Targeting the MLL complex in acute leukemia

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Professor Renato Paro
Doktor Olivier Bernard
Professor Juerg Schwaller

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Prof. Dr. Jörg Schibler
Dekan der Philosophisch-
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SUMMARY

Chromosomal rearrangements leading mostly to fusion oncoproteins of the Mixed Lineage Leukemia (MLL) gene occur in about 10% of all patients with acute leukemia and are often associated with poor clinical outcome, emphasizing the need for new treatment modalities. The MLL protein forms a ternary complex with the lens epithelium-derived growth factor (LEDGF/p75 also known as PSIP1) and another protein MENIN. Previous work has shown that LEDGF/p75 contributes to the association of the MLL multi-protein complex to chromatin. In addition, LEDGF/p75 is known for acting as a tether of the human immunodeficiency virus 1 (HIV-1) pre-integration complex to chromatin, and previous works has demonstrated that expression of the C-terminal fragment fused to eGFP (eGFP-LEDGF/p75$_{325-530}$) impaired HIV-1 replication. Here, we explored this strategy to selectively interfere with the leukemogenic activity of MLL-fusion proteins. We found that expression of the LEDGF/p75$_{325-530}$ fragment impaired the clonogenic growth of MLL-fusion gene transformed human and mouse leukemic cell lines, without affecting the growth of immortalized control cells, or normal lineage marker-depleted murine bone marrow cells. Expression of LEDGF/p75$_{325-530}$ was associated with downregulation of the known MLL target *Hoxa9* and associated with impaired cell cycle progression. Structure-function analysis revealed two small eGFP fused LEDGF/p75 peptide-sized fragments, LEDGF/p75$_{424-435}$ and LEDGF/p75$_{375-386}$ were able to phenocopy these effects. LEDGF/p75$_{325-530}$ and the smaller active peptides were all able to disrupt the LEDGF/p75-MLL interaction. Expression of LEDGF/p75$_{325-530}$ or the LEDGF/p75$_{375-386}$ fragment increased the latency period to disease development *in vivo* in a mouse bone marrow transplant model of MLL-AF9 induced acute myeloid leukemia (AML). From these studies we concluded that small peptides disrupting the LEDGF/p75-MLL interface have selective anti-leukemic activity providing a direct rationale for the design of small molecule inhibitors targeting this interaction.

Intensive biochemical and structural analysis led by our collaborators (J. De Rijck, K. Cermakova, KU LEUVEN, Belgium) further allowed the identification of two MLL-LEDGF/p75 targetable interfaces. Indeed a recently resolved partial structure revealed a potentially drugable hydrophobic pocked stabilizing the MLL-MENIN-LEDGF/p75 interface. Interestingly, our IBD-derived LEDGF/p75$_{424-435}$ fragment was targeting this interface that seems to be dependent on the interaction with MENIN and has been

successfully targeted by MENIN small molecule inhibitors. As the available X-ray data represented only a partial structure of the LEDGF/p75-MLL-MENIN complex, our collaborators used NMR spectroscopy to identify an additional MLL-LEDGF/p75 interface, which partially overlaps with the binding site of known LEDGF/p75 interactors including the HIV-1 integrase. They proved that binding of the HIV-1 integrase or MLL to LEDGF/p75 is mutually exclusive and seems to be dependent of MENIN. Importantly, the newly defined interface was directly targeted by expression of the LEDGF/p75$_{375-386}$ IBD-derived fragment we previously defined. We were then able to show that the clonogenic growth of primary murine MLL-AF9 expressing leukemic blasts was selectively impaired upon overexpression of a LEDGF/p75 binding cyclic peptide CP65, known to bind the IBD and disrupt its interaction with HIV-1 integrase. Thus collectively our data shows that this newly defined protein-protein interface represents a new target for the development of therapeutics against HIV-1 replication as well as LEDGF/p75-dependent MLL fusion oncoprotein driven leukemic disorders.

Intensive research efforts led by many groups resulted in the definition of multiple possibilities for potential interference with the leukemogenic MLL fusion protein complex. We therefore also started to explore whether targeting of the MLL complex at different nodes could result in synergistic effects to efficiently impair MLL-fusion mediated leukemia. Previous studies have shown that the histone H3 lysine 79 (H3K79) methyltransferase DOT1L is essential for MLL-fusion driven leukemogenesis. Selective small molecule DOT1L inhibitors have been generated and are currently entering first clinical trials. However, when given in monotherapy, work in mouse models has shown limited and slow responses to these compounds in many MLL-rearranged leukemia models. Collaborators from the Novartis Institute for Biomedical Research (NIBR, Basel) have performed shRNA screens in MLL-rearranged cell lines to identify sensitizing targets for DOT1L inhibitors and found that knockdown of several MLL complex components, including LEDGF/p75 significantly enhanced anti-leukemic responses. In absence of any available pharmacological agents targeting LEDGF/p75, they decided to test syngertistic activity of the DOT1L inhibitor EPZ004777 in combination with the MENIN inhibitor MI-2-2 previously shown to induce growth arrest and differentiation in MLL-rearranged leukemia cells. We therefore tested the effects of combination of MI-2-2 and EPZ004777 in our MLL-AF9 mouse AML model and found that transient and non-lethal exposure to the combination of these compounds was sufficient to permanently disable their leukemogenic infiltrating

potential *in vivo* mainly through induction of a rapid and effective differentiation of MLL-AF9 expressing leukemic blasts. Altogether, these results suggested that the EPZ004777/MI-2-2 combination might deliver synergistic and durable anti-leukemia effects in MLL-rearranged AML.

All together this work suggests that the MENIN-MLL-LEDGF/p75 complex offers several interfaces for selective therapeutic targeting for MLL-rerranged acute leukemia. In addition, our results suggest that co-targeting the MLL complex at different nodes could pave the way for selective and efficient novel therapeutic approaches for leukemic disorders mediated by MLL fusion oncoproteins. Such approaches need to be clinically tested in particular whether they will be able to overcome early relapse of the disease that is the “Achilles heel” of current applied polychemotherapeutic strategies.

TABLE OF CONTENTS

INTRODUCTION

1. BLOOD FORMATION-HEMATOPOIESIS

The hematopoietic system is responsible for numerous critical functions for mammalian physiology including oxygen and nutrients transports as well as immune defense. With around 3 x10^9 new cells produced daily in the adult human bone marrow (BM), blood is one of the most highly regenerative tissue of the human body. The entire hematopoietic system is maintained by hematopoietic stem cells (HSCs), a rare cell entity found in the BM of adult mammals. The hematopoietic system is derived from the mesoderm lineage in the developing embryo in a process called developmental hematopoiesis divided in 2 steps: (1) embryonic hematopoiesis and (2) definitive hematopoiesis (**Fig.1**) [1,2].

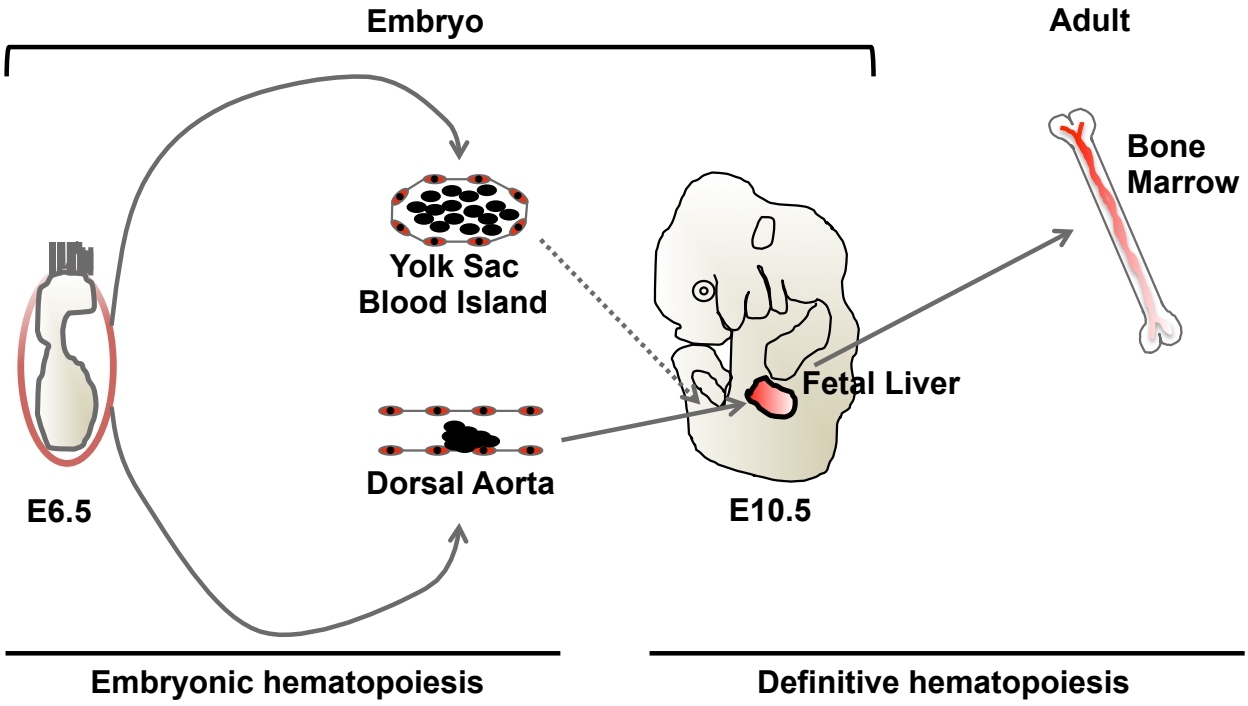

Fig. 1: Blood formation development in the mouse

Schematic representation of blood formation in the mouse embryo. The process starts at E6.5 by a phase called embryonic hematopoiesis. Here the cells migrate from the mesoderm instructed to form blood through the primitive streak to the extraembryonic region. This leads to the formation of the yolk sac where hemangioblasts are thought to differentiate into endothelial cells and primitive erythroblast, thus forming the yolk sac blood islands. A second wave of definitive hematopoiesis is initiated in the fetal liver at E10.5, when the liver rudiment is deeded by incoming HSCs though to be generated through budding of cells lining the ventral floor of the dorsal aorta. Later in fetal development hematopoiesis shifts to bone marrow, the place of adult blood cell formation. (Figure adapted from [1]).

Although adult HSCs remain mostly quiescent, they can enter the cell cycle and either self-renew or differentiate into multi-potent progenitors that give rise to a while diversity of mature blood cells, resulting in a well described hierarchy of blood cells that maintain homeostasis. This organized pyramidal representation is based on the ability of HSCs to self-renewal symmetrically, producing two daughters HSC, or asymmetrically, resulting in one HSC and another downstream progenitor that possesses a reduced capacity for self-renewal. The HSC population can also be activated and expanded in response to stress conditions such as infections or blood loss. In the hematopoietic system, stem and progenitor cells account for less than 0.01-0.05% of the cells in the bone marrow and are divided in three different populations according to their ability to self-renew. Long-term HSCs (LT-HSCs) give rise to short-term HSCs, which in turn give rise to more restricted progenitors (**Fig. 2**). During this process, the cells gradually lose their self-renewing capacity and become mitotically more active [3,4].

Cellular immunophenotyping of the blood system

In the last decade intensive efforts have been undertaken to characterize HSCs and committed progenitor cells, mostly by definition of specific surface markers that can be used for immunophenotyping to study the cells of a particular level of the hierarchy. Mature blood cells express specific markers at their surface, e.g., Mac-1 (CD11b) or Gr-1 (Ly6G/6C) for macrophages and granulocytes respectively, CD3 for T lymphocytes, B220 for B cells and Ter119 for erythroid cells. HSCs do not express substantial amounts of these lineage markers ($Lin^{-/lo}$), and this property is therefore used to enrich bone marrow for HSCs and early progenitors. At the opposite, immature progenitor cells express the antigens CD34, C-kit and Sca-1 in the mouse, and this specificity can be exploited to positively select HSCs. Inside the murine HSCs compartment, long-term (LT) HSCs can be isolated using their specific positivity for CD150 and negativity for FLK2. In Humans, the LT-HSCs pool is phenotypically characterized as lineage markers negative, $CD34^{+}$, $CD38^{-}$ and $CD90^{+}$. Metabolic criteria, such as low uptake of rhodamine dye or Hoechst stain can also be used to enrich HSCs. Additionally, individual progenitor cells that are committed to either myeloid or lymphoid fate can also be isolated by immunophenotype. For example, CLPs (Common Lymphoid Progenitors) can be isolated based on expression of interleukin- (IL)-7 receptor and their progeny can be further distinguished for B and T cell fates. Identically, CMPs (Common Myeloid Progenitors) can be distinguished thanks to expression of CD34 and FcγR for granulocyte-monocyte (GM) fate *vs*. a megakaryocytic-erythroid (Meg-E) fate. Combination of these approaches is used to isolate immature *vs*.

mature bone marrow cells and permits prospective isolation of subsets of hematopoietic cells for analysis of growth characteristics, gene expression and regulators of cells fate, survival, and differentiation[5]. A major strength of the mouse hematopoietic system is the ability to transplant distinct stem cells or committed progenitors subpopulation or even single cells using their immunophenotype specificity. *In vivo* functionality is based on the ability of transplanted cell to re-build a complete blood system in recipients that had their hematopoietic system destroyed either by exposure to gamma radiation or by cytotoxic drugs [2].

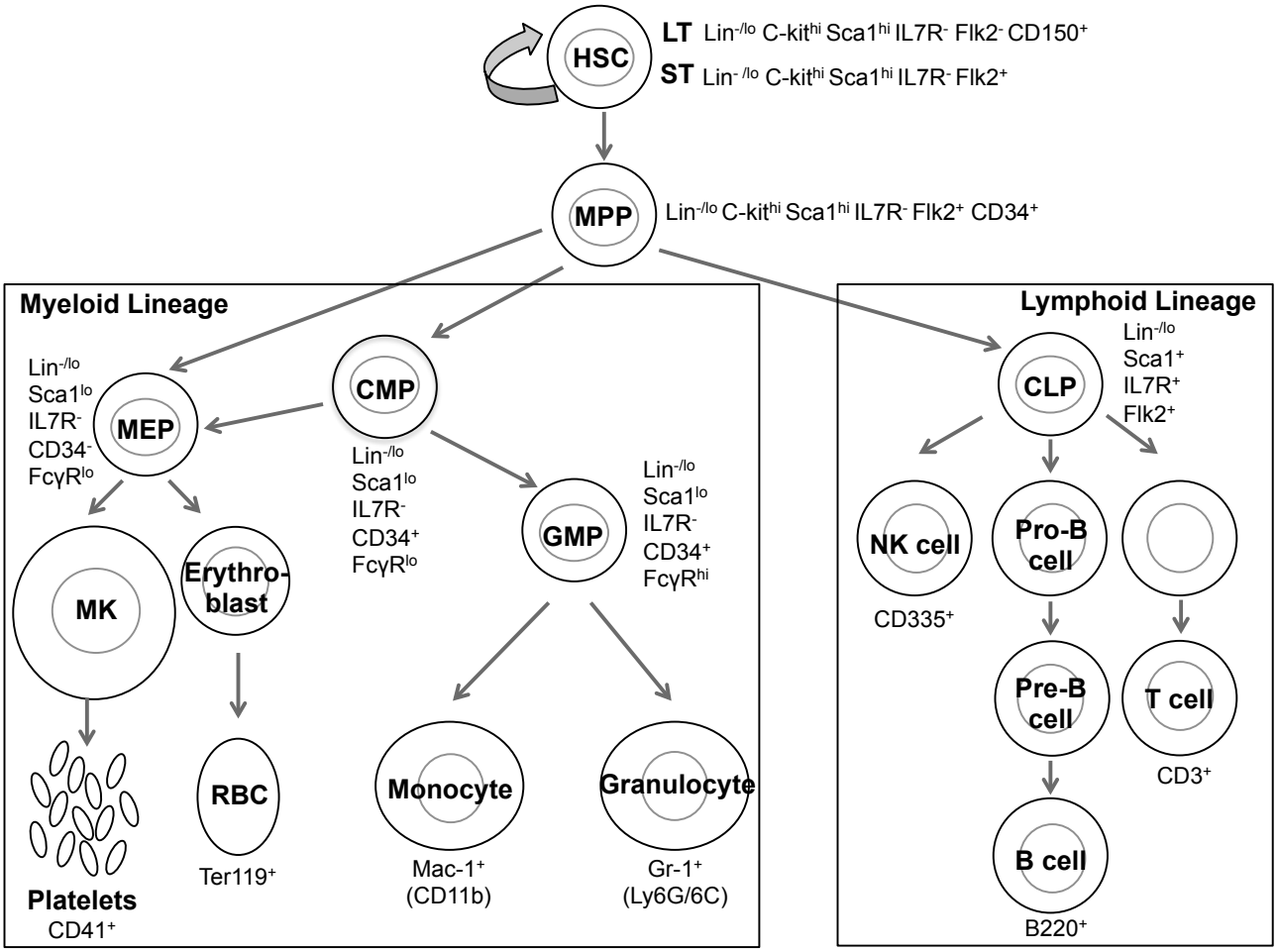

Fig. 2: HSCs differentiation tree

Illustration of the hematopoietic lineage tree. HSCs differentiate through progressively more committed progenitors giving rise to all the mature blood cell types with diverse functions divided into myeloid and lymphoid cells. The hematopoietic tree shows stable cell populations, which have been defined by surface marker expression (indicated next to the according cell population), although the exact branching points and potential of progenitors are still in debate. *HSC* hematopoietic stem cell, *LT* long-term, *ST* short-term, *MPP* multi-potent progenitors, *CLP* common lymphoid progenitors, *GMP* granulocyte monocyte progenitor, *MEP* myeloerythroid progenitor, *RBC* red blood cell, *Lin* Lineage, lo *low*, hi *high*. (Figure adapted from [2]).

Transcriptional regulation of the hematopoietic system

Hematopoietic system development and functions are both tightly controlled at the level of gene transcription. Efforts have been made to identify key regulators of the hematopoiesis developmental stages and transitions and the use of ES cells to introduce knockout gene into embryos has been invaluable [6]. For example, *Scl*$^{-/-}$ mouse embryos die at around E9.5 with an absence of hematopoietic cells, while other mesodermal lineages are unaffected. This observation constituted the first evidence that *Scl* gene (also known as *tal1*) encoding a basic helix-loop-helix protein seems to be essential for HSCs specification. In the same vein, *Runx1* (also known as *Aml1*) gene knock out in mouse is embryonically lethal at E12.5. *Scl*$^{-/-}$ embryos die of severe hemorrhaging and hematopoietic clusters fail to form in the dorsal aorta [7]. The use of specific constitutive Cre-recombinase mouse line ablators expressed at specific embryonic stages allowed defining temporal window activity of those genes [6]. Among others, *Runx1* ablation mediated by VE-Cadherin-Cre, restricted to endothelial cells of the yolk sac, causes severe defects in hematopoiesis. Conversely, its ablation upon VAV1-Cre, restricted to hematopoietic cells and downstream of *Runx1*, does not affect viability of fetal or adult derived blood cells. This study highlighted the exclusive requirement of *Runx1* in the endothelial-to-hematopoietic transition [8].

Others TFs has been found to be crucial for HSCs differentiation and blood homeostasis. Concretely, among others, *Pu-1* overexpression blocks erythroid differentiation, and its genetic disruption in mice causes embryonic lethality between days E16.5 and E18.5 mainly associated with a block in myelopoiesis and B lymphopoieisis [5]. These results strongly suggest that *Pu-1* (*Spi1*) stimulates myeloid development and block erythropoiesis. At the contrary, targeted mutagenesis in the G*ata-1* gene in mouse blocked primitive and definitive erythropoiesis highlighting the key role of *Gata-1* in erythro-megakaryocytic speciation and differentiation [9,10].

In summary, blood cell formation involves a progressive restriction to differentiation potential and the establishment of lineage-specific expression profiles relying on lineage-specific transcription factors (TFs) (**Fig. 3**).

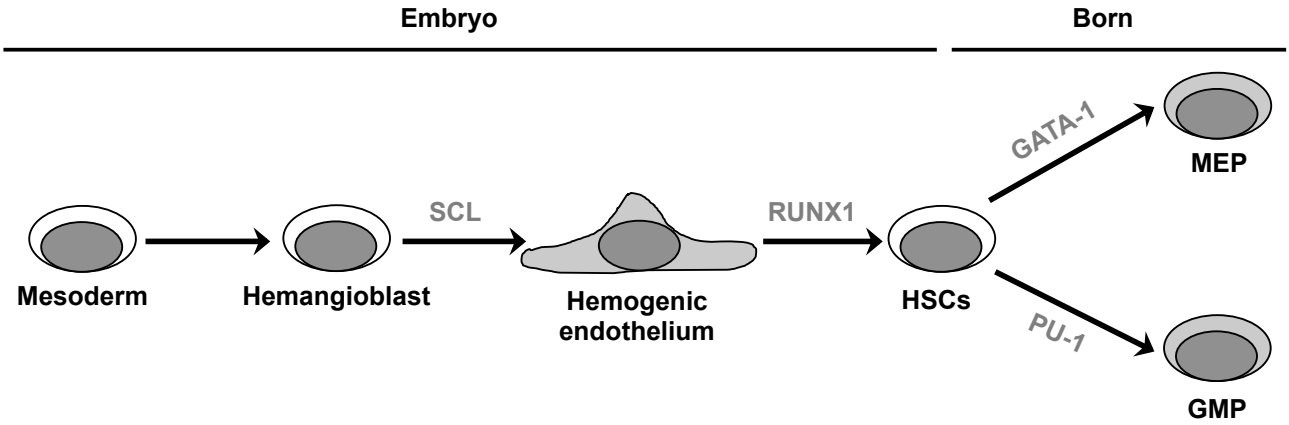

Fig. 3: Transcriptional regulation of HSCs development and differentiation

During mouse embryonic development, hematopoietic cells develop from the embryonic mesoderm through a hemangioblast intermediate. The transcription factor SCL then regulates the transition from hemangioblast to hemogenic endothelium state. The production of HSCs through budding of hemogenic endothelium is related by RUNX1 expression. From the end of embryonic stage to the adulthood, several lineage-specific transcription factors are involved in HSCs differentiation, e.g. GATA-1 is the master regulator of MEP development and erythro-megakaryocytic differentiation or PU-1 which is playing an essential role during myeloid development and differentiation (Figure adapted from [6]).

Epigenetic regulation in hematopoiesis

Another key dynamic process important for hematopoiesis is the recruitment of epigenetic modifying enzymes in order to open up chromatin and allow binding of essential TFs to the promoter/enhancers of their target genes. Remodeling of chromatin is taking place as cells differentiate, until an irreversible state is reached that is specific to terminally differentiated cell. The organization of DNA into higher order structure or nucleosomes is a central component of epigenetic gene regulation. Each nucleosome, representing the basic repeating unit of chromatin, consists of DNA that is tightly wrapped around a core of eight histone proteins including two molecules of each H2A, H2B, H3 and H4. Individual nucleosomes are linked to each other by the linker histone H1 and a short length of DNA [11]. Epigenetics is commonly used to describe the chromatin-based events including DNA methylation, histone modifications and chromatin structure that affect gene expression. If we focus on histones protein, their tail regions are subject to a large variety of post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, or adenosine diphosphate-ribosylation. These epigenetics modifications at DNA and histones levels can change chromatin structure and serve as platform for the binding of reading proteins that recruit additional chromatin-modifying proteins and enzymes. These multi-protein complexes then modulate transcriptional activation and repression, DNA replication, recombination and repair. A key role for hematopoiesis resides in the interplay between Polycomb-group (PcG) and Trithorax-group (TrxG)

complex through their histone lysine (K) methyltransferases (HKMTs) activity (**Fig. 4**). Both of these complexes have been firstly described in *Drosophila melanogaster* as important regulators of tissue homeostasis and body plan formation [12]. Indeed mutation in PcG or TrxG proteins in the fruit fly leads to loss of cellular memory and homeotic transformation involving either duplication or loss of body structures, mainly linked to deregulated expression of highly conserved bithritorax complex (*BX-C*) homeotic genes (human homolog: *Homeobox=Hox genes*) [13,14]. Concerning their functionality, the Polycomb repressive complex 2 (PRC2) is setting on chromatin the repressive histone mark H3K27 tri-methylation (me3) through EZH2 methyltransferase activity, and the TrxG complex includes Thritorax methylase (TRX) as a core component, which mediates the H3K4me3 activation mark. In hematopoiesis, the counteraction of PcG and TrxG complexes leads to proper expression of *Hox* genes in a context-dependent manner [15–17].

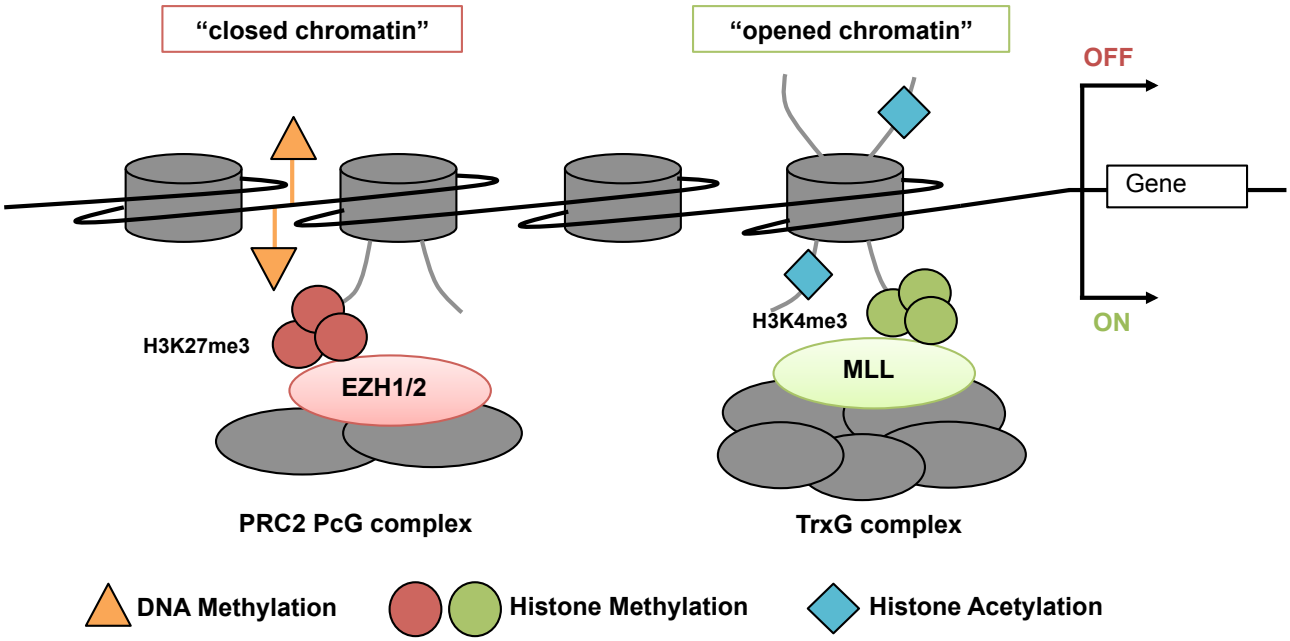

Fig. 4: Key players in epigenetic regulation of hematopoiesis

Schematic representation of a key epigenetic regulatory mechanism of hematopoiesis. DNA methylation and repressive histone marks such as H3K27me3 mediated by the EZH1/2 methyltransferases from the PRC2 are counterbalanced by histone hyperacetylation and activating histone marks like H3K4me3 exerted by TrxG proteins such as the MLL methyltransferase (human homolog of TRX). Repressive marks set by the PCR2 complex keeps chromatin in a transcriptionally inactive or "closed" state whereas TrxG complex function as an "anti-silencer" keeps chromatin in an "opened" conformation to activate the expression. The balance between PcG and TrxG of proteins allows appropriate expression of homeotic genes essential for proper blood formation. *PcG* polycomb group, *PRC* polycomb repressive complex, *me3* tri-methylation, *TrxG* trythorax group, *MLL* Mixed Lineage Leukemia protein (Figure adapted from [15,17]).

Several of the epigenetic and transcriptional factors mentioned above were identified by virtue of their involvement in chromosomal abnormalities associated with various types of hematological disorders in particular acute leukemia.

2. LEUKEMIA, A CANCER OF THE HEMATOPOIETIC SYSTEM

Leukemia is defined as a cancer of the blood formation and characterized by abnormal accumulation of white blood cells in hematopoietic organs. This malignancy is grouped in different clinical and pathological entities. Acute leukemia is characterized by an increase of immature bloods cells with aberrant differentiation that do not perform normal function, whereas chronic leukemia is defined by an abnormal accumulation of mostly terminally differentiated blood cells. According to the affected cell lineage, leukemia is subdivides into myeloid leukemia involving granulocytic, monocytic, erythroid or megakaryocytic cell lineage, *vs.* lymphocytic leukemia including B-cell, T cell or natural killer (NK)-cell lineages. Based on this classification, four main leukemia categories are defined namely acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and chronic lymphocytic leukemia (CLL). ALL occurs more frequently in children, whereas CLL affects more adults over the age of 55. AML and CML are also more frequent in the elderly affecting mostly individuals over 60y (**Table I**) [18].

