Pediatric Leukemia: Mechanisms of Initiation and Suppression

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Mohamed Eldeeb

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Abstract:

Acute leukemia (AL) is the most common cancer in children. It accounts for one-third of cancer cases in patients <16 years of age, and its frequency is slowly rising for unknown reasons. AL is a heterogeneous disease that can broadly be categorized into lymphoid (ALL) or myeloid (AML) subtypes. In children, ALL accounts for ~80% and AML for ~20% of all AL cases. AL originates from a single immature hematopoietic stem/progenitor cell (HSPC) following the acquisition of genetic changes that allow it to retain proliferative potential but lose responsiveness to normal differentiation cues. Chromosomal rearrangements that create fusion genes with aberrant transcriptional regulatory activities are frequently associated with childhood AL. The most common chromosomal translocations of pediatric leukemia, including ETV6-RUNX1 (E/R) and MLL-rearrangements (MLL-r), have a prenatal origin. MLL-r are believed to be sufficient to drive leukemogenesis without the need for secondary events. Despite this, neonatal leukemia is very rare, accounting for <1% of childhood leukemia cases, and the disease incidence rises thereafter. This led us to wonder if some prenatal signals, such as those mediated by LIN28B, could function as tumor suppressors against aggressive leukemia development.

LIN28B is a master regulator of fetal hematopoiesis with expression restricted to the prenatal period that declines shortly after birth. In Paper I, we interrogated the impact of LIN28B on MLL-r AML using a mouse model that co-expresses both genes. Our findings demonstrated that LIN28B can significantly impede MLL-r AML, even in the presence of additional mutations in RAS or MSN, the latter which was identified and characterized in Paper III. Further analysis revealed that the tumor suppressor activities of LIN28B are largely driven by positive regulation of the c-MYB suppressor MYBBP1A. Overexpression of MYBBP1A recapitulated the tumor suppressor effects of LIN28B, while its knockout eradicated them. Thus, we propose a developmentally-restricted tumor suppressor axis mediated by LIN28B that restricts MLL-r AML and perhaps other AML subtypes with MYB involvement. The natural decline of LIN28B expression postnatally provides a window for leukemogenesis.

In contrast to MLL-r that associate with few secondary mutations, a multistep genetic process is well established for E/R leukemogenesis, which can take over a decade to happen. E/R is the most common translocation in pediatric B-ALL but rarely seen in adults. In Paper II, we generated an inducible mouse model for E/R and characterized its pre-leukemic state. Induction of E/R resulted in expansion of HSCs and corrupted B-lymphopoiesis. This sharply contrasts normal HSCs activity, as we showed in Paper IV, contribute robustly to all major hematopoietic lineages in steady state. By comparing fetal and adult E/R HSCs, we found that while E/R disrupted HSC differentiation in both, fetal cells had a higher competitive advantage. This was particularly evident in a setting of viral mimicry, where the pool of fetal HSPCs was remarkably expanded and generated high numbers of B-cell progenitors. Further analysis revealed that E/R fetal HSPCs displayed higher expression and immune checkpoint activity compared to adult HSPCs. These findings suggest that pre-leukemic cells may evade immune surveillance and persist for a prolonged period before transformation, highlighting the potential of immune modulation as a therapeutic strategy.

Overall, the work presented in this thesis provides insights into the mechanisms underlying the development of pediatric leukemia and suggests new avenues for the development of innovative therapies.

Key words: ETV6-RUNX1, leukemia initiation, hematopoietic stem cells, MLL-rearrangements, LIN28B

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To my dear late grandma,
my family, and all other families
impacted by childhood cancer

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Manuscript in preparation, 2023

Abstract

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Overall, the work presented in this thesis provides insights into the mechanisms underlying the development of pediatric leukemia and suggests new avenues for the development of innovative therapies.

Abbreviations

AGM	aorta–gonad–mesonephros
AL	acute leukemia
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
BCR	B cell antigen receptor
BD	bromodomain
BM	bone marrow
CBF	core-binding factor
CFU-S	colony-forming units in the spleen
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitor
CML	chronic myeloid leukemia
CNS	Central nervous system
D	diversity
Dox	doxycycline
E	embryonic day
E/R	ETV6-RUNX1
EHT	endothelial to hematopoietic transition
EMP	erythromyeloid progenitor
FACS	fluorescence-activated cell sorting
FL	fetal liver
GM	granulocytes and monocytes
GMLP	granulocyte-monocyte-lymphoid progenitor
HDAC	Histone deacetylases

HOX	Homeobox
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cell
iAMP21	intrachromosomal amplification of chromosome 21
IFN	interferon
IgH	immunoglobin heavy chain
IgL	immunoglobin light chain
J	joining
LEDGF	lens epithelium-derived growth factor
LIC	leukemia initiating cell
Lin	mature lineage markers
LSC	leukemia stem cell
LSK	Lin+ Sca1+ Kit+
MDS	myelodysplastic syndrome
MegE	megakaryocytic-erythroid
miR	micro-RNA
MLL	Mixed-Lineage Leukemia
MLL-r	MLL rearrangements
MPN	myeloproliferative neoplasms
MPP	multipotent progenitor cells
OS	overall survival
OxPhos	oxidative phosphorylation
Ph	Philadelphia translocation
PHD	plant homeodomain
PNT	pointed N-terminal
Poly I:C	polyinosinic:polycytidylic acid
RAG	recombination activating gene
RD	repression domain
RNP	ribonucleoprotein

ROS	reactive oxygen species
RUNX1	Runt-related transcription factor-1
SCF	stem cell factor
SEC	super elongation complex
SLAM	signaling lymphocytic activation molecule
SNL	sub-nuclear localization
TAD	transactivation domain
TdT	terminal deoxynucleotidyl transferase
TLR	Toll-like receptor
V	variable
WBCs	white blood cells
WHO	World Health Organization
WT	wild type
YS	yolk sack

Background

Introduction to the Battlefield: The Hematopoietic System

Mature blood cells

Blood has aroused people's curiosity for thousands of years. In ancient cultures, mystical and divine properties were attributed to blood. The oldest known medical texts from Egypt, dating back more than 3,000 years, emphasize the fundamental importance of blood for health and disease. Over time, our perception of blood evolved from a mystical bodily fluid to a complex system composed of different types of cells. An important milestone in this change was the invention of the compound microscope in the 17th century, which allowed for a more detailed study of the cellular components of blood. The 20th century saw significant advances in laboratory techniques such as cell culture, transplantation, and flow cytometry. These innovations enabled comprehensive studies of the properties and functions of blood cells and led to a better understanding of blood and its diseases [1].

Hematopoietic (blood) cells play a critical role in maintaining overall health by carrying out a number of specialized and essential processes in the circulatory and immune systems. These cells can be broadly divided into three types: red blood cells (erythrocytes), platelets, and white blood cells (WBCs). Erythrocytes, which make up the majority of blood cells, are responsible for transporting oxygen and carbon dioxide throughout the body with their hemoglobin content. Platelets are critical for blood clotting and hemostasis, activating the coagulation cascade at the site of injury. WBCs are a diverse group of cells that play an important role in the immune system. They can be divided into two categories based on their lineage of development: myeloid and lymphoid cells. Myeloid cells include granulocytes (neutrophils, eosinophils, and basophils), monocytes, and dendritic cells. Lymphoid cells, on the other hand, include B and T lymphocytes, natural killer (NK) cells, and a subset of dendritic cells [2]. WBCs can also be divided into innate and adaptive immune cells, depending on their function. Myeloid and NK cells make up the innate immunity, which is the first line of defense that provides a rapid, nonspecific response to a variety of pathogens. These cells use mechanisms such as phagocytosis, inflammation, antigen presentation, and cytotoxicity to elicit an immune response and attract other immune cells to the site of infection. In contrast, B and T cells are usually involved in adaptive immunity,

utilizing their memory function to mount a specific and tailored response to a particular pathogen upon re-exposure. B lymphocytes produce antibodies that recognize and bind to specific pathogenic antigens, also known as humoral immunity, whereas T lymphocytes can differentiate into effector cells that kill infected cells or secrete cytokines to activate other immune cells [3]. Of note, there are certain subsets of B and T cells with innate-like characteristics, such as B1 and marginal zone B cells, and γδ T cells. These immune cells are mostly tissue-resident and have self-reactive and regulatory functions, and they are typically generated during the fetal and neonatal stages [4–6].

As our understanding of the scientific basis of blood and its functions has deepened, we have come to appreciate the true and complex wonder of this system. Today, blood continues to play a critical role in modern medicine, from life-saving blood transfusions to cutting-edge research into hematologic diseases. In this chapter, I discuss the process of blood cell formation and how it is regulated.

Hematopoiesis

The hematopoietic system is one of the most regenerative systems in the body. Throughout life, more than two million hematopoietic cells are produced every second. This number can increase dramatically during stress (infections, inflammation, and bleeding) and then return to baseline levels after recovery. This extremely plastic and intricate process of blood cell formation, known as hematopoiesis, is governed by a complex network of cell-intrinsic and -extrinsic regulators that work together to maintain a balance between proliferation, differentiation, and survival. Dysregulation of this process can lead to various non-malignant disorders, such as anemia and hemophilia, or malignant diseases, such as leukemia. Understanding the underlying mechanisms of hematopoiesis is critical for developing new therapies to treat these disorders [7].

Hematopoietic stem cells

Bone marrow (BM) is the main site of hematopoiesis in adults. Despite the heterogeneous cellular composition of hematopoietic cells, and their various lifespans that range from hours to years [8,9], almost all blood cells are derived from a unique cell type called the hematopoietic stem cell (HSC).

Stem cells are remarkable cells that have two special properties: They can self-renew and thereby produce more stem cells, and they can differentiate into more specialized cell types with different functions. Embryonic stem cells are pluripotent, which means they can generate any type of cell in the body, while adult or tissue-specific stem cells are multipotent, which means they can give rise to all cells related

to the tissue from which they originate. HSCs were the first tissue specific stem cells to be functionally identified and prospectively isolated [10].

Early experiments successfully demonstrated that BM transplantation could rescue lethally irradiated mice and restore hematopoiesis, suggesting the existence of HSCs. However, the first functional tests suggesting the presence of HSCs were performed by Till and McCulloch [11]. In their experiments, they demonstrated that a subset of BM cells could form macroscopic colonies in the spleens of lethally irradiated recipient mice 10 days after transplantation. These colonies, termed colony-forming units in the spleen (CFU-S), were composed of different hematopoietic lineages, and their numbers correlated directly with the number of cells initially transplanted. Exposure of donor BM cells to a dose of irradiation introduced random chromosomal aberrations, which allowed Till and McCulloch to confirm the clonal origin of most of these CFU-S [12]. Further transplantation of these colonies into secondary irradiated recipients revealed a capacity for self-regeneration [13]. While it was initially believed that CFU-S were derived from HSCs, the limited self-renewal capacity of these cells and the lack of evidence for long-term reconstitution potential later suggested that CFU-S were mostly derived from progenitor cells. Several studies subsequently employed retroviral integration methods and succeeded in demonstrating the existence of rare hematopoietic cells with a multilineage reconstitution potential that was sustained over a long period (> 16 weeks) [14,15]. Altogether, these pioneering experiments provided functional evidence of the ability of HSCs to regenerate the hematopoietic system with their unique properties of self-renewal and multilineage differentiation, the key defining features of stem cells, and thus laid the foundation for HSC biology and stem cell research.

Identification and isolation of HSCs

With a functional proof of HSCs activity, next steps were aimed at identifying and isolating them. This is critical for a better understanding of HSCs biology and sometimes an advantage in bone marrow transplantation, an essential treatment for several hematological malignancies. Advances in fluorescence-activated cell sorting (FACS) and the development of monoclonal antibodies have greatly facilitated HSC isolation. Several cell surface markers have been identified to distinguish HSCs from more mature progenitor cells. However, to date, no single HSC-specific marker has been identified, and a combination of markers is always required to isolate HSCs. Murine HSCs lack expression of mature lineage markers (Lin) but express stem cell antigen-1 (Sca1) and the tyrosine kinase receptor c-kit, collectively known as LSK [16,17]. The LSK compartment encompasses all functional HSC activities but remains highly impure with a majority of committed progenitor cells. For further refinement, the LSK markers are combined with the signaling lymphocytic activation molecule markers (SLAM) CD150 and CD48, and sometimes CD34 and Flt3 are also used. HSCs express CD150 but lack expression

of the rest of these markers [18–20]. Further research has revealed that the combination of the LSK SLAM markers with the endothelial protein C receptor (EPCR) allows for the isolation of HSCs with a purity suggested to exceed 60% [21–23]. Continuous efforts are aimed at finding more HSC-specific cell surface markers, with the aim of further refining the HSC population and gaining a deeper understanding of their biology. Different combinations of these surface markers also allow for the identification and isolation of various downstream hematopoietic progenitor subsets.

A hierarchical system

The classical view of hematopoiesis suggests a highly organized hierarchical structure with HSCs at the apex (Figure 1). To meet the continuous demand for mature blood cells, HSCs generate highly proliferative progenitor cells that become increasingly specialized upon differentiation and ultimately replenish all hematopoietic lineages [24]. In this way, HSCs can largely maintain quiescence while their downstream progeny handles immediate cellular demands [25]. Quiescent HSCs can maintain their self-renewal capacity for extended periods by avoiding replication-induced mutations and exhaustion. Nevertheless, the contribution of HSCs to hematopoiesis is seen over time in all hematopoietic lineages, except for some long-lived early-life derived hematopoietic subsets, such as B1a and γδ T cells (discussed in paper IV). In this classical hierarchical view, the differentiation of HSCs into mature cells is a distinct stepwise process tightly regulated by a complex network of transcription and epigenetic regulators. Transcription factors bind DNA through specific DNA-binding domains and mediate transcription activation or repression through recruitment of coactivators or repressors. This process is regulated by several chromatin remodelers and histone modifiers, including methyl- and acetyltransferases.

Studies have proposed that HSCs can maintain a balance between self-renewal and differentiation through symmetric and asymmetric divisions. Symmetric division assumes that HSCs divide into two HSCs or two differentiated daughter cells, whereas the asymmetric division proposes that each cell division gives rise to one HSC and one daughter cell [26]. Regardless of the model, the daughter cells are multipotent progenitors (MPPs) that lack self-renewal but a retain multilineage differentiation capacity [20,27]. In the most common view, a bifurcation occurs downstream of MPPs, separating the megakaryocytic-erythroid (MegE) lineage from the lymphomyeloid lineage. Pre-MegE precursors continue to differentiate and eventually generate platelets and erythrocytes, while the bipotent granulocyte-monocyte-lymphoid progenitor (GMLP) can differentiate into some myeloid and lymphoid cell types. Further refinement of GMLPs based on Flt3 expression allows for distinction between the myeloid-biased MPP3 and the lymphoid-biased MPP4. MPP3 differentiate into pre-granulocyte/monocyte progenitors (pGM) and finally

generate granulocytes and monocytes, while MPP4 differentiate into common lymphoid progenitors (CLP) to generate B, T, and NK cells [28,27].

Later studies have challenged the classical model of stepwise differentiation by demonstrating that HSCs can commit directly to the megakaryocytic lineage without passing through intermediate stages [29]. These findings suggest perhaps that HSCs are more heterogeneous than previously thought by implying that lineage priming may occur at the HSC level. The pathway of differentiation thereafter is not entirely discrete, and the roadmap of lineage specification is constantly updated, leading to overlapping nomenclatures for nearly similar populations [30]. This new perspective proposes that hematopoietic stem and progenitor cells (HSPCs) exist in a continuum of low primed undifferentiated (CLOUD) state in which the discrete differentiation steps of the classical model become less distinct [31,32].

