, expression of phenotypic markers of HSCs, *Slamf1* and *Thy1*, and more important of the cell cycle regulator p21 was not affected by the lack of RET[70,72,144,354]. Thus we postulate that rather than a direct effect of RET in cell cycle regulation, poor HSC survival followed by recruitment of quiescent cells into cycle cause the observed reduction in quiescent HSCs and the loss of repopulation capacity due to exhaustion of the HSC pool.

RET kinase receptor emerges as a new regulator of HSC survival and may be used to modulate HSC fitness. Our work suggest that RET is critical in situations of proliferative stress such as foetal haematopoiesis, chemical aggression and transplantation. Accordingly we found that *in vitro* pre-treatment of HSC with RET ligands increase their transplantation efficiency. Interestingly, we found that *Ret*$^{-/-}$ progenitors have poor CFU-s generation and fail to rapidly reconstitute the haematopoietic system of irradiated recipients. The CFU-s is a heterogeneous population of myeloid and multipotent progenitors also responsible for rapidly transient reconstitution that allow the irradiated host to survive initial haematopoietic aplasia and anaemia, before the durable engraftment provided by HSCs[72,81,82]. Thus the deficiencies observed in early reconstitution assays suggest that either defects present in *Ret*$^{-/-}$ HSCs permanently affect the subsequent more committed cells or, at least in transplantation settings, RET signals at the HSC level may empower more committed progenitors with improved surviving capacities.

Remarkably, we found that human haematopoietic progenitors also express RET. Although further studies are needed to dissect the role of RET in human HSCs, very preliminary data from our lab suggest that human haematopoietic stem cells survive better *in vitro* in the presence of GFLs. If modulation of RET signalling also favours *in vivo* human HSCs responses will be subject to future studies. Nevertheless, our work

unveils the exciting possibility of using GFLs in HSC expansion protocols to improve bone marrow and cord blood stem cell fitness.

GDNF was previously shown to promote survival and maturation of thymocytes *in vitro*. Thereafter, we investigate the role of RET signalling in thymocyte development *in vivo*, first by confirming the expression of the neuroregulatory genes *Ret*, *Gfra1* and *Gfra2* in thymocytes[331,343]. Interestingly RET ligands *Gdnf* and *Nrtn* are predominantly produced by non-hematopoietic thymic cells, which rose the interestingly possibility that RET signalling axes could control T cell development. However *Ret* deficient embryos showed normal thymocyte development, and consistently, null embryos for the RET co-receptors that provide specificity to the neurotrophic factors GDNF and NRTN, respectively *Gfra1*$^{-/-}$ and *Gfra2*$^{-/-}$, have normal T cell development. Thus, we conclude that RET and its signalling partners GFRα1 and GFRα2 are dispensable for foetal T cell development.

Sequentially we evaluate the impact of *Ret* ablation in adult thymopoiesis. We found *Gdnf* and *Nrtn* expression in the adult thymic microenvironment and expression of *Ret* and *Gfra* co-receptors in DN1 thymocytes. Interestingly, it is possible that more committed DN and DP thymocytes, which express *Gfra* co-receptors but lack *Ret* expression, control RET^{pos} thymocytes by providing soluble GFRαs. Similarly our laboratory showed that LTin cell in the embryo use RET signalling *in trans* where GFRα co-receptors are provided by neighbour cells[295]. Nevertheless analysis of adult conditional *Ret* mutant mice (*Ret*$^{fl/fl}$) bred to *hCd2*-iCre mice revealed a small reduction on the absolute numbers of DN1 thymocytes. Although *Ret* may act directly on early DN1 thymocytes, it is also possible that prior to thymus seeding, deletion of *Ret* in bone marrow progenitors by early expression of *hCd2*-iCre may contribute to the observed reduction in DN1 thymocytes. However, regardless of its origin, this reduction in DN1 thymocytes is rapidly compensated and is not translated into consecutive developmental stages, thus indicating that RET mediated signals are also dispensable for adult T cell production *in vivo*. Accordingly, positive modulation of RET-signalling (*Ret*^{MEN2B}) was not beneficial for T cell development. By transplanting *Ret*$^{-/-}$ and WT progenitors into irradiated hosts, we found that even in very sensitive competitive reconstitution assays, which ride out possible non-cell autonomous compensatory mechanisms in *Ret* deficient animals, the fitness of *Ret* deficient T cell precursors is intact and similar to WT controls. Thus, while the co-expression of *Ret*/*Gfra1* in DN1 and the decreased number of DN1 cells in *Ret*$^{fl/fl}$ mice are consistent with the previous reported survival effect of GDNF in FTOCs, it is likely that during T cell development *in*

vivo, other molecules compensate for the absence of RET[331]. Finally, it is possible that pathological or stressed conditions may resemble the *in vitro* serum free FTOCs used in the previous study, and that contrary to homeostasis, stressed thymopoiesis may actively profit from GFLs signals.