The standard treatment regimen for most forms of leukemia consists in multi-drug chemotherapy with combinations that are different for the leukemia subtypes. In combination with allogeneic or autologous bone marrow transplantation chemotherapy can lead to cure for some patients. However a large fraction of patients with acute leukemia relapse and ultimately die from their disease. Particularly, elderly patients unable to support aggressive chemotherapy regimens have limited therapeutic options. In the past decade, intensive research efforts aimed to identify compounds targeting specifically leukemic cells. Indeed the first successful anti-leukemic small molecules are protein tyrosine kinase inhibitors (TKI) like Imatinib (Gleevec®) that have revolutionized the therapy of chronic myeloid leukemia (CML) with a five-year overall survival of 90% [19]. The potency of such molecules is based on the fact that in almost all patients with CML the cells are driven by the constitutively active BCR-ABL fusion tyrosine kinase resulting from a t(9;22)(q34;q11.2) chromosomal translocation. However when CML turned into blast crisis upon acquisition of secondary mutation, kinase inhibitors are not as potent than in the chronic phase of the disease. Another good example of efficient targeted leukemia

therapy is acute promyelocytic leukemia (APL): here the leukemic cells are driven by the PML-RARα fusion protein resulting from the t(15:17)(q24;q21) chromosomal translocation. Very recent work has shown that the combination of all-trans retinoid acid (ATRA) and arsenic trioxide is able to inactivate the PML-RARα fusion and to cure >90% of the patients [20]. Beyond APL, there is currently no clinically efficient targeted therapy for other froms of acute leukemias. Nevertheless polychemotherapy achieves initial remission in about 70% of the AML patients, but the five-year survival is below 40% as most of the patients relapse within 5 years despite continued treatment [21]. In particular, patients over the age of 60 face an overall poor prognosis of 10-20% survivors for more than 5 years [22]. Facing this bottleneck, acute leukemia treatment aims to shift from a uniform approach to more personalized therapies targeting the underlying molecular alterations identified as initiator and driver of the disease.

Table I: Leukemia subtypes classification

Subtype	Age (year)	Cellular Origin	Comments
ALL	<15	HSC? B-cell > T-cell	Poorer prognosis for adults than children, >80% of B-ALL in children can be cured
AML	all, most >60	HSC Myeloblast? Promyelocyte?	Poor prognosis, relapse Heterogeneous tumor populations Best prognosis with bone marrow transplantation
CML	>50	HSC	Prognosis improved by TKI t(9;22) “Philadelphia chromosome” in >90% of cases Acceleration into “blast crisis”
CLL	>50	HSC? B-cell	Currently not cured Long course with rare conversion to acute form

First evidence from stem cell biology has provided insights into cancer biology by underlining the relationship between stem cell and tumor cells. This axe of thinking suggests that tumors might contain some cancer stem cells that are able to initiate the disease in a hierarchical manner. Interestingly in the context of leukemia, biological studies on human AML have shown that only 0.1 to 1% of leukemic cells have the capacity to initiate the disease in severe combined immunodeficient (SCID) mice [23,24]. These findings strongly suggest that similar to normal hematopoiesis, leukemia might be composed by cells with different phenotypic characteristic as well as proliferative and self-renewal potentials. Only a small number of cancer cells are able to restore themselves and maintain leukemia, whereas the majority of leukemic cells are in more “mature” state and unable to initiate the disease. This cellular entity responsible of initiating and maintaining

leukemia has been described as “leukemic stem cells” (LSCs) or leukemia-initiating cells (LICs) and share some self-renewal and differentiation capacities with normal HSCs [25]. Given this common attributes, it has been proposed that acute leukemia might be initiated by transforming events arising in HSCs. Alternatively leukemias might initiate from more committed progenitors that have acquired transforming mutations that overcome their limited self-renewal potential. Experimental evidence suggests that AML and CML arise from mutations in HSCs. Indeed for most AML categories, except for APL, the unique cells able to transplant AML in NOD/SCID (Nonobese diabetic/severe combined immunodeficient) mice have a [$CD34^+$, $CD38^-$] phenotype characteristic of the HSCs pool whereas the [$CD34^+$,$CD38^+$] leukemic cell fraction cannot transfer the disease to mice [24]. Nevertheless even if HSCs are often the target of genetic events leading to malignant transformation, committed progenitors or even mature cells may also be transformed. Actually in the case of APL, it has been shown that the APL-related aberrant fusion gene PML/retinoic acid receptor α (RARα) was present in [$CD34^-$,$CD38^+$] cell populations but not in the [$CD34^+$,$CD38^-$] HSC-enriched cell fraction [26]. This observation suggests that APL leukemogenesis might originate from a more differentiated cell type than HSC like pluripotent progenitors. In CML, the aberrant Philadelphia chromosome leading to the constitutively active $p210^{BCR\text{-}ABL}$ fusion protein kinase occurs in HSCs and GMPs might acquire additional mutations leading to acceleration and blast crisis [27,28]. To corroborate this idea, thanks to the use of promoter elements of myeloid-specific gene such as human MRP8 (hMRP8) promoter, transgenic mice expressing human leukemic oncogene specifically in committed myeloid cells have been generated. Indeed MRP8 encodes a small calcium-binding protein expressed in neutrophils, monocytes, and their immediate progenitors, CMPs and GMPs, but not in HSCs [29]. Interestingly, transgenic mice expressing *BCR-ABL* from this promoter developed CML like disease [30], and mice expressing *PML-RARα* from hMRP8 developed a preleukemic state which eventually evolves in APL similar to human patient harboring the same translocation [31].

3. ACUTE MYELOID LEUEKEMIA AS A PRODUCT OF FUNCTIONALLY COLLABORATING GENETIC AND EPIGENETIC ALTERATONS

Normal development underlies a delicate balance between self-renewal and cellular differentiation. When these two processes become deregulated or are uncoupled, acute leukemia can develop.

Developed in the 60's conventional cytogenetic analysis started to uncover the presence of chromosomal translocations, large deletions and inversions in leukemic cells. Indeed, main clues to the genetic basis of acute leukemia initially came from cloning of recurring breakpoints of mostly balanced chromosomal translocations associated with specific disease phenotypes. Successively, improved sequence analysis tools revealed the existence of smaller mutations such as point mutations, microdeletions particular in cases in which conventional cytogenetic analysis did not found any alterations. Collectively, two decades of clinical and experimental research revealed that acute leukemia is the product of a multistep process involving multiple collaborative genetic molecular aberrations. The leukemogenic potential of a large number of these genetic alterations has been characterized *in vitro* and *in vivo*. Based on their cellular activity, it has been proposed to classify leukemia-associated genetic lesions into two major categories. The "class I mutations" generally include gain of function mutations that lead to aberrant activation of signaling transduction pathways conferring cell survival and proliferation advantages. The "class II mutations" consist mostly of lesions that directly alter transcriptional programs essential for normal hematopoietic differentiation resulting in aberrant self-renewal and maturation arrest [32,33]. Here we will focus mainly on acute myeloid leukemia (AML), which is one of the best-characterized and studied developmental models for acute leukemia.

Class I mutations: protein kinases - signaling mediators

The best-studied examples of AML-associated class I mutations are alterations in genes encoding for protein tyrosine kinases (PTKs e.g. FLT3 and KIT) and the RAS protein family members. Leukemia-associated PTK mutations generally lead to constitutive activation and uncontrolled stimulation of downstream effectors such as the signal transducers and activators of transcription (STAT) family members resulting in increased proliferation and survival of the cells. In AML the most prevalent class I mutations affect the FLT3 receptor tyrosine kinase, including internal tandem duplications (ITDs) or activating point mutations in the kinase domain. FLT3-ITDs are the most frequent AML-associated PTK mutations found in up to 30% of adult patients and associated with

increased risk of relapse and/or short survival [34]. Commonly the ITDs are located in the juxtamembrane domain, but, in 28% of cases, the duplication is integrated in the tyrosine kinase domain (TKD), and associated with particularly poor outcome of the disease [35]. In addition to ITDs, point mutations mainly localized in the TKD are found in 7-10 % of cytogenetically normal AML patients [36]. Both types of FLT3 mutations lead to constitutive and enhanced activation of several downstream signals such as RAS, MAPK or STAT5. Constitutive activation of the RAS pathway can also arise from mutations in RAS itself. NRAS, KRAS and HRAS are a family of guanine nucleotide binding proteins, which are frequently found downstream of tyrosine kinase receptors and involved in development and tissue homeostasis [37]. NRAS and KRAS point mutations (typically in codons 12, 13, and 61) are found in up to 40% of AML patients. These alterations lead to inactivation of the intrinsic GTPase activity, which in turn results in constitutive activation of RAS proteins [37]. Intriguingly, multiple studies have shown that expression of “class I mutations” in murine bone marrow mostly gives rise to myeloproliferative disease (MPD) but is not sufficient to induce an AML phenotype [33]. MPD is characterized by expansion of mostly normally differentiating cells, suggesting that class I mutations do not lead to a differentiation block and aberrant self-renewal capacity characterizing AML.

Class II mutations: transcriptional regulators of hematopoietic differentiation

AML-associated “class II mutations” include mainly genetic lesions affecting transcription factors (TFs) or epigenetics regulators that are important for normal hematopoiesis. These mutations mostly lead to impaired differentiation and increased self-renewal properties of hematopoietic stem and early progenitor cells (HSPCs). Many class II mutations result from balanced chromosomal translocations that lead to chimeric fusion proteins with aberrant transcriptional activity involving key regulators essential for the differentiation program of HSPCs such as the core-binding factor (CBF) or the retinoid acid receptor alpha (RARα). CBF is a heterodimeric complex composed by the two subunits, AML1 (RUNX1) and CBFβ both essential for normal hematopoiesis development [38]. About 10-20 % of adult AML cases carry translocations involving CBF components such as t(8;21)(q22;q22) or inv(16)(p13;q22) resulting in AML1-ETO and CBFβ-MYH11 fusion genes respectively [39]. As mentioned before, translocations involving RARα on chromosome 15 are a hallmark of APL. The most common translocation in APL, which account for 4% of disease cases, is t(15;17)(q24;q21) leading to the PML-RARα fusion [40] . Functional studies suggested that fusions like AML1-ETO or PML-RARα act mainly in a

dominant negative fashion to the wild-type protein by aberrant recruitment of histone deacetylase co-repressors [41,42].

Another recurrently altered hematopoietic transcriptional regulators in AML is the mixed lineage leukemia (MLL1 also known as ALL-1) gene on the long arm of chromosome 11 [43]. MLL1 is part of over 120 different translocations leading to fusions oncogenes. Normally MLL1 is part of a large multi-protein complex that is able to regulate activation of the *Hox* genes cluster [44,45]. *Hox* cluster genes A, B and C are highly expressed in hematopoietic stem and progenitor cells and responsible for maintenance of the stem cell and progenitors pools [46–48]. Similarly, *Hox* cluster genes are themselves involved in a series of translocations implicating the nucleopore protein 98 (NUP98). Several of them including NUP98-HOXA9, NUP98-HOXD13 or NUP98-HHEX have been shown to have a leukemogenic potential in the mouse [49–51]. These observations strongly suggest a key role of aberrant *Hox* genes expression in leukemic hematopoiesis. In addition to chimeric transcription factor fusions, some, mostly “loss of function” point mutations are found in hematopoietic TFs including CEBP/α and PU-1 [5,33]. Several studies have shown that in contrast to class I mutations, retroviral or transgenic expression of class II mutations in the bone marrow of the mouse led to development of dysplastic hematopoiesis or AML-like phenotypes [36]. However, in most cases a relatively long latency was required for the induction of the disease, which suggests that the class II mutations might be crucial but by themselves not sufficient for the onset of AML.

Functional cooperation between class-I and -II mutations in acute leukemia

Several studies reported that a given AML patient often carries mutations of both classes but rarely more than one mutation of each complementation group. Indeed, 30% of the patients with APL have in addition of the hallmark PML-RARα fusion a FLT3 activating mutation. In some cases, the evolution from a chronic MPD harboring a class I mutation like the *BCR-ABL, TEL-PDGFRβ* fusions or the *JAK2*^{V617F} mutation to AML correlated with the acquisition of a class II mutation such as *AML1-EVI1, AML1-ETO* or *NUP98-HOXA9* fusion genes [52]. Even though these observations strongly proposed a “cooperation model” between class I and class II mutations for leukemogenesis they were still many patients in which no such mutations could be found.

Nevertheless functional cooperation between class I and class II mutations could be demonstrated in several AML mouse models. Cooperation between PML-RARa and FLT3-ITD was demonstrated in a transgenic mouse model. In fact, solely transgenic expression of PML-RARα leads to APL-like disease after long latency and incomplete penetrance, but

retroviral expression of FLT3-ITD in bone marrow of these mice induced APL after a shorter latency and complete penetrance [53]. Several additional publications corroborated the involvement of FLT3 mutations as a functional cooperator for AML. Retroviral co-expression experiments in murine bone marrow cells have demonstrated a potentiation effect on AML induction when FLT3-ITD was expressed in combination with several class II mutations including *CBFβ-MYH11* (inv16), *MLL-AF9*, or *NUP98-NSD1* [54–56].

Modern genomics and the clonal evolution concept in AML

The development of more powerful sequencing tools able to read the entire AML genome revealed that genetic alterations in AML seem to be somehow more complex than suggested in the simple "class I – class II complementation model". As whole genome sequence data of more and more AML patients become available a novel concept of stepwise acquisition of genetic alterations giving rise to clonal heterogeneity with subclonal architecture in AML arose [57].

The AML Cancer Genome atlas project, which characterized the somatic genomes of 50 patients with *de novo* AML and the somatic exomes of 150 others cases, represents a milestone in our understanding of hematological malignancies [58]. Hereby 2585 somatic mutations, insertions, and deletions in coding regions of AML genomes were found with a frequency of more than 5% in *de novo* AML. Strikingly, despite this high number of genetic lesions, a low average number of 13 mutations was found in a given individual. These findings enforced the concept of "driver mutations" causing leukemic transformation. However, these leading mutations occur in the same time with so-called "passenger mutations" lacking transforming capacity, but accumulating during clonal progression and carried along with the leukemic clone. 23 genes have been reported as substantially mutated in AML and have been categorized in 9 different functional classes (**Fig. 5**). There is also mutations in genes that could not be assigned to class I or class II alterations [59]. As example, mutations in the nuclear protein nuclephosmin (*NPM1*) are present in about 30% of AML patients with no leading cytogenetically detectable chromosomal alterations [57,59]. In AML the proportion of cases with normal karyotypes is quite frequent (≈50%). To better understand clonal evolution of those cytogenetically normal AML, Welch *et al.*, proposed to compare sequenced genomes of patients with known initiating mutation (*PML-RARα*) with sequenced genomes that have less known initiating events *vs.* exomes of healthy donor HSPCs [60]. This study overall suggest that most of the mutations found in AML genomes are actually random events occurring in HSPCs giving rise to a clonal signature before they acquired the initiating mutation. In many cases, only one or two additional cooperating

mutations were sufficient to generate the malignant clone [60]. Additionally, deep sequencing allows tracking and quantifying AML subclones based on the presence of different mutations. Interestingly, some mutations present in an AML sample at diagnosis are stably maintained and found back in the same AML sample upon relapse. However, most AML patients acquire additional genetic modifications during the course of the disease which are rarely lost subsequently suggesting that AML relapse originates from incomplete eradication of founder clones, rather than from development of new malignant clones [61,62]. However, two very recent studies report that preleukemic mutations in HSPCs provide a chemotherapy resistance phenotype [63,64]. These genetic alterations occur independently of the first diagnosed AML driver genetic event and target mainly epigenetic modifiers (e.g. *DMNT3*, *IDH2*). Subsequently, occurrence of these preleukemic mutations seems to allow expansion of a chemoresistant preleukemic clone that is susceptible to acquire a new initiating mutation leading in turn to disease relapse.

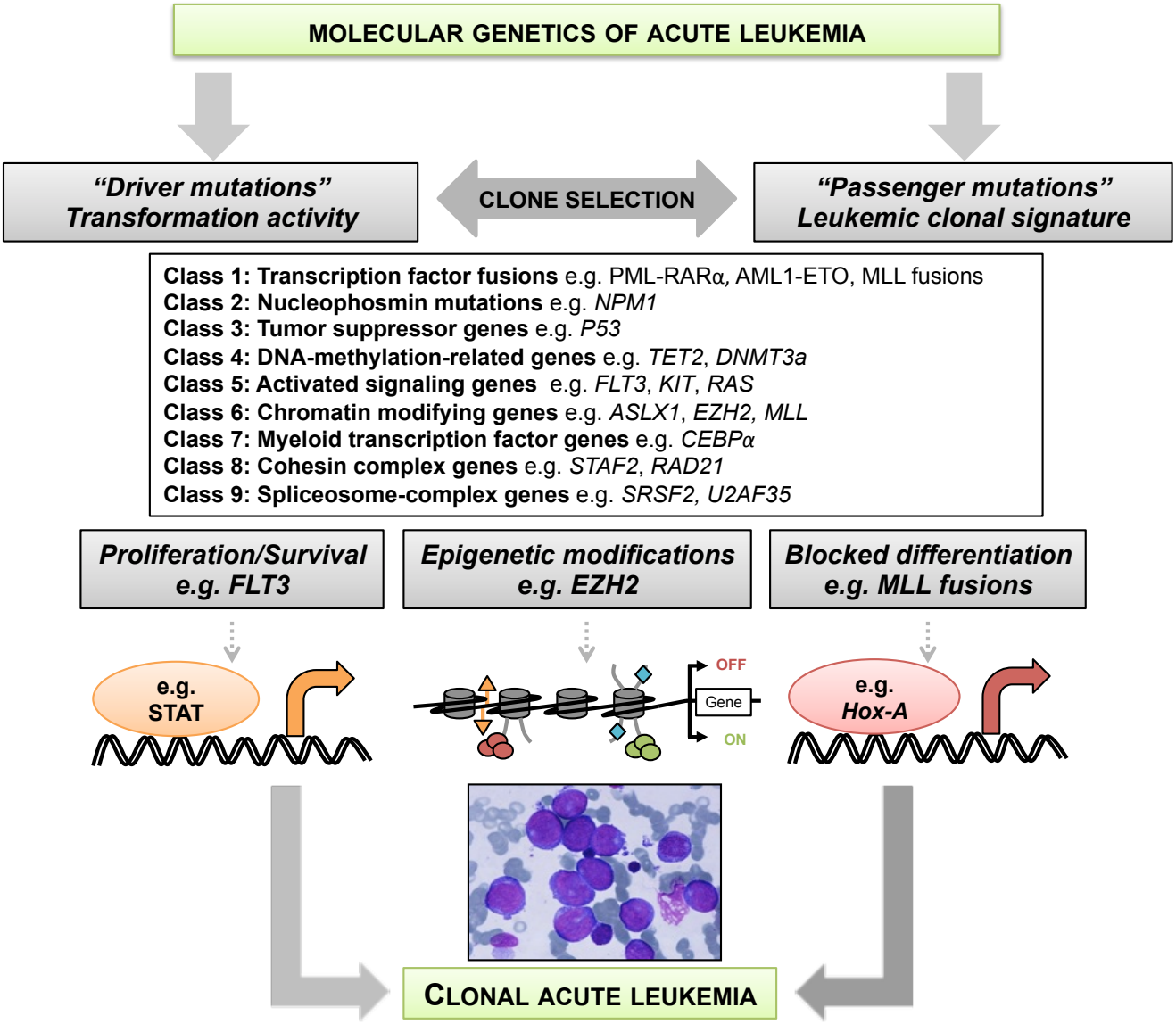

Fig. 5: The Clonal selection model in acute leukemia

In general in acute leukemia, genetic alterations can nowadays be categorized in two types: the "driver mutations" possessing the transforming activity, and the "passenger mutations" lacking leukemogenic potential but accumulating during disease progression and participating in the set up of a leukemic clonal signature associated with favorable or poor disease prognosis and chemotherapy resistance. Cooperation between these two categories of genetics alterations leads to the selection of a clone able to rapidly induce acute leukemia in mice. All recurrent human mutations have been classified in 9 categories. AML genetic alterations can target proteins kinases or other signaling proteins and provide proliferative and/or survival advantages through activation of TFs such as STAT. Several classes include recurrent mutation in epigenetic modifiers such as EZH2 and MLL1, or proteins involved in chromatin remodeling such as cohesin complex proteins, enabling aberrant expression of key oncogenic genes signature like the Hox-A cluster genes. Some single mutation in driver genes involved in key regulatory mechanisms for hematopoiesis such as translocation involving e.g. *MLL* or *AML1* are sufficient to induce a block in differentiation providing aberrant self-renewal capacity to the target cell and lead to the onset of a MDS or acute leukemia-like disease after a long latency in mouse models.

4. THE MIXED LINEAGE LEUKEMIA GENE & ACUTE LEUKEMIA

The *Mll1* (Mixed Lineage Leukemia, here referred as *MLL*) gene is one of the most frequently affected genes by class II mutations associated with aggressive human acute leukemias. In the leukemia context, *MLL* gene, also known as *All1*, *Htrx* or *Hrx*, is involved mainly in balanced chromosomal translocations resulting in novel chimeric fusion proteins containing the amino terminal part of MLL fused in-frame with the carboxy-terminal portion of over 50 different partner proteins [65]. *MLL* rearrangements affecting either the lymphoid or myeloid lineage constitute up to 80% of infant leukemia (<1 year of life), 10 % of childhood leukemia (2-18y), and approximately 5-10% of adult acute leukemias [66]. Apart from spontaneously arising leukemia, MLL fusion proteins are frequently found in patients with therapy-related leukemias particularly occurring after treatment with topoisomerase II inhibitors, which represent 5 to 10% of the MLL-associated leukemias [67]. In general, *MLL* genetic alterations are often hallmarks of early relapse and poor survival [65–67].

Functional and structural overview of the wild-type (WT) MLL gene

The *MLL* gene is located on the long arm of the chromosome 11 (11q23) and contains 37 exons spanning over 92kb of genomic DNA. The largest open reading frame leads to a transcript of around 12kb encoding for a large multi-domain protein of 430KDa recognized as the mammalian homolog of the *trithorax* gene in *Drosophila*. The evolutionarily conserved trithorax group (trxG) family of proteins is known to positively regulate transcription of Antennapedia (ANT) and Bithorax (BX) homeotic genes complexes [68]. Indeed in the fruit fly mutations in Thritorax mimic the phenotypes of loss-of-function-mutations in multiple ANT and BX complexes genes leading to homeotic transformation of the thoracic and abdominal segmentation [13,69]. MLL regulates target gene expression based on its abilities to bind to DNA and to methylate the lysine 4 residue of histone H3 (H3K4) through its C-terminal SET (Suppressor of variegation, Enhancer of zeste, Trithorax) domain. MLL is crucial for maintaining the activation of a large number of genes including the *Hox* clusters genes during embryogenesis, hematopoiesis, and neurogenesis. Indeed, homozygous deletion of the *MLL* gene in the mouse resulted in an embryonically lethal phenotype due to disturbed HOX-mediated body plan formation, highlighting MLL maintained *Hox* gene expression as an essential mechanism for embryogenesis [70]. Interestingly, in this *null* mouse, the MLL-deficient HSPCs from the yolk sac and fetal liver were impaired in self-renewal and proliferation mostly linked to decrease expression of *Hox-A* cluster genes [71–73]. Notably, using an in vitro ES-cell differentiation model it was shown that the block in hematopoiesis could be rescued by reintroducing

individually *Hoxa9*, *Hoxa10* and *Hoxb4* [72]. In contrast, a recent study report that developmentally VAVCre induced constitutive *MLL* loss in mice is deleterious only for postnatal hematopoiesis and results in global ineffective blood system formation leading to lethality 3 weeks after birth [74]. Furthermore, conditional ablation of MLL in the adult murine hematopoietic system leads to fatal bone marrow failure within three weeks. This break in hematopoiesis was reflected by impaired proliferation and response to cytokine-induced cell-cycle entry of hematopoietic progenitors and depletion of quiescent HSCs [75,76]. Taken together these observations strongly support that the key role of *MLL* appears to be maintenance of the HSPCs compartments mainly through regulation of *Hox* genes. However it remains elusive if the MLL function is essential for both developmental and definitive hematopoiesis or only for post-natal hematopoiesis.

The MLL protein is constitutes of two subunits arising from proteolytic cleavage by taspase1. *Taspase1 null* mice showed homeotic transformation due to improper *Hox* genes expression underlined the importance of cleavage for developmental functions of MLL [77]. The N-terminal MLL (MLL-N; 300/320KDa) contains several domains with transcriptional co-activator activity [e.g. plant homeodomain fingers = PHD, DNA binding motifs such as AT-hooks, CXXC-zinc finger], which set a platform for recruitment of proteins regulation of specific gene expression. The C-terminal MLL (MLL-C; 180KDa) comprises the SET methyltransferase domain [78]. Some evidence highlight the SET domain has a key role player in transcriptional activation of *Hox* genes. Actually the *MLLΔSet* mice are viable, but harbor defects in skeletal development and in several *Hox* genes expression [79]. In addition, MLL SET domain methyltransferase activity had been associated to *Hox* gene activation through direct binding of MLL on promoter sequences [45]. A more detailed structure of the wild-type MLL is depicted in **Fig. 4a**.

MLL fusion proteins in leukemia

Leukemia-associated chromosomal translocations are the product of a failure of proper DNA double strand break repair in developing HSPCs. In the *MLL* gene there is a 8.3kb breakpoint cluster region (BCR, **Fig. 4a**) between exons 8 and 13 that is the target of most *MLL* rearrangements. These rearrangements lead to an in-frame chimeric protein containing the first 8-13 exons of MLL and a variable number of exons from a fusion partner gene (**Fig. 4b**).

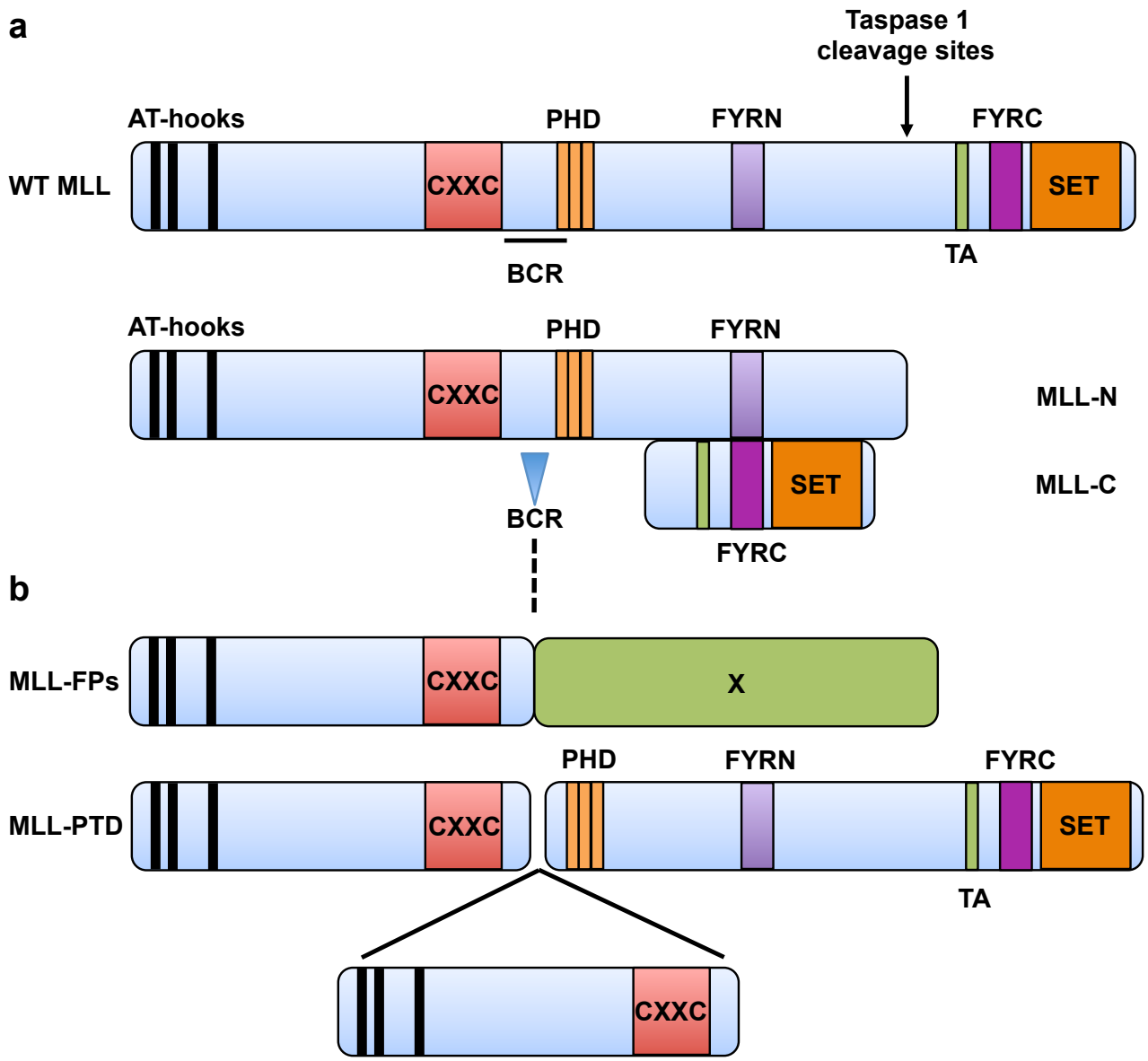

Fig. 6: Schematic overview of wild-type MLL protein and MLL fusions

(a) MLL is a nuclear protein encompassing 3969 amino acids comprising multiple functional domains. The mature MLL protein consists of two subunits MLL-N and MLL-C produced by cleavage of the nascent MLL by taspase1 and non-covalently associated through respectively FYRN and FRYC motifs. The MLL-N subunit contains AT-hooks motifs binding to the minor groove of AT-rich genomic DNA. Afterwards there is a transcriptional repression domain containing a DNA methyltransferase homology domain including histone deacetylase (HDAC) recruiting element and a CXXC zinc-finger motif binding to non-methylated DNA followed by plant homology domain (PHD) zinc-finger motifs. The MLL-C subunit contains a transcriptional activation (TA) domain that recruits co-activators such as CBP or EP300 and precedes the SET domain (homologous to fly *trithorax*) possessing the H3K4 methyltransferase activity. The breakpoint cluster region (BCR) covers exon 8 to 13. **(b)** Main of the MLL fusions proteins (MLL-FPs) contains the N terminus part of MLL encoding the first exons until the BCR and the C-terminus of an X protein that can correspond to over 50 fusion partner genes. An exception is the MLL-partial tandem duplication (MLL-PTD) resulting in a longer MLL protein consisting in the duplication and insertion of a varied number of 5 to 12 exons before exon 11 or 12. (Figure adapted from [65,80]).