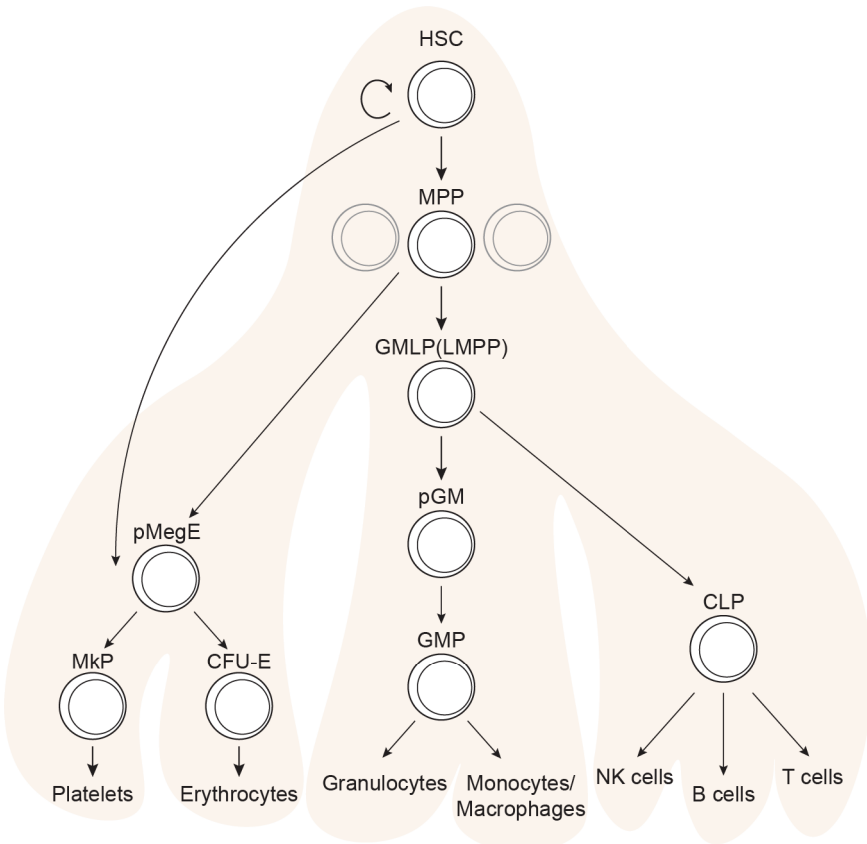

Figure 1. The hematopoietic hierarchy.
MkP: megakaryocyte progenitor, CFU-E: colony forming unit-erythroid, GMP: granulocyte-monocytes progenitors.

Lineage priming and commitment

HSPCs are constantly confronted with various fate decisions, and their differentiation trajectories are largely determined by a complex interplay of transcriptional regulators. This process is finely tuned, and the extent to which certain regulators are expressed can have radical consequences. For example, the balance between self-renewal and differentiation of HSCs is determined by the expression level of MYC. HSCs with low MYC levels tend to self-renew, whereas high expression of MYC promotes differentiation [33]. This fine balance of regulation is observed at nearly every step of fate commitment, with synergistic and antagonistic interplay between different transcriptional regulators. For instance, PU.1 (SPI1) and GATA1 have been suggested to be expressed in HSPCs at low levels. In one model, increased expression of GATA1 represses PU.1 and directs cells toward the MegE lineage, where they either upregulate KLF1 and commit to the erythroid lineage, or express FLI1 and commit to the megakaryocytic lineage [34,35]. In contrast, upregulation of PU.1 suppresses GATA1 and guides the cells towards the lymphomyeloid lineage [34]. However, later studies have found that discrepancies between mRNA and protein levels of PU.1 and GATA1, demonstrating that PU.1 and GATA1 proteins are not co-expressed in HSPCs. This refutes the notion that a switch between PU.1 and GATA1 initiates HSPC lineage decisions, and rather suggests that expression of these factors only reinforces lineage commitment once made [36]. Further elevation of PU.1 expression levels in GMLPs drives myeloid commitment by increasing CEBPA and GFI1 expression that promote neutrophils production, or EGR expression that promotes macrophages generation [37–39]. On the other hand, low levels of PU.1 drive cells towards lymphopoiesis, where they further express NOTCH, GATA3, and TCF7 and commit to the T-cell lineage, or IKZF1 (IKAROS), BCL11A, E2A (TCF3), FOXO1, EBF1, and PAX5 and generate B-cells [37,40].

B cell differentiation

B cell development is a complex process that takes place in multiple organs and involves sequential steps of assembly, expression, and signaling of the B cell antigen receptor (BCR) (Figure 2). This receptor is composed of immunoglobin heavy (IgH) and light (IgL) chains. The IgH is encoded by variable (V), diversity (D), and joining (J) gene segments, while the IgL is only encoded by V and J segments.

The process of B lymphopoiesis starts in the BM, where the expression of the transcription factors E2A, EBF1, and PAX5 triggers commitment of CLPs to the B-cell lineage (pre-pro B cell stage) [41]. At this stage, the recombination activating gene (RAG) enzymes are activated, which recognize the recombination signal sequences (RSS) in the D and J segments of IgH and introduce random double-stranded breaks [42]. To mediate ligation and increase the diversity of the BCR, the

terminal deoxynucleotidyl transferase (TdT) adds random nucleotides to the DJ junction. RAG and TdT enzymes proceed to cut and recombine the V segment to create a V-DJ-rearranged IgH, marking the pro-B stage [43]. Subsequently, the rearranged heavy chain is paired with a surrogate light chain (SLC), which leads to the formation of a pre-BCR complex and a transition to the large pre-B cell stage. During this stage, a check is conducted to ensure the productive assembly of the pre-BCR. Signaling from the pre-BCR downregulates RAG and TdT enzymes and triggers proliferation. After expansion, pre-B cells transition to the non-cycling small pre-B stage, and RAG and TdT become reactivated. This finally leads to rearrangements of the VJ segments of the light chain and the formation of the BCR [44]. Once this is achieved, the cells enter the immature B stage, marked by the expression of IgM, and a second BCR check is performed for self-reactivity [44]. Alternative splicing of the immunoglobulin heavy chain subsequently leads to the generation of IgD^+ IgM^+ transitional/mature B cells, which exit the bone marrow and migrate to the spleen for further maturation into follicular or marginal zone B cells. These cells can further differentiate into memory B cells and antibodies-secreting cells/plasma cells upon activation.

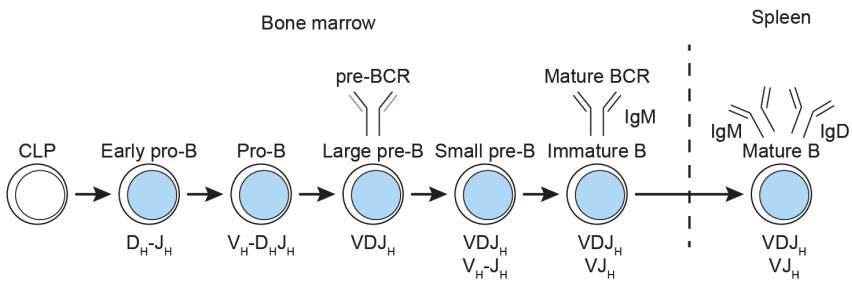

Figure 2. Stages of B cell development in the BM.

En Garde: Ontogeny of Hematopoiesis

Prenatal hematopoiesis is a complex multistep process that remains largely elusive. The fast pace of development, the multiple locations in which hematopoiesis occurs, and the ethical concerns all impose challenges to study this process in humans. Mice have contributed to a better understanding of these early stages of hematopoiesis, as we share major commonalities but with a more prolonged timescale [45].

Early hematopoiesis involves three consecutive waves that differ in time and location (Figure 3). The first wave, also known as the primitive wave, occurs in the

yolk sac (YS) on embryonic day 7.5 (E7.5) of the mouse embryo. In this wave, the blood islands in the YS emerge from hemangioblasts and generate large nucleated erythrocytes and megakaryocytes, which support the rapidly developing embryo by carrying oxygen and maintaining vasculature [46,47]. Some macrophages are proposed to arise at this stage and colonize some organs such as the brain microglia [48]. The second wave of hematopoiesis, referred to as the transient-definitive wave, starts in the YS on E8.5. In this wave, erythromyeloid progenitors (EMPs) arise from a distinct hemogenic endothelium that cannot generate HSCs. EMPs migrate to the fetal liver (FL) on E9.5 and maintain fetal erythropoiesis [49]. Concomitantly, some lymphomyeloid progenitors (LMPs) also arise in the YS then migrate to FL to support myelo- and lymphopoiesis prior to HSCs generation [50].

The HSC-independent progenitors of the first and second waves of hematopoiesis are lineage-restricted and transient. However, they generate long-lasting tissue-resident immune cells, such as brain microglia, epidermal γδ T and Langerhans cells, and B1a cells [5,51,4]. Nevertheless, lineage tracing and transplantation studies have shown that the capacity of generation of these innate-like immune cells is not restricted to LMPs, as FL HSCs are also capable of generating some of these cells [52,53].

The emergence of HSCs marks the initiation of the third and definitive wave of hematopoiesis. On E10.5, the hemogenic endothelium in the aorta–gonad–mesonephros (AGM) region undergoes an endothelial to hematopoietic transition (EHT) where the first HSCs bud off. EHT is dependent on Runx1 and Gata2 expression, while the downregulation of Notch1 and Sox17 is important at the later stages for fate determination [45]. Inflammatory signals produced by primitive macrophages are also essential for HSC-budding [54,55]. At this stage, additional sites have been shown to harbor HSCs, including the placenta and embryonic head [56–59]. Subsequently, HSCs migrate and colonize the FL on E11.5 then undergo a massive expansion from E12 to E16 in preparation to taking over hematopoiesis [60,61]. During and after this stage, hematopoietic cells colonize the spleen, thymus, and BM, and by late gestation, HSCs migrate to their final residence in the BM. Over the first 3 – 4 weeks after birth, HSCs gradually lose their fetal characteristics until they become indistinct from their adult counterparts [64,65].

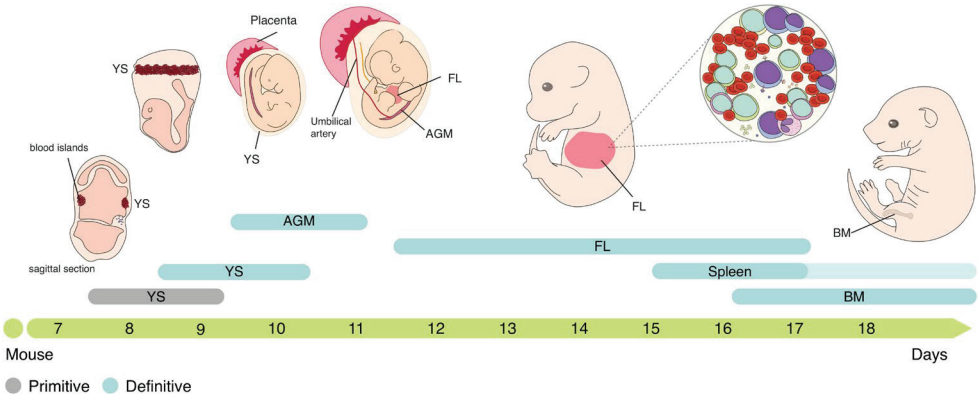

Figure 3. Ontogeny of the hematopoietic system (from Soares-da-Silva et al. [60]).

Differences between fetal and adult HSCs

Cell cycle and metabolic activity

As mentioned previously, adult HSCs maintain their steady-state quiescence by delegating the ongoing hematopoietic needs to their downstream progenitors [66]. This low cycling status of BM HSCs likely protects them from exhaustion, DNA damage, and acquisition of mutations that would otherwise be subsequently passed on to the whole hematopoietic system [67]. In stark contrast, FL HSCs expand ~100-1000 fold only within around 5 embryonic days [61]. Similarly, FL HSCs have higher protein synthesis rates than adult HSCs [68,61]. Nevertheless, the adult HSPCs proteome is more complex than the fetal one [69]. To meet these high metabolic demands, FL HSCs rely on oxidative phosphorylation (OxPhos) for energy generation [70]. This pathway is actively suppressed in adult HSCs to minimize generation of reactive oxygen species (ROS), which can subsequently lead to differentiation and exhaustion [71].

For adult HSCs, high proliferation, protein synthesis, and OxPhos rates lead to compromised activity and loss of fitness [67]. Nevertheless, FL HSCs outperform adult HSCs in transplantation assays [64,72]. How FL HSCs cope with these high levels of cellular and metabolic activity remains elusive. It has been proposed that DNA repair mechanisms are more active in FL HSCs, which might be a protective mechanism against these high-stress levels [70]. Maternal and fetal bile acids have been also suggested to reduce endoplasmic reticulum stress by inhibiting protein aggregation [68]. Nevertheless, our knowledge regarding these protection mechanisms is far from complete.

Differentiation potential and lineage biases

Ontogeny plays a significant role in shaping hematopoietic development. Although fetal and adult HSCs are capable of sustaining multilineage hematopoiesis throughout life, their differentiation potentials, lineage biases, and degrees of commitment differ. Generation of the fetal-specific lymphoid subsets B1a and γδ T cells is restricted to fetal HSCs and ceases shortly after birth [52,53]. Fetal HSCs also have a higher capacity to generate MegE and erythroid progenitors, while adult HSCs display a granulocytic bias [73]. These differences also manifest in erythropoiesis, with humans transitioning from fetal γ-globin to adult β-globin shortly after birth [74].

Moreover, fetal HSPCs seem to be less primed and more malleable in their output. Recent studies have reported that the emergence of megakaryocyte-primed HSCs coincides with the fetal-to-adult transition [75]. Furthermore, unlike adult CLPs, fetal CLPs possess both lymphoid and myeloid lineage differentiation potential [76]. Even the more committed B-cell progenitors of the fetus might have a capacity to generate myeloid cells, particularly macrophages, and can sometimes express multiple myeloid-associated genes [77–79]. This behavior is uncommon in adult progenitors, reflecting different genetic and epigenetic regulations.

Developmental regulators of hematopoiesis

Hematopoiesis is governed by distinct sets of intrinsic and extrinsic regulators that establish the unique features of each developmental stage. The transition of HSCs from a fetal-to-adult state enforces an adult cell identity marked by increased quiescence, restriction in output, upregulation of immune-associated genes, and metabolic rewiring [61]. In this section, some of the distinct regulators during these developmental stages are discussed.

Intrinsic regulators

LIN28B

LIN28 is an evolutionary conserved RNA binding protein with critical roles during development. There are two mammalian homologs of this protein, LIN28A, and LIN28B, each containing two unique RNA-binding domains: a cold-shock domain, and a CCHC zinc finger domain [80]. LIN28 post-transcriptionally regulates genes through indirect and direct mechanisms. The canonical indirect mechanism is dependent on the suppression of *let-7* micro-RNAs (miR). LIN28 binds primary and precursor *let-7* miR, thereby disrupting their maturation process [81]. This results in the de-repression of let-7 mRNA targets, which include *Lin28, Hmga2, Arid3a, Igf2bp1-3, Cyclin A2, Myc,* and *Cbx2*, as well as several tumor suppressors and DNA repair proteins, such as *Cdkn2b, Chek1, Fancd2 and Brca1/2* [82–84]. Thus, the expression patterns of LIN28B and *let-7* are inversely correlated, with

LIN28B being predominantly expressed during ontogeny, and its downregulation after birth is coupled with an increase in *let-7* expression. During early embryonic development, LIN28B is expressed in all cell types, but it becomes mostly restricted to stem and progenitor cells after E9.5 [85]. The subsequent downregulation of LIN28B around 3-4 weeks postnatally is accompanied by an increase in *let-7* levels, marking the transition from the fetal to the adult stage of hematopoiesis [86].

Apart from the indirect *let-7*-dependent mechanism, LIN28B is also able to directly bind specific mRNA targets, forming ribonucleoprotein (RNP) complexes. Most of LIN28B bound mRNAs encode for RNA-binding proteins, ribosomal proteins, and translation-associated factors. LIN28B RNP complexes can then alter the translation and stability of the bound mRNA targets [87–90]. There are several proposed mechanisms through which LIN28B might enhance the translation of its binding partners, including recruitment of polysomes and RNA helicase A, or translocation to processing bodies [90,89]. In addition, the *let-7* target IGF2BP3 contributes to the stabilization of LIN28B-bound targets [91]. However, while enhancing translation seems to be the predominant outcome of LIN28 RNP complexes, other studies have reported translational repression of some of LIN28B interactomes [88,92,93].

