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Identifikace a charakterizace nových mechanismů regulujících bazální a pohotovostní granulopoezu

Identification and characterisation of novel mechanisms regulating steady state and emergency granulopoiesis

Doctoral thesis

Supervisor: Meritxell Alberich-Jorda, Ph.D.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne 26.11.2020

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Petr Daněk

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ABSTRACT

Neutrophils are essential cells of the immune system. They engage in pathogen clearance, inflammatory response, and wound healing. Proper production and activation of neutrophils is critical for the health of an individual, since several disorders are related to neutrophilic alterations. In this thesis, we explore three previously uncharacterized mechanisms that might be involved in the regulation of neutrophilic differentiation. First, we addressed the role of the canonical Wnt signaling pathway. This signaling is executed by interaction of β-catenin with TCF/LEF transcription factors. We employed a murine model that specifically inactivates β-catenin-TCF/LEF-mediated transcription by expressing a dominant negative form of TCF4 (dnTCF4). Using this model in combination with several *in vitro* and *in vivo* assays we demonstrated that β-catenin-TCF/LEF signaling directly upregulates expression of G-CSF receptor in hematopoietic progenitors, imposing myeloid commitment and favoring neutrophilic differentiation. This appeared to be especially important during the response to systemic infection, termed emergency granulopoiesis, as dnTCF4-expressing mice showed high susceptibility to *Candida albicans* infection. Remarkably, the critical role of β-catenin-TCF/LEF signaling for neutrophil differentiation was demonstrated also in human primary cells. Second, we investigated the function of the transcription factor C/EBPγ, whose function in granulopoiesis was, unlike the function of other members of the C/EBP family, uncharacterized. To this aim, we generated a hematopoietic-specific *Cebpg* knock-out mouse. Surprisingly, our results demonstrated that C/EBPγ is dispensable for both steady-state and emergency granulopoiesis. Third, we focused on a completely unknown gene, *EVI2B*, which was found to be directly upregulated by the transcription factor C/EBPα, a master regulator of neutrophilic differentiation. With the use of *EVI2B* knock-down approaches in human and murine cell lines, primary cells, and *Evi2b* deficient mice we showed that the transmembrane protein EVI2B is necessary for proper neutrophil differentiation. Altogether, our work deepens our understanding of the processes regulating the production of neutrophils, and presents novel mechanisms that could be clinically modulated to interfere with granulopoiesis.

ABSTRAKT

Neutrofily jsou nedílnou složkou imunitního systému, zodpovědnou za zneškodnění patogenů, eskalaci zánětlivé reakce a hojení poškozených tkání. Drastické snížení krevních neutrofilů, zvané neutropenie, je život ohrožující patologie spojená s neschopností pacienta čelit běžným infekcím. Neúplná neutrofilní diferenciace zároveň vede ke vzniku fatálních meloidních leukémií. Zvýšený počet či aktivita neutrofilů je naopak zase častou příčinou patologií autoimunitních. Je tedy zřejmé, že množství neutrofilů musí být pro udržení hemostázy a správné funkce imunitního systému přesně regulováno. V rámci této práce jsme prozkoumali tři dosud nepopsané mechanismy, které by mohly neutrofilní diferenciaci regulovat. Prvním z nich je kanonická signální dráha Wnt. Aktivace této dráhy probíhá prostřednictvím interakce β-kateninu s transkripčními faktory TCF/LEF. Úlohu této signální dráhy jsme zkoumali s použitím myšího modelu, ve kterém byla transkripce, indukovaná komplexem β-kateninu s faktory TCF/LEF, inhibována prostřednictvím dominantně negativní varianty proteinu TCF4 (dnTCF4). S pomocí řady *in vitro* a *in vivo* metod jsme zjistili, že β-katenin v interakci s faktory TCF/LEF zvyšuje v hematopoetických progenitorech expresi receptoru G-CSF, molekuly, která navozuje diferenciaci myeloidních buněk. Tento regulační prvek se ukázal být důležitý zejména v průběhu odpovědi organismu na systémovou infekci, jejíž nedostatečnost se při systémové kandidémii projevila vysokou úmrtností dnTCF4 myší. V rámci druhého projektu jsme zkoumali, jakou úlohu během diferenciace neutrofilů zastává transkripční faktor C/EBPγ, jediný člen své proteinové rodiny jehož funkce v tomto procesu nebyla dosud řádně popsána. S použitím myši s tkánově specifickou ablací *Cebpg* jsme navzdory očekávání zjistili, že tento transkriční faktor není pro vývoj neutrofilů potřebný. Třetí projekt byl zaměřen na gen se zcela neznámou funckcí *EVI2B*, jehož exprese je však v průběhu diferenciace granulocytů zvýšena prostřednictvím transkripčního faktoru C/EBPα, jednoho ze zásadních regulátorů granulocytárního vývoje. Naše exerimenty na buněčných liniích a myších i lidských primárních buňkách ukázaly, že snížení exprese genu *EVI2B* pomocí shRNA vede k zástavě diferenciace granulocytů, a tedy že tento gen je pro jejich vývoj nezbytný. Naše práce tedy ve výsledku popsala dva nové, dříve neznámé mechanismy jejichž prostřednictvím je produkce neutrofilů regulována, a přispěla tedy k celkovému porozumění komplikovaného a složitého procesu neutrofilní diferenciace.

LIST OF ABBREVIATIONS

AML	acute myeloid leukemia
APC	adenomatous polyposis coli
aa	amino acid
bZIP	basic-leucine zipper
βTrCP	beta-transducing repeat containing protein
BM	bone marrow
BrdU	bromodeoxyuridine
CK1	casein kinase I
C/EBP	CCAAT/enhancer binding protein
CFU	colony forming units
Csf3r	colony stimulating factor 3 receptor / G-CSF receptor
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CLOUD-HSPCs	continuum of low-primed undifferentiated hematopoietic stem and progenitor cells
CTD	C-terminal domain
CXCL1	C-X-C motif chemokine ligand 1
CXCL12	C-X-C motif chemokine ligand 12
DAMPs	damage associated molecular patterns
DC	dendritic cell
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DVL	Disheveled
dnTCF4	dominant negative TCF4
dn	dominant negative TCF4
E2F4	E2F transcription factor 4
EGR1	early growth response factor 1

EVI2B	ectopic virus integration site 2b
EGFP	enhanced green fluorescent protein
Ery	erythrocytes
ER	estrogen receptor
ETV6	ETS variant transcription factor 6
FOXP1	forkhead box P1
FOXO	forkhead box protein O1
Fog1	friend of Gata1
FZD	frizzled
GPCR	G protein coupled receptors
GATA1	GATA binding factor 1
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
GSK3β	glycogen synthase kinase 3b
G-CSF	granulocyte colony stimulating factor
G-CSF-R	granulocyte colony stimulating factor receptor
GM-CSF	granulocyte-macrophage colony stimulating factor
GM-CSF-R	granulocyte-macrophage colony stimulating factor receptor
GMP	granulocyte-monocyte progenitor
GBS	Groucho binding domain
GFI1	growth factor 1 independent transcriptional repressor
HSPC	hematopoietic stem and progenitor cell
HSC	hematopoietic stem cell
HMG	high mobility group
HIF1α	hypoxia inducing factor alpha
ChIPseq	chromatin immunoprecipitation followed by sequencing
ChIP	chromatin immunoprecipitation
CML	chronic myeloid leukemia
Irf4	IL-4 inducing transcription factor

IgCAMs	immunoglobulin-like cellular adhesion molecules
IRF8	interferon regulatory factor 8
Il1r1	interleukin 1 receptor type 1
Il18r1	interleukin 18 receptor
IL-1β	interleukin 1β
IL-3	interleukin 3
JAK	Janus kinase
KD	knock-down
KO	knock-out
KLF1	Krüeppel-like factor 1
LAP	liver-enriched activating protein
LAP*	liver-enriched activating protein*
LIP	liver-enriched inhibitory protein
LKS	Lin^-, c-kit^+, Sca-1^+
LPS	lipopolysacharide
LRH1	liver receptor homologue 1
LT-HSC	long-term hematopoietic stem cells
LRP5	low-density-lipoprotein-receptor-related-protein 5
LRP5	low-density-lipoprotein-receptor-related-protein 6
M-CSF	macrophage colony stimulating factor
M-CSF-R	macrophage colony stimulating factor receptor
MMP-9	matrix metallopeptidase 9
MEP	megakaryocyte-erythrocyte progenitor
Mk	megakaryocytes
mRNA	messenger RNA
MPP	multipotent progenitors
MSCV	murine stem cell virus
MEIS1	myeloid ecotropic integration site 1

Mpo	myeloperoxidase
Nkd1	naked cuticle 1
NK	natural killer
NF1	neurofibromatosis type 1
Ela2	neutrophil elastase 2
NETs	neutrophil extracellular traps
NADPH	nicotinamide adenine dinucleotide phosphate
NTD	N-terminal domain
OCT4	octamer-binding transcription factor 4
CBP	p300/CREB-binding protein
PAMPs	pathogen associated molecular patterns
PB	peripheral blood
PBS	phosphate buffered saline
PE	phycoerythrin
PCR	polymerase chain reaction
PBX1	pre-B-cell leukemia transcription factor 1
PU.1	Spi-1 proto-oncogene
ROS	reactive oxygen species
RT-qPCR	reverse transcription quantitative PCR
RNA	ribonucleic acid
RNAseq	RNA sequencing
RUNX1	Runt-related transcription factor 1
shRNA	short hairpin RNA
ST-HSC	short-term hematopoietic stem cells
STAT	signal transducer and activator of transcription 3
scRNA-seq	single-cell RNA sequencing
Sp1	specificity protein 1
PU.1	spleen focus forming virus proviral integration site 1

SCF	stem cell factor
TCF/LEF	T-cell factor/lymphoid enhancer-binding factor
Th-POK	T-helper-inducing POZ/Krüeppel-like factor
TLRs	toll-like receptors
TSS	transcription start site
TNFα	tumor necrosis factor alpha
Vav-1	Vav guanine nucleotide exchange factor 1
WT^T	wild type - tdTomato
WT	wild type
wg	wingless
Wnt	wingless and int-1
WRE	Wnt responsive element

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1. INTRODUCTION

1.1. The hematopoietic system

Blood is an essential tissue that secures multiple fundamentally important processes for the organism's survival, such as tissue oxygenation, pathogen clearance, antibody production, or blood coagulation. To secure all these functions, 10^{12} functionally and morphologically diverse blood cells are produced every day in a highly dynamic process called hematopoiesis. Blood cells can be categorized into three functionally and ontologically distinct clusters – erythroid (erythrocytes), myeloid (platelets, monocytes, macrophages, dendritic cells, granulocytes, mast cells), and lymphoid (mainly T-cells, B-cells and natural killer cells) lineages. While erythrocytes are responsible for O_2 and CO_2 exchange and transport, myeloid and lymphoid cells protect the organism from pathogens and tissue malfunction or damage. Despite myeloid and lymphoid cells share their general purpose, the mechanisms they employ to protect the organism are essentially different. In particular, lymphoid cells are part of an adaptive immunity that is able to specifically respond towards new stimuli that individuals encounter during their lives. In contrast, myeloid cells provide less specific but crucial rapid pathogen clearance, death cell removal, and facilitate inflammation. The production of all these cell types needs to be in balance (or hemostasis) to maintain the organism in healthy conditions. Insufficient or extensive production of distinct cell types results in severe, life-threatening pathologies, such as anemia, thrombocytopenia, neutropenia, leukemia, or various autoimmune disorders. Thus, this massive daily blood cell production must be tightly and precisely regulated. [1] Moreover, the hematopoietic system must retain considerable plasticity in order to be able to adjust blood cell production in response to conditions of increased hematopoietic need, such as recovery after excessive blood loss or during infection. [2] Therefore, our ability to therapeutically address many blood-related pathological conditions depends on our understanding of the complex and intricate process of hematopoietic differentiation.

In this thesis, we will focus on mechanisms regulating differentiation of granulocytes, a blood cell type that is, as an important part of the innate immune system, absolutely crucial to resolve bacterial and fungal infections.

1.2. Biology of neutrophils

Together with basophils, eosinophils, and mast cells, neutrophils belong to the group of myeloid cells called granulocytes. Healthy human body produces daily approximately 10^{11} neutrophils, which makes them the most frequent leukocyte type in the blood tissue [3]. Neutrophils are predominantly characterized by their unique morphology (granular cytoplasm and rather small segmented nucleus) and expression of specific combination of surface markers (CD11b, Ly6G, and Ly6C in mice and CD11b, CD13, CD15, CD16, CD66b in humans) (Figure 1).

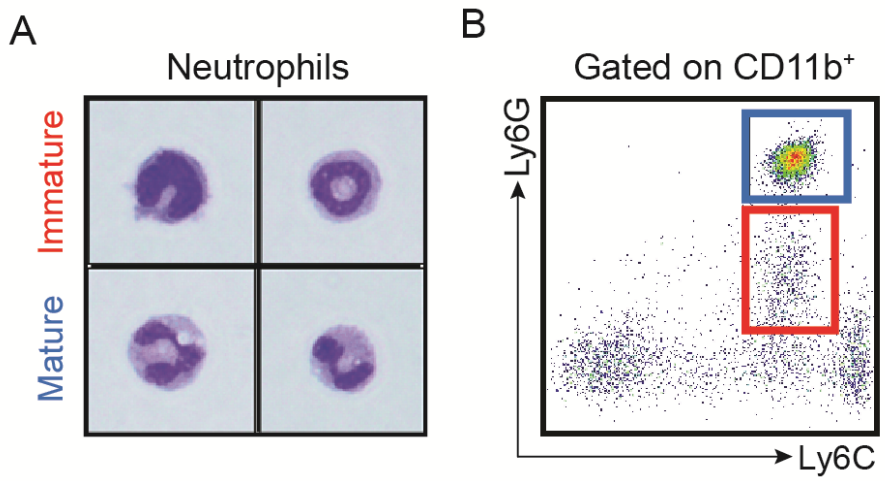

Figure 1 – Characterization of murine neutrophils by their morphology and expression of surface markers. (A) Microphotograph of May-Grunwald Giemsa stained neutrophils differentiated from 32D/G-CSF-R cell line, adapted from [4]. (B) Mature (blue square) and immature (red square) neutrophils from peripheral blood defined by expression of CD11b, Ly6G, and Ly6C surface markers by flow cytometry.