According to the different cellular location and putative function of the fusion partner, *MLL* rearrangements have been classified in several categories [65]. The first group encompassed translocations involving fusion of the N-terminal part of MLL gene with the nuclear DNA-binding proteins AF4, AF9, AF10, ENL and ELL, which all together represent more than 80% of MLL rearranged leukemia. Here the translocation t(9;11)(p22;q23) , t(11;19)(q23;p13.3) and t(4;11)(q21;q23) resulting in MLL-AF9, MLL-ENL and MLL-AF4 fusions are the most prevalent. MLL-AF9 is frequently associated with myelo-monocytic AML, whereas MLL-ENL can be found in both ALL and less frequently also in AML. On the other side, MLL-AF4 is almost exclusively linked to B-cell ALL. The second category implicates cytoplasmic proteins e.g. GAS7, EEN, AF1P, AF6 and AFX, representing more than 10% of leukemia-associated MLL fusions. An important feature of these oncogenic fusions is the presence of coiled-coil dimerization domains essential for their transformation potential [81]. A third small group of MLL fusion partners is represented by septins (SEPT2, SEPT5, SEPT6, SEPT9 and SEPT11), which are cytoplasmic proteins involved in several processes such as cell-cycle control, vesicle trafficking and compartmentalization of the plasma membrane [82]. The histone acetyltransferases EP300 and CBP form the fourth group of proteins fused to MLL and seem to keep their enzymatic activity even when they are part of the fusion [83,84]. The fusion with acetyltransferases would participate to keep chromatin on an acetylated active state necessary for transcriptional activation of MLL-target genes. The last and fifth type of *Mll* rearrangement consists of an internal tandem duplication of specific exons leading to the MLL-PTD (partial tandem duplication) fusion, found in 5-10% of cytogenetically normal AML [85].

MLL-fusions seem to exert their oncogenic activity through activation of a particular gene expression program. Because all domains within MLL-N, described as implicated in DNA binding are retained in the fusion protein, it seems likely that MLL fusions will share many target loci with wild-type MLL. As critical effectors of WT MLL regulation in embryonic development and hematopoiesis, *Hox* genes seems also to be important regulators of MLL-fusion leukemogenic activity. Indeed upregulation of *Hox* genes expression is a common feature of human *MLL rearranged* leukemias [86]. Additionally, in murine bone marrow transformation assays and animal models, co-expression of *Meis1,* a homeodomain containing co-factor, and *Hoxa9* together cause aggressive leukemia and can at least in part particularly compensate the requirement for an active MLL-ENL fusion protein [87]. Interestingly, *Hoxa9* expression alone is sufficient to cause leukemia in mice but with a much longer latency than *Hoxa9*/*Meis1* co-expression [87]. Similarly, mice transplanted with Hoxa9 deficient bone marrow cells retrovirally-expressing MLL-ENL

failed to develop leukemia [88]. However, in some circumstances *Hoxa9* might not be a key target for the oncogenicity of MLL-fusion genes. For example *Hoxa9*$^{-/-}$ mice are still vulnerable to MLL-AF9 mediated leukemogemesis and MLL-GAS7 is able to transform *Hoxa9*$^{-/-}$ bone marrow cells [89,90]. These observations suggest the importance of additional genes or pathways in MLL-fusion mediated leukemogenesis. Interestingly, MLL-rearranged leukemia also harbors an embryonic stem cell (ESC) genes signature opposed to the adult HSCs gene profiling [91]. A hallmark of this ESC signature is the activation of WNT/ßcatenin pathway that seems to be implicated mainly in the establishment of limitless self-renewing capacity but also in drug-resistance phenotype in MLL-rearranged LICs [92,93]. Despite the new discovery of several MLL-fusion specific genes, the *Hox-A* deregulation remains until now the most important factor for MLL-fusion induced leukemogenesis and the best readout used in *MLL rearranged* leukemia.

5. THE MLL COMPLEX IN NORMAL HEMATOPOIESIS AND LEUKEMIA

MLL is part of a large multi-protein complex. The first hints supporting this concept is that SET1 H3K4 methyltransferase, known as the MLL homolog in yeast, acts in a macromolecular complex of around 1MDa named “COMPASS” containing six other proteins designated according to their molecular weight on SDS-page: Cps25, 30, 35, 40, 50 and 60. Similarly, in mammalian cells, MLL functions as part of the “COMPASS-like complex” [94]. The main activity of COMPASS and COMPASS-like complexes seems to be mono-, di or trimethylation of histone3 lysine 4 (H3K4) through the conserved SET domain of the histone methyltransferase core component.

Description of the MLL macromolecular complex

After proteolysis, the MLL-C and MLL-N subunits maintain different protein-protein interactions leading to the formation of two large multi-protein complexes [95] (**Fig. 7**). The MLL-C portion associates with WRD5 (WD repeat-containing protein 5, Cps25 homolog), ASH2 (absent, small, or homeotic)-like (ASH2L; Cps60 homolog), RBBP5 (Retinoblastoma binding protein 5; Cps50 homolog) and MOF (MYST family acetyltransferase 1, KAT8) to form a COMPASS-like core complex essential for maximal enzymatic activity. In addition the MLL-C subunits can recruit transcriptional co-activators such as acetyltransferases CBP and EP300 through its TA motifs. The main role of this C-terminal complex is to prepare chromatin for efficient transcription. On the other side the N-terminal subunit guides the MLL complex to its target genes by interacting directly with DNA through its AT-hooks and CXXC motifs, but also by binding to MENIN (MEN1) and the lens epithelium-

derived growth factor (LEDGF/p75 also known as PSIP1) that makes contacts to chromatin via its PWWP domain. The CXXC domain of MLL can recruit on one hand the polymerase-associated factor complex (PAFc) and on the other hand repressive factors such as histone deacetylases (HDACs) and polycomb Group (PcG). These co-repressors recruitment appeared to be positively regulated by conformational changes initiated by the propyl-isomerase cyclophilin 33 (CYP33) enrolled through interaction with the PHD fingers motifs of the MLL protein [96,97]. Recently it also has been reported that the CXXC domain of MLL binds with high affinity to nonmethylated CpG-containing-DNA to protect *Hoxa9* promoter from silencing methylation and allows its expression [98]. Overall, the large MLL multi-protein complex orchestrates three major features of chromatin modifications: methylation, acetylation and nucleosome remodeling leading to proper transcriptional regulation of specific target genes.

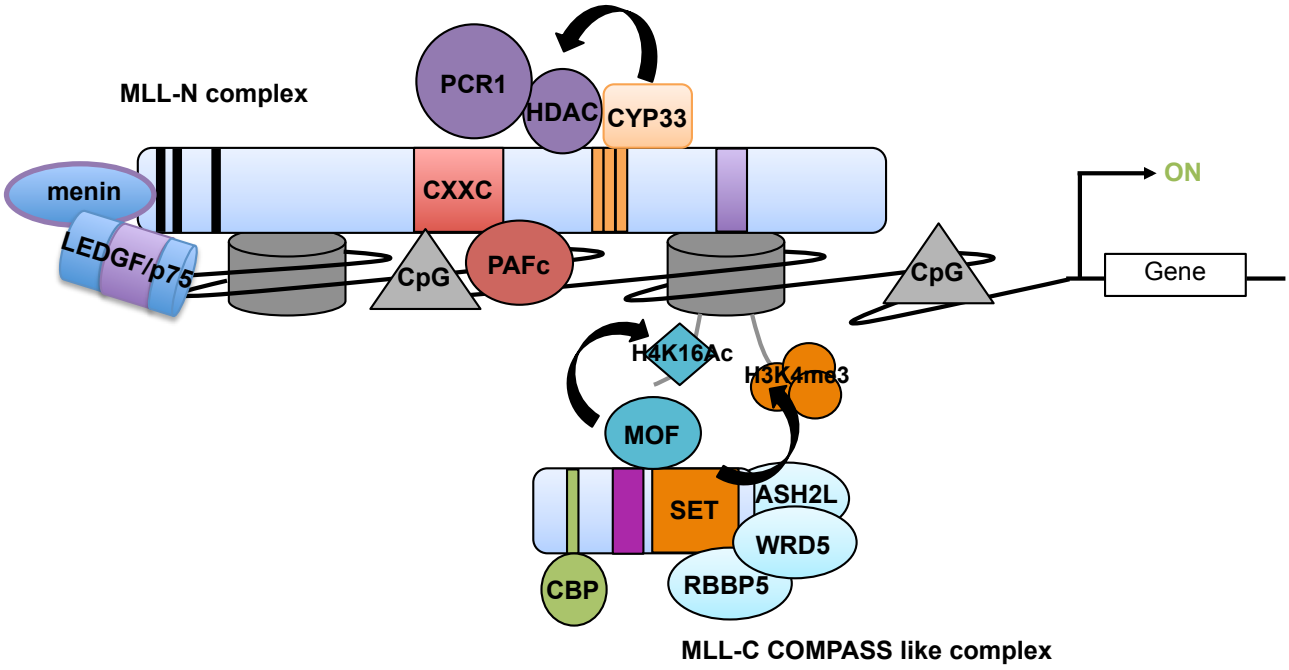

Fig. 7: The wild-type MLL multiprotein complex

MLL is present in the cell as part of a large protein complex. MLL-N complex is mainly involved in DNA binding to specific genes promoter mainly through interaction with MENIN-LEDGF/p75 complex. In addition, binding of MLL-N to non-methylated CpG-containing DNA through its CXXC domain participates to protect target genes promoters from methylation and silencing. MLL-N also interacts with transcriptional co-activators such as PAF1 complex (PAFc), but also transcriptional co-repressor such as the PCR1 or HDAC proteins. Recruitment of co-repressors is possible thanks to conformational changes exerted by the propyl-isomerase CYP33. MLL-N also tethers the MLL-C COMPASS like complex to the targeted genes promoter. MLL-C complex is mainly involved in the setting of positive DNA histone marks on the target genes promoter leading to transcription activation and elongation such as H3K4me3 and H4K16 acetylation elicited respectively by the MLL SET methyltransferase domain and MOF acetyltransferase.

MLL-fusion protein complexes function as super elongators of transcription

The large number of different fusion partners to MLL raises the question how the different fusions lead to the same disease. There is increasing experimental evidence that provided potential explanations.

First, in all common MLL-fusions the respective partners are invariably fused to MLL-N right after the CXXC domain. MLL fusions loose the PHD fingers and the SET domain, but retain the AT-hooks, CXXC domain and the interaction with MENIN and LEDGF/p75 essential for association with chromatin. Importantly, the interaction with MENIN-LEDGF/p75 complex has been shown to be essential for MLL-fusion-induced leukemia [99,100]. The MLL-fusions also keep the ability to interact with PAFc through its CXXC domain. Notably, PAFc has been shown to be essential for proper recruitment of MLL and MLL-fusions on target loci [101]. In addition, PAFc-MLL interaction has been characterized as essential for MLL-fusion driven leukemia [102]. Conversely, the PHD fingers exert a negative effect on MLL-fusion mediated leukemogenesis as artificial fusions including this domain lost their transforming capacity [103,104].

Second, the MLL fusion partner proteins fall into two classes: nuclear vs. cytoplasmic proteins (**Table II**). MLL-fusions to 5 nuclear proteins (AF4, AF9, ENL, AF10, ELL) cover over >80% of all cases of mixed lineage leukemia whereas the remaining fusion partners are much rare and mostly associated with adult AML.

Table II. Subset of MLL fusion partners with known functions or domains.

Name	Gene alias	Features	Localization
ENL*	*MLLT1*	Binds to histone H3 assembles in ENL associated proteins (EAP) elongation complex	Nuclear
AF9	*MLLT3*	ENL homolog involved as well in EAP	Nuclear
AF4	*AFF1*, *MLLT2*	Founder of AF4 family, member of EAP	Nuclear
AF5	*AFF4*, *MCEF*	AF4 homolog, found in EAP	Nuclear
LAF4	*AFF3*	AF4 homolog, found in EAP	Nuclear
ELL		Elongation factor interacting indirectly with AF4	Nuclear
AF10	*MLLT10*	Associates with the disruptor of telomeric silencing H3 methyltransferase (human: DOT1L)	Nuclear
CBP	*CREBBP*	Histone acetyl-transferase	Nuclear
P300	*EP300*	CBP homolog	Nuclear
AF1p	*EPS15*	Dimerization domain	Cytoplasmic
GAS7		Dimerization domain	Cytoplasmic
AF6	*MLLT4*	Dimerization domain	Cytoplasmic
ABI1		Associates with ENL when imported in the nucleus	Cytoplasmic
EEN		Associates with PRMT1, histone arginine methyltransferase, during nuclear import	Cytoplasmic

** The most frequent fusion partners are in bold police,* adapted from [66].

The search for a common molecular mechanism underlying MLL fusion induced leukemia revealed that the most prevalent fusion partners and close homologs ENL and AF9 were both able to interact with other fusion partners such as AF4, AF5 (AF4-homolog) and probably AF10 [105]. In addition all these proteins have been purified in a complex named EAP (ENL associated protein) complex. This complex is able to recruit the positive transcription elongation factor b (pTEFb) encompassing CDK9 (cyclin-depend kinase 9) and a cyclin T responsible of the activation of RNA polymerase II (RNA pol II) essential for efficient transcriptional elongation [106,107]. The actively elongating form of the RNA pol II is stabilized by the interaction between pTEFb and the histone acetyl interacting bromodomain-containing protein 4 (BRD4, member of the BET family of bromodomain proteins) [108,109]. All together these proteins form a so-called “super elongation complex” (SEC). On the other hand AF9 and ENL are also components of another complex named “Dot.Com” including the MLL translocation partners AF10 and AF17, along with the WNT signaling proteins TRAPP and SKP1, and the DOT1L H3K79-methyltransferase [110]. H3 methylation on lysine 79 is also introduced during transcriptional elongation [111]. Increasing evidence support the idea that recruitment of the EAP complex and DOT1L are essential for MLL-fusion protein driven leukemogenesis. Primarily, the association of ENL and AF9 with AF4 family members seems to be crucial for maintenance of mixed lineage leukemia cells as the use of small peptides disrupting these interactions impaired MLL transforming activity [112]. In the same vein, genetic studies have shown that DOT1L interaction is essential for transformation by the most prevalent MLL fusions and enrichment of H3K79 methylation is required for activation of critical effectors for MLL-AF9 mediated leukemogenesis like *Hoxa9* and *Meis1* [113–115]. In summary, the most prevalent MLL-fusion partners are involved in the recruitment of a super elongation complex comprising the EAP interactome essential for activation and elongation of transcription, and Dot.Com complex maintaining the “open chromatin” state through methylation of H3K79 by DOT1L. Dual activation of both the EAP and the Dot.com complexes seems to result in aberrant transcription and activation of MLL fusion target genes.

Interestingly, genetic studies suggested that a copy of the wildtype *MLL* allele is required for the oncogenic activity of the MLL-fusions. Indeed, it has been shown that both WT MLL and MLL-AF9 proteins are co-recruited on active *Hoxa9* gene locus through interaction with MENIN [101,116]. In addition, mobilization of WT MLL was necessary to induce and maintain MLL-AF9 mediated leukemia [116]. The same group also reported that in MLL-AF9 transformed cells, the wildtype MLL complex would be involved in transcriptional activation

of EZH2, the core H3K27 methyltransferase of the polycomb compex PRC2, which would in turn inhibit the transcription of key genes involved in proper myeloid differentiation (e.g. CEBPα) and therefore promote a “block in differentiation” state in the targeted cell [117]. These observations suggest that the presence of a normal MLL allele might be a prerequisite for stable recruitment of the MLL-fusion on *Hox* genes promoters, and to directly regulates the transcription of other genes which are promoting leukemogenesis. Nevertheless how exactly the endogenous MLL and the fusion complexes cooperatively regulate gene expression remained mostly unclear. Interestingly, very recently it has been suggested that MLL-mediated hematopoietic genes expression and MLL-AF9 driven leukemogenesis are independent of MLL intrinsic SET domain HMT activity [118]. In this study they show that the WT MLL target gene expression seems to be mainly dependent of the recruitment of MLL-associated MOF histone H4 acetyltransferase leading to H4K16 acetylation. MOF binds directly to MLL-C, but this interaction is lost upon leukemogenic translocation. Here they suggest that the de novo recruitment of pTEFb and DOT1L may override the lack of MOF activity in the MLL-fusion context [118].

Taken together these observations suggest that both partners of the fusion play distinct roles essential for leukemogenic activity: MLL on one side is involved in the binding to target gene promoters involved in hematopoiesis homeostasis and the most prevalent partner proteins are the mediators of aberrant transcriptional elongation (**Fig. 8**).

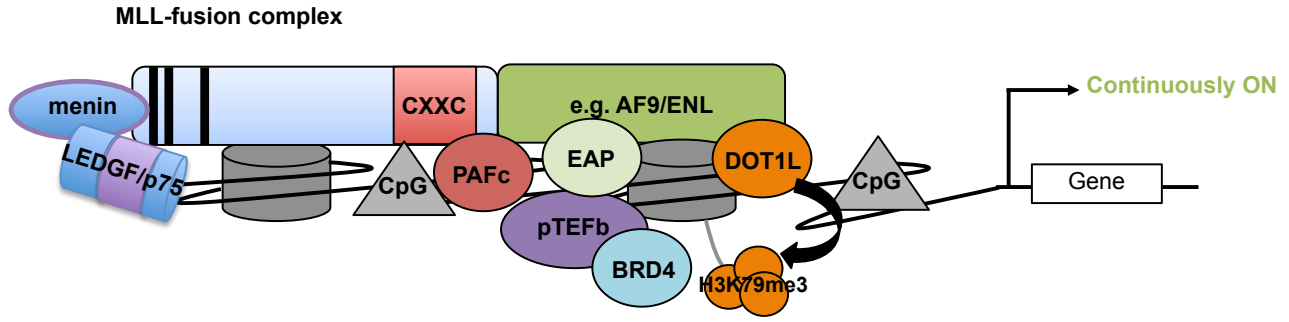

Fig. 8: MLL-fusion macromolecular complex

MLL when part of oncogenic fusion is involved imainly in proper DNA binding through interaction with MENIN and LEDGF/p75 a to non-methylated CpG-containing DNA islands through its CXXC domain. Interestingly the recruitment of transcriptional co-repressors effective in the WT MLL complex is vanished as PHD fingers are lost, but the proper recruitment of MLL-N on chromatin through interaction between its CXXC domain and PAF1 complex (PAFc) is kept. On the other end, the most prevalent partners such as AF9 or ENL are participating in the recruitment of a super elongation complex composed of the EAP complex which in turn recruits pTEFb and BRD4 both involved in RNA pol II activation and stabilization. The most prevalent MLL-fusion partners are also known to complex with DOT1L H3K79 methyltransferase. DOT1L-mediated histone methylation is associated with transcriptional activation and elongation maintenance. In all, SEC and DOT1L are tethers to DNA by MLL-fusion, which leads to continuous transcription of MLL-target genes.

6. THERAPEUTIC TARGETING OF THE MLL-FUSION PROTEIN COMPLEX

The fact that the oncogenic activity depends on interactions of components of the MLL-fusion multiprotein complexes suggests novel avenues for targeted therapeutic strategies for *MLL rearranged* leukemias. Going into this direction, some reports suggest that the efficacy of conventional chemotherapy may be increased through additional inhibition of the atypical elongation and transcription exerted by the MLL-fusion complex. Two major strategies have been taken: to inhibit the activity or interfere with binding of the MLL-complex to chromatin or with recruitment of co-activators essential for the maintenance of the aberrant leukemogenic transcription program.

Targeting the recruitment of the super elongation complex (SEC)

Several attempts have been undertake to interfere with the recruitment of the SEC complex by MLL-fusion proteins [119]. First, an AF4 peptido-mimetic "PFWT" was able to disrupt the EAP complex through competition with the AF4/AF9 interaction *in vitro* and *in vivo* [112]. Treatment of MLL-AF4$^+$ leukemic blasts with "PFWT", although at high concentrations, inhibited the proliferation and induced cell death, with only minor effects on normal HSPCs [120].

A second interesting approach is based on inhibition of the activity of proteins that are necessary to stabilize the SEC. Blocking the enzymatic activity of CDK9, a component of the pTEFb complex whose recruitment is crucial for maintenance of the transcriptional activity of the MLL fusion complex has been proposed [67,121]. This strategy is promising, knowing the potent and apparently specific inhibitory effect of flavonoids such as flavopiridol, which reached the phase II of clinical trials for several hematologic and solid tumors [122]. The main drawbacks of these compounds are their limited efficacy and high toxicity. Similarly, a large-scale RNA interference screen identified dependence of CDK6 function as specific burden of MLL-rearranged AML. In this report they showed that pharmacologic or knockdown inhibition of CDK6 induced growth defect and differentiation of human and murine MLL-fusion driven cell lines *in vitro* and delays MLL-AF9-mediated disease development *in vivo* [123]. However even if promising CDKs inhibitors are not selective for MLL-fusion mediated AML. In the same direction, selective targeting of the epigenetic reader protein BRD4 that recognizes acetylated histones participating in the stabilization of activated RNA pol II active form was proposed as potent anti-leukemic strategy [124]. Several highly specific small molecule inhibitors of the BET family of BRD

proteins (BRD3/4) showed efficient anti-leukemic effects, however these compound are also active in many other forms of human cancer [124–127].

Epigenetic targeting of the MLL fusion complex

Several histone methyltransferases (HMTs) have been identifed as critical regulators of the activity of the MLL-fusion complex. Genetic studies have shown that the DOT1L H3K79 methyltransferase is essential for the leukemogenic activity of MLL fusion genes. High-resolution structure of the catalytic domain of DOT1L resulted in the development of a small molecule inhibitor (EPZ004777) that was able to impair MLL-fusion mediated leukemogenesis *in vitro* and *in vivo* [128–130]. Subsequently more potent DOT1L inhibitors have been estabishled [131,132] and EPZ-5676, an improved version of EPZ004777 [133,134], is currently tested in first clinical trials.

Another attractive target might be the HMT activity on H3K4 of the wildtype MLL. Recent studies report the feasibility of targeting the methyltransferase activity of MLL through inhibition of a critical MLL-WRD5 interaction [135–137]. Such efforts led to the development of a MLL1 selective small molecule inhibitor (MM-404) targeting MLL-WDR5 interaction that exclusively impaired growth, induced differentiation and apoptosis of MLL-fusion transformed human and murine cell lines without affecting normal mouse bone marrow cells [138].

Targeting association of the MLL-complex to chromatin

Another potential therapeutic strategy is based on interference with the binding of the MLL-fusion complex to DNA. The first approach proposed in this regard is to target the CXXC CpG-containing-DNA binding domain of MLL. Indeed it has been shown that replacement of the MLL-AF9 CXXC domain with a lower CpG affinity CXXC domain or a methyl-CpG affinity domain leads to *Hoxa9* silencing and abolished leukemogenic activity of the fusion [98]. Interestingly, the CXXC domain activity seems to be more crucial for the oncogenic activity of the MLL-fusion than for the WT MLL protein suggesting a therapeutic window for targeting of CXXC domain [101,139]. Despite the structural resolution of the DNA non-methylated CpG-CXXC interaction defined as drugable no small molecule selectively disrupting this interface has been described yet [140].

Like interference with the CXXC domain, targeting of the MLL-MENIN-LEDGF/p75 interactome is also promising to disrupt the binding of MLL-fusion complex to chromatin. Indeed, genetic studies have shown that both MENIN and LEDGF/p75 are essential mediators of MLL-complex recruitment on the Hox gene promoters and crucial for MLL-ENL leukemogenic activity [99,100,141]. Structural studies of the MLL-MENIN interface leads to

the development of small molecules inhibitors for these interaction able to affect MLL-fusion mediated leukemogenesis [142–144]. In contrast to MENIN, it is not yet clear if it would be feasible to target LEDGF/p75 to disrupt interaction with MENIN and/or the MLL-fusion to impair the oncogenic activity.

RESEARCH BACKGROUND AND WORKING HYPTOTHESIS

My PhD project mainly focused on the functional interference with the interaction between MLL and LEDGF/p75 as a potential strategy for therapeutic targeting for MLL-fusion leukemia. LEDGF/p75 has been firstly described as a DNA-binding transcriptional co-activator and belonging to the hepatoma-derived growth factor (HDGF) family [145]. However other studies found weak transcriptional activity of LEDGF/p75 mainly involved in protection against cellular stress [146]. Until know the best characterized role of LEDGF/p75 concerns its involvement in HIV pathogenesis where it mediates tethering of the proviruses to the host cell chromatin through direct interaction with the HIV-integrase (HIV-IN) [147,148]. LEDGF/p75 consists of 530 amino acids (aa) containing a N-terminal PWWP motifs involved in chromatin binding and a C-terminal integrase binding domain (IBD). The Debyser group showed that stable overexpression of the LEDGF/p75-IBD-fragment (aa325-530) was sufficient to disrupt the interaction between HIV-IN and LEDGF/p75 and inhibit HIV-1 replication [149]. This study together with the structural resolution of the HIV-IN/IBD interface [150], led to a successful rational design of small-molecule inhibitors disrupting the LEDGF/p75-integrase interaction to impair HIV replication [151]. In the same vein, expression of phage-display screen derived LEDGF/p75 interacting cyclic peptides was able to inhibit HIV replication [152]. Interestingly, LEDGF/p75 is not only a critical scaffold for HIV replication but seem also to play an important role in human leukemogenesis. First, a chromosomal translocation t(9;11)(p22;p15) associated with AML and blast crisis CML was found to result in a NUP98-LEDGF/p75 fusion [153,154]. Second, high expression of LEDGF/p75 was reported in blasts from chemotherapy-resistant AML patients [155]. Most importantly, as mentioned above, LEDGF/p75 has been shown as essential co-factor for the MLL-Fusions complex mediated leukemogenesis [100]. ShRNA-mediated knockdown of LEDGF/p75 expression significantly impaired the maintenance of MLL-ENL driven transformation in murine bone marrow. This landmark study demonstrated that the interaction with MENIN seems to be essential for LEDGF/p75-MLL complex formation, and that the oncogenic activity of MLL-ENL seems to be fully dependent on the PWWP domain of LEDGF/p75. Considering these findings in both HIV and AML pathogenesis, we initiated collaboration with the Debyser group to figure out whether the competition between IBD-derived fragments (or LEDGF/p75-interacting peptides) with the endogenous LEDGF/p75 would disrupt the MLL-MENIN-LEDGF/p75 interface and impair MLL-fusion mediated leukemogenesis (**Fig .9**).