LIN28B is a critical regulator of fetal hematopoiesis. Several fetal-associated genetic and epigenetic regulators are downstream targets of LIN28B, including *Arid3a, Hmga2,* and *Igf2bps* [94–97]. While each of these targets has a prominent role in fetal hematopoiesis, none by itself can recapitulate the pleiotropic phenotypes of LIN28B. However, enforced expression of LIN28B in adult HSCs is sufficient to reinstate fetal programs, including increased proliferation, erythroid-bias, reactivation of fetal hemoglobin expression, and generation of B1a and γδ T cells [73,86,93,98]. In contrast, enforced expression of *let-7* in fetal cells confers an adult phenotype [94].

Given the prominent role of LIN28B in embryogenesis, it is reasonable to hypothesize that it may be involved in the development of cancer. However, the low incidence of infant tumors, which represents ~0.05% of all malignancies [99], suggests otherwise. Nevertheless, the situation is not so simple, and it might be context dependent. Paper I discusses the role of LIN28B in the context of leukemia.

SOX17

SOX17 is another important regulator of fetal and neonatal HSCs. The levels of this transcription factor peak during fetal (after FL colonization) and neonatal stages then gradually drop ~4 weeks after birth [64]. SOX17 is critical for EHT and HSC emergence [45]. Knockout of *Sox17* in fetal and neonatal mice leads to hematopoietic failure, while its depletion in young adult mice has no impact on hematopoiesis [100].

RUNX1

Runt-related transcription factor-1 (RUNX1), also known as acute myeloid leukemia 1 protein (AML1), is a member of the core-binding factor (CBF) family and a critical transcription regulator of hematopoiesis. *RUNX1* is ubiquitously expressed in all hematopoietic cells apart from erythrocytes [101]. It is part of a family of three genes (*RUNX1-3*), which share the highly conserved DNA-binding Runt homology domain. In mammals, *RUNX1* can be transcribed from distal P1 or proximal P2 promoters, generating *RUNX1*c and b isoforms, respectively, and both isoforms share nearly identical structures. There is also a shorter dominant negative isoform, known as *RUNX1*a. The different biological functions of these isoforms remain unclear, but studies have suggested that *RUNX1*b might be the dominant form until E10.5, while *RUNX1*c predominates from FL colonization and onwards [102].

RUNX1 is indispensable for fetal development, as its expression is critical for EHT and HSCs budding. However, it becomes less important after FL colonization [103,104]. This was evident as *Runx1* knockout in mice led to embryonic lethality at E12.5 due to anemia and hematopoietic failure [105]. However, these phenotypes were not recapitulated upon *Runx1* deletion in vav-expressing hematopoietic cells, i.e after the emergence of HSCs [106]. Similarly, *Runx1* loss is tolerated in adults more than during early ontogeny. Conditional deletion of *Runx1* in adult mice results in milder phenotypes, including expansion of HSPCs, reduction of long-term reconstitution activity of HSCs ~3 folds, and defects in lymphoid and megakaryocyte differentiation [107–109]. The compromised reconstitution activity has been attributed to HSC exhaustion [110]. *Runx1* haploinsufficiency does not lead to embryonic lethality but is accompanied by ~50% reduction in HSC numbers. Intriguingly, these HSCs generated a higher reconstitution output compared to wild type (WT) HSCs but with lower T cells and platelets [111]. *RUNX1* translocations and mutations are frequently found in hematological malignancies and are discussed in the next chapter and Paper II.

ETV6

ETV6, also known as TEL, is another transcription factor with ubiquitous expression and critical regulatory roles in hematopoiesis. It is crucial for embryonic development, and its homozygous deletion usually leads to lethality at E10.5 because of failed YS angiogenesis [112]. Although *ETV6* is not required for EHT, embryos that might survive past this stage die from impaired hematopoiesis by E18.5 [113]. As for adults, *Etv6* deletion results in a wipeout of HSCs, without affecting downstream progenitors [113]. While the mechanisms behind these phenotypes remain unknown, one plausible suggestion is that ETV6 provides HSCs with pro-survival signals. Notably, heterozygous deletion of *Etv6* does not affect embryogenesis or produce any obvious hematopoietic phenotypes [112,114].

Similar to *RUNX1*, translocations and mutations involving *ETV6* are very common events in leukemia and are discussed in the next chapter.

MLL1

The *MLL1* (Mixed-Lineage Leukemia) gene, also known as Lysine-specific MethylTransferase 2A (*KMT2A*), is a member of the MLL/SET (Su(var), Ezh2, Trithorax) family of methyltransferases, located on chromosome 11q23. This family consists of six histone 3 on lysine 4 (H3K4) methyltransferases and are divided into three pairs according to their sequence conservation: MLL1/MLL2, MLL3/MLL4, and SETd1A/SETd1B [115]. *MLL1* is involved in several chromosomal translocations that lead to aggressive leukemia in pediatrics and adults, and its different domains, interactions, and roles in leukemogenesis are discussed in detail in the next chapter. In normal hematopoiesis, *MLL1* is ubiquitously expressed in HSPCs and has critical roles during the fetal and adult stages. Homozygous germline deletion of *Mll1* in mice leads to embryonic lethality from E10.5 to E16.5 mainly due to hematopoietic failure and other developmental defects [116–118]. Although Homeobox (*Hox*) genes are expressed before E9, *Mll1* is needed after this stage to maintain their expression. *Mll1* null embryos have lower numbers of FL HSPCs with reduced differentiation output mainly due to low *Hox* expression [117,119]. However, as opposed to the deleterious phenotypes of *Mll1* germline deletions, Vav-Cre *Mll1*$^{-/-}$ has no impact on fetal hematopoiesis. Instead, mice die ~3 weeks postnatally from BM failure [118,120]. Notably, FL HSPCs from the Vav-Cre *Mll1*$^{-/-}$ mice are outcompeted by WT cells in competitive transplantation assays [118,120]. The differences in the fetal phenotypes between germline and Vav-Cre *Mll* deletions might indicate that *Mll1* expression in non-hematopoietic cells might provide some support to FL HSCPs. As for adult hematopoiesis, conditional deletion of *Mll1* in adult mice results in fatal BM failure within three weeks [121]. Intriguingly, while mice with CD19-Cre mediated deletion of *Mll1* display no aberrant phenotypes, Rag1-Cre *Mll1*$^{-/-}$ mice exhibit pronounced reduction in B cells around 2 – 3 weeks after birth. This is attributed to a block in the pro-B to pre-B transition because of attenuated RAS/MAPK signaling, which reduces survival downstream of the pre-BCR [122]. Collectively, while MLL1 plays a crucial role in fetal hematopoiesis, it is deemed indispensable for adult hematopoiesis.

MYB

MYB (c-MYB) is a key regulator of normal hematopoiesis and is largely implicated in several cancers. *MYB* expression is dispensable for primitive hematopoiesis but critical for the definitive wave, as its knockout leads to severe anemia and embryonic lethality at E15.5 [123,124]. However, its heterozygous deletion is well-tolerated and does not lead to obvious hematopoietic phenotypes. There is an inverse correlation between MYB and fetal hemoglobin levels. Enforced expression of *MYB* inhibits γ-globin, while its knockdown increases the levels of this fetal hemoglobin

form. MYB is also a critical regulator of both myelopoiesis and lymphopoiesis, and its knockdown disrupts differentiation [123,124].

Structurally, c-MYB has three functional domains: a DNA-binding domain, a transactivation domain (TAD), and a negative regulatory domain (NRD). These domains interact with several coactivators, such as Menin and CBP/p300, or corepressors, such as MYB binding protein 1a (MYBBP1A), which subsequently alter MYB-transcription activity. MYB target genes are known to regulate proliferation, differentiation, and apoptosis. However, while its role in leukemia is well-established, the mechanisms by which MYB mediate leukemogenesis are not fully understood [123,124].

Adult-specific regulators

Several transcription regulators are predominantly expressed in adult HSCs and mediate the transition from fetal to adult hematopoiesis in an opposing manner to LIN28B and SOX17. For instance, *CEBPA* expression levels are low in fetal/neonatal HSCs but increase ~4 weeks after birth [125]. CEBPA mediates the transition of HSCs from a proliferative fetal to a quiescent adult state by inhibiting Myc expression. Deletion of *Cebpa* in mice has no impact on fetal/neonatal HSCs but increases the proliferation of adult HSCs, re-activating certain fetal-specific programs [125]. Similarly, GFI-1 expression is critical for adult HSCs function but dispensable for fetal hematopoiesis. *Gfi-1* knockout mice show no signs of hematopoietic aberrancies until reaching adulthood, where *Gfi-1*$^{-/-}$ HSCs become functionally compromised and outcompeted [126,127]. Other important adult HSC regulators that are inessential for FL HSCs include EGR1, ASH1L, and the polycomb family members BMI1 and EED [128–130]. Recently it has been suggested that the transition from fetal to adult state is not abrupt, but rather a gradual uncoordinated process that begins at a late gestational stage and is triggered by a spike in type I interferon (IFN) expression [65].

Extrinsic regulators

Niche and cytokines

The niche is the specific microenvironment where HSCs reside and are regulated. Several cell types have been identified in the BM niche, including endothelial, mesenchymal stromal cells, osteoblasts, adipocytes, and sympathetic nerves [20,131]. These cells secrete a variety of cytokines, such as thrombopoietin (TPO), Angiopoietin-1, and stem cell factor (SCF), which bind to their respective receptors Mpl, Tie2, and cKit on HSCs and regulate quiescence and survival [132–134]. However, the exact location of HSCs in the BM remains unclear. There are two distinct proposed locations: the endosteal niche and the perivascular niche. More recent studies have found that HSCs are predominantly located close to the

endothelial and Nestin^{+} perivascular cells [131,135]. Nevertheless, ongoing research aims to ascertain the precise location and composition of these BM niches.

On the other hand, the primary site of fetal hematopoiesis is the liver, which contains multiple cell types, including desmin^{+} stellate cells, DLK1^{+} hepatocyte progenitors, and Nestin^{+}NG2^{+} pericytes of portal vessels [136,137]. These cells support HSC proliferation and maturation by secreting many cytokines and growth factors. Although earlier studies have suggested that FL HSCs are less reliant on certain cytokines such as SCF, Angiopoietins, and TPO [132–134], more recent research indicates that deletion of SCF completely depletes FL HSCs [138]. Finally, and as previously mentioned, the FL niche is unique in its ability to produce bile acids that support the high protein synthesis rates of FL HSCs by alleviating endoplasmic reticulum stress [68].

Inflammation

Inflammatory signals play critical roles in hematopoiesis at all developmental stages. Their roles start in the sterile prenatal environment where signaling downstream of the cytokines tumor necrosis factor α (TNF-α) and interferon (IFN), as well as Toll-like receptor 4 (TLR4) mediate HSCs emergence [54,139]. This wave of proinflammatory signals is mostly produced by primitive YS macrophages. A second germ-free wave of IFN-I is produced by the skin during late gestation and initiates the transition from fetal to adult hematopoiesis, leading to HSPCs expansion and gradual upregulation of major histocompatibility complex I (MHC-I) expression [65,69].

Later in life, exposure to pathogens induces inflammation and activates hematopoiesis. Pattern recognition receptors, such as TLRs, are expressed on immune cells and can bind pathogenic products, including lipopolysaccharides and viral dsRNA or its synthetic analogue polyinosinic:polycytidylic acid (poly I:C). This triggers the production of IFN and other inflammatory cytokines, which promote HSCs proliferation and elicit emergency megakaryopoiesis [140–142]. TLR activation also induces other immune responses, including increased expression of co-stimulatory molecules on antigen-presenting cells, and upregulation of MHC molecules. Intriguingly, HSCs express several TLRs, which enable them to directly bind pathogens. HSCs also express several cytokine receptors, including IFN-γR, TNF-R1, and TNF-R2 [143]. In addition, recent studies have demonstrated that HSCs retain an epigenetic memory of previous infections, which increases responsiveness to secondary exposure [144]. More recently, HSCs have been found to express MHC-II and can present exogenous antigens, thereby activating CD4 T-cell mediated immune response, which triggers HSCs differentiation [145,146].

While acute inflammation allows HSCs to return rapidly to quiescence thereafter, chronic inflammation exhausts HSCs and impairs their functional activity

[141,147]. Inflammation is also implicated in leukemia development. Childhood infections are suggested to induce an acute and dysregulated inflammatory response that triggers leukemic transformation (discussed in the next chapter). Aging also confers a state of mild chronic inflammation, referred to as inflammaging, which is associated with myeloid skewing, increased DNA damage, mutations, clonal hematopoiesis, and a higher risk of developing hematological malignancies [147].

Taken together, inflammation is continuously shaping and regulating normal and stress hematopoiesis. Depending on its intensity, duration, and stage of development, it can cause significant hematopoietic perturbations and potentially lead to leukemic transformation under certain circumstances.

The Duel: Pediatric Leukemia

Acute leukemia: an overview

Leukemia is a group of blood cancers characterized by the overproduction of immature and dysfunctional hematopoietic cells, hence its name that means white blood in Greek. These abnormal cells, also referred to as blasts, crowd out normal blood cells, leading to a range of severe complications, including anemia, infections, and bleeding. In 2020, the World Health Organization (WHO) reported nearly half a million new cases of leukemia worldwide, with over 300,000 associated fatalities [148]. Although leukemia is relatively uncommon in adults, it is the most common form of cancer in children, accounting for approximately 30% of pediatric cancer cases. The worldwide incidence of pediatric leukemia has risen by 15% from the 1980s to 2010, and it continues to rise for unknown reasons [149].

Leukemia is classified based on the speed of its progression into two types: acute leukemia (AL) and chronic leukemia. AL develops quickly and requires immediate treatment, while chronic leukemia develops more slowly and may not cause symptoms for years. Each of these subtypes is further divided according to the affected hematopoietic lineage, resulting in four main subtypes: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML). CLL and CML are very rare in children, with their incidence increasing with age, particularly in elderly. Similarly, AML is more common in older patients but can occur in children as well. In contrast, ALL accounts for ~80% of all pediatric leukemia cases, with a lower incidence in adults [150].

The exact cause of leukemia is unknown, but various environmental and genetic factors are thought to increase the disease risk. For instance, exposure to high levels of radiation, chemicals, infections, and smoking, are among the factors that might

increase the likelihood of developing leukemia [151]. The risk increases dramatically with congenital syndromes such as Down syndrome and Fanconi anemia [152,153]. Additionally, several germline genetic mutations raise the susceptibility to the disease [154]. However, these risk factors account for only a fraction of leukemia cases, as most patients develop leukemia without any identifiable risk factors.

Several factors govern disease outcome, including age, leukemia subtype, and the associated mutations. For instance, although the 5-year overall survival (OS) rate of childhood ALL is ~90%, this falls to 25% in adults [155]. Similarly, pediatric AML has a 5-year OS of ~75%, which decreases to ~60% in adults < 60 years then plunges to ~25% in patients >60 years [156]. Despite these overall differences, each leukemia subtype is heterogenous, and certain genetic and cytogenetic factors confer poor disease prognosis, regardless of the patient's age.