Neutrophils are essential executors of the innate immune system equipped with a battery of antimicrobial and pro-inflammatory molecules within their cytoplasmic granules [5] and with the ability to phagocyte. [6] They are rapidly recruited to the site of an injury or infection, [7] where they degranulate and activate an inflammatory response facilitating elimination of the pathogen and consequent tissue healing [8]. Low neutrophil numbers (neutropenia) caused either by genetic mutations, chemotherapeutic agent, or during poor

recovery after hematopoietic stem cell transplantation, is an hematological disorder that leads to life-threatening immunodeficiency [9]–[11]. On the contrary, excessive production or activation of neutrophils might have deleterious effects on a patient's health too. Pathologies derived from aberrant neutrophilic production and function include various inflammatory and autoinflammatory diseases (e.g. pyoderma gangrenosum, inflammatory bowel diseases, chronic recurrent multifocal osteomyelitis, obstructive pulmonary disease, inflammatory arthritis) or leukemia. [12]–[17] Thus, in order to successfully prevent or treat neutrophil-related pathologies, it is necessary to study and understand the mechanisms employed during neutrophil development and function.

The vast majority of neutrophils are localized in the bone marrow and peripheral blood; physically separated from the tissues they protect by the vascular endothelium. In order to overcome the endothelial barrier of microvasculature and to accumulate at the site of an injury, neutrophils, endothelial cells, and damaged tissue have to coordinate a cascade of specific signaling events. This cascade is triggered by the release of damage associated molecular patterns – DAMPs (e.g. adenosine triphosphate, extracellular histones, high mobility group box 1 – HMGB1 protein) and pathogen associated molecular patterns – PAMPs (e.g. lipopolysaccharide, flagellin, formylated peptides, etc.) from injured cells. DAMPs and PAMPs are promptly detected by pattern recognition receptors (e.g. toll-like receptors - TLRs) on the surface of tissue-residing leukocytes, triggering secretion of pro-inflammatory molecules (tumor necrosis factor α – TNFα , interleukin 1β – IL-1β), and activating endothelial cells in their proximity. [18]–[21]

Endothelial cells subsequently enhance surface expression of P- and E- groups of selectins, high affinity receptors that transiently bind glycosylated molecules on the surface of neutrophils. [22]–[28] Selectin-mediated interaction tethers neutrophils from blood stream to the inflamed endothelial wall and together with blood flow induces a process called "neutrophil rolling". [29] Neutrophils rolling on the surface of inflamed endothelial cells are subsequently exposed to secreted chemokines. [30] Upon stimulation, chemokine GPCR (G protein coupled receptors) trigger conformational changes of integrins (e.g. CD11b), enabling their firm and stable interaction with integrin ligands on the surface of endothelial cells. Integrin-ligand interaction between neutrophils and endothelial cell surface gradually

reduces the speed of neutrophil movement until it is completely arrested at the endothelium. [31]–[34] Following redistribution of the ectopic proteins on the endothelial cells navigates neutrophils towards endothelial cell junctions, [35], [36] a predominant site of transmigration from microvasculature to the inflamed tissue. [37], [38]

Once extravasated, neutrophils are guided to the site of injury by a gradient of "end-target" chemoatractants. Their activity in the afflicted site is, however, strongly dependent on the nature of the insult. While pathogen-induced damage causes strong pro-inflammatory and aggressive antimicrobial response accompanied with relatively large-scale collateral tissue damage, neutrophils responding to sterile injury rather contribute to wound healing and tissue regeneration. [39] As described above, neutrophils arriving to the afflicted tissue contain a broad palette of mechanisms that can be used to eliminate pathogens, including production of reactive oxygen species (ROS), degranulation, phagocytosis, secretion of pro-inflammatory molecules, and NETosis.

Neutrophil activation is accompanied by enhanced expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a major source of cellular ROS. [40] NADPH oxidase-mediated ROS production augmentation, sometimes referred as respiratory burst, has a potent antimicrobial activity. Mutations of NADPH oxidase prohibit human phagocytes to effectively eradicate microorganisms and are associated with severe and recurrent infections. On contrary, excessive ROS can inflict serious damage to the host tissues, cells, and macromolecules. [41], [42] Another crucial anti-microbial activity of neutrophils is degranulation, during which neutrophils release complex mixture of antibacterial agents from their granules into the environment. Granules contain for example myeloperoxidase, serine and cysteine proteases, cathepsins G, elastase, α-defensins, azorucidin, matrix metallopeptidase 9 (MMP-9), β-glucoronidase, interleukin 16, and chemokines (C-X-C motif chemokine ligand 1 and 12 - CXCL1, CXCL12). [43]–[45] Granule content has not only the potential to fight pathogens, remodel damaged tissue, and attract additional immune cells, but also to cleave and maturate pro-inflammatory interleukins of the IL-1 family, further escalating the ongoing inflammation. [46]

When the activating signal is too strong, overstimulated neutrophils might undergo an alternative type of cell death with a powerful antimicrobial properties - NETosis. During

NETosis, chromatin is decondensed, dissolved, covered with granular contents (proteases, antimicrobial and pro-inflammatory agents, etc.), and propelled from the cell forming neutrophil extracellular traps (NETs). On one hand, NETs incapacitate pathogens, restraining them from spreading, inducing their prompt clearance, and providing strong activating stimuli for other immune cells. [39], [47] On the other hand, NETs have been linked to several pathological states, such as thrombus formation, chronic inflammation, myocardial infarction, or COVID-19 related thrombosis and mortality. [48]–[50]

1.3. Hematopoietic stem cells (HSCs)

All blood cells, including neutrophils, originate from HSCs, a rare population of cells that reside in the bone marrow. [1] Despite the hematopoietic system produces millions of blood cells every minute, single HSC can go only through very limited number of cell divisions. Therefore, HSCs are kept in a dormant state and their direct contribution to hematopoiesis is rather minimal. [51] Dormant HSCs are characterized by slow cell-cycle progression, low metabolic activity, [52] energetic metabolism relying on glycolysis, [53] intracellular hypoxia, [54] and low proteosynthesis. [55] These properties protect HSCs from genotoxic stress, caused for example by DNA replication or by byproducts of basal metabolism, such as physiologically occurring reactive oxygen species (ROS). Since proper HSC function secures life-long hematopoiesis that is essential for the individual's survival, it is crucial to continuously maintain the integrity of the HSC pool by preserving their protective dormant state. Indeed, dormant HSCs have the highest reconstitution capacity in transplantation-based assays, while their proliferation or genomic instability is associated with loss of HSC function. [51], [56] Despite the importance of preserving the HSC pool, the HSC population is being slowly shortened by an infrequent cell death, eventual HSC differentiation, and bone marrow egress.

In order to compensate for this HSC loss, HSCs need to occasionally divide and propagate their population in a process called self-renewal, which is one of the unique stem cells properties. There are two major theoretical concepts of cell division that resolve self-renewal – symmetric and asymmetric. During symmetric division, the HSC goes through a cell cycle, creating two daughter cells that are functionally and morphologically indistinguishable from the maternal HSC. Asymmetric division, in contrast, produces two

functionally different daughter cells. One of them inherits properties of the original HSC, and the other gains the potential to further differentiate. [57]

Leaving aside dormancy and self-renewal, HSCs are also characterized by their multipotency – an ability to produce every blood cell type and, consequently, to reconstitute the whole hematopoietic system. Transplantation experiments clearly demonstrated that as few as one single HSC is able not only to repopulate the hematopoietic system of an irradiated recipient mouse, but also to expand the HSC pool (by self-renewal) and repopulate secondary recipients. [58], [59] Importantly, multipotency of HSC was utilized in human medicine, where HSC transplantation became a major, powerful, and up-to-day irreplaceable tool in the treatment of severe hematological disorders. [60], [61] However, despite its broad application in both clinical and experimental settings, the mechanisms regulating hematopoietic differentiation and HSC fate-decisions remain obscured.

1.4. HSC commitment and differentiation

From an ontological point of view, all hematopoietic cells can be segregated into three major hierarchically ordered categories: HSCs, hematopoietic progenitors, and differentiated cells. These categories differ mostly in their self-renewal abilities, differentiation capacity, and proliferation, with HSC being at the apex of the system. [1]

In general, commitment and differentiation to erythroid, myeloid, or lymphoid lineage is progressively acquired at the expense of multipotency and self-renewal. Dormant HSC, sometimes referred as long-term HSC (LT-HSC) for their ability to self-renew and sustain complete hematopoietic reconstitution in serial transplantation assays, differentiate with very low frequency (1% of LT-HSCs per day). As shown in Figure 2, differentiating LT-HSCs produce a population of short-term HSC (ST-HSC) that are still multipotent, but that lost their ability to reconstitute blood production in serial transplantations. On the other hand, ST-HSCs still possess high capacity to self-renew and to preserve their population without significant contribution from the LT-HSC pool. A heterogeneous population of multipotent progenitors (MPPs) descends further from ST-HSCs. MPPs show rapid proliferation, certain degree of self-renewal, lineage specification, and their production of

committed progenitors exceeds the input from ST-HSCs 280 times, suggesting MPPs are a major source of daily steady-state blood production. [62]

The classical model of hematopoietic differentiation (Figure 2), based on expression of cell surface markers, HSCs transplantations, and colony forming assays, assumes that MPPs produce two types of progenitor cells: common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). According to this model, CMPs further differentiate into megakaryocyte-erythrocyte progenitors (MEPs) or into granulocyte-monocyte progenitors (GMPs). While MEPs gradually form erythrocytes and platelets, GMPs precede the generation of monocytes and granulocytes. [63] However, recent development of novel techniques enabling analysis on a single-cell level questioned the classical hematopoietic model and the very existence of this hierarchy. For example, single-cell RNA sequencing (scRNA-seq) of CMP, MEP, and GMP populations discovered 18 transcriptionally distinct subpopulations within these progenitor pools. [64]–[66] Additionally, it has been reported that myeloid-restricted progenitors might be produced from LT-HSCs directly, bypassing the ST-HSC and MPP stages. [67] Moreover, Grinenko et al. demonstrated that HSCs can acquire myeloid commitment even without progressing through a single cell division. [68] Thus, these observations gradually raised a need for establishing a new, alternative model of hematopoietic development.

To resolve this question, Velten et al. integrated flow cytometry phenotyping, scRNA-seq, and functional *in vitro* and *in vivo* assays in unbiased analysis of human immature blood cells. [69] They showed that developing cells are not required to differentiate step-by-step through distinct defined stages, nor to do binary branching decisions, as described by the classical hematopoietic model. According to their analysis, hematopoietic stem and progenitor cells (HSPCs) rather form a continuum of low-primed undifferentiated hematopoietic stem and progenitor cells (CLOUD-HSPCs), where cells are present in resilient transitional states. In this ambiguous form, cells are continuously acquiring transcriptomic priming for multiple lineages, stochastically selecting one, and finally producing lineage-committed progenitors as an output of CLOUD-HSPCs.

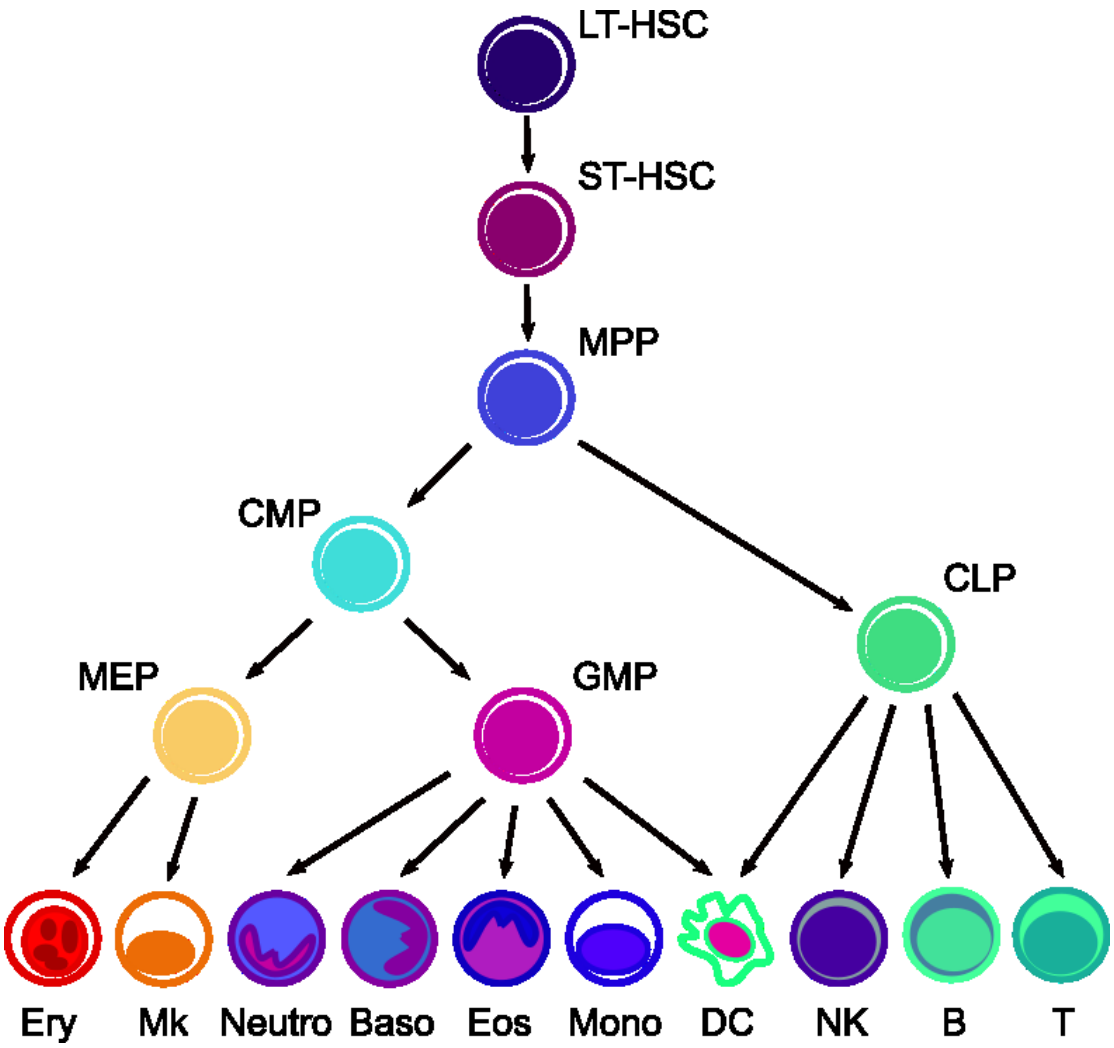

Figure 2 – The classical hierarchical model of hematopoietic differentiation. Schematic representation of the classical concept of differentiation pathways of blood cells, starting from multipotent LT- and ST-HSC. Differentiation proceeds through populations of MPP, which show the first signs of lineage specification. Consequently, the progeny of MPP bifurcate into progenitors of two separated lineages: myeloid (CMP) and lymphoid (CLP). CLP can further differentiate via stages of specifically committed precursors into dendritic cells (DC), natural killer cells (NK), B-, or T-cells (B and T, recpetively). CMP differentiate into either MEP, producing further erythroblasts (Ery) or megakaryocytes (Mk), or GMP that give rise to granulocytes (neutrophils, basophils, eosinophils) and monocytes.