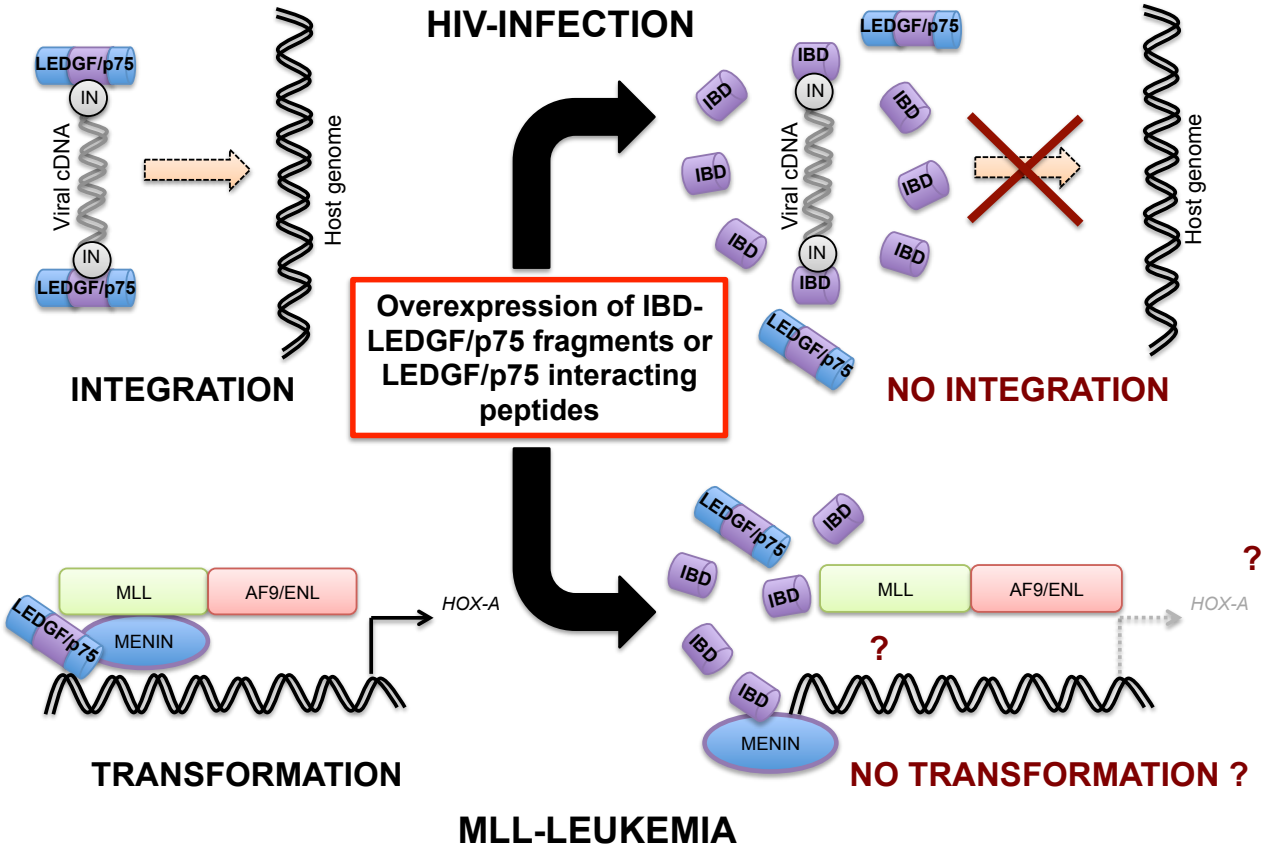

Fig. 9: Working hypothesis schematic representation

LEDGF/p75 is known to be involved in the tethering of provirus to the host chromatin through direct interaction with HIV-integrase (IN) helping to its integration, an essential step for HIV viral replication. On the other hand, in MLL-leukemia, LEDGF/p75 together with MENIN is involved in MLL-fusion complex tethering to the promoter of target genes critical for leukemogenesis. Overexpression of LEDGF/p75-IBD fragment or LEDGF/p75 interacting peptides through competition with endogenous LEDGF/p75 abolished viral genome integration and impairs viral replication. Based on this effect we proposed to use the same LEDGF/p75 competing tools in the context of MLL-leukemia to see if we could disrupt MLL-MENIN-LEDGF/p75 critical interactions and in the same time impair MLL-fusion mediated transformation.

MATERIAL AND METHODS

Cells

The human leukemia cell lines: MOLM13, (AML, MLL-AF9+); THP1, (AML, MLL-AF9+); MV4;11 (AML, MLL-AF4+); HL-60, (AML, MLL WT); Jurkat (T-ALL); and Kasumi (AML, AML1-ETO+) cell lines were purchased from DSMZ (Braunschweig, Germany). All cells were cultured in RPMI-1640 with glutamine, supplemented with 10% fetal calf serum (FCS) and 1% Penicillin/streptomycin at 37°C and 5% CO_2. IL3 independent murine Ba/F3 cells expressing the FLT3-ITD mutation (Ba/F3 FLT3-ITD) have been described previously [156]. Human kidney carcinoma 293T cells (ATCC/LGC Standards, Molsheim Cedex France) were grown in Dulbecco's modified Eagle's medium with Glutamax (Gibco, Invitrogen, Merelbeke, Belgium) supplemented with 10% FCS and 1% Penicillin/Streptomycin (Gibco).

Plasmids/vectors

pMSCV (Murine Stem Cell Virus) retroviral expression vector encoding for the MLL-AF9 fusion was provided by J. Hess (Ann Arbor) and *pMSCV-MLL-ENL-neo* was provided by Robert Slany (Erlangen)**.** The retroviral transfer plasmid expressing eGFP (*pMSCV PGK-Puro-IRES-eGFP*) was derived from *pLMP* (Open Biosystems, Fermentas GmbH, St Leon-Rot, Germany) where the backbone was removed by digestion with *SpeI* enzyme. The missing part of the PGK sequence was replaced by *SpeI* digestion of a *pMSCV-PKG-Puro* vector (Clontech, Saint-Germain en Laye, France) and ligation of this fragment into the modified *pLMP* plasmid. To create the *pMSCV* plasmid expressing eGFP-LEDGF/p75$_{325-530}$ (*pMSCV eGFP-LEDGF/p75$_{325-530}$ PGK-Puro*), eGFP-LEDGF/p75$_{325-530}$ was amplified from *peGFP-LEDGF/p75$_{325-530}$-IRES-Puro* using oligo's DR1 and DR2 (**Table 3**) [149], digested with *BamHI* and *MfeI* and subcloned into *pMSCV-PGK-Puro* (Clontech, Saint-Germain en Laye, France) digested by *BglII* and *EcoRI*. The shRNA- fragment for murine LEDGF/p75 was purchased from Open Biosystems (*V2MM_34220*) and subcloned from *pSMC* into the *pLMP* retroviral vector. The lentiviral transfer plasmids *pSFFV-eGFP-I-Puro-WS*, *pSFFV-eGFP-LEDGF/p75$_{325-530}$-I-Puro-WS* and *pSFFV-eGFP–I-Puro* 2xmi p75 WS expressing eGFP, eGFP-LEDGF/p75$_{325-530}$ and a duplicate LEDGF/p75 microRNA cassette together with eGFP respectively were made by our collaborator in Belgium as described previously [149,157].

Expression vectors for various eGFP-LEDGF/p75 deletion mutants were generated by overlap extension polymerase chain reaction (PCR) using the LEDGF/p75$_{325-530}$ subclone in *pBlueScript-KS* as template. The T3 Reverse was used as common primer to generate

the C-terminal deleted mutants and T7 Forward to generate the N-terminal deleted fragments. The smallest fragments were generated using the same strategy and using the two minimal active mutants identified LEDGF/p75$_{325-386}$ and LEDGF/p75$_{424-530}$ as templates. All primers used for the generation of the LEDGF/p75 fragments are described in **Table 3**. The generated LEDGF/p75 deletion mutants were then cloned back into *pLMP* expression vector.

Table 3: Oligo-primers used for cloning strategies

Name	Sequence 5'-3'
DR1	TTTGGATCCATGGTGAGCAAGGGCGAGGA
DR2	GGGCAATTGCTAGTTATCTAGTGTAG
DR3	CATATTGGATCCATGGTGAGCAAGGGCGAGGAG
DR4	AAAGCTAGCTCAGGCACCGGGCTTG
DR5	TTTTACTAGTACTAGCGCTACCGGACTCAG
DR6	TTTTACTAGTCAACGCGTCCCGGTGGATCC
DR7	AAAGGATCC ATGGGGCTGAAGGCCGCCC
DR8	AAAAGCTAGCTCACGCGTAGTCCGGTACGTCGTACGGGTAAGCAGCAGCGA GGCCTTTGCGCTGCCGC
DR9	TTTTGTCGACCTCAAGCTTGCGCACAGCTGTCGGTG
DR10	TTTTGCTAGCTTA GACTTTCTGGGGCTTTTCC
DR11	TTTTGAATTCGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATG AAGTCGAC TCCCCTATACTAGGTTATTGGAAAATTAAG
DR12	CCCCCTCGAG CTA ATCCGATTTTGGAGGATGGTC
DR13	TTTTCATATG GCACATAGCTGTCGTTGGCG
DR14	CCCC GGATCC TCGCACTCTGACTTCTTCATCTGAG
DR15	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGGGCTGAAGGCCGCCCAG
DR16	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGAGGCCTTTGCGCTGCCGC
410-530 For	GCAGTCCAATGACTCGAGGTAATCATGGAAAAGTCTAC
424-530 For	GCAGTCCAATGACTAAGAACATGTTCTTGGTTGGTG
450-530 For	GCAGTCCAATGACTCATGAGGCGAATAAAA
394-530 For	GCAGTCCAATGACTACAGAGATGATTACTACAC
387-530 For	GCAGTCCAATGACTATGCAACAAGCTCAGAAAC
370-530 Forw	GCAGTCCAATGACTGTGAACAGATGCATTGAGG
pBlueScript II SK T3 Rev	GGTTGGCCCTCAAAAGGG
pBlueScript II SK T7 For	TAATACGACTCACTATAGGGC
325-499 Rev	GCATCAATTGTGGTCAGTCTTCATTGCTCTCCCCGTTATG
325-449 Rev	GCATCAATTGTGGCTGTCTTTGTTCAGCAAGAGATTTATTC
325-423 Rev	GCATCAATTGTGGAAACTTGTTATACAACATTGTAG
325-409 Rev	GCATCAATTGTGGACTAACTTTGAATCGCCCTAT
325-393 Rev	GCATCAATTGTGGGTGTTTCTGAGCTTGTTGCATTG
325-386 Rev	GCATCAATTGTGTGACCTGAAGTGAAGCAAG
345-386 For	GCACTCGAGGAAACATCAATGGATTCTGCAC
355-386 For	GCACTCGAGATACATGCTGAGATTAAAAATTCAC
366-386 For	GCAGTCTCGAGGATAATCTTGATGTGAACAGATGCAAT
375-386 For	GCAGTCTCGAGGAGGCCTTGGATGAACTTGCTTCA
424-485 Rev	GCATAATTGTCATTGAGCATCAGATCCTCCATTTAG
424-449 Rev	GCATCAATTGTCACTGTCTCTTTGTTCAGCAAGAGATTTAT
424-435 Rev	GCATCAATTGTCACACGGAATCTCCTTCACCAAC

For, *forward* ; Rev, *reverse*

Lentiviral and retroviral vector production

Concentrated lentivirus was produced by our Belgium collaborators as described earlier [158]. For retroviral vector production, HEK293T cells were transiently co-transfected with *pMSCV* retroviral vectors and an ecotropic packaging vector (*pIK6*). The virus containing supernatants were collected after 48h and 72h, and only freshly harvested retroviral supernatants were used for retroviral infections of primary mouse bone marrow cells.

Generation of murine MLL-fusion expressing AML cells

Murine MLL-fusion-expressing AML cells were generated by transplantation of bone marrow cells retrovirally expressing the human MLL-AF9 or MLL-ENL fusions using a previously published protocol with some modifications [159]. In brief, bone marrow cells were harvested from 8-weeks-old (FVB/Nx129/S1) F1 or C57/BL6 mice and enriched for progenitors using a lineage-marker-depletion progenitors kit (Cell Mag Kit, R&D systems, Minneapolis, MN, USA). These cells were cultured for 24 h in RPMI-1640 (10% FCS, 1% penicillin/streptomycin, 10 ng/ml of human interleukin-6 (IL-6), 6 ng/ml of murine interleukin-3 (IL-3) and 100 ng/ml of murine stem cell factor (mSCF)) before transduction with high-titer retrovirus by spinoculation (2000 g, 90 min at 33°C) on two consecutive days. After the second spinoculation $5x10^5$ transduced bone marrow cells were injected into the tail vein of lethally irradiated (950 rad) syngeneic recipients. As described previously, 100% of the transplanted mice developed AML after a median latency of 86 and 91 days for respectively MLL-ENL and MLL-AF9 fusion.

Transduction of murine and human cells

Murine cells were transduced using the spin infection protocol described above with *pMSCV-PGK-Puro-IRES-eGFP-LEDGF/p75-fragments, pMSCV-LEDGF/p75-ShRNA-PGK-Puro* or *pMSCV-PGK-Puro-IRES-eGFP*. Cells were flow-sorted for eGFP expression 24h after the second spinoculation and used as indicated for the different assays.

Human AML cell lines were put in culture with concentrated lentiviral supernatants containing expression vectors *pSFFV-eGFP-I-Puro-WS*, *pSFFV-eGFP-LEDGF/p75-fragments-I-Puro-WS* and *pSFFV-eGFP–I-Puro 2xmi p75 WS* (LEDGF/p75 knockdown). 48h post-infection the eGFP+ cells were flow-sorted for eGFP and/or selected with puromycin as indicated.

Flow cytometry

For sorting of transduced cells, the cells were washed two times with PBS, filtered and resuspend in FACS buffer (PBS, 0.5% BSA, 1mM EDTA) containing DAPI (Gibco, Invitrogen; 1/10000). eGFP-positive/DAPI negative cells were flow sorted using a BD Influx cell sorter (BD Biosciences Europe) into tubes containing appropriate cell culture medium. After sorting cells were spin down and plated according to the desire experiment set up.

For immunophenotypic characterization of the murine retrovirally expressing MLL-fusion generated cells, frozen spleen cells isolated from MLL-AF9 mice and freshly isolated cells from a spleen of a FVB/NJx129 mouse were stained on ice for 30 minutes with an antibody cocktail containing anti- PE-CY7-C-kit PE-FcγRI/II, APC-Mac1 (eBioscience, San Diego, USA; 1/100) and APC-CY7-Gr1 (BD Biosciences Europe; 1/100). Subsequently the cells were washed and stained with DAPI (Gibco, Invitrogen; 1/10000).

To assess the effect of LEDGF/p75-IBD fragments expression on cell differentiation, MOLM13 and MV4,11 human leukemic cells were transduced with *pSFFV-IRES-eGFP-LEDGF/p75-fragments* or *pSFFV-PGK-Puro-IRES-eGFP*, and selected with 2µg/ml puromycin. $2x10^5$ cells were harvested after 5, 7 and 10 days and were stained with APC-CD11b (BD Biosciences Europe, #550019; 1 /100) and DAPI (Gibco, Invitrogen; 1/10000) for 15 min at RT as indicated in the manufacturer's protocol.

For flow-cytometric cell cycle analysis, 96 hours after transduction the cells were fixed in 2% PFA for 10 min at room temperature, washed, permeabilized with NET gel (150mM NaCL, 5mM EDTA, 50mM Tris-HCL pH7.4, 0.05% NP-40, 0.25% Lambda carrageenan (Sigma-Aldrich, St Louit, MO, USA)) to preserve membrane integrity and stained with DAPI (in PBS:0.1% Triton X-100, 1µg/ml DAPI (Gibco, Invitrogen)) for 30 min at room temperature.

For flow-cytometric detection of apopotosis, the murine leukemic cells were harvested at 3, 5, 7 and 10 days and human AML cell lines were harvested at 5, 7 and 10 days after puromycin selection and stained with an Annexin-V antibody (BD Biosciences Europe, #550475) and DAPI (Gibco, Invitrogen; 1/10000) for 15 min at RT.

For all analytic flow cytometry the samples were washed with PBS after staining procedure, filtered and run on a Cyan ADP Flow Cytometer (Dako Cytomation, Glostrup, Denmark) using Summit software. Data were analyzed using Flowjo software (Tree Star INC., Ashland, OR, USA).

Clonogenic growth assays in vitro

Colony formation was determined by plating cells in methylcellulose. MLL-fusion or WT mouse derived cells were plated in murine methylcellulose containing IL-3, IL-6 and mSCF (M3534, Stem cell technologies, Vancouver, BC) in presence of 2-4µg/ml of puromycin for selection of transduced cells. Ba/F3 FLT3-ITD cells as well as the human AML cells lines were plated in methylcellulose without cytokines (Stem Alpha.mIE, Stem Alpha, St Clement les Places, France) in the presence of 2µg/ml puromycin. Colonies and total cell numbers was counted after 5-7 days for murine cells and after 10 days for human cell lines.

Giemsa cytospin-staining

$5x10^4$ to $1x10^5$ cells were resuspended in 100 µl PBS and spun onto glass slides using a Shandon Cytospin-2 Centrifuge at 350 rpm for 5 min. The slides were air dried and stained with Wright-Giemsa using the Hematek® Stain Pak Hematology Slide Strainer (Bayer HealthCare, Zurich, Switzerland).

Growth monitoring

In all, 2 x 10^4 flow-sorted eGFP-positive cells either flow sorted or puromycin pre-selected for 24 hours were plated in the appropriate medium under 2µg/ml puromycin continuous selection. Growth was monitored for 7 days by daily counting of living cells using the Trypan Blue dye exclusion test (Gibco, Invitrogen, Life Technologies, Paisley, UK).

Reverse transcription quantitative PCR (RT-qPCR)

RNA was extracted using TRIZOL® reagent (Life technologies, Grand Island, New York City, USA) for small amount of cells (<$5x10^5$) or the NucleoSpin® RNA kit (Macherey-Nagel, GmbH & Co, Duren, Germany) for larger amount of cells (>$5x10^5$) according to the manufacturer's protocol. cDNA synthesis was carried out with the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA). The mRNA expression level was assessed in duplicate by quantitative real-time PCR using detection using SyBR Green for *Hox-cluster* genes and a TaqMan probe for the human *MLL-AF9* gene fusion. Samples were run on an ABI prism 7700 Sequence Detection System (Applied Biosystems). Results were calibrated with *Gapdh* and calculated as $\Delta\Delta$CT values normalized to vector control. The primer pairs used are described in **Table 4.**

Table 4: Primers used in quantitative RT-PCR analysis

Primers for SyberGreen detection	**Sequence 5'-3**
mouse *Hoxa7* forward	GCGCAAGCTGCACATTAGT
mouse *Hoxa7* reverse	CTCGGAGAGGCAAAGGGCAT
mouse *Hoxa9* forward	GGTTCTCCTCCAGTTGATAGAGA
mouse *Hoxa9* reverse	GAGCGAGCATGTAGCCAGTTG
mouse *Meis1* forward	CCTCTGCACTCGCATCAGTAC
mouse *Meis1* reverse	CTAAGAGAGGGAAGAGGGGGTGT
mouse Gapdh forward	ATGACATCAAGAAGGTGGTG
mouse Gapdh reverse	CATACCAGGAAATGAGCTTG
Primers for Taqman detection	**Sequence 5'-3**
human *MLL-AF9* forward	CCAGCAGATGGAGTCCACAGGATC
human *MLL-AF9* reverse	GCTCTACCAGTTCATCTAGGTATGCC
human *MLL-AF9* probe	AGGCTTAGGGATCCTTGAAGTGAAAAGTCCAATTAAAGC
mouse GAPDH primers mix	Mm99999915_g1, Cat Number 4331182, Life Technologies

Western Blots

Whole cell extracts of different cell lines (293T, human AML cells THP1 and the murine hematopoietic cell lines Ba/F3 FLT3-ITD) were made in 1% SDS, separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad, Nazareth Eke, Belgium). Membranes were blocked with milk powder in PBS-0.1% Tween 20, and detection was carried out using specific antibodies against LEDGF/p75 (Bethyl, A302-509A), or GFP (Abcam, ab6673). Detection was performed using chemiluminescence (ECL+; Amersham) and horseradish peroxidase (HRP)-conjugated secondary antibodies.

In vivo Leukemogenesis

To address effects of LEDGF/p75-IBD derived fragments expression on MLL-fusion mediated *in vivo* leukemogenic potential, $5x10^4$ eGFP-positive flow-sorted murine MLL-AF9+ cells transduced with either *pMSCV-IRES-eGFP-LEDGF/p75-fragments* or *pMSCV-PGK-Puro-IRES-eGFP*, were mixed with 10^6 normal total bone marrow cells from a healthy donor mouse and transplanted into the tail vain of lethally irradiated syngeneic (FVB/Nx129/S1) F1 recipient mice. Animals were sacrificed upon first signs of disease.

To address *in vivo* effects of the combination between DOT1L (EPZ004477) and MENIN (MI-2-2) inhibitors on leukemogenesis, MLL-AF9 expressing murine leukemic cells were pretreated for 4 days in liquid culture with either control vehicle DMSO, 3µM EPZ004777, 3µM MI-2-2, or a combination of both compounds. At day 4, $5x10^4$ living cells were injected into the tail vein of sublethally irradiated C57/BL6 syngeneic recipient mice. Mice were sacrificed upon first signs of disease. When all the control DMSO treated mice

developed AML, 3 mice from each group were analyzed for histopathology to assess disease progression. Organs of an additional mouse from asymptomatic groups were analyzed at a later time point to assess development or not of the disease. Upon sacrifice all mice were analyzed for blood counts and organ infiltration. All experiments were performed according to Swiss laws for animal welfare and approved by the Swiss Cantonal Veterinary Office of Basel (Switzerland).

Statistical Analysis

Mean values and standard deviations were calculated to estimate the degree of variation and GraphPad Prism 5.0 software was used for statistical analysis, as specified for each experiment in the figure legends.

RESULTS

1. ESTABLISHING PRIMARY MLL-FUSION DRIVEN MURINE AML CELLS

In order to test the effects of functional interference with LEDGF/p75 in MLL-fusion driven leukemia we first established MLL-fusion induced murine AMLs. As previously reported, retroviral transduction of murine bone marrow (BM) cells with a retrovirus expressing different MLL fusions found in human patients led to leukemic transformation [160–162]. Following this strategy, we produced murine MLL-fusion gene AML by transplantation of lineage depleted BM cells transduced with murine stem cell virus-based retroviral vectors (*pMSCV*) encoding human MLL-ENL or MLL-AF9 leading to an acute leukemia phenotype after a latency period of 2-4 months (**Fig. 10**). Similar to earlier reports, the murine disease was characterized by high white blood counts, hepatosplenomegaly, and extensive multi-organ leukemic infiltration, reflecting clinical features of AML patients associated with MLL-AF9 and MLL-ENL respectively (data not shown) [161,163,164]. To validate leukemic cells from these mice as an AML model we further characterized them by flow cytometry. As shown in **Fig. 11A**, MLL-AF9 leukemic cells expressed the early progenitor cell markers like C-kit and FcgRII/III as well as the myeloid markers Gr-1 and Mac-1. In addition, quantitative RT-PCR analysis revealed increased expression of known MLL target genes like the *Hox-A* cluster genes *Hoxa9*, *Hoxa7* and *Meis1* compared to control cells (**Fig. 11B**). These characteristics confirm previous reports exemplifying these MLL-fusion driven murine leukemic cells as an ideal model for the human disease[160–164].

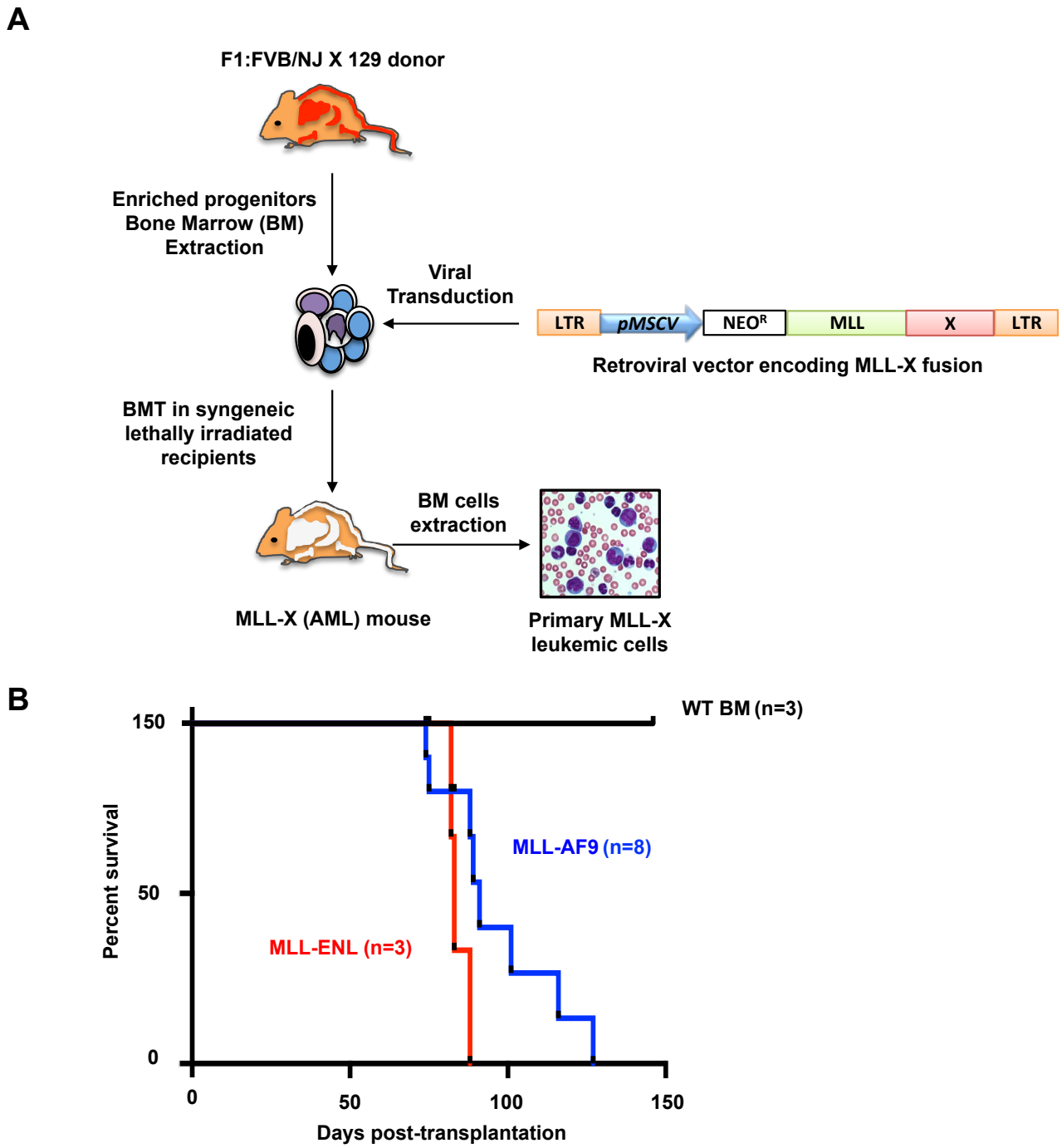

Fig. 10: Generation of MLL-X leukemic primary murine cell lines

(A) Schematic representation of the experimental setup. AML in mice was induced by primary transplantation of $5x10^5$ lineage depleted bone marrow (BM) enriched for stem and progenitors of a donor mouse retrovirally expressing MLL-ENL or MLL-AF9, as a control total bone marrow of a wild-type (WT) donor was transplanted (BMT). Mice were killed upon the first sign of illness and BM cells were extracted to constitute primary MLL-ENL and MLL-AF9 expressing leukemic cells. **(B)** Kaplan-Meier survival analysis reveals AML induction in transplanted mice receiving MLL-ENL (red line) and MLL-AF9 (blue line) expressing cells respectively after a median latency of 83 days and 91 days whereas the mice transplanted with wild-type bone marrow (WT BM, black line) were still alive at the end of the experiment.

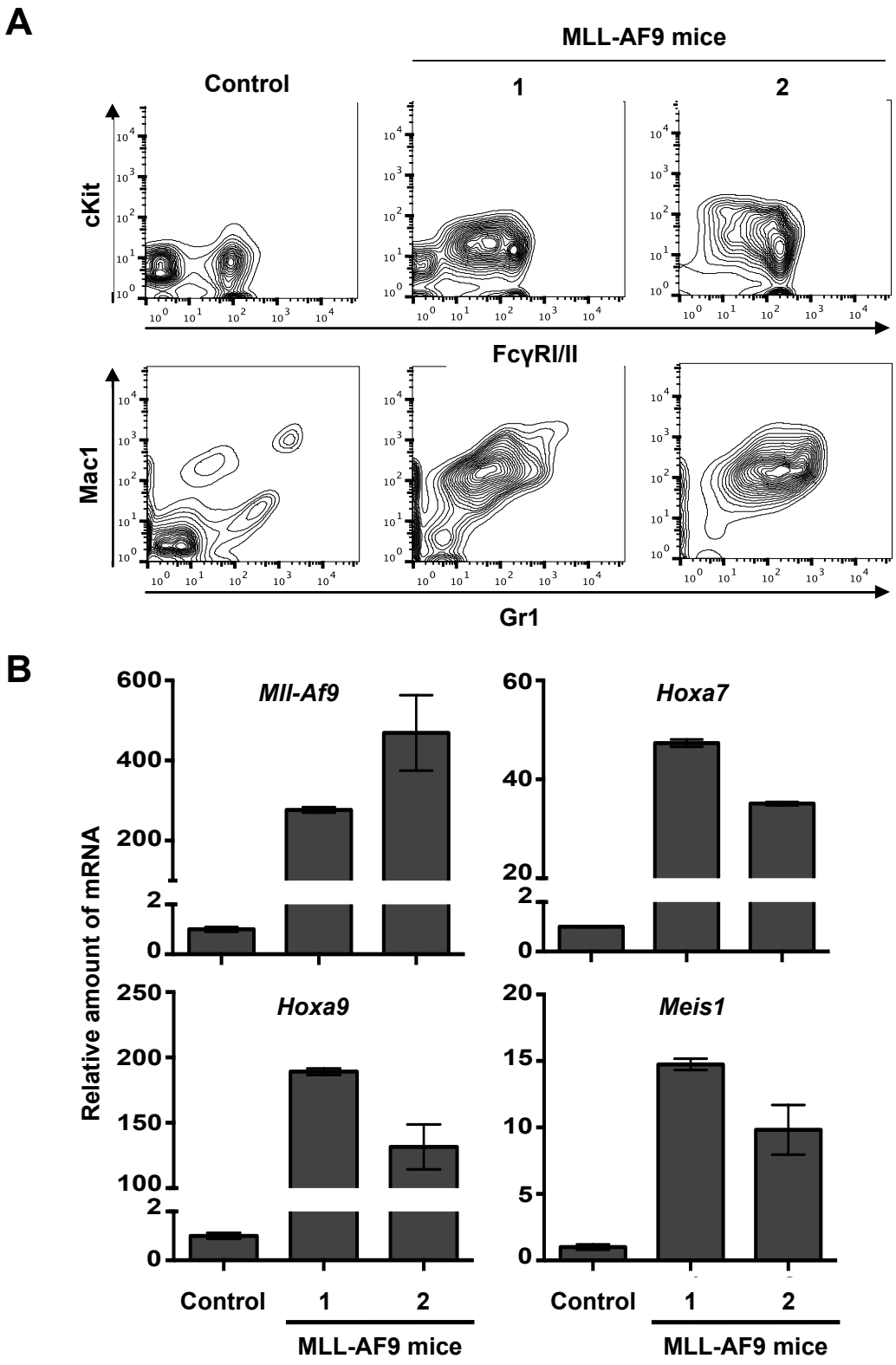

Fig. 11: Molecular characterization of MLL-AF9 primary leukemic cells

(A) Representative FACS analysis plots of murine WT and MLL-AF9 spleen cells labeled with antibody against murine markers c-Kit, FcγRI/II, Gr1 and Mac1. **(B)** Relative amount of *Mll-Af9*, *Hoxa9, Hoxa7* and *Meis1* mRNA measured in murine WT and MLL-AF9 spleen cells. The expression levels were normalized to *Gapdh* and to the value of the WT control (set to 1). Error bars represent the s.d. of a triplicate quantitative RT-PCR analysis.