Treatment strategies for AML and ALL overlap to some extent in the induction and consolidation phases. The induction phase involves high-dose chemotherapy to induce disease remission. In AML, this phase typically involves cytarabine and anthracycline [157]. While for ALL, an intensified multidrug therapy is administered, which usually contains glucocorticoids, such as prednisolone or dexamethasone, and a mix of cytotoxic drugs that involve vincristine and anthracycline or PEG-asparaginase [155]. Consolidation therapy aims to wipe out any remaining cancer cells to maintain remission and prevent disease relapse. In AML and adult ALL, particularly those with adverse prognoses, this phase involves high dose of cytarabine followed by allogeneic hematopoietic cell transplantation. However, for childhood ALL, transplantation is less required and is replaced by high doses of multiagent combination treatment that also involve steroids with cytarabine and methotrexate (reinduction therapy). Central nervous system (CNS) prophylaxis therapy is also needed in ALL, which involves 8-16 intrathecal treatments, dependent on the presence or absence of leukemic blasts in the cerebrospinal fluid at diagnosis [158]. Nowadays, additional targeted therapies are added to the treatment protocols, according to the cytogenetics of the disease, such as FLT3 and other tyrosine kinase inhibitors, or the anti-CD33 monoclonal antibody Gemtuzumab for AML ([159]. More recently, CAR-T cell therapy has been introduced as a treatment for ALL [160]. Despite the continuous improvement in survival rates, the current treatments associate with acute and long-term toxicities that impair quality of life and cause early mortality [161,162]. In addition, complete eradication of leukemic cells is not always achieved, and relapse rates are >50% in AML and adult ALL, and ~20% in childhood ALL [163–165]. Relapse is believed to be driven by a particular subset of cells that are resistant to chemotherapy and can regenerate the disease, referred to as leukemia stem cells (LSCs). Thus, understanding the cellular and molecular makeup of the disease, particularly LSCs, is critical to improving the current therapeutic strategies, allowing for better targeting approaches.

The cellular makeup of AL

As previously mentioned, AL is a very heterogeneous disease. Intertumoral variation between patients and intratumoral variation at the cellular and (sub)clonal level within the same patient are the main features of AL. This behavior is explained by the stem cell and clonal evolution models, which suggest that AL is triggered by the acquisition of genetic alterations in a single immature HSPC, the so-called cell of origin [166,167]. This cell is then unable to respond to normal differentiation stimuli, but because of its high self-renewal potential, it can proliferate and generate leukemia-initiating cells (LICs) or pre-LSCs. LICs are defined by their functional ability to initiate and maintain leukemia after transplantation in mice [168,169]. In the most common view, disease progression thereafter is a consequence of additional molecular changes leading to the emergence of fully transformed LSCs, which subsequently differentiate into leukemic blast cells. The more aggressive subclones outcompete the rest of the clones as well as normal hematopoiesis and eventually overpopulate the whole hematopoietic system. Thus, like normal hematopoiesis, leukemic cells are hierarchically organized, with LICs/LSCs residing at the apex of the hierarchy and capable of disease regeneration (Figure 4) A subset of these cells is believed to be rare and quiescent, and thus is not targeted by chemotherapy and is responsible for relapse [170]. Identifying these cells and understanding their underlying biology is crucial for the development of effective targeted therapies for complete eradication of the disease.

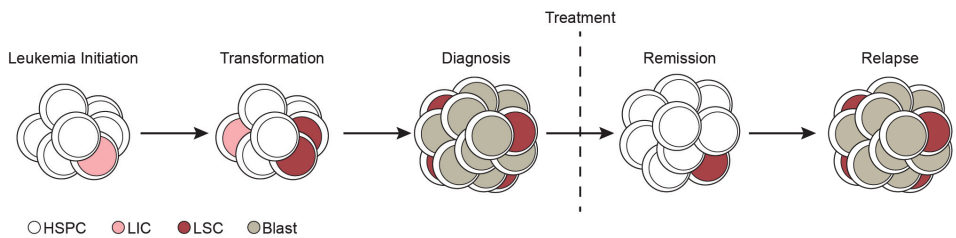

Figure 4. Composition and progression of acute leukemia.

Pediatric and adult leukemia: how do they differ?

Pediatric leukemia is the most common malignancy in children and is a leading cause of childhood mortality. Despite extensive research, the etiology of the disease remains elusive. Accumulating evidence over the past decades supports a prenatal origin for childhood malignancies [171]. However, although the incidence of neonatal and infant leukemias dramatically differ, both remain rare events, as leukemia peaks after the first two years of life [172,173]. Differences between pediatric and adult leukemias regarding their cellular, molecular, and prognostic

features suggest that they are two distinct disease entities. These differences are discussed in the following sections.

ALL

ALL is a complex disease with many subtypes that can be broadly classified into B-cell ALL (B-ALL) and T-cell ALL (T-ALL), which are further subdivided based on genetic alterations. B-ALL is by far the predominant form in children, accounting for ~85% of pediatric ALL, and is the subtype discussed here. Approximately 60% of ALL cases occur in patients younger than 20, with a peak of diagnosis around 2 – 5 years that abruptly declines afterwards [174]. Over the past five decades, the field of pediatric ALL has witnessed remarkable progress, transforming the disease from fatal to highly treatable. However, the same level of success has not been achieved in the treatment of adult ALL. This can be attributed to differences in effectiveness and tolerance to treatments as well as variations in genetic mutations/cytogenetics between pediatric and adult ALL [155]. For instance, the dose-intensified multiagent combination treatment of pediatric ALL is not very well tolerated in adult ALL, particularly in elderly patients, and leads to a significant increase in treatment toxicities and a drop in OS. In addition, adult ALL is associated with more high-risk cytogenetics, such as BCR-ABL1 (Philadelphia (Ph) translocation) which constitutes 40 - 50% of ALL cases in patients over 55 years but only 3% in children [155]. Nevertheless, despite the progress in pediatric ALL research, it remains one of the leading causes of death in children and young adults.

Genetic alterations in B-ALL

B-ALL is characterized by an early disruption of B-cell differentiation, followed by excessive proliferation of B-cell progenitors (pro/pre-B cells). The disease manifests when these cells fill up the BM and infiltrate secondary lymphoid organs, reaching in many instances the CNS. B-ALL is a multistep disease initiated by various genetic and epigenetic alterations in critical hematopoietic regulators, and the biology of the disease differs with age. The most common initiating events involve aneuploidy and chromosomal translocations, but cooperating mutations are usually needed for disease progression. Although karyotyping and fluorescence in situ hybridization (FISH) have been widely employed to detect genetic abnormalities in B-ALL, recent advances in genome analysis have yielded a more comprehensive understanding of the disease. This has allowed for further stratification of B-ALL subtypes, enabling more accurate risk assessment and better-suited treatment protocols (Table 1).

First event

Several aneuploidy subtypes are associated with B-ALL. High hyperdiploidy is a genetic abnormality characterized by a gain of at least 5 extra chromosomes. It is

present in ~25% of pediatric and only 7% of adult B-ALL patients, and generally has a favorable prognosis [175,176]. In contrast, hypodiploidy, which involves the deletion of two or more chromosomes, is very rare in children but accounts for ~10% of adult B-ALL. The low hypodiploidy subtype (32-39 chromosomes) is particularly prevalent and has a very poor prognosis [155]. Intrachromosomal amplification of chromosome 21 (iAMP21) is another high-risk aberration characterized by multiple copies of the *RUNX1* gene on a structurally abnormal chromosome 21. It accounts for ~2% of pediatric and ~11% of adult B-ALL cases [175,176]. Trisomy 21, or Down syndrome, increases the risk of pediatric B-ALL ~10-12 folds, with a diagnosis peak around the age of 10 [153].

Regarding translocations, chromosomal rearrangements involving *MLL1* (MLL-r) are the most common cause of leukemia in infants, accounting for up to 80% of B-ALL (and ~50% of AML, discussed later). The most common *MLL* fusion partners of infant B-ALL are *AF4* (*AFF1*), which accounts for ~50% of the cases, followed by *ENL* (*MLLT1*), and *AF9* (*MLLT3*) [177]. The incidence of MLL-r decreases in older children and young adults but rises again with age to constitute >10% of adult B-ALL cases. Regardless of age, MLL-r often have a very dismal prognosis.

After the age of two, the incidence of ALL starts to peak with the increased prevalence of ETV6-RUNX1 (E/R)-associated B-ALL, which constitutes ~30% of childhood but <1% of adult cases (discussed in detail later). E2A-PBX1 on the other hand occurs in 5% of children and adults B-ALL and has a favorable prognosis, unlike the rare E2A-HLF fusion gene (~1%), which has a poor prognosis [155].

As mentioned before, patients with BCR-ABL1 are mostly adults and used to have a very poor prognosis. Currently, while still unfavorable, tyrosine kinase inhibitors have significantly improved the disease outcome [155].

Aside from these well-established molecular subtypes of B-ALL, genomic analysis within the last few years identified new subtypes, including *DUX4*, *MEF2D*, and *ZNF384* rearrangements [155,178]. In addition, there are also the E/R-like and Ph-like subtypes, which express similar molecular signatures and immunophenotypes to E/R and Ph-positive subtypes but lack the expression of E/R or BCR-ABL1 fusion genes, respectively [155,176,178]. Combined, these new subtypes represent >30% of B-ALL cases in children and adults that were previously unknown (Table 1). There is also a rare subset of pediatric B-ALL with inherited genetic mutations in critical regulators such as *ETV6*, *PAX5*, and *TP53* [179].

Secondary mutations

Most of the previously mentioned first-hit mutations confer a pre-leukemic state, and the acquisition of secondary mutations is usually needed for leukemic transformation. Generally, the mutational burden in pediatric malignancies is lower than in adults, and B-ALL is no exception [180]. However, some subtypes are associated with more secondary mutations than others. For instance, MLL-r have a

very low average of additional events (~1.3 mutations) [181], while E/R and BCR-ABL1 have an average of 6 – 8 aberrations per case [182]. The relatively high mutational rate results in subclonal variation, which is commonly seen in B-ALL and governs disease progression and relapse.

Secondary mutations affecting genes that regulate B-cell development, including *PAX5*, *EBF1*, and *IKZF1*, are recurrent events found in almost two-thirds of B-ALL cases [182]. These mutations are typically loss-of-function or dominant-negative mutations that arrest B-cell differentiation. Certain mutations can significantly impact risk stratification and treatment protocols. For instance, *IKZF1* mutations usually predict a poor disease outcome, while *PAX5* mutations are less detrimental [183]. Other common mutations in B-ALL affect cell cycle regulators (*CDKN2A*), tumor suppressors (*TP53*), RAS signaling (*NRAS* and *KRAS*), protein kinases (*FLT3* and *JAK*), and *ETV6* deletions [173].

In addition, certain mutations are more common in specific B-ALL subtypes. For instance, mutations in kinase-activators, such as *ABL1*, *JAK*, *EPOR*, and *Flt3* are very common in Ph-like B-ALL, while RAS-activating mutations are the most common in MLL-r [155].

Table 1. Prevalence and prognosis of B-ALL subtypes.
Adapted from [175,176]

ALL subtype	Children	Adults	Prognosis	Frequent mutations
Hyperdiploidy	25%	7%	Good	RAS pathway, *PAX5*
Hypodiploidy (low)	Rare	~10%	Poor	*TP53, IKZF2/3*
iAMP21	2%	11%	Poor	
E/R	25 – 30%	1%	Good	*ETV6, PAX5*
MLL-r	~80% (infants)	10%	Poor	*RAS* (subclonal)
BCR-ABL1	3%	40 - 50%	Poor	*IKZF1, CDKN2A/B*
Ph-like	15 - 20%	30 – 40%	Poor	Several kinases, *IKZF1, CDKN2A/B*
E/R-like	~5%	Rare	Intermediate	*ETV6, IKZF1*
E2A-PBX1	~5%	~5%	Good	
E2A-HLF	1%	1%	Poor	*PAX5, RAS, E2A*

E/R

The t(12;21)(p13;q22) translocation results in a fusion between the transcription factors ETV6 and RUNX1. E/R is almost restricted to children and young adults and is the most prevalent chromosomal translocation in this category of patients [155]. The fusion gene is restricted to the precursor B-ALL subtypes, marked by CD19 and CD10 expression, RAG and TdT activity, and clonal rearrangement of IgH. Despite the precursor B-cell phenotype, E/R B-ALL often co-express several myeloid markers [184,185].

Patients with E/R have a favorable prognosis and current treatment protocols achieve 5-year OS rates > 90%. However, relapse occurs in >20% of the patients and it can be remarkably delayed (10 – 20 years) compared to other leukemia subtypes [186–188]. While the high incidence of E/R relapse might suggest a re-evaluation of the disease prognosis, E/R-relapsed patients respond well to therapy and have a significantly better prognosis than E/R-negative patients [188,189].

Detection of E/R translocations in neonatal blood spots (Guthrie cards) has confirmed its prenatal origin. It has been suggested that at least 1% of all neonates have cells with the E/R translocation, and with more sensitive detection methods, this frequency increased to 5% [190,191]. Additionally, the detection of identical E/R breakpoints in monozygotic twins, and in some instances common IgH rearrangements, provided further support to the in-utero origin of the disease [192,193].

Despite continuous efforts, the cell of origin in which E/R arises remains unknown. However, accumulating evidence suggests that it is an immature stem/progenitor cell before the pro-B cell stage. Retrospective analysis of neonatal blood spots from leukemia patients revealed that E/R-expressing clones can covertly persist in the BM for 15 years [194,195]. In addition, the expression of some myeloid markers in half of E/R B-ALL cases suggests a multilineage potential of the LIC [196,185]. Studies have shown that a subset of candidate stem cells with pro-B cell properties, characterized by the expression of $CD34^{+}CD38^{-/low}CD19^{+}$, were able to propagate E/R leukemia in immunocompromised mice [194,197]. However, this population was later found not to exist in all patients [198]. In further support of this notion, genetically engineered E/R mouse models with restricted expression to committed B-cell progenitors failed to generate any hematological phenotype, suggesting that the premalignant clone arises in HSPCs [199,200]. Another study that used a human pluripotent stem cell system to model embryonic B-cell lymphopoiesis has suggested an $IL7R^{+}$ HSPC as a candidate cell of origin for E/R leukemia. However, the leukemogenic potential of these cells has not been verified [79]. Several animal-model-based studies have also reported expansions in the HSPC compartment in response to E/R expression [201–203]. However, further research is still needed to unequivocally identify the potential cell that can initiate the disease.

E/R by itself is insufficient for B-ALL development and secondary mutations are required for disease progression. This is supported by the low rate of concordant leukemia in monozygotic twins (5%), and the long and variable latency between the first event and diagnosis, which can last over a decade [204,205]. Fortunately, only a small proportion of the translocation carriers (0.2-1%) develop the necessary secondary mutations for leukemic transformation [206].

Patients with E/R B-ALL have an average of 3.5 secondary mutations per case [207]. Mutations are usually sub-clonal, rendering some clones more aggressive than others [208]. Deletions of the non-rearranged *ETV6* allele are recurrent events in ~70% of the patients [209]. ETV6 acts as a tumor suppressor through dimerization and inhibition of E/R targets; therefore, its deletion contributes to E/R leukemogenesis [207,210,211]. Other common secondary events include gaining extra copies of *RUNX1* (~23%), and der(21)t(12;21), a biallelic rearrangement with a gain of an extra *RUNX1* copy [212]. Deletions of regulators of B-cell development, such as *PAX5*, *EBF1*, and *E2A*, and the cell cycle inhibitors *CDKN2A/B* are also among the most recurrent secondary events in E/R [209].

ETV6 and RUNX1 fusion on a structural level

ETV6 consists of a pointed N-terminal (PNT) domain, a central regulatory domain (repression domain), and a highly conserved ETS DNA binding domain at its C-terminal (Figure 5) [112]. Due to the C-terminal autoinhibitory activity, ETV6 forms oligomers with itself or other ETS transcription factors through the PNT domain, which is a critical step for DNA binding activity. ETV6 is a dominant transcription repressor, and it employs several co-repressors to mediate its activity, including SIN3A, NCOR, and histone deacetylases (HDACs) [112].

RUNX1 has an N-terminal region that can mediate transcriptional activation, followed by a DNA-binding Runt homology domain [213]. Other functional domains in the RUNX1 protein include a TAD that interacts with co-activators p300 and CREBBP, an inhibitory domain, and a VWRPY motif in the C-terminal region that mediates transcription repression by binding the co-repressor TLE1 (Figure 5). To exert its roles, RUNX1 heterodimerizes with CBF-β through the Runt domain. This enhances RUNX1 DNA-binding activity, allowing for the activation or repression of its target genes [214].