Regardless of the type of hematopoietic model, the developmental stage where lineage commitment primarily occurs still remains elusive. It seems probable that transcriptomic changes resulting into lineage specification and commitment are orchestrated by an intricate combination of cell intrinsic (e.g. expression of specific transcription factors and epigenetic changes) and extrinsic factors (e.g. cytokines and niche signals). It has been additionally suggested that the HSC pool is heterogeneous itself and contains a mixture of HSCs with already predisposed potential for distinct lineages which are only further propagated during differentiation. [70] Nonetheless, this hypothesis was established mostly on biased transplantation assays [71] and experiments burdened by pre-selection of phenotypically defined HSC populations, which always contain high frequency of HSC-like cells that do not possess full HSC properties. [69]

Despite the indisputable contribution of epigenetic factors [72]–[75] and metabolism, [76] the most characterized and decisive intrinsic elements driving HSC lineage commitment are transcription factors. [77] Development of certain lineage is usually associated with few specific transcription factors. For instance, generation of erythrocytes is driven by GATA binding factor 1 (GATA1), Krüeppel-like factor 1 (KLF1), and E2F transcription factor 4 (E2F4), while megakaryocyte differentiation is orchestrated by GATA1, Myeloid ecotropic integration site 1 (MEIS1) and pre-B-cell leukemia transcription factor 1 (PBX1). [64], [69] Consistently, absence of these factors results in a differentiation arrest and loss of the affected lineage, [78]–[84] while their overexpression might induce trans-differentiation of committed cells to different lineage. [85]–[89] Nonetheless, primary cell cultures and *in vitro* differentiation assays clearly demonstrate that lineage commitment and HSC fate can be imposed and driven by cell extrinsic factors as well. [90]–[92] Extrinsic factors are usually provided by the bone marrow microenvironment (mostly endothelial and mesenchymal stromal cells) and are essential for many aspects of proper HSC function including survival, self-renewal, dormancy, or homing. [93] There are various types of cell extrinsic factors, however, those regulating HSC lineage commitment and differentiation are mostly cytokines. Indeed, sole exposure to specific cytokines [94], [95] or overexpression of cytokine receptors [96] is sufficient to induce lineage switch in developing cells. Remarkably, the most important cytokines for myeloid development are macrophage colony stimulating factor (M-CSF) and granulocyte colony stimulating factor (G-CSF), as shown by

several lines of evidence. First, mice deficient in G-CSF, M-CSF, or their corresponding receptors exhibited myeloid differentiation block and showed diminished populations of neutrophils or monocytes, respectively. [97]–[101] Second, M-CSF and G-CSF were shown to directly impose myeloid comitment to HSCs. [90], [102]

1.5. Transcriptional regulation during myeloid commitment and differentiation

All cells in the organisms harbor the same genetic information. Lineage specification, commitment, and differentiation are therefore inevitably driven by changes in gene expression. These stage-specific expression patterns might be very complex and are predominantly driven by transcription factors and subsequently maintained by epigenetic changes. [103] Since this thesis is focused on the regulation of neutrophil development, we will discuss the mechanisms regulating myeloid differentiation in more detail. Interestingly, only a limited number of transcription factors (and their combinations) are critical for proper myeloid development; the most explored ones are Runt-related transcription factor 1 (RUNX1), Spi-1 proto-oncogene (PU-1), GATA1, growth factor 1 independent transcriptional repressor (GFI1), and transcription factors of the CCAAT/enhancer binding protein (C/EBP) family. [64], [69] Despite their necessity for the development of a given lineage, the expression of many transcription factors is not exclusive for one cell type or tissue and their function might substantially differ in distinct cellular contexts.

1.5.1. RUNX1

RUNX1 is necessary for the very emergence of HSCs and for the establishment of hematopoiesis during embryonic development. *Runx1* deficient mice therefore show complete lack of liver hematopoiesis and consequent embryonic lethality. [104] In order to study the function of Runx1 in adult HSC, a conditional knock-out murine model was developed. Interestingly, Runx1 was dispensable for the maintenance of adult HSCs, but it was necessary for the differentiation of more committed cells into multiple lineages. The conditional Runx1 loss was particularly characterized by impaired neutrophil and lymphocyte development, profound decline of platelet counts accompanied by block of megakaryocytic maturation, expansion of hematopoietic progenitors, and mild myeloproliferative disorder. [79], [80], [105] Consistently, *RUNX1* mutations are associated

with thrombocytopenia, myelodysplasia, and development of acute myeloid leukemia (AML) in humans. [106], [107] Given the heterogeneity of phenotypes of Runx1 deficient mice, it is clear that Runx1 is able to impose distinct transcriptional programs depending on the cellular context. In B-cell development, Runx1 supports survival of B-cell progenitors and induces expression of signaling proteins that drive pre-B-cell maturation. [108] In contrast, Runx1 regulates CD4/CD8 lineage choice via direct repression of decisive T-helper-inducing POZ/Krüeppel-like factor (Th-POK) transcription factor in T-cells. [109] In the myeloid compartment, Runx1 mediates chromatin changes that allow expression of other crucial regulators of myeloid commitment and differentiation, such as PU.1, C/EBPα, or M-CSF receptor (M-CSF-R). [110], [111] Thus, together with its function during embryonic hematopoiesis, RUNX1 seems to be a pioneer transcription factor that imposes myeloid commitment to HSCs.

1.5.2. GFI1

GFI1 is a transcriptional repressor that has multiple functions in the hematopoietic system, such as HSC self-renewal maintenance, [112] protection from genotoxic stress, [113] B-cell development, [114] and T-cell maturation. [115] Nonetheless, the most profound function of GFI1 is the promotion of neutrophil development. It was reported that *Gfi1* deficient mice showed complete ablation of neutrophils, accumulation of GMPs, and consequent fatal immunodeficiency. [81], [116] Further, *GFI1* loss-of-function mutations were consistently found in human patients suffering from severe congenital neutropenia, a genetic hematological disorder characterized by low neutrophils counts and expansion of immature cells. [117]–[119] Laslo et al. additionally described how Gfi1-dependent neutrophilic differentiation occurs. According to this study, Gfi1 imposes neutrophilic commitment to bipotential GMP progenitors (GMPs can produce either neutrophils or macrophages) by repressing macrophage-specific genes. [120]

1.5.3. GATA1

The transcription factor GATA1 affects the development of multiple hematopoietic lineages, heavily depending on the cellular context, but it is predominantly expressed in erythroid cells, megakaryocytes, eosinophils, and mast cells. Initial studies demonstrated that Gata1 deficiency caused erythropoietic arrest at the proerythroblast stage, complete loss of red blood cells, and prenatal lethality in murine embryos. [121]–[124] Later, more specific models showed that loss of Gata1 activity impairs also maturation of eosinophils, [125] mast cells, [126] and megakaryocytes. [127] Despite it seems that Gata1 promotes rather terminal maturation of cells, [128] it possesses the striking ability to reprogram and transdifferentiate various blood progenitors to erythroid, megakaryocytic, and eosinophilic lineages when overexpressed. [87], [129]–[131] How can a single transcription factor instruct commitment to such different lineages is not completely understood. The most current hypothesis assumes that different cell types provide distinct binding partners that are able to shift Gata1 activity towards specific promoters and enhancers. Erythroid maturation is, for instance, driven by Gata1 in complex with its cofactor Friend of Gata1 (Fog1); disruption of this complex or Fog1 deficiency results in an erythroid differentiation arrest observed also in Gata1 loss-of-function models. [132], [133] Additionally, Gata1 activity can be enhanced on specific promoters by its synergistic interaction with other erythroid-specific transcription factors, such as (Specificity protein 1) Sp1 or Klf1. [134]

In contrast, GATA1 expression can be suppressed by transcription factors specific for different lineages. For instance, GATA1 and the transcription factor PU.1, an essential regulator of neutrophil and B-cell development, exerts reciprocal inhibitory activity. In other words, presence of PU.1 results in the absence of GATA1 and vice versa. [135] This functional antagonism and mutual exclusivity therefore illustrates how a sole stoichiometry of two instructive factors can impose the commitment to a specific lineage in HSPCs.

1.5.4. PU.1

The transcription factor PU.1 is absolutely essential for the entire hematopoietic system, as PU.1 null mice suffer from lethal multi-lineage hematopoietic failure and die shortly after a birth. Interestingly, erythroid and megakaryocytic lineages, regulated by PU.1

antagonist GATA1, [135] were preserved in these mice. [82], [136] In HSCs, PU.1 expression is initiated by the transcription factor RUNX1 [137] and preserves HSCs dormancy and function by balancing cell cycle regulators. [138] In more committed populations, PU.1 is involved in the production of CLP and CMP progenitors, CMP to GMP transition, and further maturation of monocytes and granulocytes. [139], [140] Additionally, high PU.1 expression is necessary for B-cell lineage commitment and formation of early B-cell progenitors, while its downregulation is a critical event during T-cell development in thymus. [141], [142] This pleiotropic PU.1 activity and its capacity to regulate differentiation of multiple lineages is secured by its variable DNA binding properties. [143] Thus, PU.1 coordinates cell cycle regulators in HSCs, [138] upregulates G-CSF-R, M-CSF-R, and granulocyte-macrophage colony stimulating factor receptor (GM-CSF-R) in myeloid cells, [144], [145] while it suppresses genes involved in T-cell receptor production [141] and stimulates expression of interleukin-7 receptor (IL-7-R) in lymphoid lineage. [146] The binding affinity of PU.1 towards specific gene regulatory regions depends mostly on PU.1 concentration [143], [147] and binding partners. The early myelopoietic program, for instance, is thought to be initiated by PU.1 interacting with members of the C/EBP family of transcription factors. [148]

1.5.5. C/EBP transcription factors

C/EBP transcription factors (C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ) are widely expressed proteins belonging to the basic-leucine zipper (bZIP) superfamily, which regulate crucial processes such as cell differentiation, proliferation, energy metabolism, or inflammation. They harbor highly variable N-terminal transactivation domains and a conserved C-terminal region containing dimerization and DNA binding domains (bZIP). Since the C-terminal region is conserved in all C/EBP family members, it is not surprising they all have a similar consensus binding sequence, i.e. TTACGTAA. But despite this very similar DNA binding sequence, each C/EBP factor has distinct tissue and developmental-stage specific functions. This functional heterogeneity of C/EBP transcription factors is therefore ascribed to a) their N-terminus that is variable in sequence, length, posttranslational modifications, and interacting partners; and b) their ability to form

intra- and interfamily homo- and heterodimers that provide each dimer with unique DNA binding specificity. [149]

1.5.5.1. C/EBPα

C/EBPα is the most extensively studied C/EBP transcription factor in the hematopoietic system and it is an essential "master" regulator of early neutrophil development. *Cebpa* deficiency is manifested by downregulation of myeloid-related genes and complete loss of granulocytic lineage from GMPs to further stages.[150]–[153] Consistently, inactivation of an hematopoietic-specific C/EBPα enhancer located 42 kb upstream from *Cebpa* gene resulted in loss of C/EBPα expression and consequent ablation of GMPs and neutrophils. [154] Mechanistically, C/EBPα binds to the *cis*-regulatory elements that were made accessible by PU.1, and orchestrates CMP-to-GMP transition, after which C/EBPα is dispensable for terminal stages of granulopoiesis. [155] Nevertheless, others reported that C/EBPα is also necessary for proper HSC maintenance. In these studies, *Cebpa* deficient HSCs showed loss of C/EBPα-mediated quiescence [156] followed by functional impairment and exhaustion of the HSC pool. [154], [157]

Moreover, mutations in *CEBPA* that prevent proper granulocytic differentiation are frequently identified in patients suffering from AML, a disease characterized by a granulocytic differentiation arrest. [158] *CEBPA* mutations were detected in 8.8 % of AML cases. A broad spectrum of mutation types occurred in these patients including various insertions, deletions, and nonsense mutations. However, particularly interesting and the most frequent point mutations were nonsense mutations localizing in the second N-terminal ATG codon of *CEBPA*. These mutations enabled augmented translation of a truncated isoform of C/EBPα marked as p30, whereas full-length C/EBPα is called p42, based on their molecular mass. [159] Despite p30 lacks only one N-terminal transactivation domain, it exerts an ability to counteract the function of full length p42 C/EBPα in a dominant negative manner and possesses a diminished DNA binding activity. Consequently, a monoallelic p30 mutation is able to completely hinder C/EBPα activity in the cell and to provoke a differentiation arrest resulting in leukemia onset. [160]

The transcriptional program induced by C/EBPα is rather strong. C/EBPα overexpression can, in coordination with PU.1, even trigger T- or B-cell transdifferentiation to the myeloid lineage. [85], [161] The set of genes directly upregulated by C/EBPα, called C/EBPα signature, was obtained by combining data generated by chromatin immunoprecipitation followed by sequencing (ChIPseq) with transcriptomic analyses and was found to be downregulated in AML patients harboring *CEBPA* mutations. [162], [163] Despite C/EBPα is primarily a transcriptional activator, a recent study revealed it can also actively repress genes driving differentiation into other lineages. [64]

Although the transcriptomic analyses searching for C/EBPα target genes identified genes that were previously known to be involved in granulopoiesis, they also discovered a novel set of C/EBPα target genes with so far uncharacterized role in the process of myeloid differentiation. An interesting target was the ectopic virus integration site 2b (*EVI2B*).

1.5.5.1.1. EVI2B

EVI2B was originally identified as a common viral integration site in murine leukemias, suggesting it might be involved in leukemogenesis. [164] *EVI2B* gene is localized within an intron of neurofibromatosis type1 gene (*NF1*) and encodes 448 amino acid residues long transmembrane glycoprotein. The highest EVI2B expression was detected in the bone marrow, but it is also expressed on the surface of B- and T-cells, granulocytes, macrophages, DCs, and NK cells. [165], [166] Despite the function of EVI2B in hematopoiesis is completely uncharacterized, several studies indicate it might play a role in cell differentiation and leukemia. First, *EVI2B* is involved in the differentiation of certain non-hematopoietic cells, specifically melanocytes and keratinocytes. [167] Second, elevated expression of *EVI2B* is associated with poor prognosis in patients with chronic lymphocytic leukemia (CLL) [168] and colorectal cancer. [169]

Together, the evidence that *EVI2B* is a direct target of C/EBPα with high expression in granulocytes and with the potential to regulate cell differentiation and leukemia outcome allowed us to speculate that EVI2B might be also involved in the process of granulocytic development. Thus, one of the goals of this study is to elucidate in detail how is *EVI2B*

regulated by C/EBPα and what is the function of EVI2B in the differentiation of granulocytes.