2. EXPRESSION OF LEDGF/P75–IBD DERIVED FRAGMENT (LEDGF/P75$_{325-530}$) IMPAIRED SELECITVELY MLL-FUSION LEUKEMOGENIC ACTIVITY

Our collaborators have previously shown that expression of an eGFP (enhanced Green Fluorescent Protein) fused C–terminal fragment of LEDGF/p75 (eGFP-LEDGF/p75$_{325-530}$, **Fig. 12A**) containing the integrase binding domain (IBD) was able to compete with the endogenous LEDGF/p75 leading to impaired HIV-1 replication [149]. To experimentally address whether expression of this fragment would also affect the growth of MLL-fusion driven leukemic cells we used the MLL-AF9 and MLL-ENL expressing cells isolated from the diseased mice and transduced them with *pMSCV* vectors expressing either eGFP-LEDGF/p75$_{325-530}$, eGFP together with a LEDGF/p75 specific shRNA ("LEDGF/p75-KD") or eGFP alone. As a control for specificity of this approach, we also transduced normal lineage marker-depleted mouse bone marrow cells and a murine hematopoietic cell line rendered IL-3 independent by expression of the human *FLT3-ITD* gene mutation ("Ba/F3 FLT3-ITD"). 48h after transduction, eGFP-positive cells were flow-sorted and either seeded in methylcellulose to determine the effect on clonogenic growth, or plated in transplant medium ("TM") to address the impact on cellular proliferation and expression of MLL-fusion targets. In both cases, the cells were cultured in presence of puromycin to select for cells carrying the virus expressing the LEDGF/p75-fragment. To test the effect on leukemia induction *in vivo,* we transplanted MLL-AF9 expressing leukemic cells expressing eGFP-LEDGF/p75$_{325-530}$ or eGFP solely into syngeneic recipient mice (**Fig. 12B**).

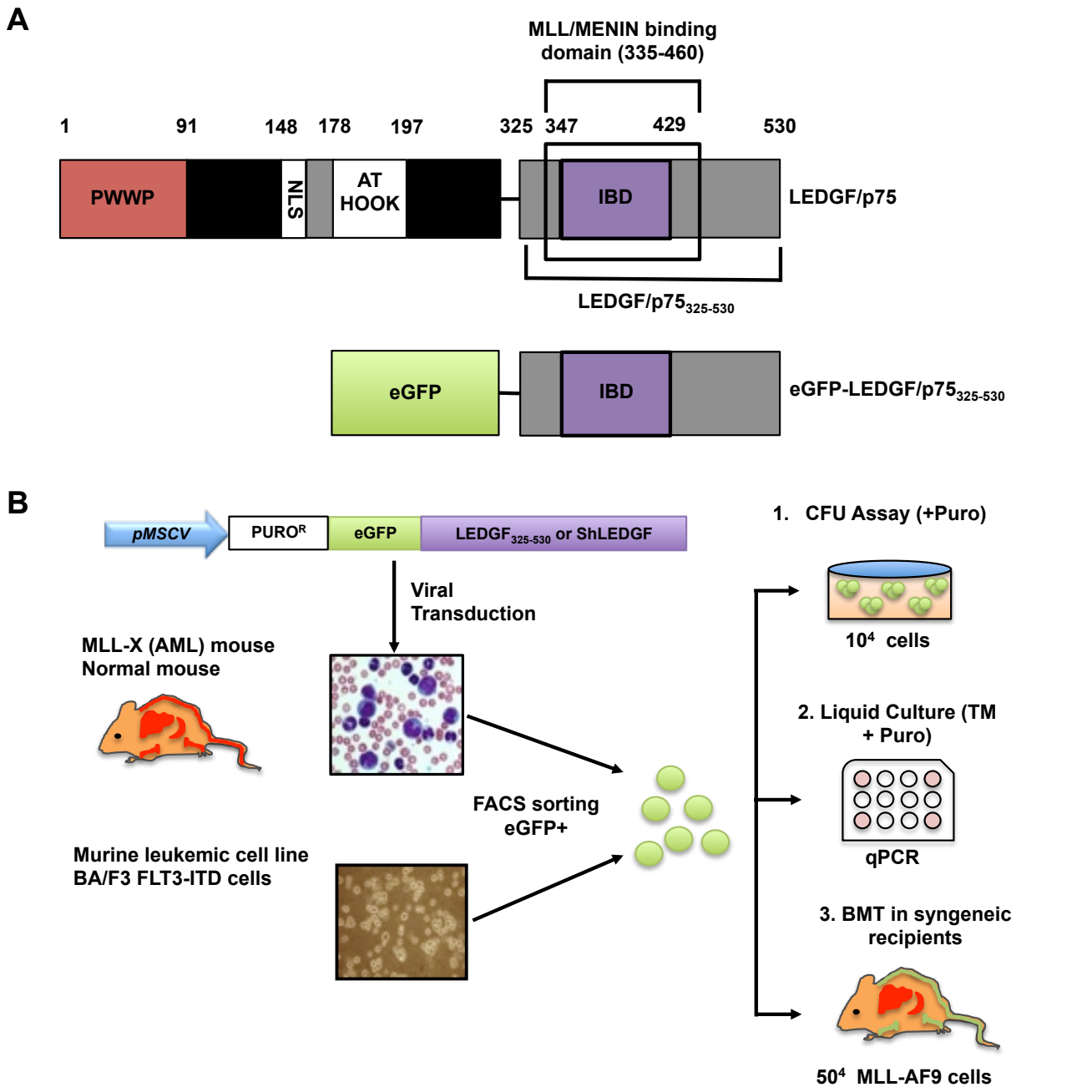

Fig. 12: Experimental strategy set up for testing the impact of LEDGF/p75 functional interference in MLL-X primary leukemic cells.

(A) Schematic overview of LEDGF/p75 and eGFP fused LEDGF/p75$_{325-530}$. The relative positions of LEDGF/p75$_{325-530}$, the IBD and the MLL/MENIN-binding domain are indicated. The numbers refer to amino acids positions of the reference sequence (GenBank accession number, NP_001121689.1). **(B)** Schematic representation of the experimental setup. Effect of the expression of eGFP-LEDGF/p75$_{325-530}$ was assed on eGFP transduced and flow-sorted primary MLL-X leukemic cells, normal mouse lineage depleted progenitor cells (WT BM) and BA/F3 FLT3/ITD cells. For each cell type, clonogenic growth capacity was checked by colony-forming unit (CFU) assay of 10^4 plated cells. *Hoxa9* MLL target gene expression was assessed by quantitative PCR (qPCR) in WT BM and MLL-X leukemic cells kept in culture 72h after sort. Effect on MLL-fusion related AML induction *in vivo* was tested by secondary transplantation of $5x10^4$ eGFP positive MLL-AF9 murine leukemic cells together with 10^6 supportive total WT BM cells, into lethally irradiated syngeneic recipient.

As shown in **Fig. 13A & B**, expression of eGFP-LEDGF/p75$_{325-530}$ significantly reduced the number of murine MLL-AF9 (77%) or MLL-ENL (56%) expressing colonies which was comparable to the effect of shRNA-mediated knockdown of LEDGF/p75 (68 and 40%, respectively). In contrast, colony formation by normal bone marrow stem and progenitor cells or Ba/F3-FLT3-ITD cells was not significantly affected (**Fig. 13A**). Quantitative RT-PCR analysis revealed that expression of LEDGF/p75$_{325-530}$ or shRNA-mediated LEDGF/p75 knockdown both led to a similar and significant reduction of *Hoxa9* mRNA levels, in MLL-AF9 (47%) and MLL-ENL (34%) fusion transformed cells (**Fig. 13C**). These results indicate that overexpression of the LEDGF/p75 IBD is able to impair the transformed state induced by the MLL-AF9 and MLL-ENL fusions in mouse bone marrow cells *in vitro*. Upon transplantation of the transduced cell in irradiated syngenic mice we observed a significantly longer latency period to disease development in the group receiving eGFP-LEDGF/p75$_{325-530}$ expressing cells (78-96 days; median 88.5 days) in comparison with the eGFP control group (48-78 days; median 55 days) (**Fig. 13D**). All animals ultimately succumbed to AML characterized by elevated blood counts, hepatosplenomegaly and multi-organ infiltration as described before (not shown).

To strengthen these observations from the mouse model, we analyzed the effects of eGFP-LEDGF/p75$_{325-530}$ expression in several human leukemia cell lines. Hereby we have selected lines with a known MLL-status, that can be efficiently virally transduced and that are able to form countable colonies in methylcellulose including THP-1 (AML, MLL-AF9+) and MOLM-13 (AML, MLL-AF9+, FLT3-ITD), and as a negative control HL-60 (AML, MLL WT) [162,165]. Those cell lines were transduced with concentrated supernatant containing spleen focus-forming virus (*SFFV*)-based lentiviral particles expressing either eGFP-LEDGF/p75$_{325-530}$, a LEDGF/p75 specific shRNA(LEDGF/p75 KD), or eGFP alone. FACS-sorted eGFP-positive cells were plated into methylcellulose without cytokines or kept in liquid medium. As shown in **Fig. 14A**, similar to murine AML cells, expression of eGFP-LEDGF/p75$_{325-530}$ or LEDGF/p75 knockdown significantly impaired the clonogenic growth of THP-1 (71% and 47% respectively) and MOLM-13 (38% and 57% respectively) without affecting colony formation of HL-60 cells. In addition functional LEDGF/p75 depletion, by either expression of LEDGF/p75$_{325-530}$ or LEDGF/p75 knockdown, resulted in a growth defect in liquid medium as highlighted by an increase of cells in G_1 phase, and a decrease of cells in the G_2 stage of the cycle as observed in THP-1 but not in HL-60 (**Fig. 14B**). Collectively, this data shows that overexpression of the LEDGF/p75 IBD is able to impair the MLL-fusion induced transformed state not only in mouse but also in human leukemic cells *in vitro*.

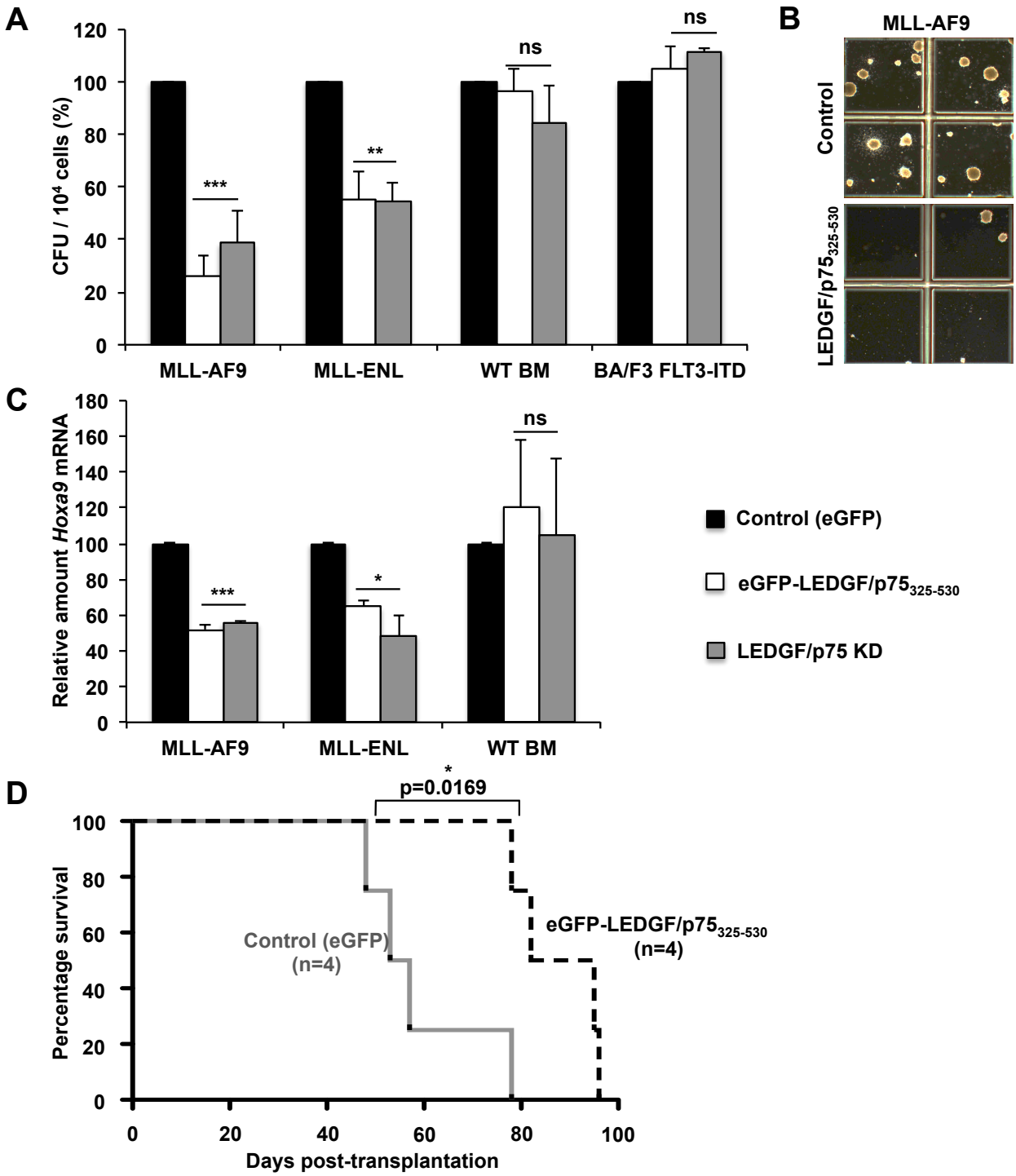

Fig. 13: Expression of eGFP-LEDGF/p75$_{325-530}$ selectively impairs leukemogenic activity of MLL fusion expressing murine AML cells *in vitro* and *in vivo*

Error bars represent the s.d. of at least two independent experiments for each cell type. *, P<0.05; **, p<0.005; ***, p<0.001, One-way ANOVA with Tukey's Multiple Comparison Test. **(A)** Colony forming units (CFU) per 10^4 eGFP flow-sorted MLL-X murine leukemic cells, normal lineage marker-depleted bone marrow cells (WT BM) or FLT3-ITD transformed Ba/F3 cells expressing eGFP-LEDGF/p75$_{325-530}$, LEDGF/p75 shRNA (LEDGF/p75 KD) or eGFP/vector control (set arbitrarily as 100%).**(B)** Representative picture of colonies for murine MLL-AF9 expressing cells transduced with eGFP/vector control or eGFP-LEDGF/p75$_{325-530}$. **(C)** Relative amount of *Hoxa9* mRNA measured in the eGFP+ cells described in (A) by quantitative RT-PCR 72 hours after flow-sorting. Expression levels were normalized to *Gapdh* and to the value of the vector control (set to 100%). **(D)** Kaplan-Meier survival analysis of syngeneic recipient mice injected with MLL-AF9+ blasts expressing eGFP-LEDGF/p75$_{325-530}$ compared to eGFP/vector control transduced cells (* p<0.05, Log-rank test).

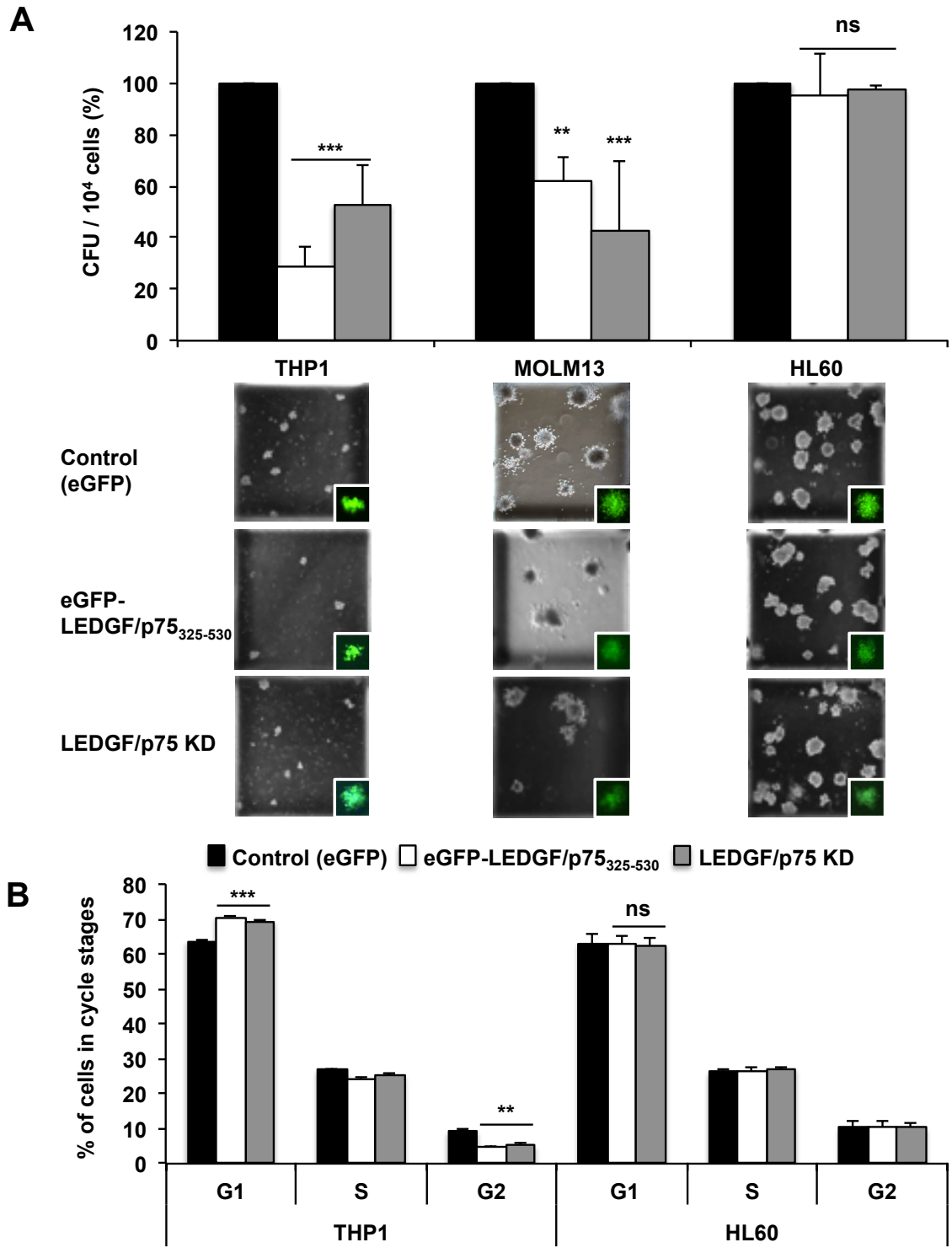

Fig. 14: Expression of eGFP-LEDGF/p75$_{325-530}$ selectively impairs colony-forming capacity and cell cycle of MLL-AF9+ human AML cell lines

P<0.05; **P<0.005; ***P<0.001, two-way ANOVA with Bonferroni post-tests compared with eGFP/vector control. **(A)** Colony-forming units (CFU) per 10^4 eGFP flow-sorted THP1, MOLM13 and HL60 cells transduced with eGFP-LEDGF/p75$_{325–530}$, LEDGF/p75 shRNA against (LEDGF/p75 KD) or a eGFP/vector control (set as 100%). Error bars represent the s.d. of two independent experiments with all cell lines. A representative picture of colonies for each condition is shown under the corresponding histogram cell line serie. **(B)** Evaluation of the proportion of eGFP+ THP1 and HL60 cells expressing eGFP-LEDGF/p75$_{325–530}$ or a shRNA against LEDGF/p75 (LEDGF/p75 KD) *vs.* eGFP/vector control in the G1, S and G2 cell cycle stage (%). Cells were harvested 96h post-transduction. The error bars represent the s.d. of three independent experiments.

3. IDENTIFICATION OF REGIONS IN LEDGF/P75-IBD THAT ARE ESSENTIAL TO IMPAIR TRANSFORMED STATE

We next aimed to identify a minimal domain of LEDGF/p75$_{325-530}$ that might be sufficient to impair the transformation potential of MLL fusions. For this purpose we applied PCR-based cloning to generate smaller fragments using LEDGF/p75$_{325-530}$ fragment as a template and fused them to eGFP. As a first functional test, we assayed for the cellular localization of these eGFP-fused fragments by transient expression in adherent HeLa cells. We generated 6 C- and 6 N-terminal eGFP-fused deletion mutants covering the full-length LEDGF/p75$_{325-530}$ fragment (**Fig. 15**).

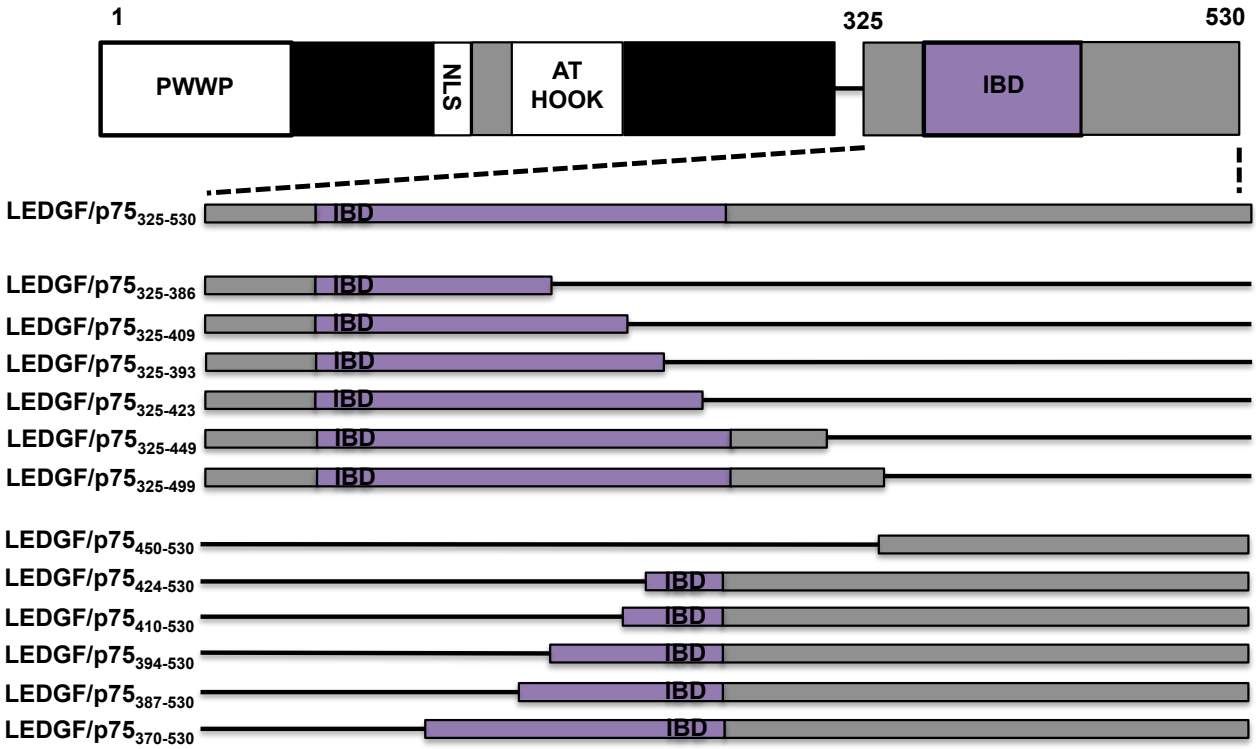

Fig. 15: PCR-based generation of LEDGF/p75$_{325-530}$ deleted mutants

Schematic representation of the C-and N-term LEDGF/p75 deleted mutants series generated by polymerisation chain reaction (PCR) using LEDGF/p75$_{325-530}$ fragment as a template.

Similar to the eGFP-LEDGF/p75$_{325-530}$, all C-terminal deletion mutants (LEDGF/p75$_{325-386}$, $_{325-393}$, $_{325-409}$, $_{325-423}$, $_{325-449}$ and $_{325-499}$) were localized mostly in the nucleus (**Fig. 16A**). In contrast, four of the N-terminal deletion mutants (LEDGF/p75$_{370-530}$, $_{387-530}$, $_{394-530}$ and $_{410-530}$) were excluded from the nucleus suggesting that the domain (LEDGF/p75 370-410aa) might play an essential role for nuclear translocation (**Fig. 16B**).

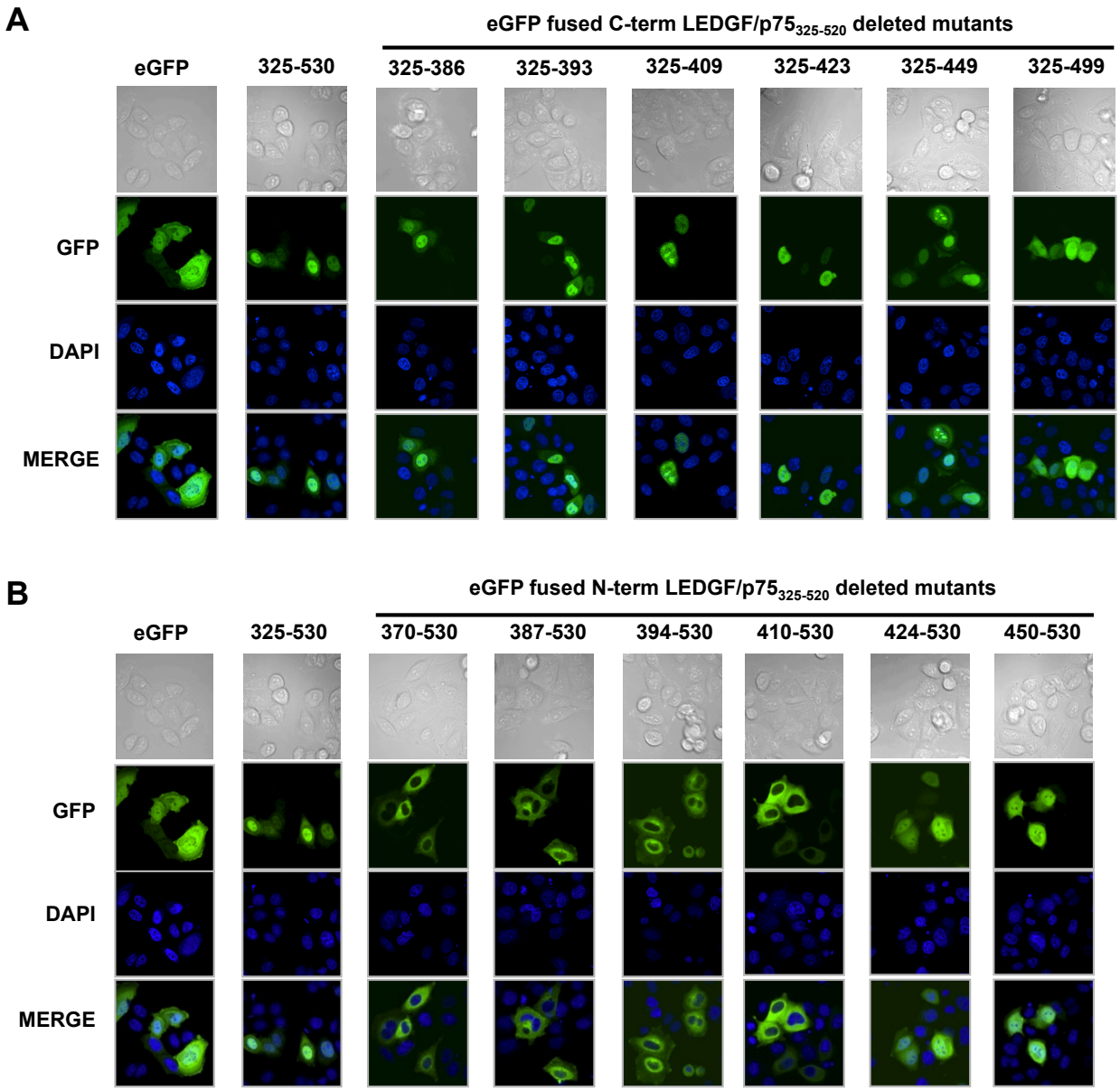

Fig. 16: Cellular localization of eGFP fused LEDGF/p75$_{325-530}$ C-and N-term deleted mutants

(A-B) Cellular localization of eGFP fused C-term (A) and N-term (B) deleted designed mutants serie in Hela cells by confocal microscopy using GFP fluorescence. Cells nuclei were stained with DAPI. The numbers correspond to the amino acids positions of LEDGF/p75 protein.