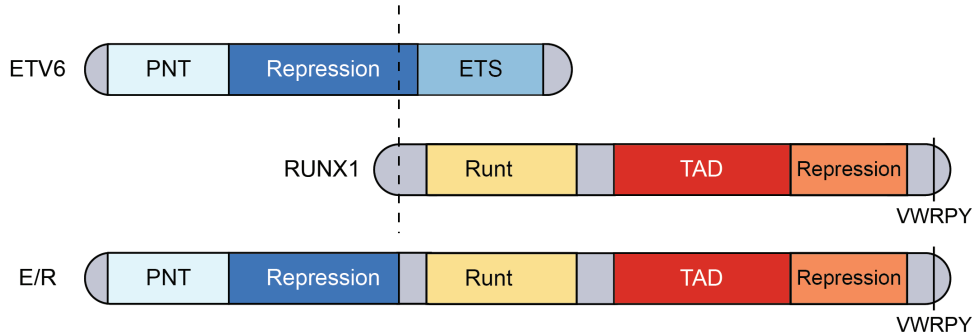

Figure 5. Schematic depiction of the structure of *ETV6*, *RUNX1*, and the fusion gene E/R.

As shown in Figure 5, the t(12;21) translocation fuses most of the RUNX1 gene, encompassing exons 1 and 2, with the first five exons of ETV6 [215]. The translocation typically results in the loss of the ETS domain of ETV6. Thus, the DNA-binding activity of the resulting E/R fusion protein is mainly mediated by the DNA-binding domain of RUNX1. Nonetheless, the retained PNT and inhibitory domains of ETV6 keep recruiting the co-repressors SIN3A, NCOR, and HDAC, leading to transcriptional repression of RUNX1 target genes [216,217]. Furthermore, the PNT domain enables dimerization of E/R and ETV6, which disrupts ETV6 activity [218]. Indeed, there is an inverse correlation between E/R expression levels and the transcription repression activity of ETV6, highlighting the importance of ETV6 targets in propagating ALL [218]. ETV6 has been shown to repress the expression of several genes implicated in the survival and proliferation of leukemia cell lines, such as *SPHK1*, *PTGER4*, and *CLIC5* [176].

Molecular implications of E/R fusion

The mechanisms whereby E/R initiates B-ALL remain elusive. However, studies on ETV6 and RUNX1 dysregulation shed some light on the implications of the E/R fusion gene. As discussed earlier, both ETV6 and RUNX1 are critical regulators of hematopoiesis, and their deletions lead to significant hematopoietic perturbations. Thus, it is not surprising to find these genes mutated in several hematological malignancies or fused with different binding partners.

Most *RUNX1* mutations are located in the Runt domain and affect its DNA-binding ability. In addition, RUNX1 is frequently involved in chromosomal translocations, with > 50 binding partners identified. In these events, one of *RUNX1* alleles is usually disrupted [219]. The most common translocations that involve RUNX1 include E/R, RUNX1-ETO, RUNX1-MECOM, and RUNX1-CBFA2T3. Except for E/R, all these translocations are associated with AML. Notably, a unique feature of E/R is that it retains the C-terminal and Runt domains of RUNX1, unlike the other translocations.

RUNX1 mutations usually result in impaired differentiation and increased proliferation of HSPCs [219]. While this increases the propensity to leukemia, *RUNX1* mutations/translocations are usually heterozygous and complete deletion is very uncommon [220]. In addition, recent studies have suggested the importance of the non-rearranged *RUNX1* for optimal leukemia growth, and knockdown of this gene or its partner CBF-β can abrogate both ALL and AML [221,222]. The criticality of the non-rearranged *RUNX1* extends to MLL-r leukemias, where *RUNX1* expression has been shown to promote the survival of leukemic cells [223,224].

On the other hand, loss of function mutations or hemizygous deletions in *ETV6* increases genetic susceptibility to both ALL and AML [225]. Similar to *RUNX1*, there are more than 30 chromosomal rearrangements involving *ETV6* [112]. ETV6 rearrangements can drive transformation through multiple mechanisms, such as dysregulation of transcription, activation of kinase activity when fused to protein kinases, and disruption of the expression and function of the non-rearranged *ETV6* gene [226].

Apart from the regulatory roles of ETV6 and RUNX1, several molecular pathways are directly dysregulated by the E/R fusion gene. For instance, a recent study has shown that E/R can directly bind and increase RAG1 expression [227]. Previous studies have endorsed the role of RAG1 in inducing secondary mutations and promoting transformation [228,229]. This suggests that E/R might be implicated in both leukemia initiation and progression [227]. In addition, E/R can also activate STAT3, which has been shown to positively regulate early B-cell progenitors, and induce MYC activity [230,231]. E/R also binds EPOR and increases its expression, which enhances survival through activation of the JAK/STAT5 and PI3K/AKT/mTOR downstream pathways [232]. PI3K/AKT/mTOR as well is directly activated by E/R, since knock down of E/R in REH cell (a human cell line of E/R B-ALL) has been reported to abrogate PI3K/AKT/mTOR activity and reduce proliferation and survival [233].

In addition, E/R transactivates MDM2, which is the predominant negative regulator of p53 [234]. Suppression of p53 activity is a key step in E/R leukemogenesis, and inhibition of MDM2/p53 interaction leads to cell-cycle arrest and apoptosis [234]. Furthermore, upregulation of RNA binding protein IGF2BP1 is frequently associated with E/R B-ALL and proposed as a diagnostic biomarker for this specific subtype of ALL [235,236]. IGF2BP1 is suggested to mediate leukemogenesis partly by binding and stabilizing E/R and STAT3 mRNA levels [237].

The discrepancies between the high incidence rate of the first hit and the lower rates of disease development confirm that the acquisition of secondary mutations is the rate-limiting step in E/R leukemogenesis. However, what triggers this step remains unknown. E/R pre-leukemic cells have been suggested to have elevated ROS levels, which can induce genome instability and lead to mutations [238]. As mentioned

previously, RAG activity is proposed as a main mechanism by which secondary mutations are acquired, and exposure to infections is suggested to trigger its activity [239,240]. In addition, E/R reduces the sensitivity to TGF-β-mediated inhibition of proliferation, which suggests that the pre-leukemic clones might gain a competitive advantage during pathogenic infections. This highlights the role of infection in the process of transformation from covert to overt leukemia.

Infection hypothesis

The concept that infections could be a causative factor in childhood ALL has been around for almost a century. Childhood ALL are suggested to arise as a result of an abnormal immune response to infections. This hypothesis provides the most plausible explanation for the patterns of childhood ALL. Evidence from epidemiological and modeling studies has yielded two models that explain the link between infections and childhood ALL: the population mixing hypothesis by Kinlen, and the delayed infection hypothesis by Greaves [240,241].

Kinlen's hypothesis arose from the observation of a temporary surge in childhood leukemia cases following mass migration events in rural areas. The introduction of new low-antigenic pathogens to non-immune populations was postulated as the underlying cause [241].

Greaves' hypothesis proposes that common infections might have opposing effects, depending on the time of exposure. Early exposure to common antigens can have a prophylactic effect against ALL, as it shapes the microbiome, builds immune tolerance, and has long-term impacts on immune function [242,243]. Infants who lack this experience may develop a dysregulated immune response upon encountering common infections later in life, which can be a trigger for transformation. This hypothesis is supported by studies that have found a lower incidence rate of ALL in children who attended nursery during their first year [244]. Additionally, it may also explain the increasing rates of childhood leukemia in modern societies, which could be attributed to increased hygiene levels [240].

Although the two hypotheses may appear distinct, they both propose that leukemia arises from an aberrant immune response triggered by infections in an inexperienced immune system (Figure 6). However, the mechanisms behind this remain largely unknown, and further research in this area could lead to the development of preventive therapies.

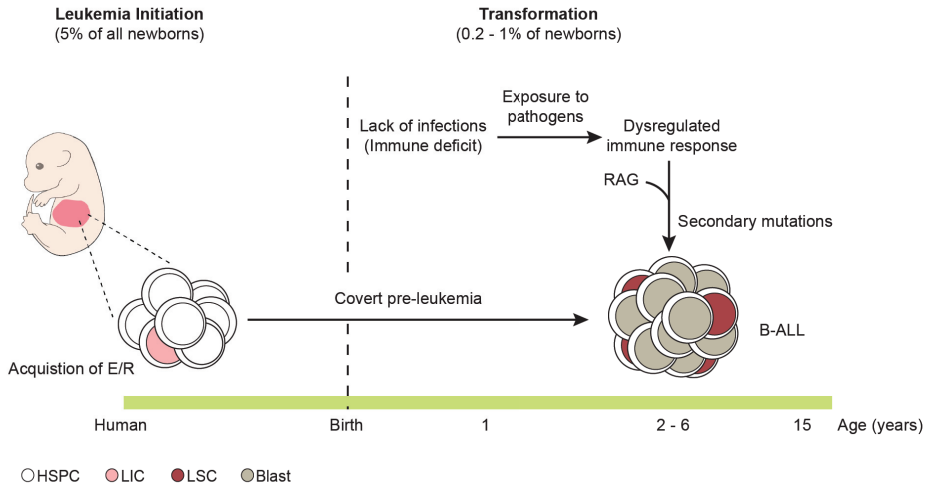

Figure 6. Overview of the potential role of infection in E/R leukemogenesis.

Relapse

Genomic studies have demonstrated a significant clonal diversity during the initial diagnosis of E/R B-ALL, suggesting complex and branched trajectories of clonal evolution that govern disease development and relapse. E/R relapses are characterized by their late onset, as most cases occur three years after diagnosis but can occasionally exceed 10 years [187–189]. The simplest pattern of relapse is when the initial dominant clone re-emerges after treatment. In such cases, IgH rearrangements of the dominant clone are expected to be the same at diagnosis and relapse [245,246]. However, variations in IgH rearrangements are sometimes found in relapse clones, which indicates that they might be derived from a lingering pre-leukemic ancestral clone [247–249]. Relapse can also be derived from minor clones that preexisted at the time of diagnosis, which is a commonly observed event in E/R B-ALL [250–252]. These minor clones might possess some mutations that confer poor response or resistance to treatment. Additional mutations can also emerge later on and accelerate disease progression; hence, the higher mutational burden in relapse cases [180]. The most common events found in relapse involve loss of function mutations/deletions in *NR3C1/2*, which encode for glucocorticoid receptors, *CREBBP*, a histone acetyltransferase, *CDKN2A/B*, regulators of the cell cycle, and *IKZF1* [207,209]. Understanding how relapse happens and the involved molecular pathways can be critical for the development of targeted therapy approaches.

AML

AML is a highly aggressive malignant disease characterized by uncontrolled proliferation of immature myeloid precursor cells. It is the most common AL form in adults, accounting for ~80% of the cases, and its incidence particularly increases above 60 [253]. AML also comprises ~20% of pediatric leukemias, with high incidence during infancy and adolescence [254]. However, there are significant distinctions between pediatric and adult AML that they can be almost considered two separate diseases. Nevertheless, AML generally has a relatively poor prognosis in comparison to other leukemia subtypes, accounting for ~60% of all leukemic deaths [255]. The 5-year OS rates of AML in children and adults are between 60 – 70% and 25 – 30%, respectively [255,256].

Classification of AML

AML is a very heterogeneous disease that can be broadly categorized based on disease etiology into de-novo, therapy-related, and secondary AML, the latter of which evolves from prior myelodysplastic syndrome (MDS) or myeloproliferative neoplasms (MPN). However, AML classification can also be based on morphology, immunophenotype, and genetic features. The historical French American British (FAB) system was developed in the 1970s and classified AML into 6 categories based on morphology (M1 – M6). Later, when immunophenotyping was incorporated, two more subtypes were added (M0 – M7). However, with the advancement of genomic analysis techniques, the WHO system was introduced, which incorporates recurrent genetic and cytogenetic abnormalities for a more accurate classification. The latest update to the WHO AML classification, the 5th edition, was released in 2022 and is summarized in Table 2 [257].

Unlike the 2016 edition, the latest edition recognizes nucleophosmin (*NPM1*) and monoallelic *CEBPA* mutations as AML-defining subtypes. However, the group for *RUNX1* mutations has been removed as they overlap with molecular features that define other AML subtypes.[257]. The previous classification systems used a blast count threshold of 20-30% for AML diagnosis in most cases. However, the 2022 update lowers the threshold to 10%, as the detection of specific genetic or cytogenetic abnormalities is now considered sufficient for the diagnosis of de novo AML. Exceptions to this rule include *CEBPA* mutations and the ‘’AML with myelodysplasia-related changes’’ category, which includes *SRSF2, SF3B1, U2AF1, ZRSR2, ASXL1, EZH2, BCOR, STAG2* mutations and other unbalanced translocations. In these cases, a blast count >20% is required to exclude MDS and MPN. This same cut-off also applies to BCR-ABL1 for excluding CML [257]. It is worth noting that *NPM1* mutations were previously associated with clonal hematopoiesis and MDS, but due to their rapid progression to AML, they are now categorized as AML-defining genetic mutations. These updates allow for a more accurate diagnosis and classification of AML.

Table 2. WHO 2022 classification of AML subtypes and the associated prognosis [257–259].

AML classification	Prognosis
AML with defining genetic abnormalities:	
PML-RARA (promyelocytic)	Favorable
RUNX1-RUNX1T1	Favorable
CBFB-MYH11	Favorable
KMT2A-r	Adverse
DEK-NUP214	Adverse
RBM15-MRTFA (megakaryoblasic)	Intermediate
BCR-ABL1	Adverse
MECOM-r	Adverse
NUP98-r	Adverse
NPM1 mutations	Favorable
CEBPA mutation	Favorable
AML with myelodysplasia-related	Adverse
AML with other defined genetic alterations	Adverse
AML defined by differentiation	
AML post cytotoxic therapy e.g AML with *TP53* mutations or MLL-r	Adverse

Role of genetic abnormalities in AML pathogenesis

Knudson's two-hit hypothesis has significantly contributed to our understanding of leukemia pathogenesis. AML develops in immature cells through the sequential acquisition of mutations. These mutations were initially classified into two classes, with at least one mutation from each class required for AML development. Class I mutations provide a proliferative advantage to the cells, while class II mutations impede cell differentiation and enhance self-renewal [260]. However, this classification was incomplete, and subsequent studies identified additional mutations that did not fit into either class. Advances in genomic profiling techniques have revealed that AML is a complex disease, with numerous mutations contributing to clonal and subclonal variations. The prevalence of these mutations can change with disease progression, further highlighting the complexity of AML [261]. This heterogeneity builds up the architectural mosaic of AML.

AML typically has a lower mutational burden compared to other adult cancers, with an average of 13 mutations per patient [262]. However, this varies between AML subtypes, with some initiating events requiring more accompanying mutations than others. Moreover, advanced age, prior exposure to cytotoxic therapy, and underlying MDS or MPN, all increase the average number of mutations. Recurrent mutations in AML can be classified into nine categories based on their biological function. Activating mutations in signaling pathway components, such as *FLT3, RAS, KIT, CBL, NF1,* and *PTPN11*. Mutations in myeloid transcription factors such as *CEBPA, RUNX1,* and *PU.1*. Mutations in DNA methyltransferases (*DNMT3A*), demethylase genes (*TET2, WT1, IDH1,* and *IDH2*), *NPM1*, spliceosome-complex genes, cohesin-complex genes, and tumor suppressor genes such as *TP53*. Finally,

transcription-factor fusions such as PML-RARA, RUNX1:RUNX1T1, CBFB:MYH11, and RBM15-MRTFA [262].

Certain mutations exhibit patterns of cooccurrence or mutual exclusivity. For instance, *NPM1* mutation frequently cooccurs with *FLT3, DNMT3A, TET2,* and *IDH1/2* mutations [262,263]. In contrast, most chromosomal translocations are mutually exclusive of *NPM1* and *DNMT3A* mutations [262,263]. Strong associations between certain mutations and age further add to the complexity of the disease.