1.5.5.2. C/EBPβ

Interestingly, increased expression of neutrophil-stimulating cytokines (G-CSF, GM-CSF, and IL-3) in the hematopoietic system of *Cebpa* deficient mice was able to overcome the complete neutrophilic differentiation arrest and to produce functional neutrophils. It was shown that these cytokines (present also in the blood during systemic infection) enhance expression of C/EBPβ. Consistently, C/EBPβ loss impaired neutrophil production during systemic infection but not in steady-state hematopoiesis, demonstrating C/EBPβ is crucial for emergency granulopoiesis. [170]

1.5.5.2.1. Emergency granulopoiesis

Until this chapter, the thesis was focused on mechanisms driving the development of neutrophils in unperturbed hematopoiesis. However, there are situations during an individual's life requiring enhanced and rapid production of new neutrophils via a process called emergency granulopoiesis. These situations typically involve reconstitution of the hematopoietic system after severe bleeding, bone marrow injury (by chemotherapeutics or ionizing radiation), or during the course of systemic microbial infections. [171]

As shown by Boettcher et al., pathogen presence is detected by TLRs expressed on the surface of endothelial cells. [172] Endothelial cells respond to this stimulus by releasing a potent stimulant of neutrophilic production, G-CSF, into the blood stream. G-CSF is a crucial signaling molecule and has multiple roles during emergency granulopoiesis. First, it induces egress of neutrophils from bone marrow to peripheral blood. And second, it stimulates G-CSF-R-expressing HSPC, skewing their differentiation towards GMPs and, consequently, augmenting *de novo* production of neutrophils. [173], [174]

The importance of G-CSF in emergency granulopoiesis is well documented in both humans and mice. First, elevated levels of G-CSF were detected in septic patients in numerous studies. [175]–[178] Second, *Csf3* and *Csf3r* deficient mice were not able to expand their neutrophil pool and resolve infections effectively. [97], [98] However, it is

important to note that mice lacking G-CSF or its receptor still produce neutrophils and are able, to some extent, to enhance their production in response to the presence of pathogens. This is probably not caused by the anticipated functional redundancy of G-CSF, GM-CSF, and M-CSF, since neutrophils are still present in mice deficient in all three aforementioned factors. [101] According to other studies, it is more likely that cytokine-independent mechanisms might be involved in myelopoiesis enhancement. Nagai et al. for example showed that HSC are able to detect and respond to PAMPs directly with their own TLRs. [179]

Mechanistically, stimulation of G-CSF-R activates downstream JAK/STAT signaling that provides pro-survival and pro-proliferative signal, and induces expression of the key regulator of emergency granulopoiesis, the transcription factor C/EBPβ. [170], [180] Three different C/EBPβ isoforms referred as liver-enriched inhibitory protein (LIP), liver-enriched activating protein (LAP), and liver-enriched activating protein* (LAP*) are sequentially produced from *Cebpb* mRNA. The first isoform that occurs in the HSC upon stress induction is LIP. LIP activates expression *c-myc* and promotes cell cycle progression and an expansion of HSC. Thereafter, LIP is replaced with LAP and LAP* isoforms, that induce rapid myeloid differentiation and secure enhanced granulocyte production. [181], [182] Interestingly, gradual increase of C/EBPβ expression is accompanied by declined expression of C/EBPα in G-CSF-stimulated HSPCs. [183] Since C/EBPα blocks cell cycle progression, this antagonism is probably essential for the HSPC expansion and sufficiently enhanced production of neutrophils.

Despite that C/EBPβ is the key driver of emergency granulopoiesis, a recent study demonstrated that expanding GMPs form clusters in the bone marrow and activate β-catenin signaling, suggesting that canonical Wnt signaling might be involved in the regulation of emergency granulopoiesis as well. [184]

1.5.5.3. C/EBPδ

Other C/EBPs, such as C/EBPδ, have been linked to granulopoiesis as well. C/EBPδ, a less prominent member of the family, synergizes with C/EBPβ and helps to stimulate cytokine synthesis during response to an infection. [185] Additionally, C/EBPδ is

considered as a tumor suppressor. C/EBPδ is transcriptionally silenced in 35 % of AML patients, [186] its low expression is associated with blast crisis stage of chronic myeloid leukemia (CML), [187] and experimental restoration of C/EBPδ expression promoted differentiation of leukemic blasts. [188]

1.5.5.4. C/EBPε

Unlike previously described C/EBP transcription factors, C/EBPε is crucial for later stages of granulopoiesis, and was shown to be required rather during GMP transition to neutrophil committed progenitors called preNeu. C/EBPε deficient mice accordingly showed accumulation of GMPs, loss of preNeu progenitors and neutrophils, and higher susceptibility to infections. [78], [189]

1.5.5.5. C/EBPγ

Remarkably little is known about C/EBPγ transcription factor and its function in the hematopoietic system. C/EBPγ is the shortest, ubiquitously expressed member of the C/EBP family that lacks the N-terminal transactivation domains. [149] Consistently, C/EBPγ has not been reported to have trans-regulatory activity on its own. It was suggested that C/EBPγ heterodimerizes with C/EBPα and C/EBPβ to inhibit their activity as a dominant negative regulator. [190], [191] Available mouse studies, however, provide rather incoherent and limited information. The first murine model, *Cebpg* full-body knock-out (KO) mice, showed neonatal lethality caused by respiratory failure. To overcome this issue, chimeras of wild type (WT) and *Cebpg* deficient bone marrow were constructed. Using this approach, the role of C/EBPγ was investigated in the lymphoid lineage and it was reported that *Cebpg* deficient cells exhibited reduced cytotoxic activity of NK cells. [192] The overexpression of C/EBPγ, in contrast, affected and impaired fetal liver erythropoiesis in mice. [193]

Considering these conflicting lines of evidence, the function of C/EBPγ in the hematopoietic system remains rather elusive. However, there are several independent observations pointing towards its role in the granulocytic differentiation. First, C/EBPγ belongs to the family of transcription factors that predominantly regulate granulocyte

differentiation. [149], [194] Second, it was suggested that C/EBPγ might act as a dominant negative inhibitor of C/EBPα and C/EBPβ. [190], [191] Third, Alberich-Jorda et al. showed that *Cebpg* expression is downregulated during granulocytic differentiation, while its overexpression effectively blocks this process. Authors of this study also consistently observed augmented *CEBPG* expression in a subset of AML patients harboring granulocytic differentiation arrest and *CEBPA* hypermethylation. [195]

In order to further elucidate the potential role of C/EBPγ in granulopoiesis, we generated blood-tissue specific *Cebpg* knock-out mice. In this thesis, we analyzed how its loss affects HSC function and granulocytic development in steady-state and emergency granulopoiesis.

1.6. The Canonical Wnt signaling pathway

The canonical Wnt signaling was originally discovered as a pathway regulating organ development in *Drosophila melanogaster*. Its name is derived from wingless (*wg*) gene which is necessary for the wing development and pattern formation in fruit flies. [196] Previous research gradually discovered one canonical and two non-canonical Wnt signaling pathways (planar cell polarity and Wnt/calcium pathways), and it was demonstrated that Wnt signaling has a crucial role in the development and homeostasis of mammalian tissues. [197], [198]

The Wnt signaling pathway has primarily a paracrine or autocrine character. The initiating event in the pathway activation is binding of secreted Wnt glycolipoprotein to one of the Frizzled receptors (FZD) [199], [200] and their co-receptors, low-density-lipoprotein-receptor-related-protein 5 or 6 (LRP5 and LRP6 respectively), on the surface of the receiving cell (Figure 3). [201], [202] Wnt ligands are 40 kDa large hydrophobic proteins that need to be palmitoylated by palmitoyl transferase Porcupine prior to secretion. [200], [203], [204] Interestingly, Wnt ligands are not secreted in a free form, but rather incorporated into exosomes. [205]–[207] The complexity of this system is vast. Human genome contains 19 Wnt ligands harboring different types of lipid modifications further increasing the variety of their biochemical properties, 10 FZD receptors with various activities and specificities for canonical and non-canonical pathway activation, and 2 LRP tissue-specific co-receptors.

Moreover, a battery of secreted antagonists and scavengers which affect availability of Wnt ligands or FZD receptors is involved as well. [208] Thus, this signaling system has the capacity to induce large number of diverse context-dependent outcomes. In case of the canonical Wnt signaling pathway, all these different signals are integrated to a single molecule, the scaffold protein β-catenin. [209]

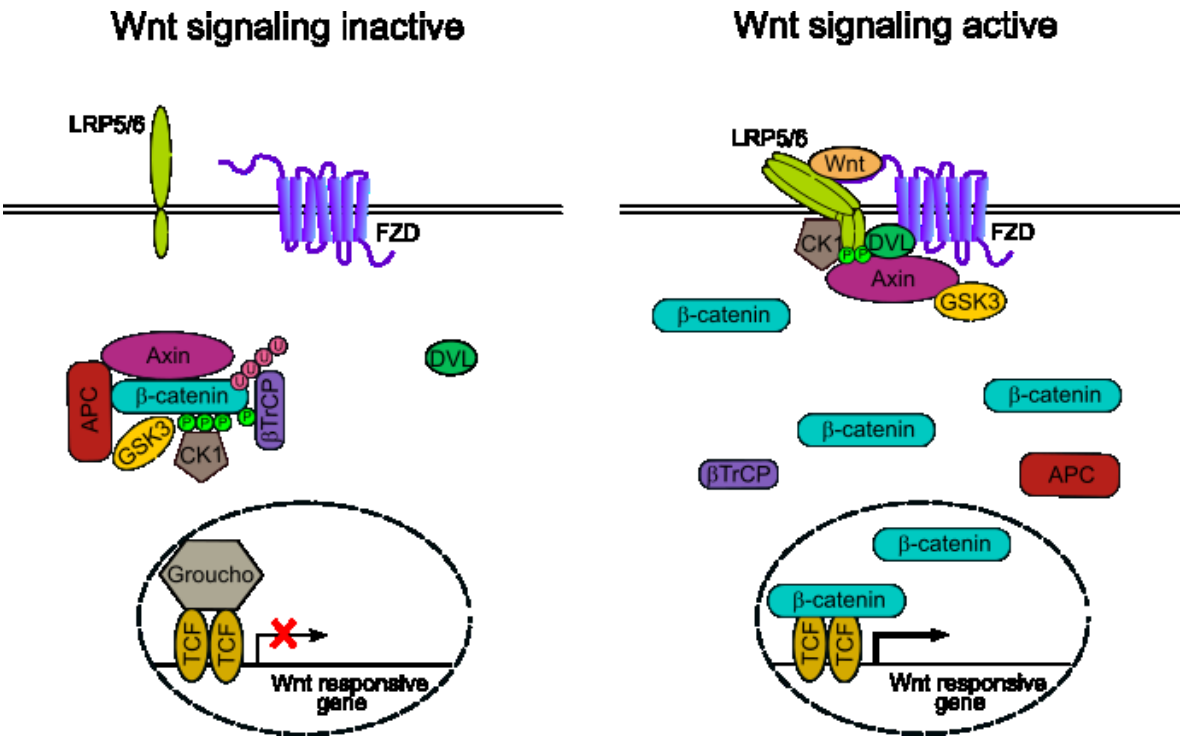

Figure 3 – Schematic description of canonical Wnt signaling pathway. Unless Wnt ligand binds to FZD receptor and its co-receptor LRP, the β-catenin destruction complex is assembled. Scaffold proteins Axin and APC bring β-catenin into proximity of GSK3β and CK1 kinases. Subsequent β-catenin phosphorylation provides a binding site for E3 ligase β-TrCP, that ubiquitinates β-catenin and targets it for proteasomal degradation. Thus, TCF/LEF transcription factors remain in their repressive form and prohibit transcription of Wnt responsive genes. Structural changes occurring upon Wnt ligand recognition enable LRP-DVL interaction. Following LRP aggregation gradually induces CK1-mediated phosphorylation of LRP, scavenging Axin and GSK3β, and, therefore, disintegrating the β-catenin destruction complex. Stabilized, unphosphorylated β-catenin accumulates, translocates to the nucleus, where it interacts with TCF/LEF transcription factors, replaces Groucho, and activates transcription of Wnt responsive genes.

β-catenin is a 781 amino acids (aa) long protein composed of short (approximately 140 aa), structurally flexible N- and C-terminal domains (NTD and CTD) flanking the central superhelical core. The core of β-catenin is composed of 12 armadillo repeats that shape the positively charged groove, providing a binding site for the majority of β-catenin interacting partners (Figure 4). [210] The majority of β-catenin localizes to the focal adhesions where it interacts with E-cadherin and facilitates the interaction with the actin cytoskeleton. Residual unbound β-catenin serves as signaling molecule that transduces signals from FZD receptor to the nucleus. When the canonical Wnt signaling pathway is not active, free β-catenin is rapidly targeted for proteasomal degradation by catenin-destruction complex (Figure 3). [211] The key components of catenin-destruction complex are: scaffold proteins AXIN and adenomatous polyposis coli (APC), casein kinase I (CK1), and glycogen synthase kinase 3β (GSK3β). [212] APC attracts β-catenin to the destruction complex through binding to its core groove. [213] CK1 subsequently phosphorylates β-catenin NTD on Ser45, providing a priming signal for GSK3β-mediated phosphorylation of β-catenin on Thr41, Ser37, and Ser33. [214] These phosphorylated residues form a binding site for beta-transducing repeat containing protein (βTrCP) E3 ligase, [215] that finally ubiquitinilates β-catenin and targets it for degradation. [216] In other words, free β-catenin is constantly degraded and can not mediate signal transduction in the absence of Wnt-FZD-LRP interaction. In this scenario, the transcription of Wnt responsive genes is repressed by T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors that occupy their regulatory regions [217] in association with transcriptional repressors, such as Groucho protein. [218], [219]

Binding of Wnt ligand to a FZD-LRP5/6 receptor complex triggers a sequence of signaling events that gradually disassemble the catenin-destruction complex, and therefore stabilize free cytoplasmic β-catenin. Since β-catenin is constantly synthesized in the cells, its stabilization causes immediate accumulation and subsequent nuclear translocation. Mechanistically, FZD receptors, structurally altered by Wnt binding, recruit protein Disheveled (DVL) which induces aggregation of LRP co-receptors, enabling LRP phosphorylation by CK1. Phosphorylated LRP aggregates sequester AXIN and GSK3β and, consequently, deconstruct the catenin-destruction complex. [220] Unphosphorylated β-

catenin accumulates in the cytoplasm and translocates to the nucleus, [221] where it interacts with the TCF/LEF family of transcription factors.