As for the entire LEDGF/p75-IBD domain, we expressed the smaller mutants in MLL-AF9 driven murine AML cells and determined the effects on colony formation in methylcellulose. We selected those deletion mutants that were able to enter the nucleus. The LEDGF/p75$_{325-499}$, $_{325-393}$, and $_{325-386}$ C-terminal deletion mutants potently reduced clonogenic growth (**Fig. 17A**). LEDGF/p75$_{424-530}$ N-terminal mutant significantly reduced colony growth, but no activity was observed upon expression of the N-terminal deletion mutants LEDGF/p75$_{410-530}$ located in the cytoplasm and LEDGF/p75$_{450-530}$ located in the nucleus **(Fig. 17A)**. We further validated the LEDGF/p75$_{424-530}$ and LEDGF/p75$_{325-386}$ as the smallest N- and C-terminal deleted mutants significantly impairing colony growth of MLL-AF9 and MLL-ENL murine AML cells (**Fig. 17B**). Reduced colony formation was also associated with a significant reduction of *Hoxa9* mRNA expression (**Fig. 17C**). These observations suggested that two regions of LEDGF/p75, 325-386aa (containing the very N-terminal part of the IBD) and 424-449aa (starting just at the C-terminal end from the IBD) might be critical to keep the MLL-fusion complex functional (**Fig. 17D**).

4. NARROWING DOWN THE CRITICAL REGIONS IN THE LEDGF/P75-IBD TO PEPTIDE-SIZE FRAGMENTS IMPAIRING MLL-FUSION MEDIATED LEUKEMOGNESIS

Starting from the two regions defined above, we generated additional deletion mutants and expressed them in MLL-AF9 espressing murine leukemic cells (**Fig. 18A**). Hereby we were able to identify two peptide-sized fragments, LEDGF/p75$_{424-435}$ and LEDGF/p75$_{375-386}$, that both impaired colony formation (**Fig. 18B**). Cellular expression of LEDGF/p75$_{424-530}$ and LEDGF/p75$_{325-386}$ and of the two peptide-sized fragments, LEDGF/p75$_{424-435}$ and LEDGF/p75$_{375-386}$ was demonstrated in BA/F3 cells by western blot at the protein level (**Fig. 18C**).

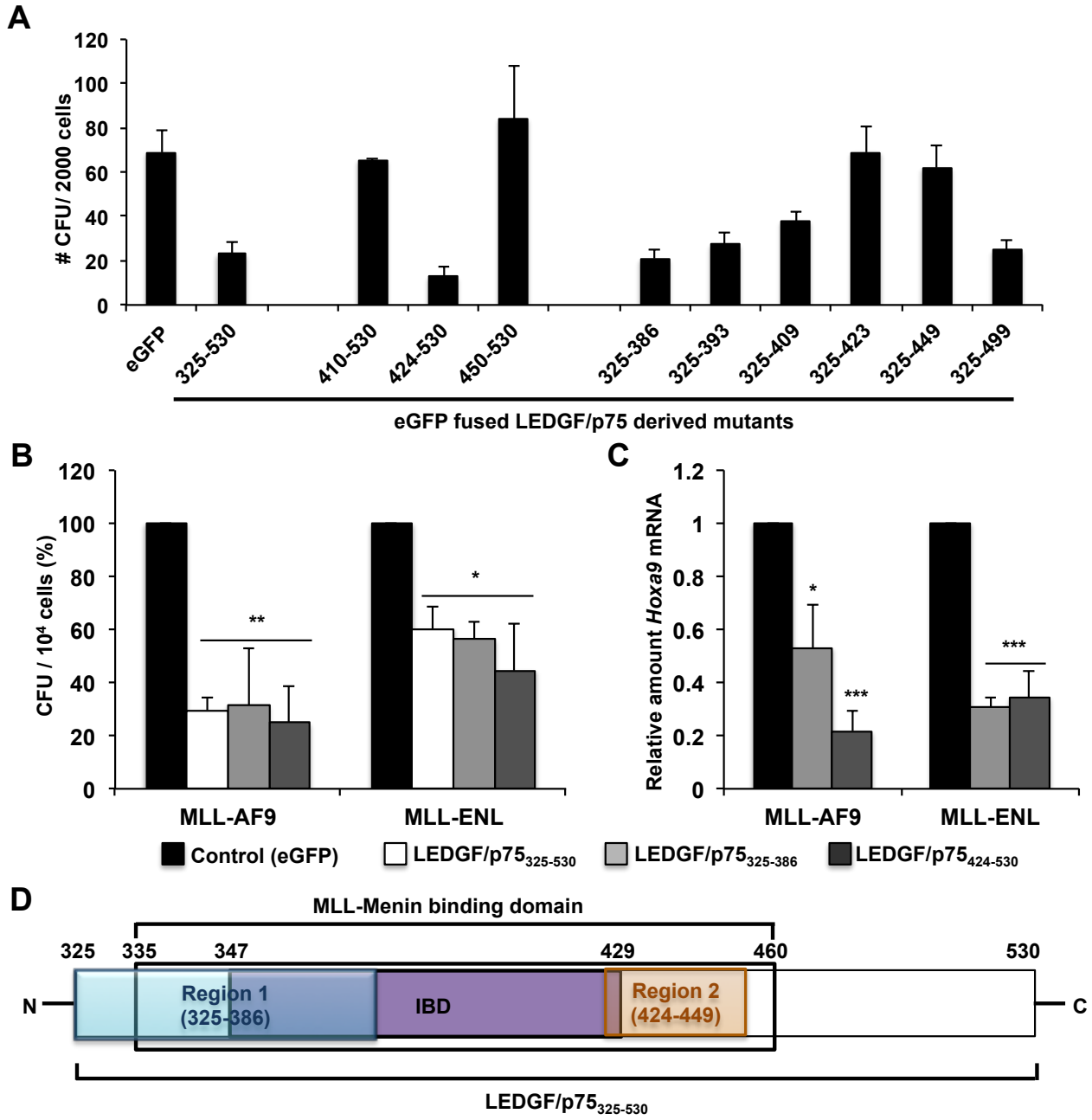

Fig. 17: Identification of critical LEDGF/p75 regions for MLL-fusion leukemogenic activity

Full-length eGFP-LEDGF/p75$_{325-530}$ and eGFP/vector are used respectively as a positive and negative control. Error bars represent the s.d. of two independent experiments for each cell type. *, P<0.05; **, p<0.005; ***, p<0.001, One-way ANOVA with Tukey's Multiple Comparison Test. **(A)** Colony forming units (CFU) per 2000-plated eGFP flow-sorted MLL-AF9 murine cells expressing the eGFP-LEDGF/p75 N-term and C-term deletion mutants described in Fig. 15. The error bars represent the s.d. of a duplicate experiment. The numbers on the X-axis correspond to LEDGF/p75 amino acids positions. **(B)** CFU per 10^4 eGFP-flow sorted MLL-X murine leukemic cells expressing the two smallest eGFP fused N-terminal and C-terminal active deletion mutants identified in (A) LEDGF/p75$_{325-386}$ and $_{424-530}$ respectively (eGFP/vector is arbitrarily set at 100%). **(C)** Relative amount of *Hoxa9* mRNA measured in cells described in (B) by quantitative RT-PCR 72 hours after flow-sorting. The expression levels were normalized to *Gapdh* and to the value of the eGFP/vector control (set at 1). **(D)** Schematic representation of LEDGF/p75$_{325-530}$ indicating the position of the IBD, the MLL-MENIN binding site and the 2 identified critical regions for MLL-fusion leukemogenic activity *in vitro*.

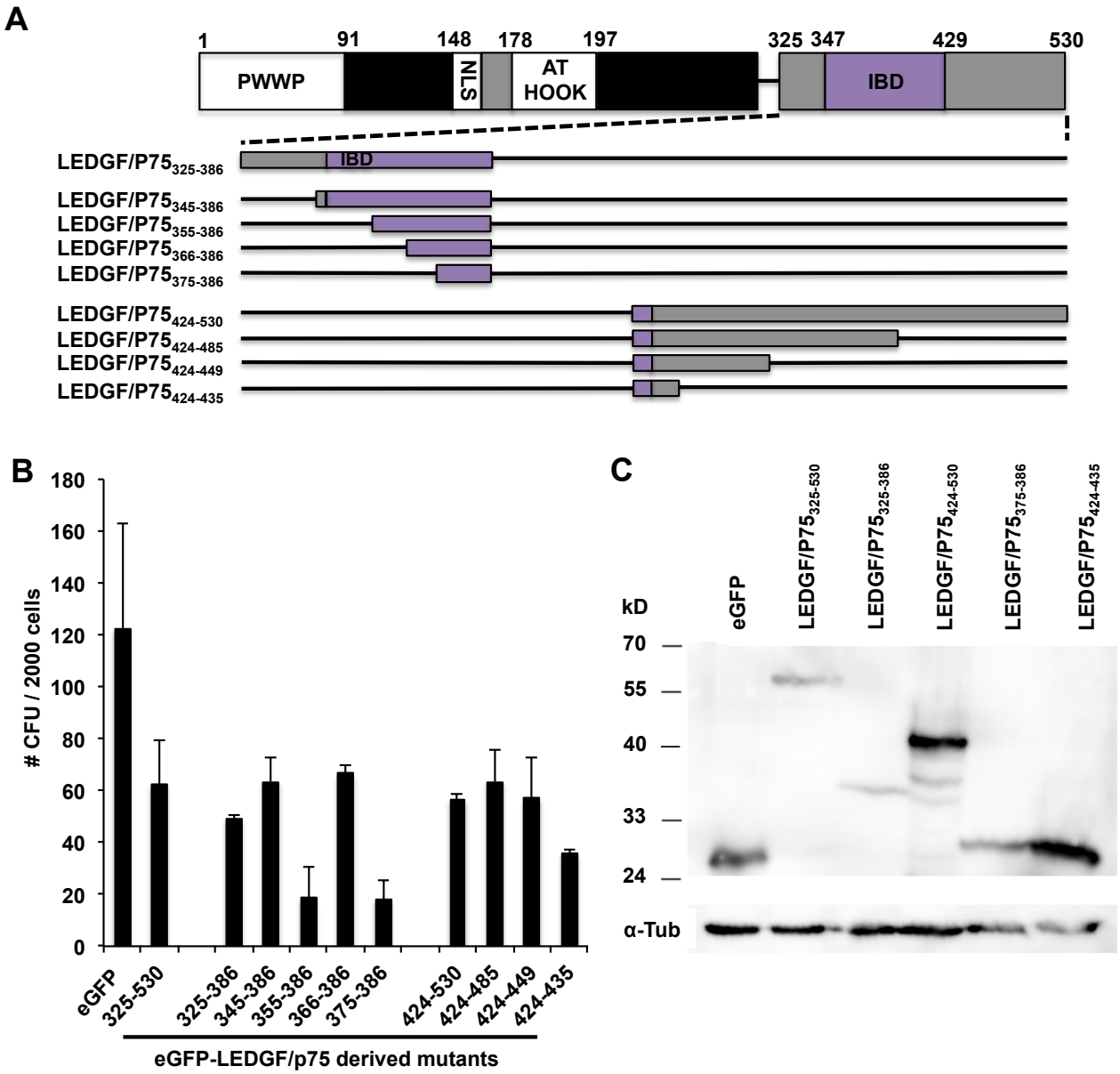

Fig. 18: Narrowing down the minimal LEDGF/p75 domains necessary for MLL/MENIN interaction to active peptide-size fragments

Full-length eGFP-LEDGF/p75$_{325-530}$ is use as a positive control and eGFP/vector is use as a negative. **(A)** Schematic representation of the eGFP fused LEDGF/p75 mutants series derived from LEDGF/p75$_{325-386}$ and LEDGF/p75$_{424-530}$ fragments. **(B)** Colony forming units (CFU) per 2000 plated eGFP flow-sorted MLL-AF9+ murine leukemic blasts expressing the eGFP fused LEDGF/p75 deletion mutants described in (A). The error bars represent the s.d. of a duplicate experiment. The numbers on the X-axis correspond to the LEDGF/p75 amino acids positions. **(C)** Western Blot analysis of BA/F3 cells transduced with *pMSCV* derived vectors expressing eGFP, or the indicated eGFP fused LEDGF/p75 fragments. Proteins were detected using an eGFP antibody. Equal loading was controlled with a α-tubuline antibody (α-Tub).

Expression of the small peptide-sized fragments LEDGF/p75$_{375-386}$ and LEDGF/p75$_{424-435}$ reduced the clonogenic activity of MLL-AF9 and MLL-ENL murine AML cells by at least 40% (**Fig. 19A**). Both fragments also significantly reduced expression of *Hoxa9* in MLL-AF9+ and MLL-ENL+ leukemic cells (**Fig. 19B)**. In addition, expression of LEDGF/p75$_{424-435}$ and LEDGF/p75$_{375-386}$ also impaired the growth of MLL-AF9+ cells in liquid culture by 3 to 4 fold (**Fig. 19C**). Similarly to LEDGF/p75$_{325-530}$, their expression was associated with altered cellular and nuclear morphology in murine MLL-AF9 cells mostly characterized by increased cell size with larger cytoplasm and nuclei altered chromatin structure (**Fig. 20A)**. However expression of any LEDGF/p75-fragments in murine MLL fusion-driven leukemic cells never resulted in significant apoptosis (**Fig. 20B**).

The effect of the peptide-sized LEDGF/p75 fragments was also addressed in human leukemic cells. Protein expression of the smaller LEDGF/p75 fragments was demonstrated in MLL-AF9+ human THP-1 AML cells (**Fig. 21A**). As observed for eGFP-LEDGF/p75$_{325-530}$, expression of eGFP-LEDGF/p75$_{424-435}$ and eGFP-LEDGF/p75$_{375-386}$ reduced colony formation of at least 40% in MLL-AF9+ THP-1 and MOLM-13 cells opposed to the HL-60 (MLL, WT) cells (**Fig. 21B**). In addition, expression of eGFP-LEDGF/p75$_{424-435}$ and eGFP-LEDGF$_{375-386}$ resulted in diminished cell cycle progression of THP-1 cells line but not of HL-60 cells (**Fig. 21C**). We also observed a growth defect of MV-4;11 cells (MLL-AF4+) by 2–3 fold in liquid culture, while other acute leukemia cell lines without any known MLL-gene alterations including JURKAT (T-ALL) or KASUMI-1 (AML1-ETO+ AML) were slightly or not affected (**Fig. 22**). However, we did not observed any significant effects of the peptide sized LEDGF/p75 fragments on apoptosis or on terminal differentiation of MLL-fusion+ human cells lines. Indeed MOLM-13 and MV-4;11 human AML cells transduced with LEDGF/p75 fragments were not expressing at their surface increased level neither of phosphatidyl-serine know as an apoptotic marker detected by annexin-V binding, nor of CD11b macrophage differentiation marker when compared to control eGFP transduced cells (**Fig. 23**). Taken together, our data show that expression of two LEDGF/p75-derived eGFP-fusion peptides (LEDGF/p75$_{424-435}$ and LEDGF/p75$_{375-386}$) impairs the leukemogenic capacity of murine and human MLL-fusion expressing leukemic cells *in vitro.*

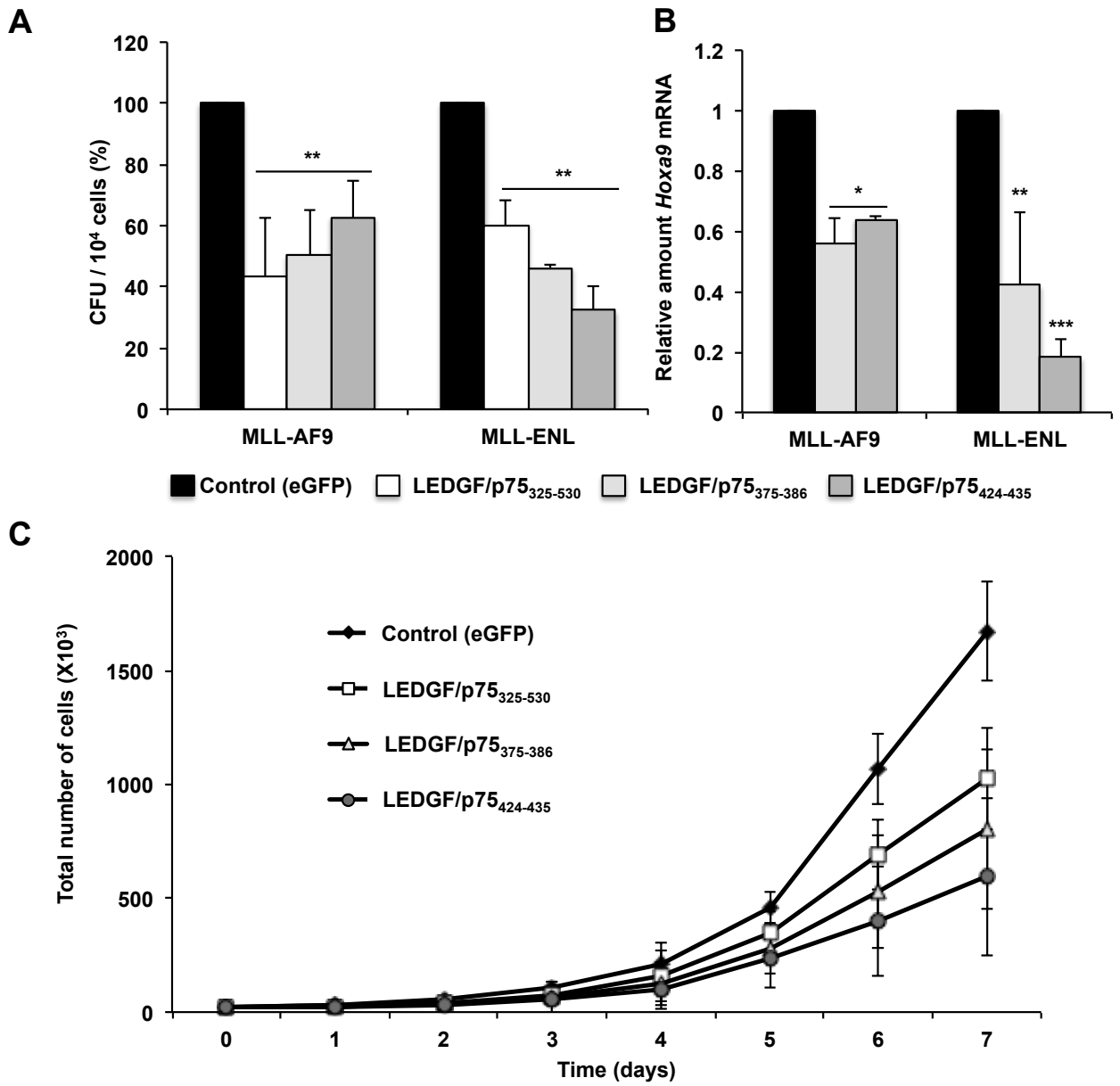

Fig. 19: LEDGF/p75$_{375-386}$ and $_{424-435}$ peptide-size fragments mimic the LEDGF/p75$_{325-530}$ mediated defects in murine MLL-fusion+ AML cells

Full-length eGFP-LEDGF/p75$_{325-530}$ is use as a positive control and eGFP/vector is use as a negative. *, p<0.05; **, p<0.005; ***, p<0.001, One-way ANOVA with Tukey's Multiple Comparison Test. Error bars represent the s.d. of at least two independent experiments for each cell type. **(A)** CFU per 10^4 eGFP flow-sorted MLL-AF9 or MLL-ENL murine leukemic cells expressing the two smallest peptide-size active deletion mutants LEDGF/p75$_{375-386}$ and $_{424-435}$ identified in Fig. 7 (eGFP/vector is arbitrarily set at 100%). **(B)** Relative amount of *Hoxa9* mRNA measured in the eGFP positive cells expressing eGFP-LEDGF/p75$_{375-386}$ or $_{424-435}$ by quantitative RT-PCR 72 hours after flow-sorting. The expression levels were normalized to *Gapdh* and to the value of the eGFP/vector control (arbitrarily set at 100%). **(C)** Growth curve of MLL-AF9 murine leukemic cells expressing the indicated eGFP-LEDGF/p75 fragments or eGFP as a control.

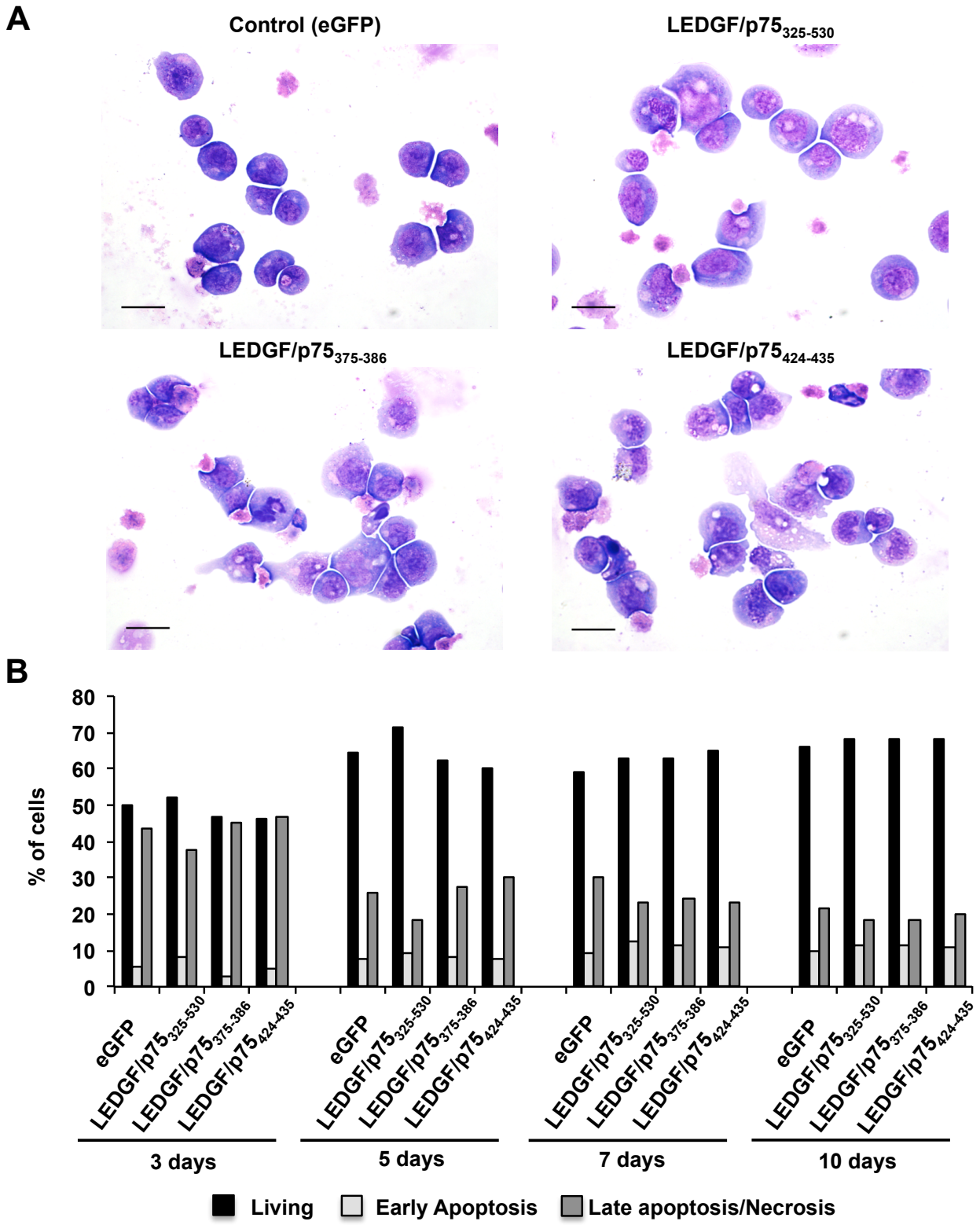

Fig. 20: LEDGF/p75 derived fragments expression is able to induce cellular and nuclear morphological changes but not apoptosis in murine MLL-AF9 AML cells

MLL-AF9 cells transduced with the indicated eGFP fused LEDGF/p75 fragments or eGFP/vector control were grown in the presence of puromycin (2mg/ml) and analyzed at the indicated time points.
(A) Representative Wright-Giemsa-stained cytospin preparation 10 days after transduction with the indicated. For each picture, the black scale bar in the bottom left is representative of 20 µm. **(B)** Evaluation of the proportion of living (Annexin-V^-/$DAPI^-$), early apoptotic (Annexin-V^+/$DAPI^-$) and late apoptotic /necrotic (Annexin-V^+/$DAPI^+$) cell fractions by FACS analysis.

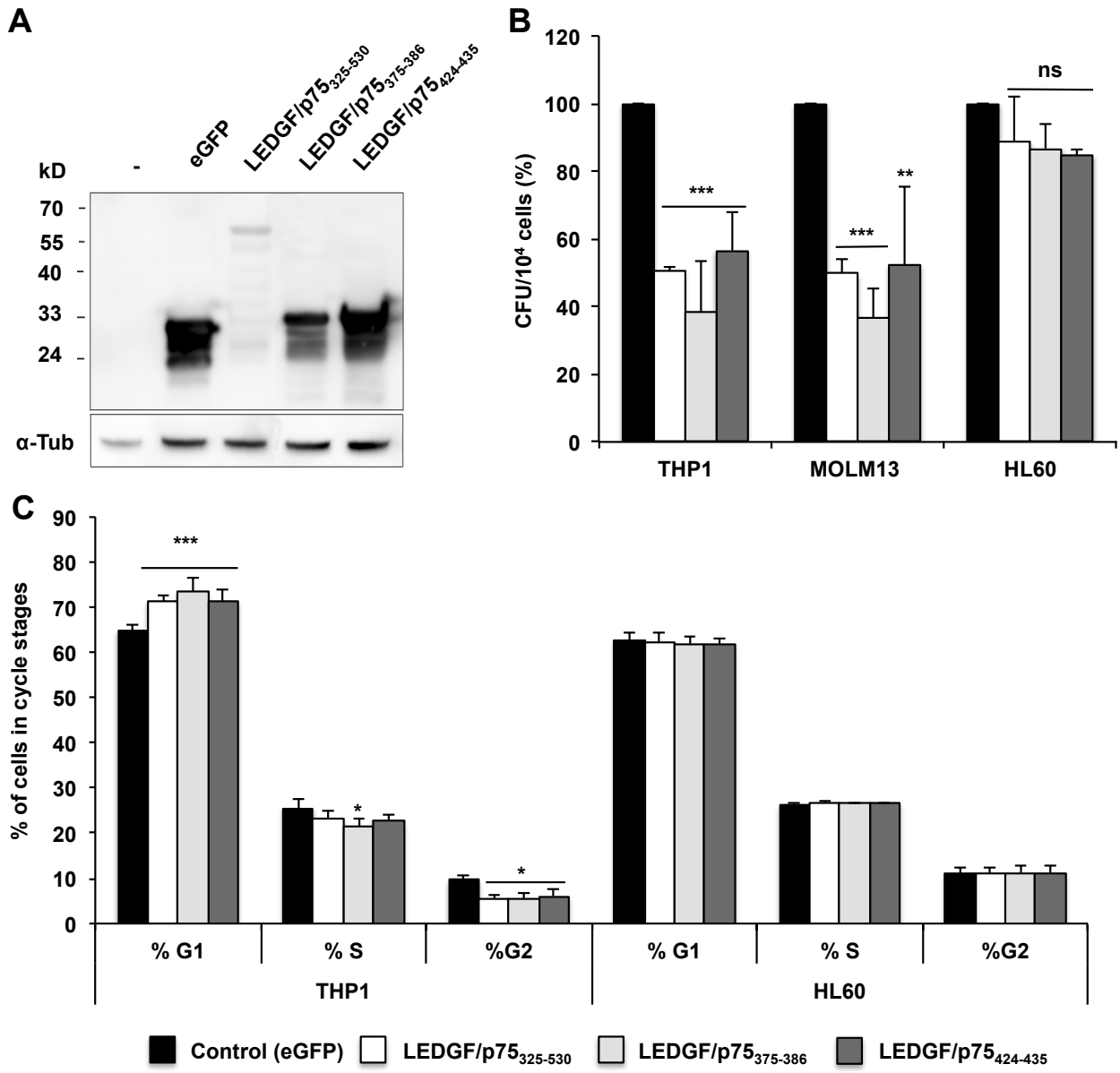

Fig. 21: Expression of LEDGF/p75-IBD peptide size derived fragments impairs growth of MLL-fusion+ human AML cell lines *in vitro*

Full-length eGFP-LEDGF/p75$_{325-530}$ and eGFP/vector are used respectively as a positive and negative control. *P<0.05; **P<0.005; ***P<0.001, two-way analysis of variance (ANOVA) with Bonferroni post-tests compared with control (eGFP). **(A)** Western Blot analysis of THP1 cells transduced with lentiviral vectors expressing the indicated eGFP fused LEDGF/p75 fragments. Fragments were detected with an eGFP antibody. Equal loading was controlled with an anti-tubuline antibody (α-Tub). **(B)** Colony-forming units (CFU) per 10^4 eGFP flow-sorted plated THP1, MOLM13 or HL60 cells expressing the indicated eGFP fused LEDGF/p75 fragments or eGFP/vector control (set to 100%) in the presence of puromycin. Error bars represent the s.d. of two independent experiments with all cell lines **(C)** Evaluation of the proportion of eGFP positive THP1 and HL60 cells expressing the indicated eGFP fused LEDGF/p75 fragments or eGFP/vector control in the G_1, S and G_2 cell cycle stage. Cells were harvested 96 hours post transduction. Error bars represent the s.d. of three independent experiments.