Genetic abnormalities in pediatric and adult AML

There are several fundamental differences between pediatric and adult AML regarding disease etiology and genetic landscape. While de novo cases account for over 95% of pediatric AML, a large proportion of adult AML cases arise from an underlying MDS/MPN (approximately 20%) [258]. Moreover, aging-related phenotypes, including myeloid-lineage bias, functional decline in HSCs, clonal hematopoiesis, and genome instability are all predisposing factors exclusive to adult AML. These differences are further highlighted by the contrasting genetic landscapes of pediatric and adult AML, with the former having an average of around 5 somatic mutations per sample, while the latter has approximately 13 mutations per sample (Figure 7) [264,265]. Despite the low mutational burden in pediatric AML, cytogenetic abnormalities are frequently found in ~80% of these patients, compared to < 50% in adult AML. While *FLT3* mutations are the most commonly occurring mutations across all age groups, other genetic aberrations exhibit age-related distribution [265,266]. For instance, mutations in the RAS pathway, *KIT*, and *WT1* mutations are commonly found in pediatrics. In contrast, *DNMT3A, TET2,* and *TP53* mutations are almost exclusive to adults, and together with *NPM1* and *IDH* mutations, they constitute the majority of events in adult AML. As for fusion genes, PML-RARA, RUNX1:RUNX1T1, CBFB:MYH11, and MLL-r are found in ~50% of pediatric but only ~25% of adult AML cases. Most of these translocations, except for MLL-r, are associated with a favorable prognosis, which could partly explain the higher OS rates observed in childhood leukemia [258,267]. Taken together, this suggests that the pathogenesis of AML in pediatric patients differs significantly from that in adults.

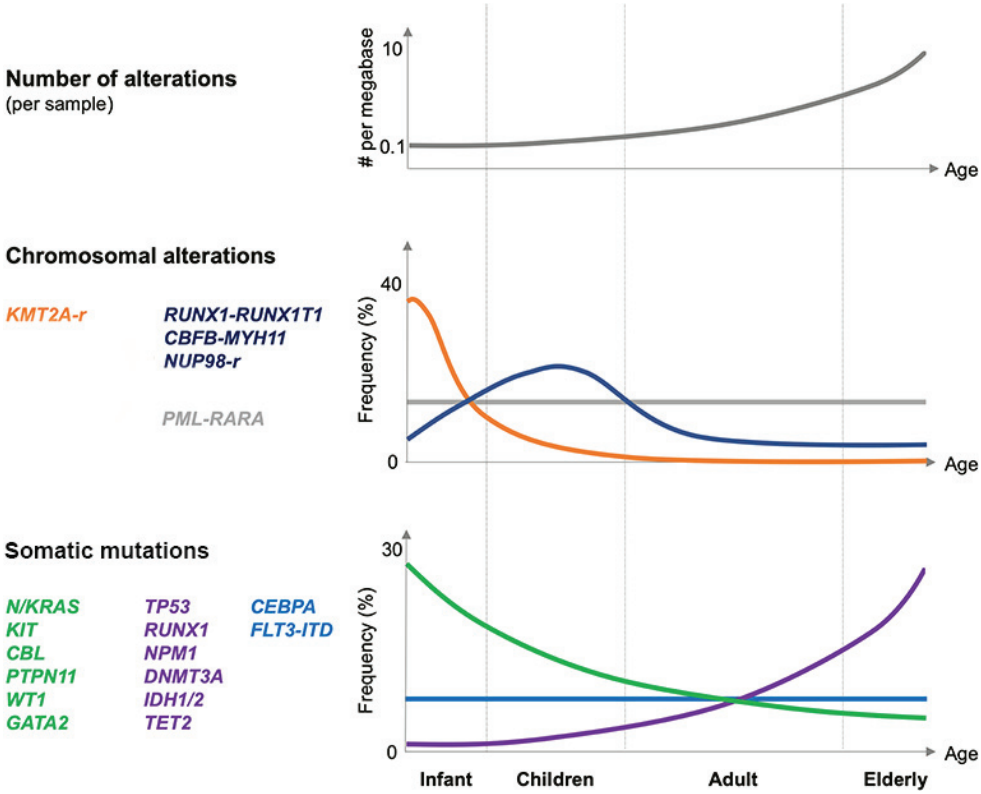

Figure 7. Frequency and distribution of genetic alterations in AML across ages (from Mercher and Schwaller, 2019 [266]).

MLL translocations

As mentioned previously, translocations involving *MLL1* generate one of the most aggressive forms of AML, ALL, or mixed lineage leukemia. The incidence rate of MLL-r differs between pediatrics and adults, and variations exist even within each age group. For instance, MLL-r account for ~50% of AML and ~80% of ALL infants, but their incidence drops in older children to 15% of AML and <5% of ALL cases [268,269]. As for adults, MLL-r constitute 10% of all leukemia cases, and a large fraction of those are therapy-related leukemias, which have been attributed to exposure to topoisomerase II inhibitors [269]. Regardless of the patient's age, the presence of MLL-r usually confers an adverse prognosis and is associated with a higher risk of resistance to treatment and relapse.

In infants, MLL-r typically arise during fetal development and have been detected in neonatal blood spots of leukemia patients [270,271]. The prenatal origin of MLL-r is further supported by studies on monozygotic twins, which have found identical

MLL fusion breakpoints and a high rate of concordant leukemia, approaching 100% [271,272].

Despite the prenatal origin of MLL-r, the incidence of congenital/neonatal leukemia is very low, accounting for less than 1% of all pediatric leukemia cases [172,273]. MLL-r leukemias usually develop postnatally and are presented with very few cooperating mutations, if any. An average of 1 – 2 mutations per case has been reported, which are often subclonal and lost in relapse [265,181,274]. Together, this suggests that the fusion gene by itself can be sufficient for disease development. The most recurrent mutations in MLL-r involve *KRAS, NRAS, PTPN11, BRAF,* and *FLT3* [181,265,274]. Understanding the structure and interactome of MLL1 can provide valuable insights into the molecular mechanisms underlying the aggressive nature of MLL-r leukemia.

Structure and interactions of MLL1

MLL1 encodes for a nuclear protein with multiple functional domains that interact with a diverse range of proteins and molecules to regulate gene expression (Figure 8). The N-terminal region contains DNA-binding AT hooks, sub-nuclear localization domains (SNL1 and 2), and repression domains (RD). This region also contains a DNA methyltransferase homology domain (CxxC), which binds non-methylated CpG-rich DNA regions. Adjacently, there are four plant homeodomain fingers (PHD), with embedded bromodomain (BD). PHDs bind H3K4me3 regions and recruit proteins to regulate the expression of MLL1. The C-terminal region encompasses a TAD and the highly conserved SET domain, which mediates mono-, di- or tri-methylation of H3K4 [115,275].

Proteolytic cleavage of MLL1 by Taspase 1 generates two fragments: $MLL1^{C}$, which comprises the TAD and SET domains, and $MLL1^{N}$, which has all the remaining domains [275]. Each of these fragments has specific functions, and together they form a complex with several other proteins to regulate chromatin accessibility and gene expression. The N-terminal fragment interacts with Menin, which further facilitates binding to the lens epithelium-derived growth factor (LEDGF). The formation of this ternary complex is critical for the activation of downstream target genes, such as *HOX* and *MEIS1*, which mediate the functions of both WT and rearranged *MLL1* [276–278]. On the other hand, the H3K4 methyltransferase activity of MLL1 is mediated by the C-terminal SET domain through interaction with RBBP5, ASH2L, WDR5, and DPY30, known as the WRAD complex [115,279]. It has been shown that homozygous deletion of SET has no impact on normal hematopoiesis or leukemogenesis, suggesting that the methyltransferase activity of MLL1 is dispensable. In contrast, the recruitment of the histone acetyltransferases MOF by the MLL complex has been found to be critical for the expression of MLL1 target genes [280].

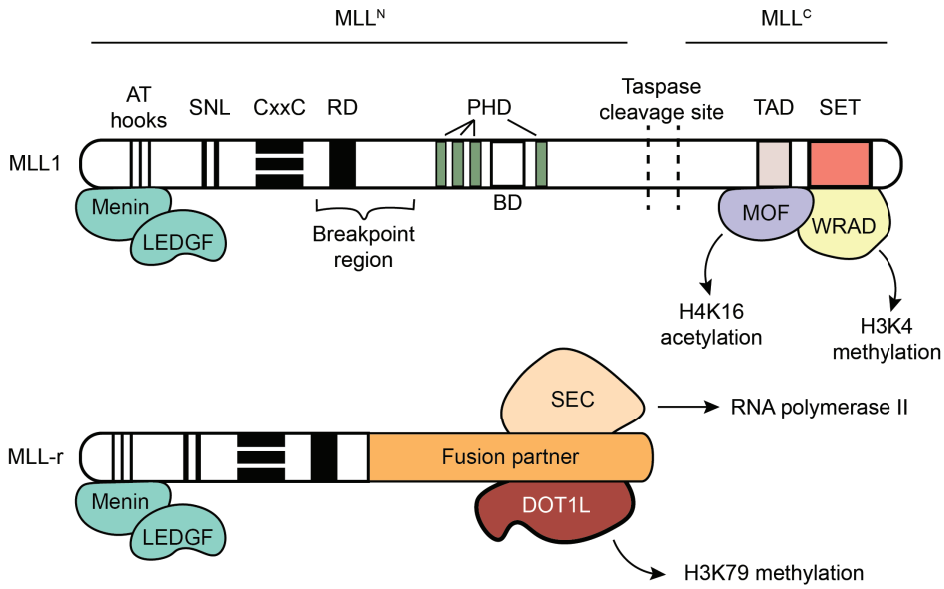

Figure 8. Structure and interactions of *MLL1* (top) and MLL-r (bottom).

MLL fusion partners

MLL translocations result from monoallelic double-stranded breaks at exons 8-13 of *MLL1* followed by fusion with one of over 80 partner genes [275]. The necessity of the WT *MLL1* allele in leukemogenesis has been debated, with studies reporting conflicting results [281–284]. The *MLL1*-rearranged allele lacks the PHD fingers domain and the entire MLLC region (Figure 8). By itself, the truncated *MLL1* gene is devoid of any transformative ability, highlighting the functional importance of the fusion partner [285]. Among the multiple identified fusion partners, there are 5 that constitute ~80% of the MLL-r cases, which are *AF4* (36%), *AF9* (19%), *ENL* (13%), *AF10* (8%), *ELL* (4%) [268]. Certain fusion partners seem to impact the leukemia subtype and have apparent age-related distribution. For instance, MLL-AF4 is mostly associated with ALL, while MLL-ELL is predominantly found in AML, but both are found in all age groups. In contrast, MLL-AF9, MLL-ENL, and MLL-AF10 are found in both AML and ALL, but with distinct age association patterns. In infants, MLL-AF9 and MLL-AF10 are commonly linked to both AML and ALL, while MLL-ENL is predominantly found in ALL. However, in older children and adults, MLL-ENL can generate both AML and ALL, whereas MLL-AF9 and MLL-AF10 are more frequently associated with AML [268]. While the identity of the fusion partner can influence leukemia subtype, age-related differences in the microenvironment can also play a role in conferring lineage bias [286]. For instance, a fetal microenvironment may be more permissive to ALL, whereas an adult microenvironment may be more conducive to AML [286].

Furthermore, there are indications that certain fusion partners may influence the outcome of the disease, like MLL-AF9 which has a better prognosis in pediatric AML compared to other MLL-r [287]. While most of these fusion partners are transcription elongation factors, it is not completely clear how their distinct regulome might differentially contribute to leukemogenesis.

Molecular Mechanisms of MLL-r

Despite the large variety of MLL fusion partners, MLL-r leukemias share a distinct transcription signature, suggesting a functional overlap between MLL fusion partners [288–290]. Indeed, most of these fusion genes belong to the super elongation complex (SEC), which includes AF4, AF9, ENL, ELL, and positive transcription elongation factor b (P-TEFb) [291]. When MLL fuses with any of these partners, it recruits SEC and enables efficient transcription of MLL-r target genes (Figure 8) [292]. This process is mediated by the interaction between SEC and the H3K79 methyltransferase DOT1L, which is crucial for the expression of MLL-r targets [293,294]. Increased levels of H3K79 methylation on the promoters of Hoxa9 and Meis1 provide evidence for this [295,296]. DOT1L activity compensates the loss of H3K4 methyltransferase activity of the SET domain after translocation, and its inhibition impairs MLL-r leukemogenesis [275,296].

The N-terminal region of MLL remains crucial for the fusion gene activity, and is regulating *HOX* and *MEIS1* expression through recruitment of Menin [297]. HOXA9/MEIS1 subsequently activate their downstream target c-MYB, which is essential for MLL-r driven AML [298–300]. Targeting these indispensable components of the MLL-r machinery has been the aim of several lines of research, which allowed for the development of several drugs, including DOT1L and Menin inhibitors that are currently undergoing clinical trials.

Aims of the thesis

The overall goal of this thesis work was to understand the cellular and molecular mechanisms governing acute leukemia in children. While most of the genetic events that elicit childhood leukemia are often acquired in utero, congenital leukemia is very rare, and transformation usually occurs postnatally. This begs several questions: Are there fetal-restricted tumor suppressor pathways that protect neonates from leukemic transformation? What triggers transformation postnatally? Why do many children never develop leukemia despite the existence of a prenatal pre-leukemic clone?

We approached these matters using transgenic mice that model MLL-r and E/R, two of the most common fusions in pediatric leukemia, with the following specific aims in mind:

1) To investigate the influence of fetal hematopoietic programs, regulated by the fetal master regulator LIN28B, on the development of MLL-r AML (Paper I).

2) To identify and characterize the most recurrent mutation that drives MLL-r AML progression (Paper III).

3) To characterize HSCs activity both in steady state and in E/R-driven pre-leukemia (Paper II and IV).

Summary of included papers

Paper I

At a genetic level, human AML is a highly heterogeneous disease and this heterogeneity influences disease severity and preferred treatment. In both children and adults, balanced translocations that involve the MLL1/KMT2A gene (MLL-rearrangements; MLL-r) generate some of the most aggressive forms of AML. The very low mutational burden in pediatric patients suggests that MLL-r might be sufficient to drive transformation, without the need for additional secondary events, challenging Knudsen's classical two-hit theory on tumor formation. However, although pediatric MLL-r arise in utero, congenital/neonatal leukemia is very rare, with transformation typically occurring postnatally. These patterns of pediatric leukemogenesis allude to a fetal-specific tumor suppressor activity that declines after birth.

The expression of LIN28B, a master regulator of fetal hematopoiesis, is largely restricted to the prenatal stages of development, with an abrupt drop in expression a few weeks after birth. While a few studies have previously attempted to address the link between LIN28B and leukemia, there have been conflicting views emerging, with LIN28B being described both as an oncogene that gets upregulated in AML and as a tumor suppressor that restricts transformation. In light of this discrepancy, we interrogated cohorts of pediatric and adult AML patients and found that the vast majority of the samples lack the expression of LIN28B.

Since AML patients are admitted to the clinic at advanced stages of the disease, it becomes difficult, if at all possible, to study AML initiation and early stages of disease development in humans, including the stages at which LIN28B and MLL-r cooccur in LICs. Therefore, we here used highly defined transgenic models to explore the influence of LIN28B on MLL-ENL (ME) leukemogenesis.

Our results revealed that LIN28B interferes with AML initiation, evident by the complete abrogation of the disease in ~60% of the mice. Furthermore, when combined with activating RAS mutations, which generate very aggressive AML, LIN28B interfered with AML progression.

Transcriptional profiling revealed that LIN28B expression associates with significant downregulation of MLL-r and LSC signatures and upregulation of fetal lymphoid differentiation and apoptosis signatures. More importantly, we observed

depletion of c-MYB target genes. c-MYB activity is critical for MLL-r leukemogenesis, and its transient or partial suppression completely impedes AML. Therefore, we sought to identify how LIN28B might be interfering with c-MYB activity.