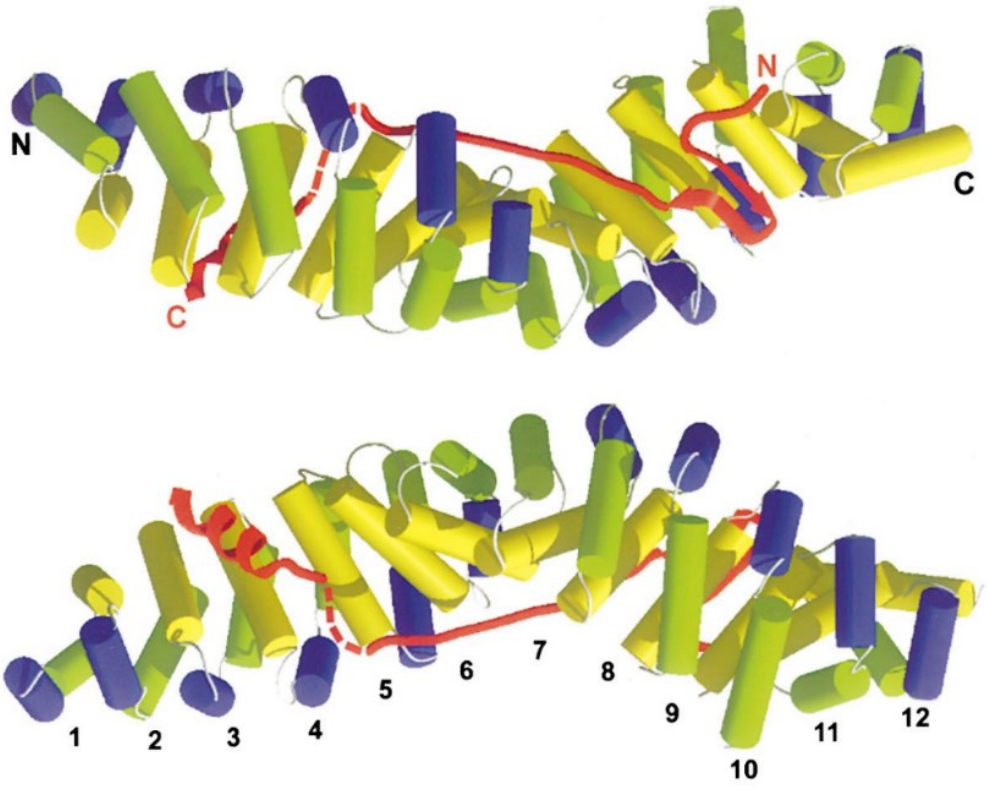

Figure 4 – Structure of β-catenin interacting with N-terminal β-catenin binding domain of TCF/LEF transcription factors. Figure shows structurally disordered N-terminal β-catenin binding domain of TCF/LEF transcription factors (red ribbon) docking to positively charged core groove of β-catenin from two angles. Black "N" and "C" letters in the upper panel depicts N- and C-terminal end of β-catenin. Numbers in the bottom panel (1-12) mark the order of individual armadillo repeats. Figure was adapted from [222]

TCF/LEF transcription factors, belonging to the high mobility group (HMG) protein family, and are exclusive effectors of the canonical Wnt signaling pathway. [217] The vertebrate genomes harbor four different TCF/LEF genes, designated as *TCF7*, *TCF7L1*, *TCF7L2*, and *LEF1*, which encode for TCF1, TCF3, TCF4, and LEF1 proteins, respectively. [223] TCF/LEF transcription factors contain five conserved domains: N-terminal β-catenin

binding domain, Groucho binding sequence (GBS), HMG, basic tail functioning as nuclear localization signal, and C-clamp. [224] The β-catenin binding domain functions generally as a transactivation domain. [222] GBS is a binding site for transcriptional repressors of the Groucho family, which mediate gene repression preceding TCF/LEF transactivation. [218] The HMG domain, together with the basic tail, is responsible for the DNA binding activity of TCF/LEF transcription factors, [225], [226] recognizing the ACATCAAG sequence, also known as Wnt responsive element (WRE). [227], [228] Finally, the C-clamp, presented in TCF1 and TCF4, functions as an additional DNA binding domain specific for GC rich "Helper site" that can be present in close proximity to WRE, favoring binding of specific TCF1 and TCF4 factors. [229]

TCF/LEF factors work as molecular switches between repression and activation of Wnt-responsive genes (Figure 3). [219] In their repressive state, TCF/LEF factors suppress the expression of Wnt-responsive genes by interacting with Groucho transcriptional repressors. [218] In order to change their activity from repressive to active state and to induce expression of Wnt-responsive genes, TCF/LEF transcription factors need to interact with β-catenin. This interaction is facilitated by the β-catenin core groove and by highly conserved, structurally disordered N-terminal β-catenin binding domain of TCF/LEF transcription factors (Figure 4). [222], [230] The β-catenin-TCF/LEF interaction induces conformational changes of TCF/LEF factors that subsequently displace Groucho repressors from the GBS domain, [231] allowing association with general transcription factors and p300/CREB-binding protein (CBP) histone deacetylases which induce transcriptional activation of target genomic loci. [232]–[234]

Nonetheless, this is rather simplistic view obtained from invertebrates which contain only one TCF factor per genome. Genes coding for TCF/LEF transcription factors in vertebrates are, in contrast, equipped with numerous alternative promoters that enable intricate production of many alternatively spliced TCF/LEF isoforms with remarkably altered properties. [235]–[237] Not only that particular TCF/LEF isoforms have distinct functions on their own, but their final signaling activity is also regulated by their ratios and stoichiometry. For instance, enhanced production of shorter TCF4 isoform results in transcriptional de-repression of regulated regions. [238]

Moreover, a genome-wide ChIPseq analysis identified a whole set of loci enriched for β-catenin scattered across the genome that were not occupied by TCF/LEF factors, suggesting that β-catenin possess an additional TCF/LEF-independent transactivating function. [239] In particular, it was shown that β-catenin can interact and activate transcription through octamer-binding transcription factor (OCT4), hypoxia inducing factor 1-alpha (HIF1α), androgen receptor, liver receptor homologue 1 (LRH1), or forkhead box protein O1 (FOXO) transcription factors. [240]–[244]

1.6.1. The canonical Wnt signaling pathway in hematopoiesis

Several studies addressing the function of the canonical Wnt signaling pathway in the hematopoietic development have been conducted. However, its function in hematopoiesis remains controversial and no scientific consensus has been reached. [245] The majority of these studies targeted β-catenin or other upstream pathway components. Studies relying on β-catenin gain-of-function approaches demonstrated that constitutive activation of β-catenin has a deleterious effect on the entire murine hematopoietic system. Introduction of stable, constitutively active β-catenin resulted in erythroid, myeloid, and lymphoid differentiation arrest and loss of HSCs function. [246], [247] In line with this evidence, similar hematopoietic failure was observed also in APC-deficient mice whose β-catenin-destruction complex was permanently disintegrated. [248] Intriguingly, authors of another study using APC-deficient mice came to different conclusion. On one hand, Famili et al. observed, in agreement with aforementioned study, diminished HSC reconstituting ability. On the other hand, they found that stable β-catenin rather promoted myeloid differentiation instead of impairing it. [249]

Moreover, experiments using β-catenin loss-of-function approaches showed no major differentiation defects. For instance, blood tissue-specific β-catenin knock-out mice exhibited reduced ability of HSC to reconstitute hematopoiesis in recipient mice after transplantation, but did not cause any hematopoietic abnormalities in steady-state conditions in young adult mice. [250] In contrast to this observation, HSC harboring inducible double knock-out of β- and γ-catenin (γ-catenin can potentially compensate for β-catenin loss) showed no functional deficiency. [251] Additionally, the hematopoietic system of mice

whose secretion of Wnt ligands was genetically inhibited was indistinguishable from wild type control, suggesting that Wnt signaling is dispensable in the hematopoietic system. [252]

TCF/LEF transcription factors are in the context of the hematopoietic system, according to phenotypes of individual TCF/LEF deficient mice, most frequently described as important regulators of lymphoid development. [253], [254] Nevertheless, several studies suggested that TCF/LEF factors might be involved also in the regulation of granulocytic differentiation. For instance, Skokowa et al. proposed that LEF1 can directly regulate expression of CEBPα. [255] Additionally, enhanced secretion of Wnt ligands [256] and canonical Wnt pathway activation [184] were observed during hematopoietic stress.

Together, the conflicting evidence summarized in the previous paragraphs might be explained by several technical or biological limitations of the experimental approaches that were employed. First, β-catenin loss- and gain-of-function experimental approaches do not allow to distinguish between TCF/LEF-dependent and -independent β-catenin activity. Second, different level of Wnt activation is probably needed in different cell types and overactivation or complete loss of canonical Wnt signaling might result in artificial and unspecific effects. Third, deletion of TCF/LEF factors does not cause a specific loss of β-catenin-TCF-LEF-mediated transcription, but rather permanent de-repression of regulated regions. Thus, it is necessary to employ more specific models in order to investigate the function of the canonical Wnt signaling pathway in the hematopoietic system.

Thus, to elucidate the specific function of the canonical Wnt signaling pathway mediated by β-catenin-TCF/LEF signaling axis, it will be necessary to employ a model system inactivating specifically and exclusively this terminal part of the pathway; for instance by disrupting β-catenin-TCF/LEF interaction. Disintegration of the β-catenin-TCF/LEF transcription mediating complex can be achieved by multiple strategies. First, by small molecular inhibitors that bind to the β-catenin core groove and block its interaction with TCF/LEF transcription factors. [257]–[259] Second, by cell penetrating peptides derived from the N-terminal β-catenin binding domain of TCF/LEF transcription factors that compete with TCF/LEF for the interaction with β-catenin. [260] And third, by introducing a truncated, dominant negative form of TCF/LEF transcription factors lacking β-catenin

binding domain. These truncated TCF/LEF factors occupy WRE but as they can not interact with β-catenin, they prevent β-catenin-mediated transcription activation. [261], [262] Since the last option allow us to investigate effects of canonical Wnt signaling *in vivo* without any invasive insults, in this thesis we decided to investigate the function of β-catenin-TCF/LEF transcription mediating complex in the hematopoietic system with the use of this approach.

2. OBJECTIVES

The overall goal of this thesis is to characterize novel molecular mechanisms that might be involved in the regulation of neutrophil differentiation during physiological conditions and hematopoietic stress. We will focus on three distinct, but inevitably interrelated, topics divided into three aims. Importantly, data from each aim were published as scientific articles in peer reviewed journals; these publications can be found attached to this work.

Aim 1 – The role of the β-catenin-TCF/LEF complex during neutrophilic differentiation in both steady-state and emergency granulopoiesis and its importance for the biology of hematopoietic stem and progenitor cells.

The canonical Wnt signaling pathway, mediated by the β-catenin-TCF/LEF complex, is crucial for maintenance of many types of somatic stem cells and proper tissue architecture. In the hematopoietic system, however, the function of this pathway remains obscured. Here, we will determine what is the specific function of β-catenin-TCF/LEF-mediated transcription in steady-state and emergency granulopoiesis. In particular, we will decipher whether and how this pathway affects distinct populations of hematopoietic stem and progenitor cells, their stemness, proliferation, and ability to differentiate into neutrophils. We will additionally investigate how the canonical Wnt signaling regulates neutrophilic differentiation during systemic infection, and how it affects the ability of the organism to fight the infection. Finally, we will determine whether our conclusions, derived mainly from murine models, are relevant in human primary cells.

Aim 2 – To elucidate the role of the transcription factor C/EBPγ on differentiation of neutrophils during steady-state and emergency granulopoiesis.

The transcription factors of the C/EBP family are crucial and essential regulators of neutrophilic development. While C/EBPα and C/EBPβ are important for the differentiation of granulocytic progenitors, other members of the family, such as C/EBPε, are employed at later stages of neutrophil maturation. Interestingly, little is known about C/EBPγ and its function in steady-state or emergency granulopoiesis. Therefore, we will generate a

conditional KO mouse model and assess how C/EBPγ deficiency affects the murine hematopoietic system, its ability to maintain hemostasis, the potential to produce functional neutrophils, and the capacity to clear pathogens.

Aim 3 – To investigate how C/EBPα regulates expression of *EVI2B*, and determine the function of this transmembrane protein during granulocyte differentiation.

EVI2B is a transmembrane glycoprotein with largely uncharacterized function. Recently, *EVI2B* was identified as a gene directly upregulated by an essential regulator of neutrophil development, the transcription factor C/EBPα. Additionally, EVI2B was previously associated with the differentiation of keratinocytes, melanocytes, and leukemia. Together with high EVI2B expression in granulocytes, these lines of evidence allow us to hypothesize that EVI2B is involved in the regulation of granulocytic differentiation. To investigate this possibility, we will use *Evi2b* knock-down in cell lines, murine and human primary cells, and *Evi2b* knock-out mouse strain and show how *Evi2b* deficiency affects functionality of HSPC and myeloid development.

3. RESULTS AND DISCUSSION

The overall goal of this thesis is to identify novel molecular mechanisms regulating production of neutrophils, critically important cells of the innate immune system. Three distinct potential regulatory mechanisms were investigated in the scope of this thesis. These include: the β-catenin-TCF/LEF transcription-mediating complex, the transcription factor C/EBPγ, and the transmembrane glycoprotein *EVI2B*.

3.1. Aim 1 – The role of the β-catenin-TCF/LEF complex during neutrophilic differentiation in both steady-state and emergency granulopoiesis and its importance for the biology of hematopoietic stem and progenitor cells.

Here, I present the main results of the thesis. They were compiled in a publication entitled "β-catenin-TCF/LEF signaling promotes steady-state and emergency granulopoiesis via G-CSF receptor upregulation". I am the first author of this publication and I contributed with the experimental design, performing most of the experiments, data analysis and interpretation, figure design, and writing the manuscript. For additional experimental details, please refer to the manuscript included in Chapter 7.

3.1.1. Description and validation of hematopoietic-specific dnTCF4 transgenic mice

The choice of an appropriate model system for the investigation of canonical Wnt signaling, mediated by β-catenin-TCF/LEF interaction in hematopoiesis, is not trivial. By definition, TCF/LEF transcription factors are the sole effectors of canonical Wnt signaling. [217] However, β-catenin harbors both TCF/LEF-dependent and -independent activities and is involved in various biological processes. [240]–[244] Thus, a model system allowing specific inactivation of β-catenin-TCF/LEF mediated gene expression is needed. Genetic manipulation of β-catenin is not suitable, as it would debilitate all β-catenin activities. Knock-out of genes coding for TCF/LEF factors is not appropriate either. First, because TCF/LEF factors display high degree of functional redundancy, the construction of quadruple KO mouse would be necessary. Second, this complete loss of TCF/LEF factors

would cause genome-wide de-repression of TCF/LEF-regulated regions. Third, loss of all TCF/LEF factors would disturb their β-catenin-independent activity as well.