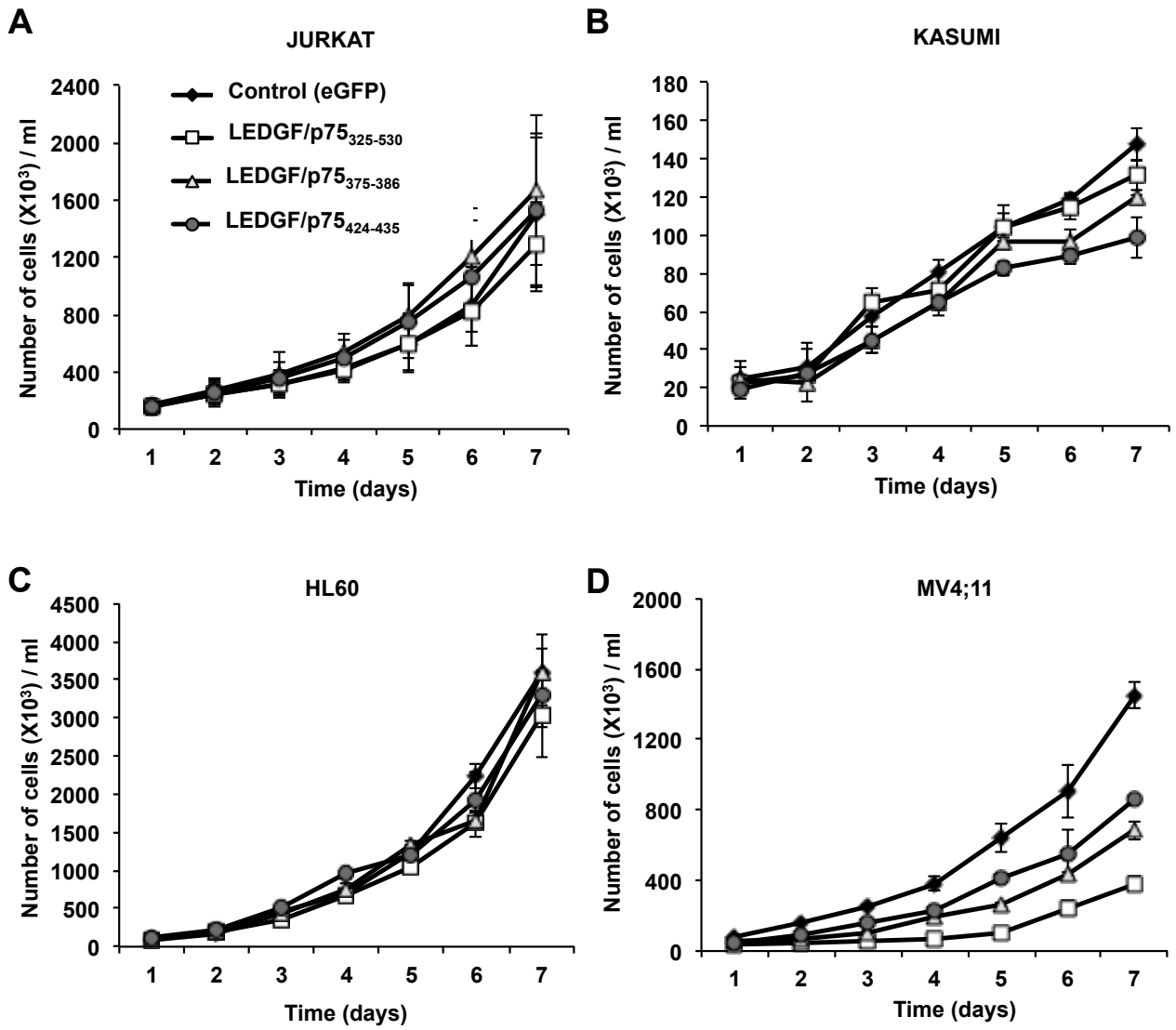

Fig. 22: LEDGF/p75-IBD peptide size derived fragments mediated growth defect is selective for MLL-fusion+ human AML cell lines *in vitro*

Cell proliferation in liquid culture of (**A**) Jurkat (T-ALL), (**B**) Kasumi (AML1-ETO+; AML), (**C**) HL-60 (c-myc amplification; AML) and (**D**) MV4;11 (MLL-AF4+; AML) cells, transduced with lentiviral vectors expressing the indicated eGFP fused LEDGF/p75 fragments. Cells were selected with puromycin for 72 hours and plated at the same concentration in liquid medium at day 1 in presence of antibiotic. Error bars represent the standard deviation of two independent experiments. Full-length eGFP-LEDGF/p75$_{325-530}$ and eGFP/vector are used respectively as a positive and negative control.

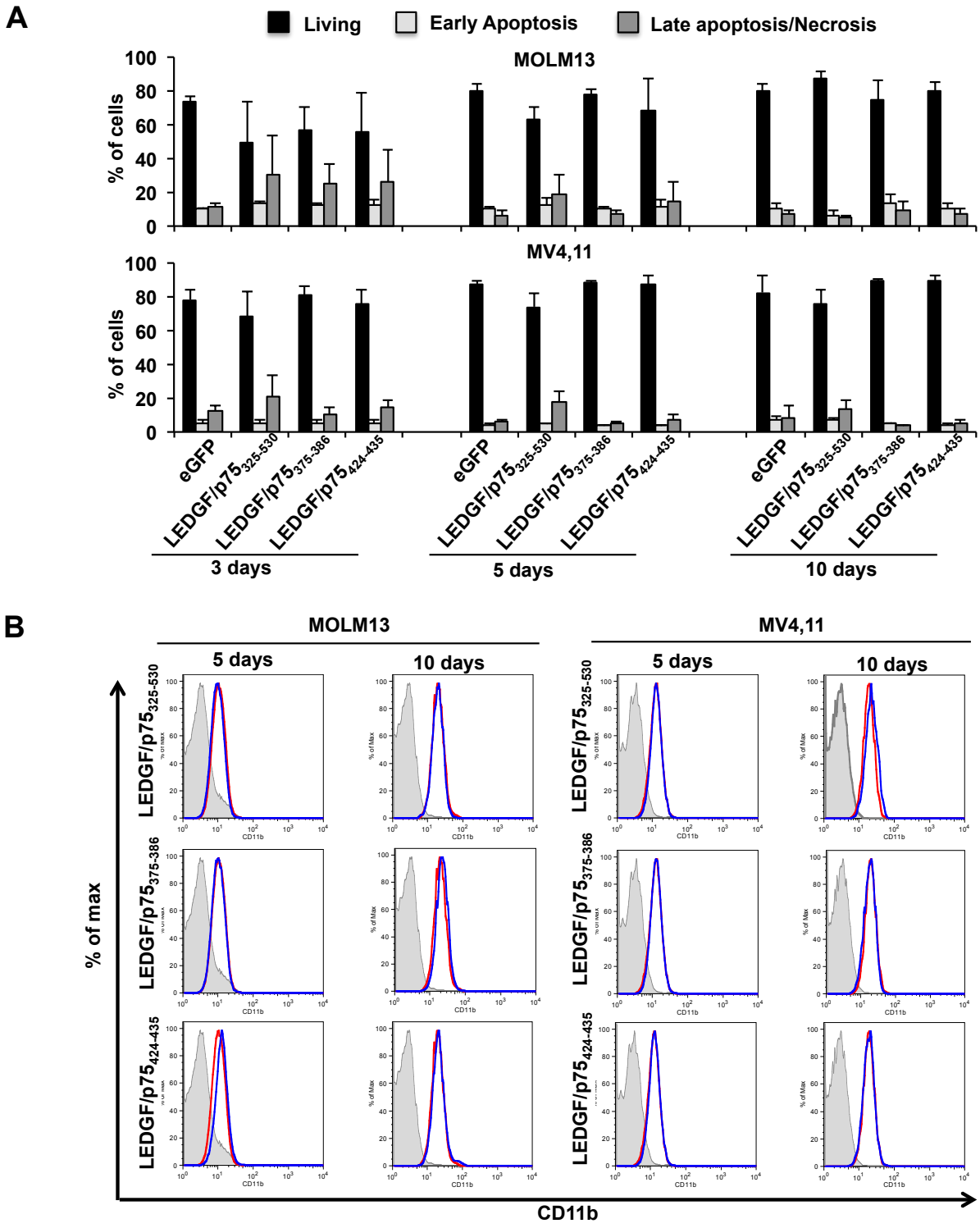

Fig. 23: Expression of eGFP-LEDGF/p75-IBD derived fragments does not increase apoptosis neither CD11B expression in MLL-fusion driven leukemic cell line

Full-length eGFP-LEDGF/p75$_{325-530}$ and eGFP/vector are used respectively as a positive and negative control. Cells were flow-sorted and puromycin selected after transduction, and harvested at the indicated days **(A)** Evaluation of the proportion of MOLM13 and MV4;11 cells expressing the indicated eGFP-LEDGF/p75-fragments or eGFP as a control in living (Annexin-V-/DAPI-), early apoptotic (Annexin-V+/DAPI-) and late apoptotic /necrotic (Annexin-V+/DAPI+) stages. Error bars represent the standard deviation of two independent experiments. **(B)** Representative FACS analysis histogram of CD11b expression in MOLM13 and MV4;11 cells transduced with the indicated eGFP-LEDGF/p75-fragments or eGFP/vector control. For each graph, the grey histogram represent unstained cells, the red line histogram eGFP/vector control and the blue line histogram the according eGFP fused LEDGF/p75-fragment.

We also investigated the effect of eGFP-LEDGF/p75$_{375-386}$ and eGFP-LEDGF/p75$_{424-435}$ on *in vivo* leukemogenesis using the bone marrow transplantation model (**Fig. 24A**). Hereby we observed that expression of eGFP-LEDGF/p75$_{375-386}$ resulted in a moderately but significant prolonged latency of MLL-AF9-induced AML compared to control (median eGFP-LEDGF/p75$_{375-386}$ = 45.5 days; median eGFP = 38 days) (**Fig. 24B)**. Surprisingly, despite the potent anti-leukemogenic effect *in vitro*, expression of eGFP-LEDGF/p75$_{424-435}$ did not delay disease induction *in vivo* (median eGFP-LEDGF$_{424-435}$ = 34 days; median eGFP = 38 days) (**Fig. 24B)**. All animals ultimately succumbed to AML characterized by elevated white blood counts, hepatosplenomegaly and multi-organ infiltration (data not shown).

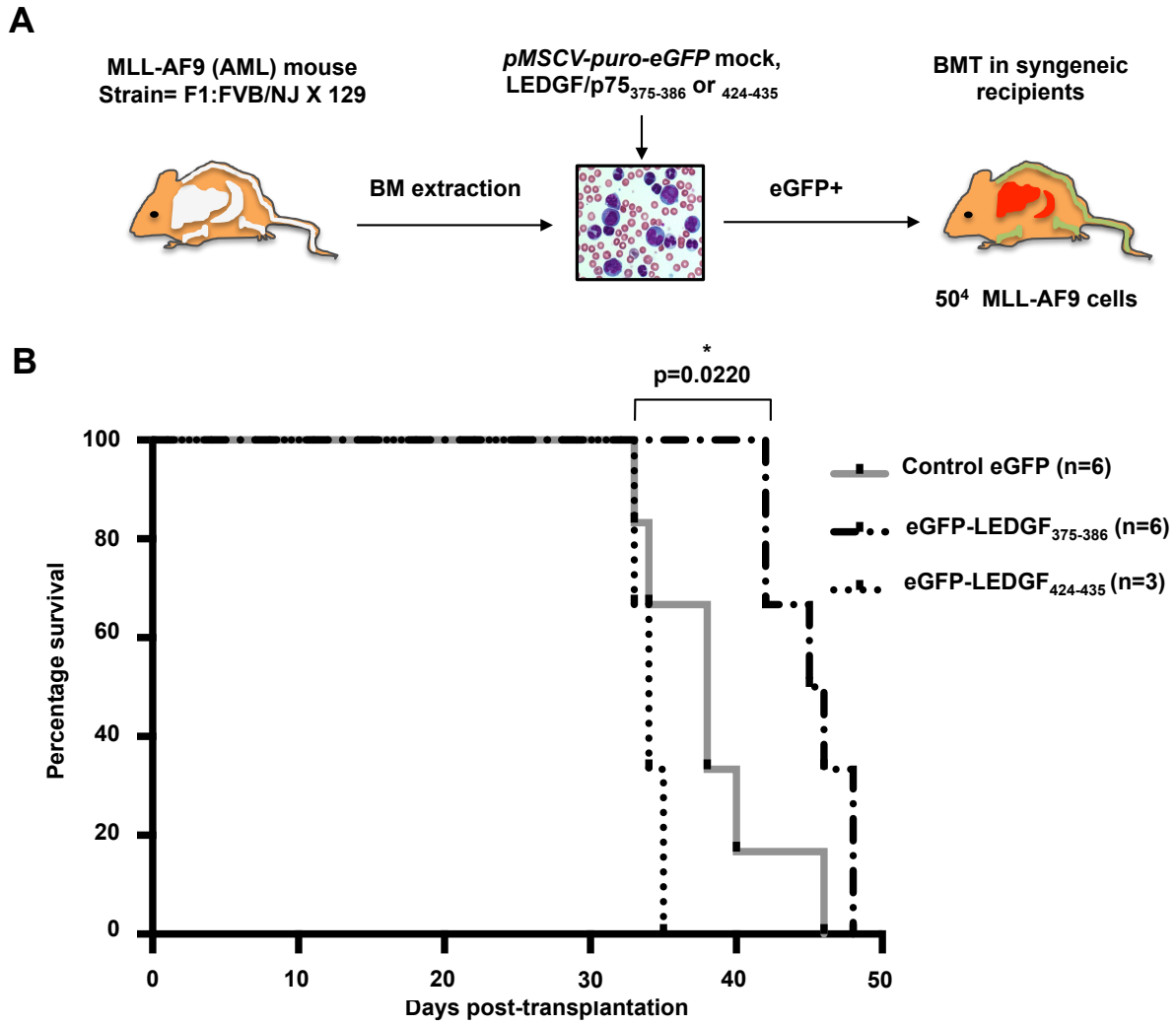

Fig. 24: Expression of LEDGF/p75$_{375-386}$ impairs MLL-AF9 acute leukemia *in vivo*

(A) Schematic representation of the experimental setup. Effect on MLL-fusion related AML *in vivo* induction was tested by secondary transplantation of 50^4 eGFP+ MLL-AF9 murine leukemic cells expressing either LEDGF/p75$_{375-386}$ or $_{424-435}$ eGFP fused fragments *vs* eGFP/vector together with 10^6 supportive total WT BM cells, into lethally irradiated syngeneic recipient. **(B)** Kaplan-Meier survival analysis of syngeneic recipient mice injected with MLL-AF9+ blasts expressing eGFP fused LEDGF/p75$_{375-386}$ or LEDGF/p75$_{424-435}$ compared to eGFP/vector control transduced cells (* p<0.05, Log-rank test).

5. EXPRESSION OF LEDGF/P75-IBD DERIVED FRAGMENTS DISRUPTS MLL-MENIN-LEDGF/P75 INTERACTION

To explain the observed anti-leukemic effects of LEDGF/p75-IBD fragments, we hypothesized that eGFP-LEDGF/p75$_{325-530}$ could compete with endogenous LEDGF/p75 and thereby interfere with the formation of a ternary MLL/MENIN-LEDGF/p75-complex. To validate this idea, our collaborator Jan de Rijck, (University of Leuven, Belgium) performed the following co-IP experiment: he transiently co-expressed an N-terminal flag-tagged MLL fragment (aa 1-330, Flag-MLL$_{1-330}$) comprising both the LEDGF/p75 and MENIN binding motifs with C-terminally HA-tagged MENIN in 293T cells (MENIN-HA). To test whether the LEDGF/p75$_{325-530}$ fragment can outcompete LEDGF/p75 from the MLL/MENIN complex he then added different amounts of purified recombinant Maltose Binding Protein-tagged LEDGF/p75$_{325-530}$ (MBP-LEDGF/p75$_{325-530}$) or MBP alone in excess of endogenous LEDGF/p75 prior to co-IP of MLL/MENIN. While LEDGF/p75 was detected in the absence of MBP-LEDGF/p75$_{325-530}$ or in the presence of MBP, LEDGF/p75 was either partially or completely displaced from the complex in the presence of 10 or 50nM of MBP-LEDGF/p75$_{325-530}$, respectively (**Fig. 25A**). The capacity of LEDGF/p75$_{325-530}$ to displace LEDGF/p75 was confirmed *in vitro* using an AlphaScreen and purified recombinant proteins. Next to full length His-Thioredoxin-MENIN (H-Trx-MENIN) and Flag-LEDGF/p75, the N-terminal part of MLL containing the first 160 amino acids fused to Glutathione S-Transferase (MLL$_{1-160}$-GST) was purified. To analyze whether LEDGF/p75$_{325-530}$ competes with LEDGF/p75 for MLL binding, increasing amounts of MBP-LEDGF/p75$_{325-530}$ were added to the MLL$_{1-160}$-GST/Flag-LEDGF/p75 AlphaScreen assay. As shown in **Fig. 25B**, MBP-LEDGF/p75$_{325-530}$, but not MBP alone, displaced LEDGF/p75 from MLL with an apparent IC$_{50}$ of 120.5nM (95% CI [93.9; 154.7]) confirming the data obtained by immunoprecipitation. Together these results strongly suggest that LEDGF/p75$_{325-530}$ can outcompete LEDGF/p75 from the MLL/MENIN complex.

Using an identical AlphaScreen setting our collaborators similarly analyzed whether LEDGF/p75$_{424-435}$ and LEDGF/p75$_{375-386}$ peptide-size fragments could disrupt the LEDGF/p75-MLL complex. For this purpose, recombinant His-Thioredoxin (HT) fused LEDGF/p75$_{424-435}$ or LEDGF/p75$_{375-386}$ fragments were purified. Both proteins inhibited the direct interaction between MLL and LEDGF/p75 in the AlphaScreen assay with IC$_{50}$ values of 52.2 and 108.6nM respectively, while an unrelated control peptide was unable to disrupt the complex **(Fig. 25C)**. In the same assay he also tested the activity of untagged peptides. While the untagged LEDGF/p75$_{424-435}$ peptide did not block the MLL-LEDGF/p75

interaction, LEDGF/p75$_{375-386}$ clearly abrogated the interaction with an IC_{50} of 5.8µM (**Fig. 25D**). These last observations suggest the possibility that steric hindrance of the tag helps LEDGF/p75$_{424-435}$ to disrupt the complex. Taken together, these biochemical experiments support the idea that LEDGF/p75-IBD-derived fragments interfere with MLL-LEDGF/p75 through « outcompetition » of the endogenous protein from the MLL-MENIN complex.

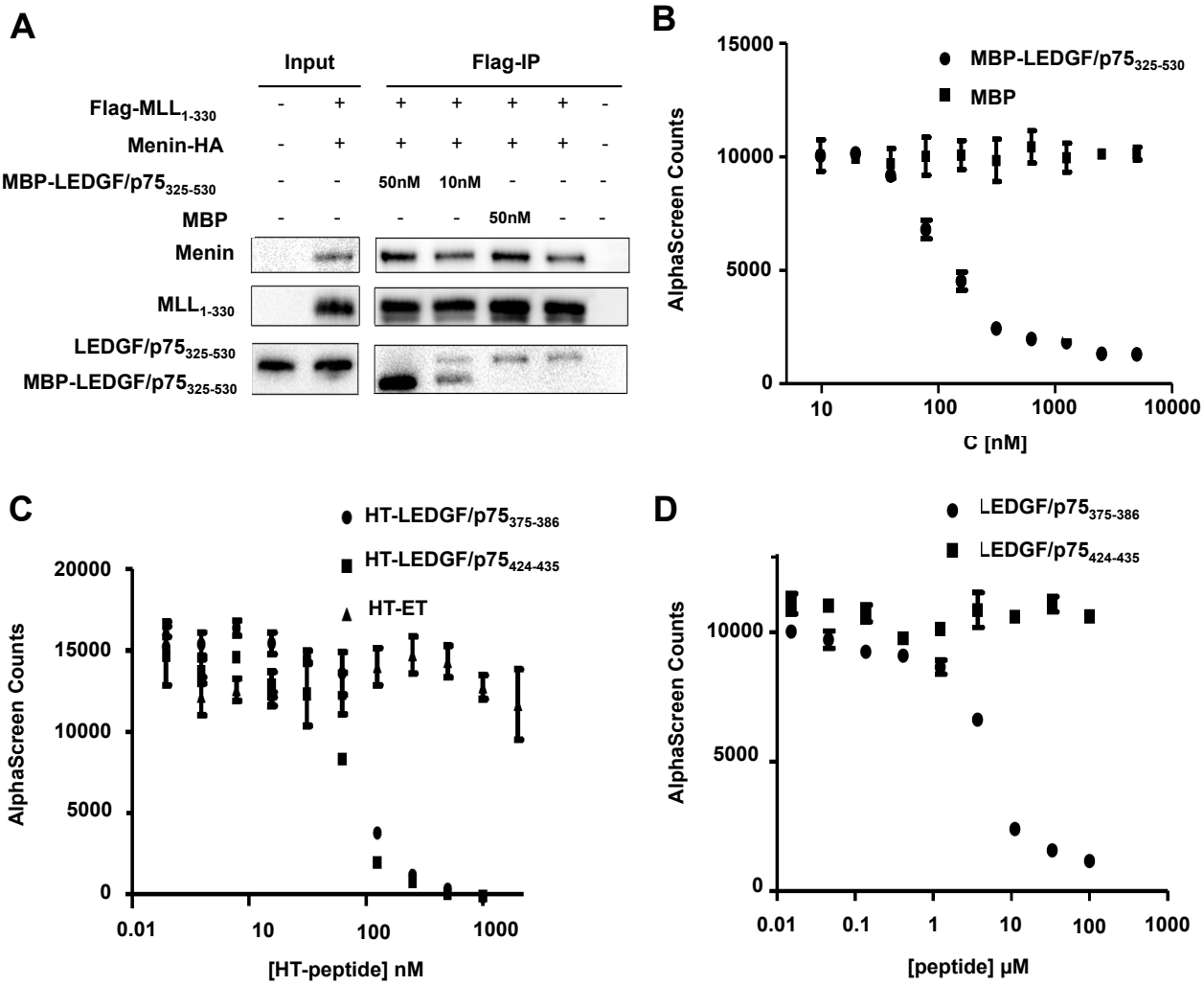

Fig. 25: LEDGF/p75-IBD derived fragments interfere with endogenous LEDGF/p75 and disrupt MLL-LEDGF/p75 interaction *in vitro*

(A) 293T cells were transfected with Flag-MLL(1-330) and MENIN-HA expression constructs as indicated. Recombinant MBP-LEDGF/p75$_{325-530}$ or MBP protein was added to the IP to compete with LEDGF/p75 from the complex. Flag-MLL(1-330) was immunoprecipitated using anti-Flag beads. Precipitated proteins were analyzed through western blot. A HA and a Flag antibody was used for detection of HA-MENIN and Flag-MLL(1-330) respectively. Endogenous LEDGF/p75 and MBP-LEDGF/p75$_{325-530}$ were detected with a LEDGF/p75 antibody. **(B)** The interaction between recombinant purified Flag-LEDGF/p75 and MLL$_{1-160}$-GST was monitored in an Alphascreen assay upon addition of an increasing amount of MBP (Maltose binding protein) or MBP-LEDGF/p75$_{325-530}$. Error bars indicate s.d. of triplicate measurements. One representative experiment out of three is shown. **(C-D)** The interaction between purified recombinant Flag-LEDGF/p75 and MLL$_{1-160}$-GST was monitored in an AlphaScreen assay. Increasing amounts of His-Thioredoxin (HT) fused LEDGF/p75-derived peptides (C) or untagged peptides (D) were added. As a control an unrelated HT fused peptide (ET) was used. Error bars indicate s.d. of triplicate measurements. In each case, a representative titration out of three independent experiments is shown.

6. EXPLORING THE EFFECTS OF LEDGF/P75 INTERACTING CYCLIC PEPTIDES IN MLL-AF9 EXPRESSING LEUKEMIC CELLS

Based on our observation that LEDGF/p75-derived fragments competing with the endogenous LEDGF/p75 are able to impair MLL-fusion mediated leukemogenesis, we wondered whether expression of cyclic peptides known to directly interact with the LEDGF/p75 IBD (here called “CP63”, “CP64” and “CP65”) previously shown to be able to impair the cellular replication of the HIV-1 virus, would phenocpy the anti-leukemic effects of IBD-derived fragments [152]. To abundantly express these peptides in leukemic cells, we cloned viral vectors encoding for N-terminal eGFP-fused CP63, CP64 or CP65. As shown in **Fig. 26A**, expression of these peptides significantly reduced colony formation of MLL-AF9+ murine AML cells in methylcellulose over 2 rounds of consecutive plating. Importantly, a CP65 mutant with the potentially inactivating single W to A exchange in the middle of the peptide completely lost its activity in this assay. Likewise expression of the normal CP65 (wt) but not a W to A mutant impaired colony growth of murine MLL-AF9+ AML cells but did not affected colony formation of normal lineage marker-negative mouse bone marrow stem and progenitor cells (**Fig. 26B & D**). Expression of CP65 was associated with a significant reduction (80%) of *Hoxa9* mRNA levels. The mutant peptide also reduced *Hoxa9* mRNA expression to 60% probably due to residual binding of the peptide (**Fig. 26C**). Although at the time we performed these experiments, we did not knew the binding sites of these peptides on the LEDGF/p75 IBD, our observations suggested that these cyclic peptides might also disrupt interaction of LEDGF/p75 with MLL and/or MENIN impairing the transcriptional and leukemogenic activity of the MLL fusion complex.