LIN28B exerts its effects through two primary mechanisms: 1) inhibition of *let-7* microRNA biogenesis, which in turn results in upregulation of *let-7* targets; and 2) direct binding to specific mRNA targets, thereby altering their translation. Disruption of *let-7* activity in ME LICs had no impact on AML progression, which indicated that the tumor suppressor activities of LIN28B are largely driven by its direct binding activity. Thus, to identify the mRNA targets bound by LIN28B, we performed individual-nucleotide resolution UV cross-linking and immunoprecipitation followed by sequencing (iCLIP-seq). We screened the RNA binding partners of LIN28B for potential MYB regulators and identified MYBBP1A. The interaction between LIN28B and MYBBP1A mRNA results in a remarkable increase in MYBBP1A protein levels.

MYBBP1A has been previously described as a tumor suppressor in several non-hematopoietic malignancies. It exerts its function through binding c-MYB and inhibiting its targets. Loss of MYBBP1A has been reported to increase MYB activity and induce a metabolic shift toward oxidative phosphorylation; both of which have also been proposed to be critical for LIC maintenance. Indeed, enforced expression of MYBBP1A captured to some extent the tumor suppressor activity of LIN28B, and its knockout restrained it.

Combined, this work describes a novel fetal-associated tumor suppressor axis involving LIN28B→ MYBBP1A –| MYB that restricts MLL-r AML and perhaps also other tumors with a MYB involvement. The developmentally restricted expression of LIN28B provides a natural protection against MYB-dependent tumors, while its abrupt decline a few weeks after birth presents an opportunity for leukemogenesis. This uncovers a molecular layer of protection against leukemogenesis and adds significant new functional and molecular insights into the roles of LIN28B during development, which can be exploited further for therapeutic benefits against AML of all ages.

Paper II

E/R is the most common chromosomal translocation in pediatric cancer and is exclusively associated with B-ALL. Although the fusion gene arises in utero and can be detected in about ~5% of all newborns, only a few individuals acquire the additional mutations/events necessary for transformation. The time between the initial and secondary events can span over a decade. However, in the absence of secondary mutations during childhood, the pre-leukemic clones become extinct,

which is evident by the sharp decline in the incidence of E/R B-ALL in young adults. Despite the generally favorable prognosis of E/R B-ALL, relapse rates are relatively high. Many aspects of the disease, such as the cell of origin, the mechanisms underlying the persistence of pre-leukemic clones in patients for years, and the triggers for transformation, remain largely elusive. By understanding these cellular and molecular mechanisms, new targeted or even prophylactic therapies can be developed.

Studying early events of E/R-driven leukemogenesis in children is very challenging, and such events cannot be deduced from established tumors at diagnosis. Here, we generated a mouse model (iE/R) that can reversibly induce physiologically relevant expression levels of E/R in primary cells. We also confirmed that E/R expression can significantly accelerate B-ALL development when combined with *Pax5*$^{+/-}$ and *Ebf1*$^{+/-}$, which are among the most common mutations in E/R B-ALL.

With the model at hand, we directed our work towards understanding E/R-driven pre-leukemia. Induction of iE/R, which simulates the first event in E/R leukemogenesis, resulted in a marked expansion of candidate HSCs and early block in B lymphopoiesis. Previous studies have suggested that HSCs expand in response to E/R expression [201,203], and results from human-based models have provided some support to this notion [197,79]. However, the permanent expression of E/R oncogene in all these models confounds experimental work aimed at defining HSC function. This is because 1) the fusion gene might impose changes in the unique properties of HSC, such as multilineage differentiation, which is a hallmark for appropriate HSC identification. 2) Fusion genes such as E/R might induce an aberrant cellular phenotype that could make cells phenotypically resemble HSCs. With the benefits offered by our model, which permits switching off E/R expression, we were able to confirm that candidate HSCs expanding in response to E/R possess serially transplantable long-term multilineage reconstitution potential.

Additional evidence supporting the role of HSCs in E/R leukemogenesis was obtained from BM transplantation experiments. In these experiments, E/R expression impaired overall hematopoietic reconstitution, particularly affecting lymphopoiesis. However, E/R-expressing HSCs were able to persist in the BM for extended periods with retained multilineage reconstitution potential. These cells were then outcompeted by WT cells in competitive transplantation assays. These findings suggest that HSCs may represent the cell of origin for E/R-driven leukemia and demonstrate that E/R clones are poorly competitive.

Consistent with the disease patterns, E/R fetal cells appeared to possess a higher competitive advantage than their adult counterparts. Transcription profiling of HSPCs revealed that E/R expression suppressed RUNX1 and MYC targets, but induced several inflammatory pathways, including interferon-gamma and alpha responses, TNF-alpha, JAK/STAT, and MHC-I antigen presentation-associated pathways. More importantly, E/R fetal HSPCs possessed much higher expression

and activity of the immune modulators PD-L1 (CD274) and CD200r, which might explain the persistence of E/R prenatal clones that are otherwise poorly competitive.

In light of these findings and the proposed connection between infections and E/R transformation, we finally tested whether E/R pre-leukemic cells might have an advantage in a context of viral mimicry. Indeed, E/R fetal and adult cells exhibited drastically different responses to poly I:C. Unlike adults, E/R fetal HSPCs were significantly more competitive and able to generate large numbers of corrupted B-cell progenitors. Taken together, apart from suggesting immune modulation and checkpoint inhibitors as potential treatments for E/R leukemia, our results begin to explain the tight link between ontogeny and E/R leukemia.

Paper III

AML arises from a serial acquisition of primary (epi)genetic changes in an immature blood cell. The primary lesion endows the target HSPC with the capacity to initiate leukemia (LIC). Transformation thereafter occurs following the acquisition of additional/secondary mutations, which leads to tumor growth in a highly deregulated and aggressive manner. The identity and actions of the secondary mutations can thereby shed light on the pathogenesis of AML and provide molecular candidates for therapeutic targeting. However, AML is a highly heterogeneous disease with distinct genetic lesions influencing disease subtype, severity, and preferred treatment. Balanced chromosomal translocations involving MLL-r generate one of the most aggressive AMLs. Previous studies have shown that MLL-r are usually accompanied by very few secondary mutations, particularly in younger patients, and these mutations are mostly subclonal. Established AML samples provide little information on the early stages of disease development and the stepwise process of transformation. Thus, to approach this, we used a defined murine transgenic model, in which MLL-ENL can be induced as the primary genetic lesion, and we aimed to identify the secondary events that underly AML progression.

First, we established the relevance of our model regarding AML disease patterns and clonality, mimicking human AML progression. Next, we performed exome sequencing to retrospectively identify candidate secondary driver events upon transformation. While recurrent secondary driver events were rare, a notable exception was a codon-changing mutation (Arg295Cys) in the ERM protein Moesin (MSN) that occurred in ~65% of the samples. Further experimental validation confirmed that the R295C mutant MSN can dramatically accelerate MLL-r AML progression, and its downregulation abrogates the disease.

MSN is known to regulate cell polarity, adhesion, and migration. While not previously reported in human AML, MSN mutations and/or expression

dysregulation have been found in several cancer contexts, including anaplastic large-cell lymphoma, melanoma, and breast cancer, and its loss of function mutation has been linked to an X-immunodeficiency syndrome. However, our structural modelling and biochemical validation results indicate that the R295C mutation is rather a gain of function mutation.

MSN contains a FERM domain that has three subdomains, F1 – F3, which form a cloverleaf conformation through interaction between F1 and the R295 residue in F3. This protein conformation is critical for homodimerization and inactivation of MSN [301]. Our results confirmed that the R295C mutation disrupts this, increasing the monomer form of MSN. Surveying human cancer samples in the TCGA, we observed two other R295 MSN variants: S295 and H295, both of which accelerated AML in our MLL-ENL mice. Intriguingly, we observed a direct correlation between the positive charge on the substituted residues and the pathogenicity of the disease.

Mass spectroscopy revealed only minor differences between the protein interactome of WT and mutant MSN. However, we found that mutant MSN converges on enhanced MAPK/ERK signaling, which is frequently hyperactivated in MLL-r AML.

Overall, this study offers valuable insights into the molecular mechanisms underlying MLL-r AML, emphasizing the significant role that MSN dysregulation plays in driving AML development and progression.

Paper IV

HSCs are characterized by their unique ability to establish hematopoiesis through their high self-renewal and multilineage differentiation capacities. Transplantation experiments are the gold-standard assay for assessing the functionality of HSCs and have contributed significantly to our understanding of HSC biology. However, these experiments are highly non-physiological, forcing HSCs to proliferate and restore the whole hematopoietic system in a lethally irradiated microenvironment. Thus, there is a need to approach the behavior of HSCs under homeostatic conditions.

Advancements in sequencing techniques and mouse models have enabled the identification of HSC-specific markers that allowed for evaluation of HSCs activity in native settings. Lineage tracing studies have yielded conflicting views on the contribution of HSCs to adult steady-state hematopoiesis. Several studies have suggested that unperturbed hematopoiesis is largely driven by progenitor cells rather than HSCs [302,303], while others have supported the view that HSCs have contribute to adult hematopoiesis [304].

To address these uncertainties, we employed an HSC-specific lineage tracing approach, using the previously generated Fgd5 reporter mice (Fgd5-CreERT2

ZsGreen) [305]. First, we confirmed that Fgd5 expression is restricted to HSCs on the single-cell transcriptomic level. Next, we crossed the Fgd5-CreERT2 mice with Tomato^{LSL} reporter mice to generate the Fgd5-CreERT2Tomato^{LSL} strain, which allowed us to label-trace HSCs progeny. BM analysis 48h after Tamoxifen injection confirmed the restricted expression of Tomato^{LSL} to HSCs.

With a validated model in hand, we set out to assess the contribution of HSCs to native hematopoiesis. For this, we induced the mice with Tamoxifen food for 16 weeks to ensure complete labelling of HSCs, followed by an extensive chase period (up to 41 weeks). Our results revealed that HSCs robustly contributed to all hematopoietic lineages but with distinct kinetics. For instance, platelets were the fastest to be generated from HSCs, followed by granulocytes and erythrocytes. However, lymphoid subsets took longer to acquire the label, with T cells being the slowest. These kinetics remained the same when 5 doses of Tamoxifen were administered instead of Tamoxifen food. However, label progression and robustness were lower when only 1 injection was administered.

As for progenitors, MPPs showed the fastest kinetics, reaching equilibrium to HSCs label only after 4 weeks. The MPP2 compartment was next, which reached equilibrium to HSCs label after ~32 weeks. Further characterization of MPP2 revealed that they were biased towards the MegE lineage. Similarly, and in line with the labelling trends in peripheral blood, MkPs showed equivalent labels after 32 weeks, followed by other myeloerythroid progenitors. In contrast, MPP3/4 acquired the label with much slower kinetics.

We also analyzed label progression in tissue-resident immune subsets, including Langerhans cells, microglia, and B1a cells, and found no contribution from adult HSCs to these cells. This supports their fetal origin and ability to self-maintain.

Finally, we investigated the contribution of HSCs to hematopoiesis in aged mice. This revealed a striking reduction in label progression from HSCs to MPPs and subsequently mature cells, providing further confirmation of the age-related decline in HSC function.

Taken together, this study provides further evidence of the continuous contribution of HSCs to steady-state hematopoiesis.

General discussion and future perspectives

The fetal period is a remarkable stage of extensive proliferation and growth, orchestrated by intricate gene regulatory networks. When reactivated later in life, most of these networks often exhibit oncogenic activity. Yet, the incidence of cancer during the congenital and neonatal stages is very low compared to all other life stages. What further adds to this paradox is that many childhood cancers arise in utero but only develop at a later stage postnatally. This raises the possibility that there may be a previously unexplored side to some of these developmentally restricted regulators capable of restricting cancer growth.

In Paper I, we identified a prenatal tumor suppressor axis driven by the master regulator of fetal hematopoiesis, LIN28B, which serves as a barrier against AML development. The ectopic expression of LIN28B in MLL-r mouse model dramatically impeded AML development and progression even in the presence of powerful cooperating mutations in RAS or MSN (Paper III). Further investigation revealed that MYBBP1A, a tumor suppressor associated with fetal hematopoiesis and a known c-MYB suppressor, is a key driver of LIN28B's tumor suppressor actions.

Previous studies have proposed a dramatically opposing role for LIN28B, suggesting it as a downstream oncogenic target of MLL-r that becomes reactivated in AML [306–308]. However, we interrogated ~1700 pediatric and adult AML patients and found almost no expression of LIN28B. In the few cases where LIN28B was expressed (12 patients, ~0.7%), its levels were very low. Several other studies have corroborated our findings, albeit with little mechanistic explanations [265,309,310]. While we have shown that the tumor suppressor effects of LIN28B are largely *let-7* independent, there is a plethora of mRNA targets bound by LIN28B that might be synergistically cooperating with MYBBP1A to abrogate AML, and future research will likely reveal more about this.

We believe that we have only scratched the surface of this tumor suppressor axis, and there is certainly more to it than we have explored. For instance, besides the role of MYBBP1A in suppressing c-MYB activity, low expression levels of this tumor suppressor protein have been shown to increase ROS levels, which can further contribute to leukemogenesis. Other studies have shown that MYBBP1A

can directly activate TP53 and regulate cell cycle progression and DNA repair [311]. However, most of these studies have been conducted in a non-hematological context, and further research is needed to identify the roles of this multifunctional protein in normal and malignant hematopoiesis.

Blood and solid tumors have distinct cellular architecture, vasculature, and oxygen levels, which can largely alter their metabolic programs and response to therapies. For instance, although its role was also debated at first, the hypoxia inducible factor (HIF) is known to act as a tumor suppressor in AML but promotes tumor growth in neuroblastoma [312–314]. Similarly, LIN28B, which we propose as a tumor suppressor in AML, has been linked to poor outcomes in neuroblastoma [315]. This suggests that the activity of LIN28B is most likely context dependent.

The role of LIN28B in ALL is an intriguing line of research that has yet to be fully explored. Fetal and adult hematopoiesis significantly differ in terms of lineage biases and differentiation capacities. Our findings in Paper IV confirm that adult HSCs continuously and robustly contribute to all adult hematopoietic lineages in steady state, yet they are incapable of generating fetal-derived immune cells. The ability of LIN28B to instate fetal hematopoiesis in adult HSCs, including the generation of early-life B1a and γδ T cells, is well-established [86,94]. Furthermore, studies have suggested that myeloid and MegE arise following the Lin28b/let-7 developmental switch [73,75]. Thus, while we can only speculate here, one hypothesis might be that LIN28B could contribute to pediatric ALL development by conferring a bias towards fetal lymphopoiesis. Previous studies have attempted to assess the impact of LIN28B on lineage distribution and activities of multipotent progenitors. These studies reported a slight skewing towards the lymphoid lineages, with the most significant observation being re-acquisition of the capacity to generate these developmentally restricted immune cells [86,98]. Other studies have shown that the differences in lymphoid/myeloid biases between fetal/neonatal and adult cells are rather cell-extrinsic, governed by the developmental stage of the hematopoietic niche [286]. [286]. Thus, the hypothesis that LIN28B might increase susceptibility to ALL development by conferring a lymphoid bias is rather unlikely.

The second hypothesis suggests that LIN28B expression might interfere with ALL by a similar or different mechanism from the one described in Paper I. We lean more towards this hypothesis for several reasons. First, we have not observed significant expression of LIN28B in pediatric ALL patients (TARGET cohort). In fact, although infrequent, there were 15 patients with deletions in LIN28B compared to only one with an activating mutation. Second, MYB activity seems to be also important for ALL maintenance [316–318], which might suggest that MYBBP1A could potentially interfere with ALL development as well. Third, the previously discussed patterns of development of pediatric leukemia, with a prenatal initiation and postnatal transformation, would not align with an oncogenic role of LIN2B. Nevertheless, these are all speculations and future investigation is needed.

The progression patterns of childhood ALL cannot be solely attributed to a prenatal tumor suppressor preventing disease progression. This is because the most frequent initiating events in childhood ALL are weak oncogenes that need secondary mutations for transformation, and this process might take years. For instance, despite the well-established prenatal origin of E/R, leukemia peaks after 2 – 5 years, with many instances in older children and teenagers. However, the frequency of E/R ALL sharply declines in adults.