Therefore, we employed a previously published [261], [262] approach of overexpressing a truncated dominant negative form of human TCF4 (dnTCF4) that lacks the N-terminal β-catenin binding domain. When expressed, dnTCF4 occupies WRE in the regulatory regions of Wnt-responsive genes and since it can not bind β-catenin, it constantly keeps these regions in a transcriptionally repressed state.

To generate dnTCF4 mice repressing TCF/LEF transcription in the hematopoietic system, we employed the following strategy. As described by Janeckova et al., [262] the *dnTCF4* cassette, harboring floxed *tdTomato* (followed by transcriptional blocker) and *dnTCF4-EGFP* fusion gene, was inserted into murine *Rosa26* allele. In this system, dnTCF4 can be expressed only after Cre-mediated excision of the transcriptional blocker. The excision is marked by the switch from tdTomato to EGFP expression in Cre^+ cells. We have crossed dnTCF4 mouse to a Vav-iCre mouse strain, expressing Cre recombinase from the hematopoietic-specific *Vav-1* promoter.

In this thesis, we used three different genotypes of experimental animals. One, expressing dnTCF4 ($Rosa26^{dnTCF4}$ $Vav\text{-}iCre^+$, referred as dnTCF4 or dn), and two types of controls lacking dnTCF4 expression. The first control had Cre recombinase along with wild type *Rosa26* allele ($Rosa26^{wt}$ $Vav\text{-}iCre^+$, referred as WT). The second control contained the *dnTCF4* cassette while lacking Cre recombinase ($Rosa26^{dnTCF4}$ $Vav\text{-}iCre^-$, referred as WT^T for its tdTomato expression). Importantly, we demonstrated that *dnTCF4* is expressed in a gene-dose dependent manner. While no *dnTCF4* transcript or protein was detected in WT animals, homozygotes harboring two alleles of *dnTCF4* displayed twice as high *dnTCF4* expression as heterozygotes with one wild-type $Rosa26^{wt}$ and one $Rosa26^{dnTCF4}$ allele. EGFP or tdTomato expression levels detected by flow cytometry were in agreement with the expected genotype and dnTCF4 expression. To test whether dnTCF4 presence represses transcription of Wnt responsive genes in our system, we treated primary murine myeloid progenitors (defined by expression of c-kit surface marker, referred as c-kit^+) with a Wnt activator (inhibitor of GSK3β - CHIR99021) and measured the expression of the Wnt-responsive genes *Axin2* and *Nkd1*. As expected, dnTCF4 cells did not respond to the

treatment, while expression of Wnt responsive genes in WT^T cells was elevated. Thus, we validated that dnTCF4 transgenic mice are functional and suitable for subsequent phenotypic analysis.

3.1.2. β-catenin-TCF/LEF signaling promotes neutrophil differentiation

First, we have analyzed peripheral blood (PB) and bone marrow (BM) of WT and dnTCF4 mice by flow cytometry. dnTCF4 mice showed reduced frequency of neutrophils (defined as $CD11b^+$ $Ly6G^+$ $Ly6C^+$) when compared to WT (27.5 % vs 52.5 %, respectively), while frequencies of other blood cells were not altered. This observation was additionally confirmed by establishing neutrophil cell counts in PB with the use of a veterinary hematological analyzer; neutrophil cell count for dnTCF4 mice was 0.72×10^9/L while WT contained 1.05×10^9/L of PB neutrophils. Accordingly, we observed reduced neutrophil frequency and counts also in the BM of dnTCF4 mice. In contrast, frequencies and numbers of B- and T-cells were similar in WT and dnTCF4 animals. This might be surprising as TCF/LEF factors are necessary for proper lymphocyte development, [253], [254] however, our data are in line with the evidence showing that it is rather the repressive TCF/LEF activity that needs to be present during lymphocyte development. [263], [264]

In depth analysis of the immature cell compartment within the BM discovered significantly increased numbers of ST-HSC (Lin^-, $c\text{-}kit^+$, $Sca\text{-}1^+$, $CD48^+$, $CD150^-$), LKS (Lin^-, $c\text{-}kit^+$, $Sca\text{-}1^+$), $c\text{-}kit^+$(Lin^-, $c\text{-}kit^+$), MEP (Lin^-, $c\text{-}kit^+$, $Sca\text{-}1^-$, $CD34^-$, $CD16/32^-$), CMP (Lin^-, $c\text{-}kit^+$, $Sca\text{-}1^-$, $CD34^+$, $CD16/32^-$), and GMP (Lin^-, $c\text{-}kit^+$, $Sca\text{-}1^-$, $CD34^+$, $CD16/32^+$) populations in dnTCF4 mice. Previous studies [250], [265] showed the importance of β-catenin-TCF/LEF signaling for various type of stem cells, including HSC. To our surprise, we did not observe any changes in the abundance of phenotypically defined LT-HSC (Lin^-, $c\text{-}kit^+$, $Sca\text{-}1^+$, $CD48^-$, $CD150^+$) in dnTCF4 mice. Deficiency of mature neutrophils accompanied by accumulation of myeloid progenitors therefore implies that inhibition of β-catenin-TCF/LEF mediated transcription causes partial block of neutrophilic differentiation. Accordingly, a flow cytometry panel designed by Satake et al. [183] that allows to discriminate different stages of neutrophilic development revealed accumulation of cells in myeloblast and promyelocyte stages in the dnTCF4 bone marrow. However, to rule out the

possibility that expansion of myeloid precursors in the BM of dnTCF4 animals is not caused by their enhanced proliferation, we performed *in vivo* BrdU (bromodeoxyuridine) incorporation assays and *in vitro* cell cycle analysis by pyronin Y/Hoechst flow cytometry staining. Percentages of $BrdU^+$ LT-HSC, ST-HSC, and c-kit^+ cells were comparable in WT and dnTCF4 BM as well as proportions of WT and dnTCF4 c-kit^+ cells in G_0, G_1, and $S/G_2/M$ phases, demonstrating that proliferation of HSPC is not affected by inhibition of β-catenin-TCF/LEF signaling.

The ability of WT and dnTCF4 progenitors to differentiate was further assessed by colony forming assays. In this assay, the number of functional progenitors is reflected by the number of present colonies (progenitors are therefore evaluated as colony forming units – CFU). Colonies can be harvested every 7-10 days, re-plated, and cellular composition of the culture can be evaluated by assessing cellular morphology (May-Grünwald Giemsa staining) and by flow cytometry. Consistently to our previous results, the dnTCF4 BM formed significantly more colonies compared to WT. WT cells also differentiated mostly to mature neutrophils during 2^{nd} plating, while dnTCF4 culture retained rather an immature phenotype with enhanced expression of elastase and cathepsins G, markers expressed typically in myeloblasts and promyelocytes. [266] Finally, WT cells did not form any colonies during 3^{rd} plating (as all immature cell were already differentiated), whereas dnTCF4 cells showed differentiation towards macrophages, but not granulocytes.

Together, these results demonstrate that inhibition of β-catenin-TCF/LEF mediated transcription by dnTCF4 causes a partial block of neutrophilic differentiation reflected by accumulation of myeloid progenitors and mild neutropenia. In other words, our data show that β-catenin-TCF/LEF signaling promotes neutrophilic differentiation of myeloid progenitors. Consistently, it was previously shown that expression of LEF1 is dramatically reduced in patients suffering from congenital neutropenia. It is interesting that these patients, coherently with our data, displayed a differentiation arrest in the promyelocytic stage. On the other hand, LEF1 decrement was also associated with decreased proliferation and enhanced apoptosis of HSPC, which we did not observe in dnTCF4 mice. [255] Nonetheless, it is important to mention that the vast decrement of LEF1 expression might lead to TCF/LEF isoform imbalance, unspecific de-repression of LEF1-controlled regions, [238], [267], [268],

and loss of β-catenin-independent activity of LEF1. [269] Specific disruption of the β-catenin-TCF/LEF complex (e.g. by dnTCF4) might therefore inflict a less severe phenotype. Additionally, it is important to take into account that the necessity of intact β-catenin-TCF/LEF signaling for the development of neutrophils might differ in humans and mice.

3.1.3. β-catenin-TCF/LEF is dispensable for proper function of HSC

As mentioned in the previous chapter, the frequency of LT-HSC assessed by flow cytometry was comparable in dnTCF4 and WT mice. However, presence of cells with expression of surface markers typical for LT-HSC does not necessarily reflect their functionality and "stemness". Therefore, we performed a series of transplantation experiments to assess functional properties of dnTCF4 expressing HSC.

First, we sorted LT-HSC from WT and dnTCF4 animals (Ly5.2) and transplanted distinct doses (10, 20, or 40 LT-HSC) into lethally irradiated congenic (Ly5.1) recipients together with the supporting BM (Ly5.1). Four months later, we assessed the number of animals which were successfully reconstituted with cells of Ly5.2 origin. Subsequently, we used a poison distribution statistics algorithm [270] to quantify numbers of functional HSC within the phenotypically defined LT-HSC compartment. WT contained 1 functional HSC per 29.7 LT-HSC and dnTCF4 1 HSC per 25.2 LT-HSC ($p = 0.688$, $chisq = 0.168$), suggesting that loss of the β-catenin-TCF/LEF-mediated transcription does not compromise functionality of LT-HSC. However, cell sorting is dependent on the expression of cell surface markers. To overcome this issue, we transplanted whole BM from WT and dnTCF4 mice (Ly5.2) into lethally irradiated congenic recipients (Ly5.1) in a competitive manner. Frequency of donor derived (Ly5.2) WT and dnTCF4 cells was similar 16 weeks after transplantation, when contribution of LT-HSC to hematopoiesis should be visible. Interestingly, we observed transiently enhanced engraftment of dnTCF4 BM 1 month after transplantation, reflecting elevated numbers of progenitors in the dnTCF4 BM detected previously by flow cytometry.

Taken together, we can conclude that inhibition of β-catenin-TCF/LEF-mediated transcription is dispensable for proper function of HSC. This conclusion is in contrast with a previously published study by Zhao et al., where loss of β-catenin functionally impaired LT-

HSC activity and reduced their engraftment after transplantation. [250] In their study, β-catenin was ablated from embryonic day 13.5 further, suggesting it might be important for the function of fetal HSC. [271], [272] Thus, functional impairment of adult LT-HSC observed by Zhao et al. could be a reminiscent effect of their aberrant embryonic development. Since dnTCF4 is in our model also expressed from embryonic day 13.5 in, it is probable that fetal HSC require rather TCF/LEF-independent β-catenin activity in order to functionate properly. Our conclusions are additionally supported by the fact that inducible β-catenin KO mice showed no functional deterioration of LT-HSCs in adult mice. [251]

3.1.4. β-catenin-TCF/LEF signaling directly upregulates G-CSF-R in HSPC

In order to decipher mechanisms through which β-catenin-TCF/LEF signaling promotes neutrophil differentiation, we performed a transcriptomic analysis of WT and dnTCF4 ST-HSC, the earliest population during hematopoietic development affected by the presence of dnTCF4. Using RNAseq, we identified 743 downregulated and 754 upregulated genes (log2 fold change > 0.5, $p < 0.05$) in dnTCF4 ST-HSC compared to WT ST-HSC. As dnTCF4 acts primarily as a transcriptional repressor, we focused on the list of downregulated genes. Several genes involved in myeloid development were downregulated in dnTCF4 ST-HSC (e.g. *Csf3r*, *Irf4, Il1r1,* or *Il18r1*). Interestingly, the list of differentially expressed genes in dnTCF4 ST-HSC was significantly ($q < 10^{-8}$) enriched for genes deregulated in murine C/EBPα deficient progenitors (GSE61468), further supporting our hypothesis that β-catenin-TCF/LEF signaling promotes neutrophilic differentiation. A particularly interesting gene that was downregulated in dnTCF4 is *Csf3r*, a gene encoding a crucial regulator of neutrophil development, G-CSF-R.

First we confirmed *Csf3r* downregulation (3.5 % of *Gapdh* in WT vs. 1.9 % of *Gapdh* in dnTCF4) on mRNA level in c-kit$^+$ by RT-qPCR. To ascertain whether *Csf3r* transcript reduction translates also into reduced expression of G-CSF-R on the surface of the cells, we treated c-kit$^+$ and LKS populations with biotinylated G-CSF, stained them with streptavidin-PE conjugate, and analysed its abundance at the cell surface by flow cytometry. Indeed, dnTCF4 LKS and c-kit$^+$ cells diplayed significantly reduced G-CSF-R levels compared to WT. Importantly, when stimulated by G-CSF, dnTCF4 c-kit$^+$ and LKS populations exhibited

an attentuation of downstream [273] Stat3 phosphorylation without any alteration in Stat5 phosphorylation levels. Since Stat3 phosphorylation promotes differentiation of HSPC [274] while Stat5 phosphorylation induces cell cycle progression, [275] this result is in agreement with our previous observation that dnTCF4 presence causes block of neutrophilic differentiation but has negligible effect on the proliferation rate of HSPC. To finally proove that these G-CSF-R expression changes do have a biological consequence, we subjected WT and dnTCF4 BM to colony forming assays containing only two cytokines: G-CSF (supporting differentiation of neutrophils) and stem cell factor (SCF; supporting survival of HSPC). dnTCF4 BM formed more colonies (34 CFU in WT vs. 60 CFU in dnTCF4), contained more c-kit^{+} cells (2 % in WT vs 10 % in dnTCF4), and less neutrophils (55 % in WT vs. 30 % in dnTCF4), demonstrating dnTCF4 HSPC display diminished G-CSF-induced differentiation.

Next, we asked whether the β-catenin-TCF/LEF signaling pathway regulates G-CSF-R levels directly. We analyzed publicly available ChIPseq data showing genomic occupancy of TCF4 in various human cell lines (HEK293, HepG2, MCF7, PANC, and HeLa; available under following accession numbers: ENCSR000EUY, ENCSR000EVQ, ENCSR000EWT, ENCSR000EXL, ENCSR000EVF, and ENCSR000AOF, respectively). TCF4 was enriched in *Csf3r* transcription start site (TSS) and putative enhancer located -3.5 kb from TSS. Because non of these cell lines is of the hematopoietic origin, we performed ChIP-qPCR assessing enrichment of LEF1 in *Csf3r* TSS and putative enhancer (-3.5 kb) in K562 cells. Of note, LEF1 was selected as a representative member of the whole TCF/LEF family as it is highly expressed in K562 cells and there is an available ChIP-grade antibody against it. Indeed, we detected LEF1 enrichment in both TSS and putative enhancer, but not in control region (located -6.1 kb from TSS) or no-antibody control. These results suggest that β-catenin-TCF/LEF signaling pathway directly regulates G-CSF-R expression on transcriptional level in hematopoietic cells.