GDNF signalling independent of RET was also shown, particularly by activation of the neural cell adhesion molecule (NCAM) in neurons[298]. A similar effect in thymocytes could explain the survival effect reported in GDNF treated *in vitro* cultures. Nevertheless, GFL signalling still need its cognate co-receptor. However, we found no significant differences in GFRα1 and GFRα2 deficient embryos, thus RET independent GFL signalling is unlikely to have an important role in thymopoiesis.

RET and its signalling partners were shown to be expressed in several haematopoietic cells, particularly by mature T cells; thereafter it may impact T cell function while being dispensable for T cell development[325]. Thus, it would be interesting to evaluate the importance of RET signals in specific T cell responses.

The kinase receptor RET has a myriad of functions that include the provision of migratory and survival signals to several neuronal subpopulations, regulation of spermatogenesis, renal development and enteric lymphoid organogenesis. Herein we have shown that while RET is critical to HSC response to physiological demand, it is dispensable for proper T cell development *in vivo*. Interestingly, previous studies in animal models and human patients showed that sustained or deregulated RET activation may cause several non haematopoietic cancers, particularly thyroid carcinomas. Importantly, *Ret* expression has been also detected in haematopoietic cancers, and RET anti-apoptotic downstream targets BCL2 and BCLxL have oncogene potential. However our results showed that transient RET signalling significantly improves HSC transplantation but does not affect haematopoietic cell differentiation. Thus the possible application of GFLs treatment to improve HSC transplantation should not be damped by the fear of establishment of haematological malignancies.

Pharmacological inhibition of the RET signalling pathway has been considered in severe malignancies, such as medullary thyroid cancer[348,349]. Although some haematopoietic perturbations may occur from RET signalling inhibition, we observe that adult HSCs in conditional *Ret* deficient animals seem to be less dependent of RET than active proliferating foetal or stressed HSCs. Consequently, inhibition of RET signalling is expected to affect mainly RET-dependent cancers, which normally have increased RET activity due to mutations in the *Ret* gene, such as in patient with MEN 2

syndromes. Importantly, as *Ret* deficiency has no impact in thymopoiesis, inhibition of RET signalling is not likely to compromise the development of T cells, crucial to maintain protective immunity.

The work presented in this thesis illustrates how molecular mechanisms historically associated with specific tissues are used by cells with different origins. We showed that haematopoietic stem cells receive neurotrophic survival signals through the RET receptor. Signals from nervous cells were already shown to regulate haematopoiesis by different mechanisms. Adrenergic neurotransmitters regulate HSC niche components, such as osteoblasts and mesenchymal stem cells, indirectly influencing HSC responses like proliferation and BM egress[212,217-219]. Nervous fibres were also seen in association with MSCs and HSCs and can controls HSCs directly, as both human and mice HSCs express adrenergic receptors[221,222]. Moreover, glial cells ensheathing non-myelinatined autonomic nerves in BM control HSC maintenance and quiescence by regulating activation of latent TGFβ[220]. Still, neurotrophic factors are not neuronal signals but crucial growth factors to maintain several nervous populations. The finding that HSCs also respond to GFLs suggests that HSCs and neuronal cells use common regulatory pathways. The importance of this shared mechanism in HSCs microenvironments is still not understood and should be the focus of futures studies. Nevertheless we should consider the hypothesis that RET signalling components may be part of an active crosstalk mechanism between these two systems. As several players in HSCs niche produce GFLs one may conceive that in these microenvironments neurotrophic signals are used to maintain both haematopoietic progenitors and nervous cells. Moreover we can imagine the possibility that HSCs directly impact neuronal functions by consumption of neurotrophic factors. HSCs might regulate neuronal cells by keeping a determine amount of available GFLs. Furthermore, the amount of available GFLs in the HSC niches might be read by nervous cells as an indicator of the haematopoietic status, since with our results we would expect that active proliferating cells, in which RET signalling plays a crucial role, cause a reduction in GFLs; whereas cell aplasia would increase GFL availability.

Interestingly, the proposed crosstalk probably have a complex subtle architecture as we found that HSCs respond to multiple RET ligands, whereas different nervous populations where shown to depend on specific GFL-GFRα-RET axes, thus expanding the possibilities of HSC-nervous cell-microenvironment interactions.

In addition to non-haematopoietic stroma cells in the HSC niche, increasing evidence shows involvement of mature haematopoietic cells in HSC regulation.

Recently, T cells were shown to be required for parathyroid hormone derived HSC expansion[355]. Importantly, while we found no requirement for RET signals in T cell differentiation; mature T cells also express RET signalling molecules. Therefore, an interaction between T cells and HSCs using RET mediated signals cannot be excluded and should be taken in consideration when analysing the impact of RET in mature T cell populations.

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