In Paper II, we generated a novel mouse model to investigate how E/R pre-malignant cells can persist in the BM for extended periods before transformation, and why E/R ALL is very rare in adults. Our findings suggest that E/R leukemogenesis originates from HSCs, as we were able to demonstrate their ability to expand significantly in response to E/R and persist in the BM for an extended period. However, we think that these phenotypes are largely influenced by the developmental stage of HSCs. This is because despite E/R generally impairing HSCs capacity to compete and generate B cells, these phenotypes were milder in fetal compared to adult cells. These findings are in line with the previously reported low frequencies of E/R clones in neonates (~ 10^{-4}) and the even lower frequencies in adults (10^{-5} to 10^{-6}) [190,191,319]. Thus, our results endorse the notion that E/R pre-leukemic clones become outcompeted upon reaching adulthood in the absence of secondary events, explaining the scarcity of E/R ALL cases in adults.

While there is substantial epidemiological evidence supporting Greaves' delayed infection hypothesis, only a few studies have experimentally validated it. One study exposed Pax5 haploinsufficient mice to common pathogens, resulting in B-ALL development in ~20% of the mice with a latency of 6-16 months [320]. Another study used a mouse model in which E/R expression was driven by the Sca1 promoter, restricting E/R expression to HSPCs while B-cell progenitors lacked any expression. When these mice were exposed to infections one month after birth, a few instances of B-ALL (~10%) were observed, but with a latency exceeding 20 months [321]. Although a dysregulated immune response has been suggested as the trigger for E/R transformation, the exact mechanisms behind this remain unclear [240].

In our study, we examined the effects of a viral-infection mimicry on E/R pre-leukemia (Paper II). Our results showed that fetal HSPCs responded differently to poly I:C stimulation compared to adult HSPCs, exhibiting a greater ability to compete and generate early B-cell progenitors that could potentially undergo transformation. Whether these responses could be even more pronounced in the setting of an actual viral infection remains to be explored. Additionally, our findings revealed that E/R expression in fetal HSPCs led to a more significant upregulation of MHC-I and immune checkpoint-related pathways compared to adult HSPCs.

Taken together, these observations highlight the critical role of ontogeny in determining the susceptibility of pre-malignant cells to malignant transformation

and underscore the potential for immune evasion by pre-malignant cells. This evasion may enable pre-malignant cells to persist and gain a competitive advantage over normal HSPCs during infection. Although our findings are consistent with recent sequencing studies on E/R B-ALL patients [206,322,323], further research is required to validate the potential of immune modulatory drugs and checkpoint inhibitors as promising therapies for these patients.

Popular scientific summary

The blood system is one of the most regenerative systems in our body. Throughout life, more than two million blood cells are being produced every second. Blood cells can be divided into three main types: red blood cells, white blood cells, and platelets. Each of these types is critical for our survival. Red blood cells carry oxygen to all our body cells, white blood cells establish the immune system that fights infections, and platelets are for blood clotting and wound healing. All these blood cells are generated by a unique and rare type of cell called blood stem cells. In Paper IV, we used a mouse model in which we can specifically mark blood stem cells and thereafter track the cells they generate. This allowed us to study the behavior of blood stem cells and the rate at which they generate each blood cell type. Our results show that while the rates differ depending on the identity of the types of cells produced, most blood cell types are continuously generated from blood stem cells.

The process of blood cell production from stem cells is very complex and tightly controlled. Errors in these processes might result in benign or malignant diseases. Leukemia is a the most common blood cancer, characterized by abnormal and uncontrolled expansion of dysfunctional blood cells that crowd out normal cells, leading to a variety of clinical symptoms. It is the most prevalent cancer in children. It is believed that most childhood leukemias originate in the womb due to a genetic error/mutation. Some of these mutations, like MLL fusions, are very aggressive and are expected to generate leukemia very quickly. Despite this, the incidence of leukemia during the prenatal and neonatal stages is very low (~1% of all childhood leukemia cases), and leukemia usually develops at a later stage after birth. Thus, we hypothesized there might be a protein that acts as a tumor suppressor during these early stages of life, hindering leukemia development.

LIN28B is a master regulator of fetal blood cell formation. Its expression peaks during the prenatal period and declines abruptly after birth. In paper I, we used a mouse model that always generates leukemia, and we tested the impact of LIN28B expression on leukemia development. Indeed, LIN28B abrogated leukemia in more than half of the mice, and the disease took a long time to occur in the rest. Moreover, when we introduced additional mutations, like MSN, that generate more aggressive disease, we found that LIN28B was still able to interfere with leukemia development. Further analysis revealed that LIN28B impedes critical programs for leukemia development, and these actions are mainly driven by another tumor suppressor protein called MYBBP1A. Thus, our study proposes that the expression

of the tumor suppressor LIN28B before birth hinders leukemia initiation, and when it gets inhibited after birth, the protection wanes off, with an opportunity for leukemia development. This suggests potential new therapies for leukemia of all ages.

In contrast to MLL fusions leukemia, ETV6-RUNX1 (E/R) leukemia is a less aggressive but more prevalent type of leukemia in older children. The first mutation also happens in the womb, but more mutations are always needed for the development of E/R leukemia. The time difference between the first and second mutations might span over 15 years. However, the incidence of E/R leukemia sharply declines in adults. How these pre-malignant cells can persist in the body for all this time without being recognized and wiped out by the immune system, and why its incidence declines in adults, are still unanswered questions. To answer some of these questions, we modelled E/R in mice and found that these pre-malignant expand and persist in the bone marrow by activating certain pathways to evade the immune cells. We also found that exposure to infection triggers the expansion and growth of these pre-leukemic cells that are derived from fetal but not adult origin. This might explain the disease patterns and suggest potential immune modulatory treatments for targeting the disease before its development.

Populärvetenskaplig sammanfattning

Blod är ett av de mest regenerativa systemen i vår kropp. Under hela livet produceras mer än två miljoner blodkroppar varje sekund. Blodkroppar kan delas in i tre huvudtyper: röda blodkroppar, vita blodkroppar och blodplättar. Var och en av dessa typer är avgörande för vår överlevnad. Röda blodkroppar transporterar syre till alla våra kroppsceller, vita blodkroppar utgör immunförsvaret som bekämpar infektioner och blodplättar är till för koagulering och sårläkning. Alla dessa blodkroppar genereras av en unik och sällsynt typ av celler som kallas blodstamceller. I Artikel IV använde vi en mus-modell där vi kan markera blodstamceller och följa alla celler som de genererar. Detta gjorde det möjligt för oss att studera beteendet hos blodstamceller och hur snabbt de genererar varje blodcellstyp. Våra resultat bekräftar att även om hastigheten kan skilja sig, genereras de flesta blodcellstyper kontinuerligt från blodstamceller.

Processen för produktion av blodceller från stamceller är mycket komplex och noggrant kontrollerad. Om det går fel där kan det leda till sjukdomar, som kan vara godartade eller elakartade. Leukemi är den vanligaste blodcancern. Den kännetecknas av onormal och okontrollerad expansion av felfungerande blodkroppar som tränger ut normala celler, vilket leder till en mängd olika kliniska symtom. Det är den vanligaste cancersjukdomen hos barn. Man tror att de flesta barnleukemier har sitt ursprung i livmodern och sker på grund av ett genetiskt fel eller en mutation. Vissa av dessa mutationer, exempelvis MLL-fusioner, är mycket aggressiva och förväntas generera leukemi mycket snabbt. Trots detta är förekomsten av leukemi under det prenatala och neonatala stadiet mycket låg (~1% av alla barnleukemifall), och leukemi utvecklas vanligtvis i ett senare skede efter födseln. Därför antog vi att det kan finnas ett protein som fungerar som en tumör-hämmare under dessa tidiga skeden av livet, vilket hindrar utvecklingen av leukemi.

LIN28B är en av spindlarna i nätet som reglerar fostrets blodcellsbildning. Den uttrycks mest under prenatalperioden och avtar abrupt efter födseln. I artikel I använde vi en mus-modell som alltid genererar leukemi, och vi testade effekten av LIN28B-uttryck på leukemiutveckling. Faktum är att LIN28B upphävde leukemi hos mer än hälften av mössen, och det tog lång tid för sjukdomen att uppstå i resten. Dessutom, när vi introducerade ytterligare mutationer, som MSN (vilket genererar mer aggressiv sjukdom), fann vi att LIN28B fortfarande kunde störa utvecklingen av leukemi. Ytterligare analys avslöjade att LIN28B hindrar kritiska program för leukemiutveckling, och dessa åtgärder drivs huvudsakligen av ett annat tumör-

hämmande protein som kallas MYBBP1A. Således visar vår studie på att uttrycket av tumör-hämmarande LIN28B före födseln hindrar leukemiinitiering, och när det hämmas efter födseln avtar skyddet och det resulterar i en möjlighet för leukemi att utvecklas. Detta ger upphov till potentiella nya terapier för leukemi i alla åldrar.

I motsats till MLL-fusionsleukemi är ETV6-RUNX1 (E/R)-leukemi en mindre aggressiv men mer utbredd typ av leukemi hos äldre barn. Den första mutationen sker också i livmodern, men fler mutationer behövs för att utveckla E/R-leukemi. Tidsskillnaden mellan den första och andra mutationen kan sträcka sig över 15 år. Förekomsten av E/R-leukemi minskar dock kraftigt hos vuxna. Hur dessa pre-maligna celler kan finnas kvar i kroppen under hela denna tid utan att kännas igen och utplånas av immunsystemet, och varför dess förekomst minskar hos vuxna, är fortfarande obesvarade frågor. För att svara på några av dessa frågor modellerade vi E/R i möss och fann att dessa pre-maligna expanderar och kvarstår i benmärgen genom att aktivera vissa vägar för att undvika immuncellerna. Vi fann också att exponering för infektion utlöser expansion och tillväxt av dessa pre-leukemiska celler som härrör från foster (till skillnad från vuxet ursprung). Detta kan förklara sjukdomsmönstren och inspirera potentiella immunmodulerande behandlingar för att rikta in sig på sjukdomen innan den utvecklas på riktigt.

ملخص باللغة العربية

الدم هو أحد أكثر أجهزة الجسم تجددًا. يتم إنتاج أكثر من مليوني خلية دم كل ثانية. يمكن تقسيم خلايا طوال الحياة. الدم إلى ثلاثة أنواع رئيسية: خلايا الدم الحمراء وخلايا الدم البيضاء والصفائح الدموية. كل نوع من هذه الأنواع ضروري لبقائنا. تنقل خلايا الدم الحمراء الأكسجين إلى جميع خلايا الجسم، وتشكل خلايا الدم البيضاء جهاز المناعة الذي يحارب الالتهابات، أما الصفائح الدموية فهي المسؤولة عن التخثر والتئام الجروح. يتم إنشاء جميع خلايا الدم هذه بواسطة نوع فريد ونادر من الخلايا يسمى خلايا الدم الجذعية. في ورقة البحث الرابعة، استخدمنا نموذج للفئران يمكننا من خلاله تحديد وتمييز خلايا الدم الجذعية وجميع الخلايا التي تنتجها. سمح لنا هذا بدراسة سلوك خلايا الدم الجذعية ومدى سرعة إنتاجها لكل نوع من خلايا الدم. تؤكد نتائجنا أنه على الرغم من اختلاف المعدل ، إلا أن جميع أنواع خلايا الدم تتولد باستمرار من خلايا الدم الجذعية.

عملية إنتاج خلايا الدم من الخلايا الجذعية معقدة للغاية ويتم التحكم فيها بعناية. إذا ساءت الأمور هناك، فقد يؤدي ذلك إلى أمراض حميدة و خبيثة. اللوكيميا هي أكثر أنواع سرطان الدم شيوعًا. تتميز اللوكيميا بالتوسع غير الطبيعي وغير المتحكم فيه لخلايا دم مختلة. هذه الخلايا الفاسدة تزاحم الخلايا الطبيعية، مما يؤدي إلى مجموعة متنوعة من الأعراض السريرية، منها الإرهاق، الأنيميا، كثرة الإصابة بالعدوى والنزيف. اللوكيميا هي أكثر أنواع السرطان شيوعًا عند الأطفال. يُعتقد أن معظم اللوكيميا في مرحلة الطفولة تنشأ في الرحم وتحدث بسبب خطأ / طفرة جينية. بعض هذه الطفرات، على سبيل المثال إندماج MLL مع جينات أخرى، تكون شديدة العدوانية ومن المتوقع أن تولد اللوكيميا بسرعة كبيرة. على الرغم من وجود هذه الطفرات ، فإن معدل الإصابة بسرطان الدم خلال مرحلتي ما قبل الولادة وحديثي الولادة منخفض جدًا (حوالي 1 ٪ من جميع حالات سرطان الدم لدى الأطفال)، وعادة ما يتطور سرطان الدم في مرحلة لاحقة بعد الولادة. لذلك، افترضنا أنه قد يكون هناك بروتين يعمل كمثبط للورم خلال هذه المراحل المبكرة من الحياة، مما يعيق تطور سرطان الدم.

LIN28B هو منظم رئيسي لتكوين خلايا دم الجنين. مستويات هذا البروتين تبلغ ذروتها خلال فترة ما قبل الولادة وتنخفض بشكل مفاجئ بعد الولادة. في الورقة البحثية الاولى، استخدمنا نموذجًا للفأر يولد دائمًا لوكيميا شديدة بسبب طفرة إندماج MLL ، واختبرنا تأثير LIN28B على تطور المرض. بشكل مثير، أبطل LIN28B سرطان الدم في أكثر من نصف الفئران. علاوة على ذلك، عندما أدخلنا طفرات إضافية، مثل MSN (التي تولد مرضًا أكثر عدوانية، كما هو مذكور في الورقة البحثية الثالثة)، وجدنا أن LIN28B لا يزال قادرًا على تعطيل تطور سرطان الدم. كشفت المزيد من التجارب أن LIN28B يعيق البرامج الحاسمة لتطوير اللوكيميا، وأن هذه العملية مدفوعة بشكل أساسي ببروتين آخر مثبط للورم يسمى MYBBP1A. وهكذا ، توضح دراستنا أن التعبير عن مثبط الورم LIN28B قبل الولادة يمنع بدء اللوكيميا، وعندما تقل مستوياته بعد الولادة، تتضاءل الحماية وتصبح فرصة لتطور اللوكيميا. تشير هذه الدراسة إلى علاجات جديدة محتملة لسرطان الدم في أي عمر.

على عكس سرطان الدم الناتج عن إندماج MLL ، فإن سرطان الدم ETV6/RUNX1 (E/R) هو نوع أقل عدوانية ولكنه أكثر انتشارًا، خاصةً لدى الأطفال الأكبر سنًا. تحدث الطفرة الأولى أيضًا في الرحم ، ولكن هناك حاجة إلى مزيد من الطفرات لتطوير سرطان الدم E/R. يمكن أن يمتد الفارق الزمني بين الطفرة الأولى (في الرحم) والطفرة الثانية لأكثر من 15 عامًا. ومع ذلك، فإن معدل الإصابة بسرطان الدم E/R ينخفض بشكل حاد عند البالغين. كيف يمكن لهذه الخلايا ما قبل الخبيثة أن تستمر في الجسم طوال هذا الوقت دون أن يتعرف عليها الجهاز المناعي ويمحوها ، ولماذا ينخفض حدوثها عند البالغين ، لا تزال أسئلة بلا إجابة. للإجابة على بعض هذه الأسئلة، قمنا بنمذجة E/R في الفئران (ورقة البحث الثانية) ووجدنا أن هذه الأورام الخبيثة تتوسع وتستمر في نخاع العظام عن طريق تنشيط مسارات معينة لتفادي الخلايا المناعية. وجدنا أيضًا أن التعرض للعدوى يؤدي إلى توسع ونمو هذه الخلايا المشتقة من أصل جنيني وليس من أصل بالغ. قد يفسر هذا أنماط المرض ويقترح علاج تعديل المناعة لاستهداف المرض قبل تطوره.

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