Together, we demonstrated that the β-catenin-TCF/LEF complex directly regulates expression of G-CSF-R through TCF/LEF trancription factors that are present in *Csf3r* regulatory regions. Inactivation of the pathway by dnTCF4 resulted in both, reduction of *Csf3r* transcript and G-CSF-R surface protein levels in HSPC. Further, decrease of G-CSF-

R on HSPC provoked attentuation of downstream Stat3 signaling and, consequently, reduced response to G-CSF.

Our conclusions are strongly supported by scientific literature. Remarkably, the populations of neutrophils in mice lacking G-CSF or G-CSF-R are reduced 2-10 times, but are not eliminated completely. [97], [98] These residual neutrophils are probably produced by yet unidentified compensatory mechanism. [101] Therefore, a decrease of G-CSF-R expression is consistent with the rather mild reduction of neutrophils that we observed in dnTCF4 mice. Interestingly, inhibition of β-catenin-TCF/LEF signaling reduced G-CSF-R levels in the very immature population of LKS cells. The fact that cytokines (namely G-CSF and M-CSF) are able to impose distinct lineage comittment to HSPC and affect the whole course of blood cell production [90], [102] allowed us to speculate that the β-catenin-TCF/LEF-mediated G-CSF-R upregulation imposes neutrophilic commitment to LKS cells. Additionally, Skokowa et al. reported that LEF1 acts dowstream from G-CSF-R during neutrophil development. [276] Thus, our data together with this observaton suggest an existence of a previously uknown positive feedback loop between TCF/LEF factors and G-CSF-R.

3.1.5. β-catenin-TCF/LEF signaling is essential for emergency granulopoiesis

G-CSF is an important mediator of emergency granulopoiesis that promotes egress of neutrophils from BM to PB and induces augmented myeloid differentiation. The enhanced differentiation is mediated by stimulation of G-CSF-R, subsequent downstream STAT3 phosphorylation, and expression of C/EBPβ transcription factor in HSPC. [170], [174], [180] Taking into account our finding that β-catenin-TCF/LEF signaling regulates expression of G-CSF-R, we next elucidated whether this transcriptional complex plays a role during emergency granulopoiesis. To simulate a systemic infection, we intravenously injected 35 μg of bacterial lipopolysaccharide (LPS) or PBS twice into WT and dnTCF4 animals and analyzed their response to the stimulus 72 hours later (as described in [174]). Both mature ($Ly6G^{hi}$, Figure 1B) and immature ($Ly6G^{lo}$, Figure 1B) neutrophils egressed BM in a comparable way in WT and dnTCF4 mice, suggesting that dnTCF4 neutrophils are not functionally impaired. Indeed, ROS production, migration, and phagocytosis of dnTCF4

neutrophils were comparable to WT. However, we observed a decline in c-kit$^+$ myeloid progenitors and immature neutrophils in the BM of dnTCF4 animals compared to WT. Moreover, dnTCF4 animals failed to skew the ratio of myeloid progenitors towards the GMP population, which was defined as a hallmark of emergency granulopoiesis, [174] and which was accordingly present in WT mice.

This observation suggests that neutrophilic commitment imposed to HSPC by β-catenin-TCF/LEF-G-CSF-R signaling axis is critical for massive *de novo* production of neutrophils during emergency granulopoiesis. This hypothesis is supported by work of Hérault et al., showing β-catenin activation in proliferating GMPs during chemically induced emergency granulopoiesis. [184] Consistently with this study, dnTCF4 mice injected with myeloablative agent 5-fluorouracil showed insufficient myeloid recovery compared to WT animals. Moreover, repetitive administration of the drug and resulting hematopoietic stress was fatal for dnTCF4 mice while all WT animals were able to recover.

Based on our observation, we hypothesized that emergency granulopoiesis in mice with inactive β-catenin-TCF/LEF signaling should not be sustainable during continuous infectious events. Consequently, continuous and progressive sub-optimal production of neutrophils would compromise effective pathogen clearance and would inevitably result in higher susceptibility of dnTCF4 mice to infection. To test our hypothesis, we injected WT and dnTCF4 animals with a low dose of *Candida albicans* and assessed their survival. Despite neutrophils of dnTCF4 mice were functional, dnTCF4 mice displayed increased sensitivity to *Candida albicans* and significantly higher mortality rate compared to WT, confirming further our hypothesis.

3.1.6. β-catenin-TCF/LEF-mediated transcription is essential for differentiation of human neutrophils

Next we investigated whether β-catenin-TCF/LEF mediated transcription is crucial also for the differentiation of human neutrophils. We isolated primary human CD34$^+$ cells from frozen cord blood samples, transduced them with either dnTCF4-containing or control empty MSCV retroviral vector, and subjected them to *in vitro* differentiation as described by Jie et al. [91] Expression of neutrophil markers was assessed before, and after 15 days of

differentiation by flow cytometry. Cells transduced with dnTCF4 showed reduced expression of CD11b, CD18, and CD66b neutrophilic markers compared to empty vector control at differentiation day 15. To demonstrate that the observed block of differentiation is not just an artifact of our genetic model, we differentiated primary cord blood $CD34^+$ cells into neutrophils in the presence of cercosporin, a small molecule inhibitor of β-catenin-TCF/LEF interaction, or vehicle control (DMSO). Consistently, cercosporin-treated cells showed reduced expression of CD11b, CD15, CD16, and CD66b neutrophilic markers when compared to vehicle-treated cells, demonstrating that both genetic and pharmacological inactivation of β-catenin-TCF/LEF mediated transcription impairs proper differentiation of human neutrophils in cell cultures assays. Finally, primary cord blood $CD34^+$ cells cultivated in colony formation assays treated with Wnt activators BIO or CHIR99021 harbored increased CD11b expression when compared to DMSO treated cells, suggesting that activation of β-catenin-TCF/LEF signaling can also enhance differentiation of human neutrophils in culture. Together, our result suggest that β-catenin-TCF/LEF signaling promotes differentiation of human HSPCs into neutrophils. Our conclusion is additionally supported by several studies that associate the absence of nuclear LEF1, a member of the TCF/LEF family of transcription factors, with reduced neutrophilic differentiation in congenital neutropenia patients. [255], [276], [277]

Taken together, we have employed a novel murine model that allowed us to specifically inhibit β-catenin-TCF/LEF mediated transcription by overexpressing *dnTCF4* transgene. These mice exhibited mild neutropenia accompanied by accumulation of HSPC in their bone marrow. In following colony forming assays we demonstrated that dnTCF4 HSPC tend to retain their immature phenotype and possess partial block of neutrophilic differentiation. Subsequent transcriptomic and ChIP analyses revealed that β-catenin-TCF/LEF signaling directly regulates expression of *Csf3r*, a gene coding the potent regulator of neutrophil production, G-CSF-R, in HSPC. Importantly, dnTCF4-mediated downregulation of G-CSF-R on the surface HSPC resulted in attenuation of downstream signaling and reduced biological response to G-CSF stimuli. Consistently with the fact that G-CSF-R is important player during emergency granulopoiesis, our results suggest that β-catenin-TCF/LEF mediated G-CSF-R upregulation is necessary for the continuous massive

de novo production of neutrophils during emergency granulopoiesis. In other words, β-catenin-TCF/LEF signaling promotes neutrophilic differentiation of HSPC via direct upregulation of G-CSF-R, which seems to be especially crucial in the conditions of increased hematopoietic need, or during systemic infection.

3.2. Aim 2 – To elucidate the role of the transcription factor C/EBPγ on differentiation of neutrophils during steady-state and emergency granulopoiesis

In this aim, I contributed to design and performance of the experiments described in paragraphs 3.2.1. and 3.2.2. To place my contribution into scientific context, I present the whole project in paragraphs 3.2.1. - 3.2.4. Experimental details and extended results can be found in the manuscript entitled "C/EBPγ is dispensable for steady-state and emergency granulopoiesis", attached in Chapter 7.

3.2.1. Generation and validation of hematopoietic-specific *Cebpg* knock-out mice

To investigate the function of a rather unexplored transcription factor of the C/EBP family, C/EBPγ, in granulocyte development, we have constructed a tissue specific *Cebpg* KO mouse strain. A targeting vector, containing tdTomato reporter, neomycin resistance gene, and the whole *Cebpg* coding sequence flanked by LoxP sites, was inserted into C57Bl/6NCrl by homologous recombination. The resulting *Cebpg*$^{fl/fl}$ mice were crossed to a mouse strain expressing Cre recombinase from the hematopoietic tissue-specific Vav-1 promoter (*Vav-iCre* mouse), providing 2 types of progeny. First, *Cebpg*$^{fl/fl}$ *Vav-iCre*$^{+}$ (referred as KO) and second, *Cebpg*$^{fl/fl}$ *Vav-iCre*$^{-}$ (referred as WT). Excision of *Cebpg* was validated using PCR. Importantly, *Cebpg* transcript (assessed by RT-qPCR) or protein (assessed by western-blotting) was not detected in KO cells.

3.2.2. C/EBPγ is dispensable for steady-state granulopoiesis

First, we analyzed the BM composition of WT and KO animals. Surprisingly, both absolute numbers and frequencies of mature neutrophils (CD11b^{+}, Ly6G^{+}), B-cells (B220^{+}), and T-cells (CD3^{+}) were comparable in WT and *Cebpg* KO mice. In order to investigate different stages of neutrophil development in greater detail, we segregated SSC^{int}, CD3^{-}, CD19^{-}, B220^{-}, Ter119^{-} cell population into 5 distinct developmental stages based on their surface expression of c-kit and Ly6G markers as described by Satake et al. [183] Nonetheless, the process of neutrophilic development was comparable in WT and *Cebpg* deficient mice. The most immature BM compartment, including LKS, c-kit^{+}, CMP, MEP,

and GMP populations, was not altered in *Cebpg*-deficient mice neither. Following colony forming assays consistently showed no functional alteration of *Cebpg* KO BM cells compared to WT, suggesting C/EBPγ is not involved in myeloid development in steady-state conditions. Importantly, WT and *Cebpg* KO mice were also monitored during the course of ageing, but we did not observe any age-related or progressive phenotypes.

Alberich-Jorda et al. described *Cebpg* as a negative regulator of granulocytic differentiation that is upregulated in C/EBPα deficient mice and whose knock-down was sufficient to restore arrested granulopoiesis in this model. [195] Based on this study, we originally anticipated that *Cebpg* loss would alter HSPC populations and enhance differentiation of granulocytes. The unaltered hemostasis of our *Cebpg* KO mice is therefore surprising, however, this discrepancy can be explained by different experimental approaches that were applied. Alberich-Jorda et al. showed that *Cebpg* blocks granulocytic differentiation solely in immortalized cell lines and primary leukemic cells harboring overexpression of C/EBPγ. It is therefore possible that C/EBPγ overexpression contributes to the granulocytic differentiation arrest in leukemic cells, while its endogenous levels are dispensable for healthy unperturbed granulopoiesis.

Nevertheless, not all members of the C/EBP family are involved in differentiation *per se.* While C/EBPβ and C/EBPδ are employed rather during stress conditions of emergency granulopoiesis, [78], [170], [185] C/EBPε is for example essential for the functionality and proper composition of neutrophil granules. [278] To test whether C/EBPγ loss affects these processes, we assessed morphology and granule content of WT and *Cebpg* KO granulocytes. Nonetheless, the cell morphology and expression of *Mmp9*, cathepsins G, lactoferrin, neutrophil elastase 2 (*Ela2*), and myeloperoxidase (*Mpo*) was comparable in WT and *Cebpg* KO granulocytes. Taken together, our data document that C/EBPγ is dispensable for proper granulocytic differentiation and maturation in steady-state conditions.

3.2.3. C/EBPγ is dispensable for emergency granulopoiesis

As discussed in the previous paragraph, C/EBPβ and C/EBPδ are critical during emergency granulopoiesis, but their contribution to steady-state hematopoiesis is rather insignificant. [78], [170], [185] Interestingly, we have detected C/EBPγ-C/EBPβ

heterodimers present in nuclear extracts from murine BM and spleen by electrophoretic mobility shift assay, suggesting C/EBPγ might regulate emergency granulopoiesis by modulating C/EBPβ activity. Therefore, we decided to investigate the function of C/EBPγ during situations of enhanced granulocytic need. To simulate a systemic bacterial infection, we repeatedly injected WT and *Cebpg* KO mice with LPS or PBS as a control. [174] The response to the LPS administration was, however, comparable in WT and *Cebpg* KO animals.

During emergency granulopoiesis, endothelial cells from the vascular tissue detect LPS though TLR4 and respond by G-CSF secretion into the blood stream, mediating egress of granulocytes from BM to PB and induction of C/EBPβ expression in myeloid progenitors. [170], [172], [174], [183] Since LPS simulates only the infection by gram negative bacteria, we additionally stimulated the emergency granulopoietic program in WT and *Cebpg* KO mice by direct intravenous injection of general inducer of emergency granulopoiesis - G-CSF. [170], [174] G-CSF administration decreased BM cellularity (by granulocyte egress) and markedly elevated numbers of immature granulocytes ($CD11b^{+}$ $Gr1^{lo}$) as a result of their enhanced *de novo* production. However, these changes were indistinguishable in WT and *Cebpg* KO mice.

In order to challenge the hematopoietic system of C/EBPγ deficient mice with a more physiologically relevant stimulus, we induced acute systemic candidemia in WT and *Cebpg* KO mice. Interestingly, *Cebpg* KO displayed slightly enhanced numbers of PB granulocytes in comparison to WT after *Candida albicans* infection but the biological relevance of this observation is undermined by two facts. First, other measured parameters (neutrophil counts in different developmental stages, BM cellularity, and numbers of MEP, CMP, and GMP progenitors) were comparable between WT and *Cebpg* KO mice. Second, kidney burden of infected animals and their overall survival of candidemia was similar in WT and *Cebpg* KO mice. Thus, our thorough analysis demonstrated that C/EBPγ is probably not involved in the regulation of emergency granulopoiesis in mice.

3.2.4. C/EBPγ does not regulate functionality of HSC

On one hand, our results demonstrated that the absence of C/EBPγ does not impair the development and maturation of granulocytes. On the other hand, *Cebpg* expression culminates in immature LKS cells and is gradually diminished as these cells differentiate into granulocytes. [195] It is therefore possible that C/EBPγ might be important for the maintenance and functionality of HSC.

To assess the functionality of *Cebpg* deficient HSC, limiting dilution transplantation assays were performed. We sorted 50, 100, 150, 200, and 1000 LT-HSC from WT and *Cebpg* KO mice (both of Ly5.2 genetic background), transplanted them into lethally irradiated congenic (Ly5.1) recipients, and analyzed the number of repopulated individuals 4 months after the transplantation. Surprisingly, the algorithm using poison distribution statistics [270] did not discover any difference in the functionality of WT and *Cebpg* KO HSC. Contribution of WT and *Cebpg* KO donor cells to different hematopoietic lineages was also comparable. Moreover, transcriptomic analysis of WT and *Cebpg* KO LT-HSC discovered only minor changes in gene expression. Considering these results, we concluded that C/EBPγ is dispensable for the functionality of HSC. Nevertheless, based on this experiment we can not rule out the possibility that C/EBPγ might play a role in HSC self-renewal and maintenance. In order to investigate the function of C/EBPγ in this process, secondary transplantation assays with WT and *Cebpg* KO donor BM need to be carried out in the future.

Taken together, using a tissue specific murine model of C/EBPγ deficiency, we showed that C/EBPγ is dispensable for differentiation and maturation of granulocytes in both steady-state and emergency granulopoiesis induced by LPS, G-CSF, or *Candida albicans*. Additionally, we showed that despite its relatively high expression in LKS cells, C/EBPγ does not affect the functionality, differentiation capacity, or lineage commitment of HSC. Therefore, we did not confirm our previously anticipated [195] function of C/EBPγ in hematopoiesis. Nonetheless, we can not exclude that a potential hematopoietic phenotype of *Cebpg* deficient mice might be masked by an unknown compensatory mechanism. The structural and sequential similarity of the bZIP domains of C/EBP factors and their ability to bind the same binding sequence suggest that there is a certain level of functional redundancy

within the C/EBP protein family members. [149] It is therefore possible that another C/EBP transcription factor might compensate for *Cebpg* loss in our murine model.

3.3. Aim 3 – To investigate how C/EBPα regulates expression of *EVI2B*, and determine the function of this transmembrane protein during granulocyte differentiation.

In this section, I have contributed by designing and performing experiments described in sections 3.3.1. and 3.3.2. Experimental details and extended results can be found in two manuscripts attached to this thesis in Chapter 7. I have contributed as a coauthor to the first manuscript entitled "EVI2B is a C/EBPα target gene required for granulocytic differentiation and functionality of hematopoietic progenitors" and as a shared first author to the second manuscript entitled "Proliferation and Differentiation of Murine Myeloid Precursor 32D/G-CSF-R Cells". In both manuscripts I contributed by performing experiments, analyzing data, figure design, and writing the manuscript.

3.3.1. EVI2B expression is directly induced by the myeloid master regulator C/EBPα

C/EBPα is a well-studied driver of early neutrophilic development. Malfunction or loss of C/EBPα activity is associated with block of neutrophil differentiation [150]–[153] and consequent leukemogenesis. [158]–[160] In search for C/EBPα-driven transcriptomic changes, microarray and ChIPseq analyses identified 33 genes that are directly upregulated by C/EBPα. *EVI2B*, almost a completely uncharacterized gene, was among them. [162] To validate this observation, we employed the K562 cell line overexpressing full-length C/EBPα fused to estrogen receptor (p42 C/EBPα-ER), truncated non-functional isoform of C/EBPα fused to estrogen receptor (p30 C/EBPα-ER), or estrogen receptor alone (ER). Treatment with β-estradiol, triggered nuclear translocation of ER-fused proteins and induced expression of EVI2B on both mRNA and protein levels in p42 C/EBPα-ER expressing cells. Truncated p30 C/EBPα-ER or ER alone were not able to induce EVI2B expression. We performed ChIP-qPCR on the same cell lines and demonstrated p42 C/EBPα-ER enrichment in *EVI2B* promoter region upon β-estradiol treatment. Luciferase assays additionally confirmed that p42 C/EBPα-ER is able to activate luciferase expression from a reporter vector containing *EVI2B* promoter, while p30 C/EBPα-ER is not.

Taken together, these results demonstrate that EVI2B is directly upregulated by functional full-length C/EBPα binding to its promoter. This allowed us to hypothesize that *EVI2B*, as a C/EBPα target gene, might be important for the execution of C/EBPα-driven neutrophilic differentiation. This hypothesis is also supported by our observation that EVI2B is abundantly expressed in mature human and murine granulocytes.

3.3.2. EVI2B is essential for proper granulopoietic development *in vitro* and *in vivo*

To investigate EVI2B function in granulocyte development, we employed murine 32D/G-CSF-R cells, whose granulocytic differentiation can be induced by G-CSF administration. First, we observed that both C/EBPα and EVI2B are upregulated in 32D/G-CSF-R cells upon G-CSF stimulation and their expression was gradually increasing as the differentiation progressed. Second, *Evi2b* knock-down by two different shRNAs was sufficient to effectively block granulocytic differentiation of these cells (based on cellular morphology). In order to find out whether *Evi2b* knock-down can block granulocytic differentiation in primary cells as well, we sorted murine LKS cells, transduced them with *Evi2b* shRNA containing lentiviral particles, and assessed their ability to differentiate in colony formation assays. Interestingly, *Evi2b*-silenced cells formed less colonies that were also smaller in size and produced almost no mature granulocytes compared to non-silencing controls. Next, we transplanted *Evi2b*-silenced LKS cells ($Ly5.2^+$) to lethally irradiated congenic recipient animals ($Ly5.1^+$) and observed significant reduction in number of *Evi2b* shRNA-transduced granulocytes compared to non-silencing controls *in vivo*.

Since *in vitro* and transplantation-based experimental systems are biased, [71] we developed *Evi2b* full body KO mouse strain (referred as *Evi2b* KO) and use it to study Evi2b function in hematopoiesis. Taking into account previous results, it is surprising we did not observe any abnormalities in the frequencies of hematopoietic populations in steady-state hematopoiesis in *Evi2b* KO mice. It is possible that an unknown compensatory mechanism was applied to cope with long-term Evi2b deficiency during development. In agreement with this speculation, we observed that when subjected to colony formation assays in the presence of G-CSF, *Evi2b* KO BM cells formed less colonies, produced fewer granulocytes, and harbored reduced clonogenic capacity.

To elucidate whether EVI2B is essential also for human granulopoiesis, we transduced human primary $CD34^+$ cells with lentiviral vectors expressing non-silencing control or two shRNAs targeting human *EVI2B* mRNA. Transduced cells were sorted, cultured for 10 days in semi-solid medium supporting myeloid differentiation, and the differentiation status of these cells was assessed by expression of CD11b and CD15 granulocyte markers. Consistently with our murine data, *EVI2B* shRNA-transduced $CD34^+$ progenitors produced significantly less $CD11b^+$ and $CD15^+$ cells, suggesting that EVI2B is important for proper differentiation of human granulocytes.

Together, these results provide novel evidence that *EVI2B*, a membrane glycoprotein with previously unknown function, is necessary for granulocytic differentiation in both mice and humans. Its expression is directly induced by full-length functional myeloid master regulator C/EBPα and is gradually increasing during the course of differentiation. Additionally, binding motifs of other transcription factors (other C/EBP family members, GFI-1, and PU.1) involved in granulopoietic development are located within *EVI2B* promoter, suggesting that *EVI2B* can be upregulated also by other regulators of granulocyte differentiation. EVI2B loss or downregulation was sufficient to block the differentiation of granulocytes *in vitro* and *in vivo*. These observations therefore illustrate the importance of EVI2B for proper differentiation of both murine and human granulocytes. Nonetheless, the mechanistic explanation how a transmembrane glycoprotein that has no similar counterpart within the mammalian genome contributes to the granulopoiesis remains obscured.

Despite the exact mechanism how EVI2B regulates myeloid differentiation is not known, the observation that one of the most frequent mutated forms of *CEBPA* (p30 C/EBPα) lacks the ability to induce *EVI2B* expression is particularly interesting in relation to the biology of certain types of AML. Publicly available transcriptome analysis of 529 AML patients [279] indeed showed that loss of C/EBPα activity was associated with reduced *EVI2B* expression. Since we demonstrated that EVI2B deficiency hinders granulocytic differentiation, it is possible that the ablation of EVI2B contributes to the pathogenesis of AML characterized by C/EBPα activity loss. Though, it would be necessary to perform additional experiments to further explore this possibility. Contrary to our hypothesis, other studies associating EVI2B with a cancer revealed that EVI2B presence has rather negative

consequences. In detail, EVI2B expression was identified as a diagnostic marker in acute lymphoblastic leukemia [280] and was associated with a poor prognosis of patients suffering from CLL and colorectal cancer. [168], [169] However, the function of transmembrane glycoprotein EVI2B might substantially differ in myeloid and lymphoid cells and further investigation is needed in order to decipher its functions in these cell lineages.

4. CONCLUSIONS

The objective of this thesis was to dissected three previously unknown mechanisms that might have been, according to our hypotheses, involved in the regulation of granulocytic differentiation. In particular, we focused on the function of β-catenin-TCF/LEF signaling, transcription factor C/EBPγ, and transmembrane glycoprotein EVI2B.

The first aim of this thesis was to describe the function of β-catenin-TCF/LEF signaling during neutrophil development in steady state and emergency granulopoiesis. Here we showed that β-catenin-TCF/LEF signaling promotes differentiation of HSPCs into neutrophils via direct G-CSF-R upregulation. The influence of β-catenin-TCF/LEF signaling on neutrophil differentiation from HSPC was rather moderate during steady state conditions. However, it was absolutely critical during the second step of emergency granulopoiesis, where a massive *de novo* production of neutrophils occurs. Loss of β-catenin-TCF/LEF activity and consequent reduction in G-CSF-R levels resulted in insufficient neutrophil generation and fatal inability of the organism to regenerate after hematopoietic injury or to fight the infection.

The second objective was to investigate the function of the vastly uncharacterized transcription factor C/EBPγ, a member of the C/EBP family of essential regulators of granulocyte differentiation. Despite we employed a plethora of experimental approaches to characterize multiple aspects of granulocyte development, we surprisingly did not find any abnormalities upon C/EBPγ loss. Therefore, it seems that C/EBPγ is the only member of the C/EBP family which is not involved in neutrophil production during steady state, nor emergency granulopoietic programs.

The third aim of this thesis was to characterize the role EVI2B during granulocyte differentiation. First, we described that *EVI2B* is directly upregulated by C/EBPα during the course of granulocytic differentiation. Interestingly, this upregulation was abrogated in AML patients harboring loss of functional C/EBPα. Next we demonstrated that *EVI2B* knock-down is sufficient to impair differentiation of granulocytes in both murine and human primary cells *in vitro* and *in vivo*. Despite we did not manage to provide mechanistic insight

into the molecular function of EVI2B, our data clearly show that EVI2B is crucial for the establishment and the execution of granulocytic differentiation program.

Together, our work extends the understanding of the molecular mechanisms that contribute to granulocyte generation. In this thesis, we demonstrate that by activating or inhibiting certain proteins or pathways in stem and progenitor cells we can diminish the granulocytic production, whereas by activating them we can potentiate granulopoiesis. Interestingly, this possibility to fine-tune granulocytic differentiation opens a new venue for potential clinical interventions, in which modulation of granulocyte counts needs to be adjusted.

However, it is not clear how these novel regulatory modules described in this thesis interact to ultimately orchestrate granulocytic differentiation. Thus, further investigation needs to be conducted in order to identify their potential interplay and to describe this regulatory network as a whole.

5. PUBLICATIONS

This thesis was based on the following list of publications. Full texts of these articles can be found enclosed to this thesis in Chapter 7.

β-catenin-TCF/LEF signaling promotes steady-state and emergency granulopoiesis via G-CSF receptor upregulation.
Danek P, Kardosova M, Janeckova L, Karkoulia E, Vanickova K, Fabisik M, Lozano Asencio C, Benoukraf T, Tirado-Magallanes R, Zhou Q, Burocziova M, Rahmatova S, Pytlik R, Brdicka T, Tenen DG, Korinek V, Alberich Jorda M. ***Blood***. 2020

Author contribution:
The author of this thesis performed all in vitro experiments with human and murine primary cells, bone marrow and stem cell transplantations, phenotyping of dnTCF4 mice, cell cycle, proliferation assays, RNAseq including library construction and data analysis, murine survival experiments, data analysis, and figure design. In addition, the author contributed extensively to the writing of the manuscript.

C/EBPγ is dispensable for steady-state and emergency granulopoiesis.
Kardosova M, Zjablovskaja P, **Danek P**, Angelisova P, de Figueiredo-Pontes LL, Welner RS, Brdicka T, Lee S, Tenen DG, Alberich-Jorda M. ***Haematologica***. 2018

Author contribution:
The author of this thesis performed experiments validating *Cebpg* KO model and colony forming assays.

EVI2B is a C/EBPα target gene required for granulocytic differentiation and functionality of hematopoietic progenitors.
Zjablovskaja P, Kardosova M, **Danek P**, Angelisova P, Benoukraf T, Wurm AA, Kalina T, Sian S, Balastik M, Delwel R, Brdicka T, Tenen DG, Behre G, Fiore F, Malissen B, Horejsi V, Alberich-Jorda M. ***Cell Death Differ***. 2017

Author contribution:
The author of this thesis performed western blotting and ChIP-qPCR assays using K562 cells.

Proliferation and Differentiation of Murine Myeloid Precursor 32D/G-CSF-R Cells.
Zjablovskaja P*, **Danek P***, Kardosova M, Alberich-Jorda M**.,** ***J Vis Exp***. 2018
*authors contributed equally

Author contribution:
The author of this thesis contributed to this publication by retrovirus production, transduction of the 32D/G-CSF-R cell line, assessment of granulocytic differentiation, figure design, and writing the manuscript.

Additional publication by Petr Daněk that was not included in the thesis:

Chromosome 21 gain is dispensable for transient myeloproliferative disorder driven by a novel GATA1 mutation.
Lukes J Jr, **Danek P**, Alejo-Valle O, Potuckova E, Gahura O, Heckl D, Starkova J, Stary J, Mejstrikova E, Alberich-Jorda M, Zuna J, Trka J, Klusmann JH, Zaliova M., ***Leukemia***, 2020

I hereby confirm that the author of the thesis, Petr Daněk, has substantially contributed to the publications listed above. In the case of his first-author publications, he performed the major part of experimental work and contributed to the manuscript preparation.

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Meritxell Alberich-Jorda, Ph.D.

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7. REPRINTS OF PUBLICATIONS