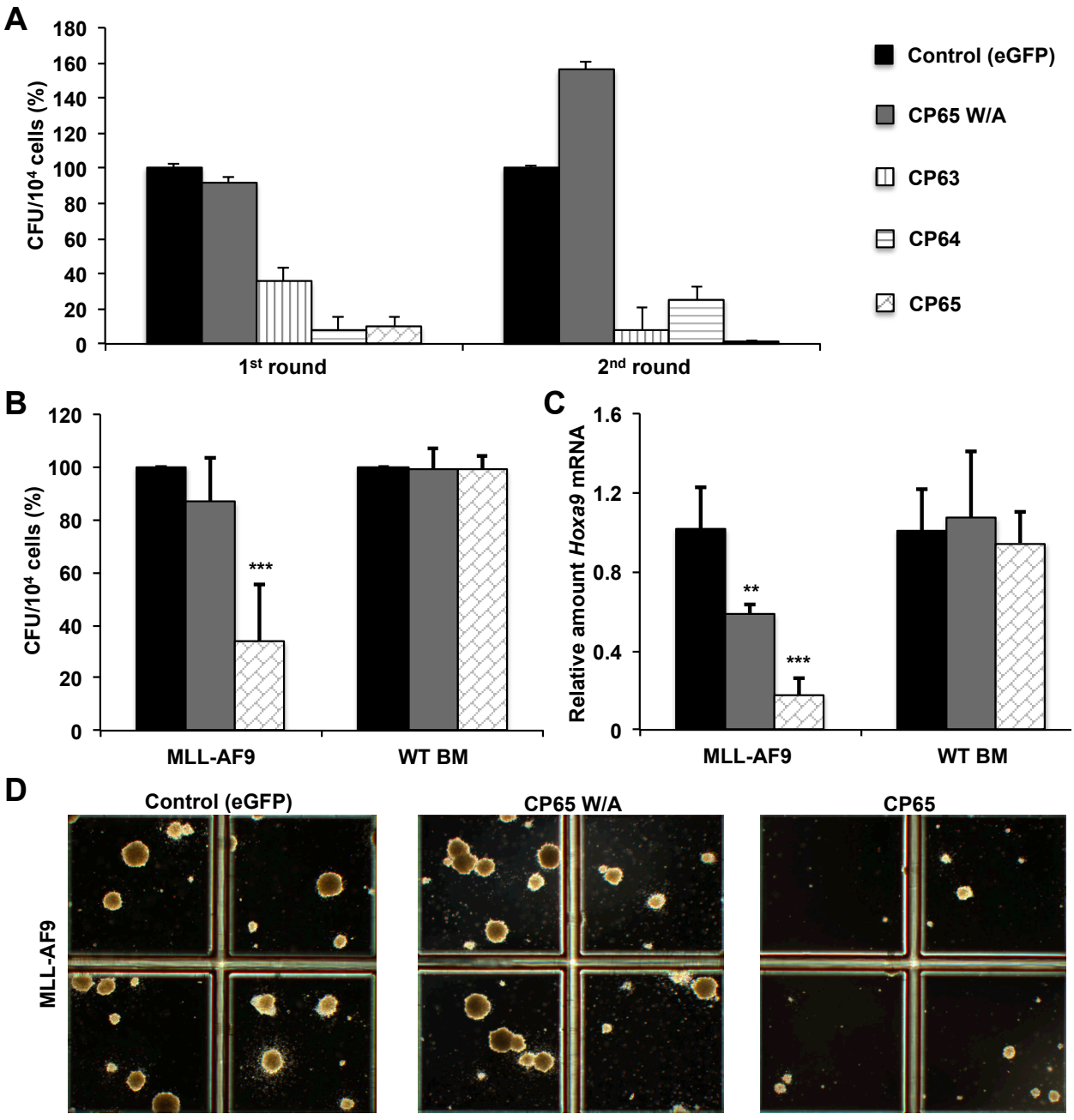

Fig. 26: Expression of LEDGF/p75-IBD interacting cyclic peptides phenocopy the *in vitro* anti-leukemic effects of small LEDGF/p75-IBD fragments

*P<0.05; **P<0.005; ***P<0.001, two-way analysis of variance (ANOVA) with Bonferroni post-tests compared with eGFP/vector control. **(A)** Relative Colony-forming units (CFU) per 10^4 eGFP-flow-sorted plated cells for MLL-AF9 murine leukemic blasts expressing N-term eGFP fused CP63, CP64, CP65 vs W to A inactive mutant form of CP65 or eGFP/vector control (arbitrarily set at 100%) after 2 round of consecutive plating. The error bars represent the s.d. of a duplicate experiment. **(B)** Relative Colony-forming units (CFU) per 10^4 eGFP flow-sorted plated cells for MLL-AF9 murine AML blasts and murine WT Lineage negative bone marrow (WT BM) expressing CP65 vs its W to A inactive mutant form or eGFP/vector control (arbitrarily set at 100%). The error bars represent the s.d. of at two independent experiments. **(C)** Relative amount of *Hoxa9* mRNA analyzed by quantitative RT-PCR after the first plating in cells described in (B). Expression levels are normalized to *Gapdh* and expressed relative to eGFP/vector control value (arbitrarily set at 1). Error bars represent the s.d. of two independent experiments. **(D)** Representative picture of colonies for MLL-AF9 murine AML cells described in (B).

7. CHARACTERIZATION OF CRITICAL INTERFACES OF THE LEDGF/P75-MLL-MENIN COMPLEX THAT ARE ESSENTIAL FOR MLL-FUSION ONCOGENIC ACITIVITY

From the structural point of view, our results suggest that two protein-protein interfaces might be critical for stabilization of the interaction between LEDGF/p75 and MLL: one inside in the IBD ("interface-1") impaired by the competing fragment LEDGF/p75$_{375-386}$, and one at the very end of the IBD ("interface-2") weakened by the LEDGF/p75$_{424-435}$ competing fragment. While we were performing our experiments with the LEDGF/p75-derived fragments, a partial crystal structure of the MLL-LEDGF/p75-MENIN complex was published [166]**.** Their work strongly suggested the existence of an interface composed of the α2 helix of MLL and the α4 helix of MENIN sequestrating the α5 (also αE) of LEDGF/p75 IBD. Interestingly the α5 helix of the IBD contains the aa 413-428 so this structural model somehow validated the proposed "interface-2" based on our experiments. Interestingly our observations suggest the existence of an MLL-LEDGF/p75 interface impaired by IBD interacting cyclic peptides identified as disruptors of HIV integrase-LEDGF/p75 interface. Based on these results our collaborators in Belgium aimed to characterize this potential interface in more details. They conducted intensive structural biochemical analysis in order to define the unstructured part of MLL involved in this interaction. This work led to the identification of an interface consisting in the MLL α1-α2 helix anchored, via F148 and F151, between the two inter-helical loops of LEDGF/p75 IBD containing respectively aa 363, 368, 403, 406, 407 408 and aa 359, 360, 363, 399, 402, 403 [167]. Interestingly they also found that part of the HIV integrase-LEDGF/p75 interface is sharing a strong similarity with the MLL-LEDGF/p75 interface-1. Indeed both proteins MLL and HIV integrase share interaction with K360, L363, I365, L368, K402, I403, F406 and V408 of the LEDGF/p75 IBD [150,167]. These observations strongly suggest that the anti-leukemic activity of CP65 we observed is linked to disruption of the MLL-LEDGF/p75 complex by targeting the interface-1 we already defined by overexpression of competing LEDGF/p75$_{375-386}$ IBD-derived fragments.

In conclusion integrating all these findings together, our work identified two interfaces that seem to stabilize the MLL-LEDGF/p75 interaction, and that are critical for the transforming activity of most prevalent MLL-fusion genes (**Fig. 27**). Our work provided the fundament to use modern structurally guided screens for small molecules that might be able to phenocopy the effects of the LEDGF/p75-derived fragments and/or the LEDGF/p75 interacting peptides.

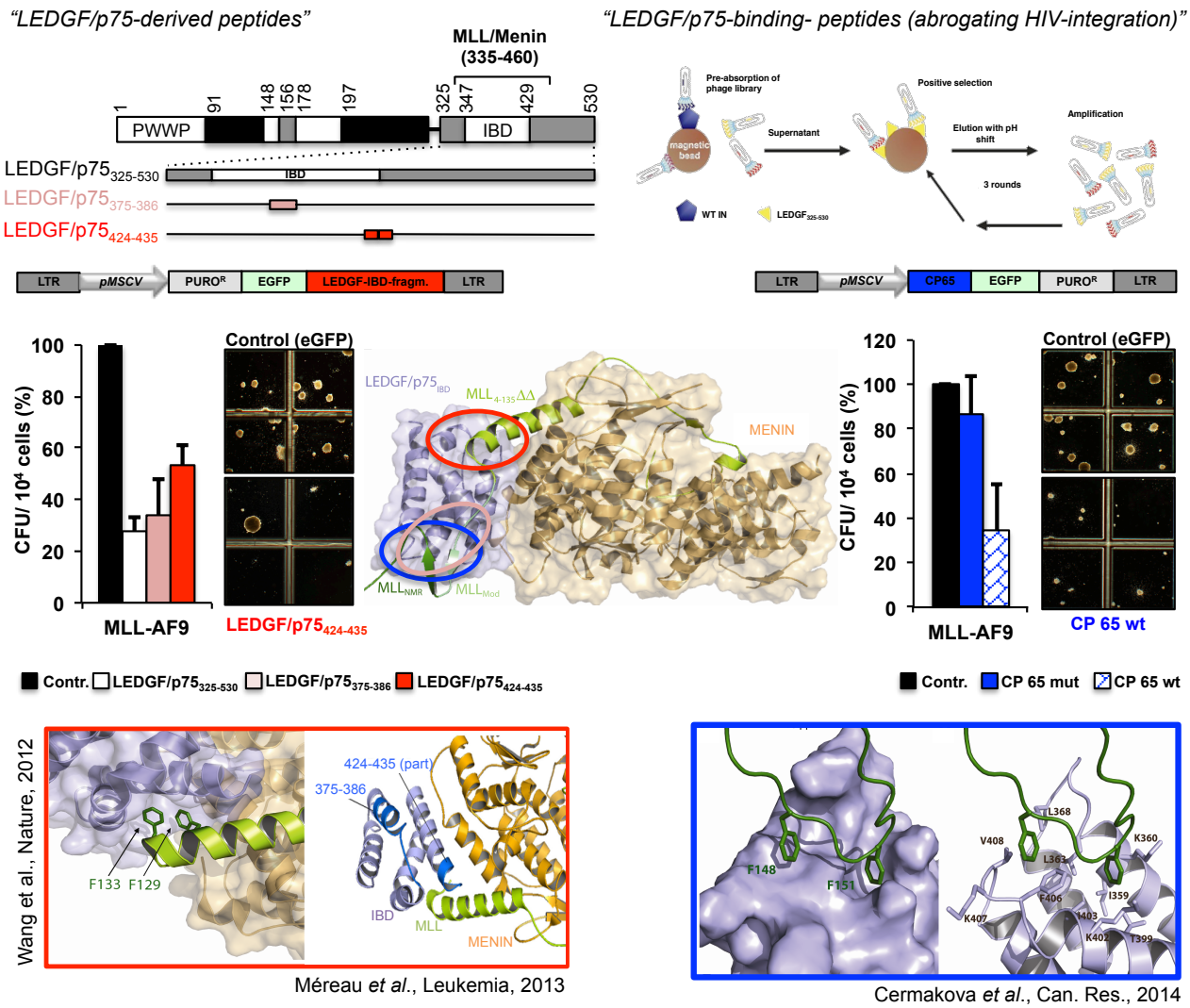

Fig. 27: Dissection of MLL-LEDGF/p75 interfaces essential for MLL-fusion oncogenicity

Dissection of critical LEDFD/p75 IBD interaction domains essential for MLL-fusion leukemogenic activity through design of LEDGF/p75-IBD derived competing peptide size fragments allowed identification of two MLL-LEDGF/p75 interfaces (left side of the figure). One involving LEDGF/p75-IBD, MENIN and MLL has been resolved in the published structure by Wang *et al*. (left side of the figure) and can be disrupted by the fragment LEDGF/p75$_{424-435}$ (strong red circle on the structural scheme) leading to impaired clonogenic activity in MLL-AF9 expressing cells. The other one absent of the published structure has been characterized further and involve LEDGF/p75-IBD and MLL only (right side of the figure). The fragment LEDGF/p75$_{375-386}$ (light red circle on the structural scheme) and the LEDGF/p75 interacting cyclic peptide CP65 (blue circle on structural scheme) can disrupt this interaction and impair clonogenic activity of MLL-AF9 fusion.

8. EFFICIENT INHIBITON OF MLL-AF9 GENE DRIVEN LEUKEMIA MURINE LEUKEMIA BY COMBINATION OF SMALL MOLECULES INHIBITORS TARGETING DOT1L AND MLL-LEDGF/P75-MENIN COMPLEX FORMATION

Previous studies have shown that treatment of MLL-fusion gene transformed cells with small molecule DOT1L inhibitors leads to differentiation rather than cell death. However, this effect seemed rather slow and dependent of relatively high doses of the compound [133]. Collaborators from the Novartis Institute for Biomedical Research (NIBR, Basel, R. Tiedt, V. Craig) therefore aimed to search for targets that are able to sensitize the cells to a DOT1L inhibitor (EPZ004777) by performing shRNA based knockdown screen focusing on epigenetic regulators in MLL-fusion positive human leukemic cell lines. Interestingly, MLL and LEDGF/p75 poped up as top hits suggesting that MLL complex can be targeted at different nodes to enhance the effects of DOT1L inhibition. Although MENIN was not covered in the used shRNA library, the MLL-MENIN interaction can be selectively interrupted by small molecule inhibitors such as MI-2-2 [168,169]. In absence of any available pharmacological agents targeting LEDGF/p75, they therefore aimed to test combinations of the EPZ004777 DOT1L inhibitor and the MI-2-2 MENIN inhibitor instead. Having different MLL-leukemia models at hand, we decided to test the combination treatment in our retrovirally induced MLL-AF9 AML mouse model.

As a first step we assayed the compounds *ex vivo* in methylcellulose colony formation assays at two different concentrations, 3μM and 6μM in MLL-AF9+ leukemic cells and WT lineage depleted bone marrow progenitor cells (**Fig. 28**). Treatment of MLL-AF9 expressing cells with 3μM of each compound alone led to an increase in differentiation but only mildly reduced colony numbers. In contrast the combination treatment significantly reduced their ability to form colonies in methylcellulose and significantly induced cellular and nuclear morphological changes associated with monocytic and granulocytic differentiation of the leukemic cells (**Fig. 28A & B**). Likewise, a higher dose of 6μM EPZ004777 further reduced the colony-forming potential of MLL-AF9 expressing murine cells, compared to the corresponding individual compound treatments (**Fig. 28A**). Importantly, wild-type bone marrow progenitor cells did not show a substantial inhibition of colony formation or any significant morphological alterations at 3μM (**Fig. 28C-D**). However, the 6μM combination treatment resulted in a reduction of number of colonies in

methylcellulose (**Fig. 28C**). Considering these results, we decided to select the concentration of 3µM for further testing to minimize significant cytotoxic effect to WT cells.

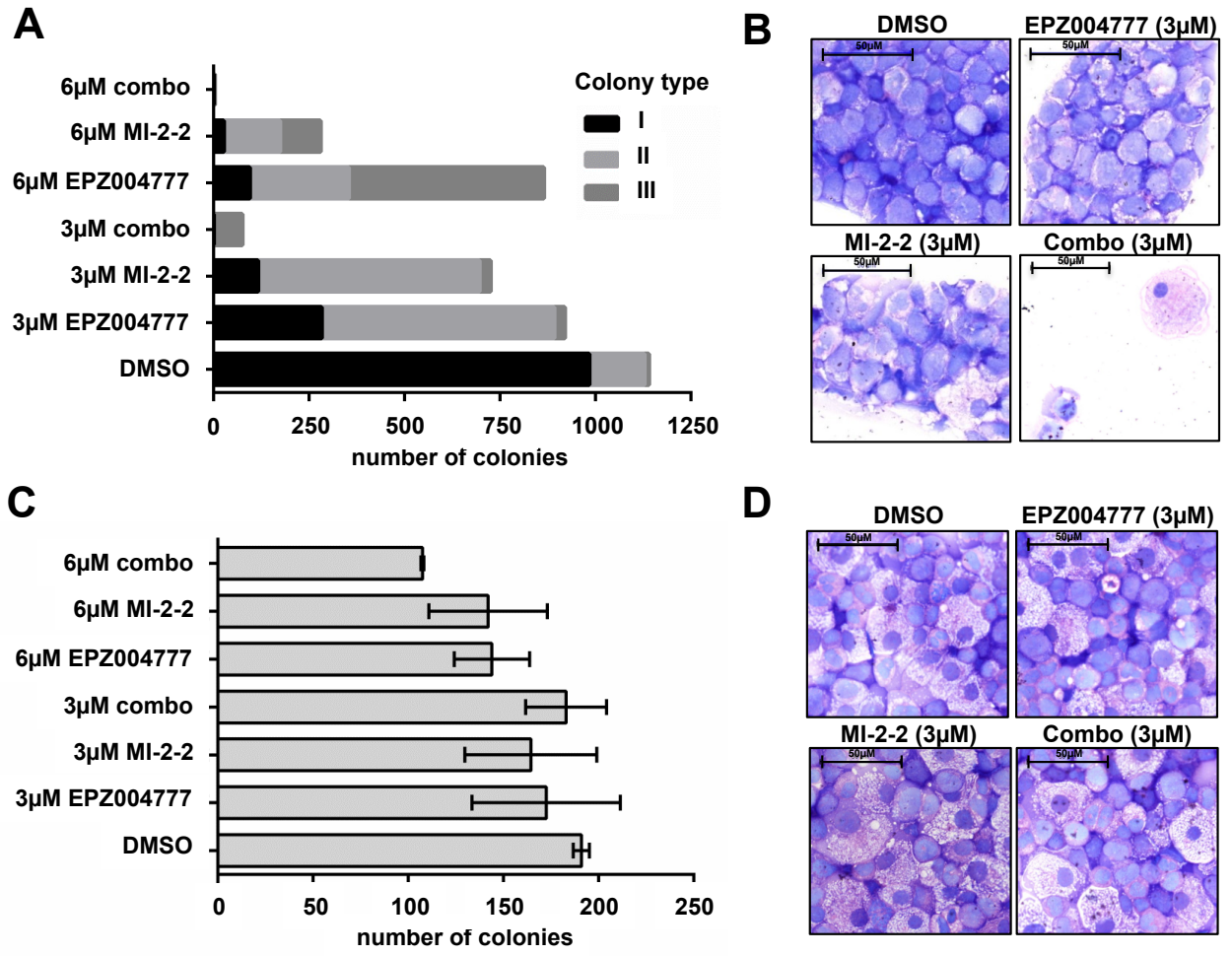

Fig. 28: DOT1L/MENIN inhibitors combination treatment selectively impairs clonogenic growth of murine MLL-AF9 expressing cells

(A) Number and classification of colonies per 10^4 plated cells recorded after treatment of primary murine MLL-AF9 cells for 5 days in methylcellulose with the indicated compound concentrations. Data represent mean values for duplicates. **(B)** Representative May-Grünwald Giemsa stained cytospin preparations of the cells depicted in (A), Scale bar 50 µM. **(C)** Colony formation per 10^4 plated wild type lineage depleted bone marrow cells after 5 days of the indicated compound treatment. Data represent mean values for duplicates ± s.d. **(D)** Representative May-Grünwald Giemsa stained cytospin preparations of the cells depicted in (C), Scale bar 50µM.

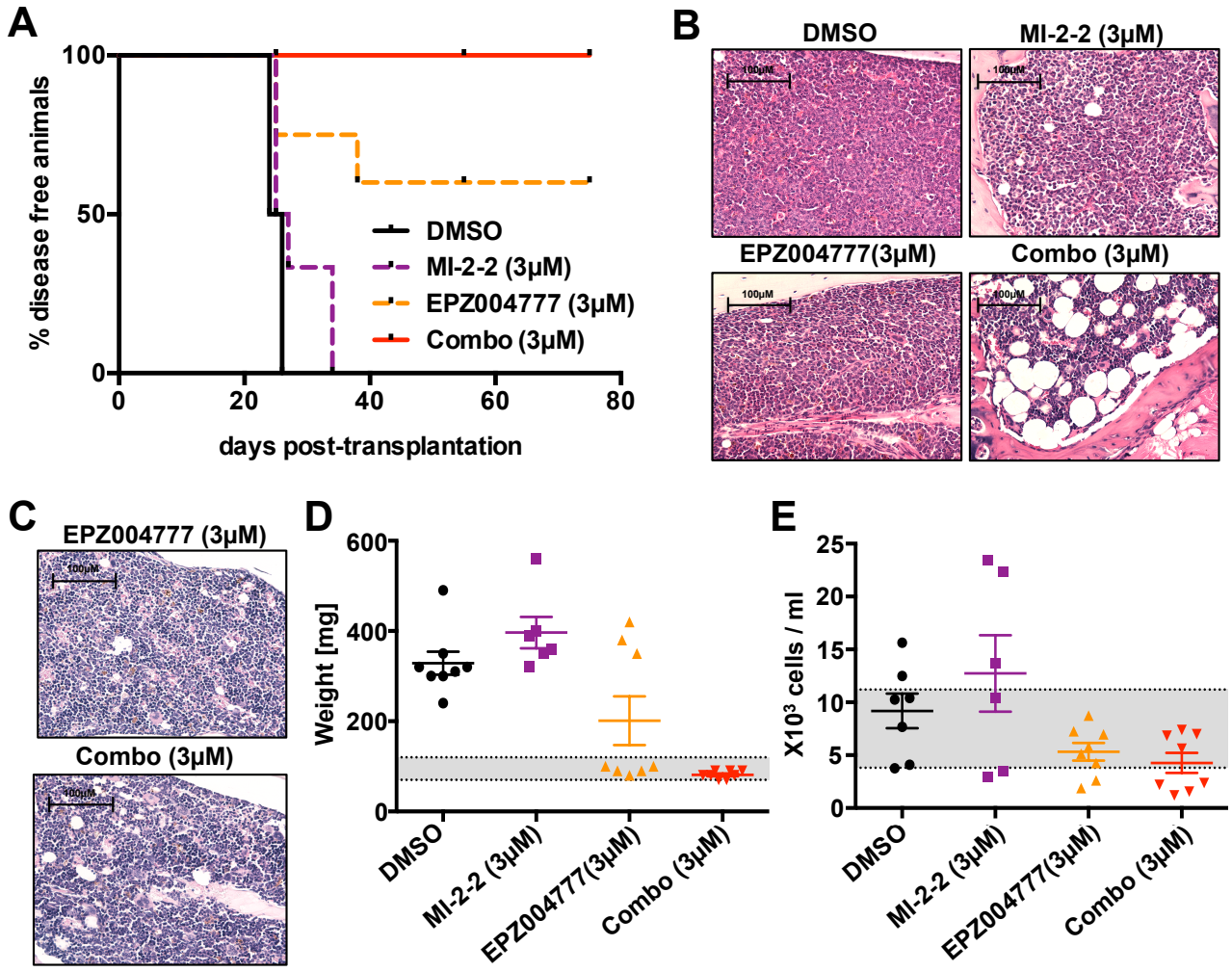

Fig. 29: Combination of DOT1L/MENIN inhibitors ex vivo pre-treatment abolished murine MLL-AF9 leukemic blast expansion *in vivo*

(A) Kaplan-Meier survival analysis of secondary recipient mice injected with $5x10^4$ MLL-AF9 leukemia cells that were treated ex vivo for 4 days with the indicated compound concentrations. Percent of disease-free animals upon sacrifice are represented. **(B)** Representative hematoxylin and eosin-stained bone marrow sections for each treatment group. Analysis was performed after all DMSO control animals showed signs of disease, scale bar 100μM. (**C**) Hematoxylin and eosin–stained bone marrow sections from an asymptomatic recipient mouse injected with EPZ00477 or combination treated cells taken at a later time point (55 days) to assess disease development/progression, scale bar 100μM. **(D-E)** Spleen size measurements (D) and white blood cell (WBC) counts (E) for all animals involved in the study.

To address the potential synergy of the compounds *in vivo*, we *ex vivo* pretreated MLL-AF9 expressing murine leukemic cells for 4 days in liquid with either control vehicle DMSO, or 3μM of EPZ004777, MI-2-2, or combination of both compounds. A pretreatment schedule is necessary as both compounds have a very short half-life *in vivo*, and we have no possibility to use intravenous pumps in mice. At day 4, $5x10^4$ living cells were injected into the tail vein of sublethally irradiated C57/BL6 syngeneic recipient mice. Mice were sacrificed upon first signs of disease. When all the control mice (DMSO treated cells) developed AML, 3 mice from each group were analyzed for progression of the disease by histopathology. All mice transplanted with control cells (n=8) or MI-2-2 treated cells (n=6)

developed the classic MLL-AF9 induced AML after short latency (**Fig. 29A**). Leukemia formed also in 3 out of 8 mice transplanted with EPZ004777 pretreated cells. In contrast, none of the mice transplanted with cell pretreated with the combination (n=8) developed any symptoms of disease (**Fig. 29A**). Histopathology revealed that bone marrow integrity was preserved in all mice that received the cells pretreated with the combination (**Fig. 29B**). They also presented white blood cells and spleen size in a normal range (**Fig. 29D-E**). One asymptomatic mouse from the EPZ00477 and from the combination group was analyzed for histopathology at a later time point (day 55 post-transplant) to assess potential disease progression but no signs for progression was found with normal bone marrow in both animals (**Fig. 29C**). Except the mice transplanted with the combination, all symptomatic mice showed excessive multi-organ infiltration of leukemic cells (**Fig. 30A**). In contrast, no clear blast infiltration was observed in the asymptomatic mice even when analyzed at a later time point, except a unique suspicious spot in the liver of a mouse injected with EPZ004777 treated cells (**Fig. 30B**). The experiment was terminated when the remaining animals in the EPZ004777 and combination group reached 3 times the latency that was observed for disease development of the control (DMSO-treatment) group (25 days). Further characterization of the pre-treatment setting indicated that single or combination treatment of MLL-AF9 expressing cells ex vivo did not significantly impaired their growth or viability (**Fig. 30C-D**). However we observed significant morphological signs of differentiation, such as lighter cytoplasm with granulation and donut shape nucleus, particularly after combination treatment (**Fig. 30E**). This data suggested that transient and non-lethal exposure of leukemic cells to the combination treatment is sufficient to permanently disable their leukemogenic infiltration potential. Altogether, these results demonstrate that the combination of EPZ004777/MI-2-2 has synergistic and durable anti-leukemia effects in an MLL-AF9 driven AML mouse model.

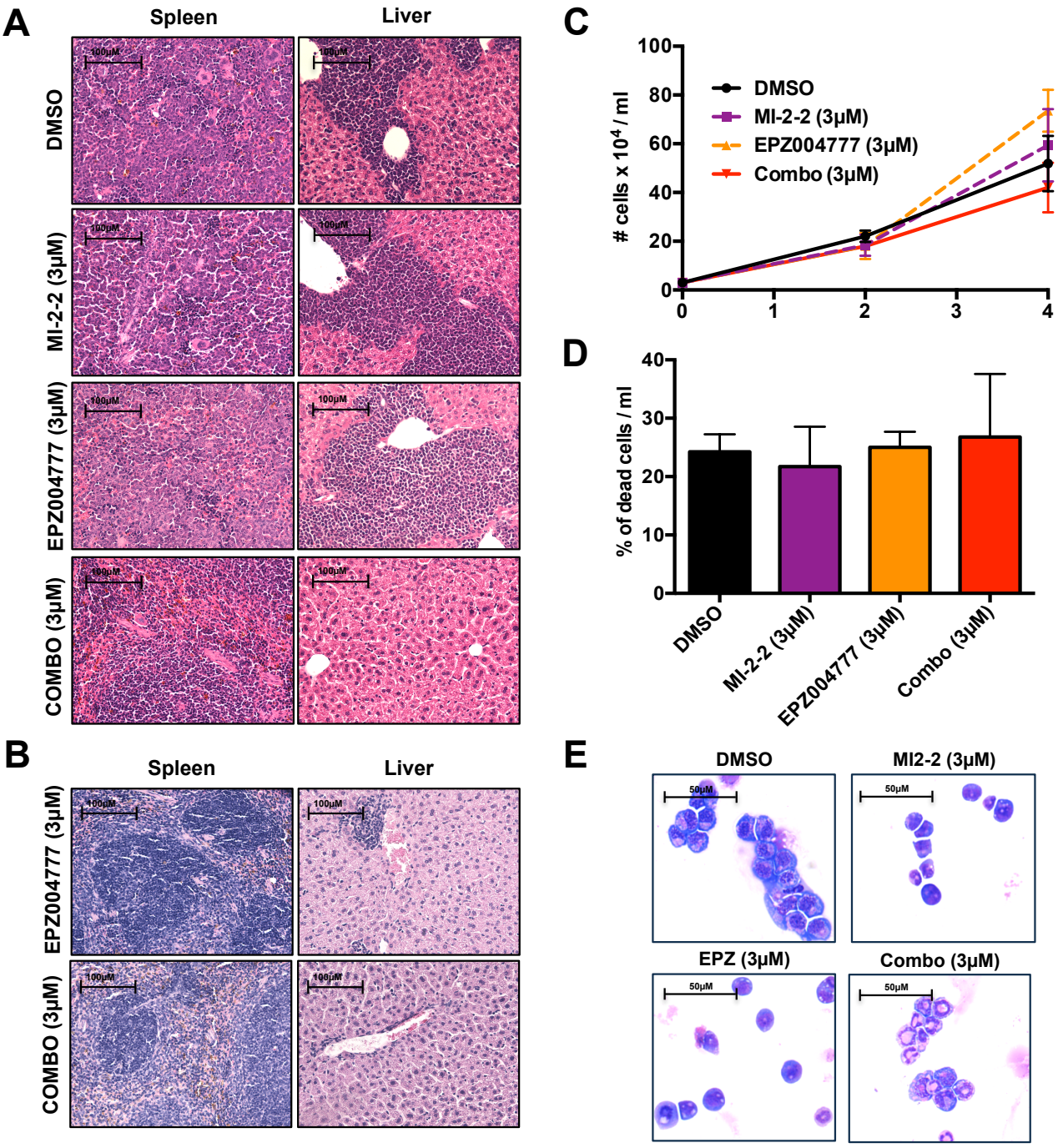

Fig. 30: DOT1L/MENIN inhibitors combination ex vivo pre-treatment induces non-lethal differentiation of MLL-AF9 leukemic blasts abolishing leukemogenic infiltration *in vivo*.

(A-B) Hematoxylin and eosin–stained organs sections from (A) mice analyzed when all the DMSO control mice came with disease and (B) an asymptomatic recipient mouse injected with EPZ00477 or combination treated cells taken at a later time point (55 days) to assess disease development/progression, scale bar 100μM. **(C-D)** Growth curve analysis (C) and percentage of trypan blue counted dead cells (D) recorded for MLL-AF9 cells after 4 days of treatment. **(E)** Representative May-Grünwald Giemsa stained cytospins of murine MLL-AF9 cells after 4 days of treatment in liquid culture with DMSO, 3μM EPZ004777, 3μM MI-2-2 and combination, scale bar 100μM.

DISCUSSION

MLL-fusion gene expressing acute leukemia represents a genetically and clinically distinct subset of leukemic disorders that are often associated with a poor prognosis and a high relapse frequency. The availability of reliable human and mouse models for MLL-fusion driven leukemia as well as intensive biochemical and structural studies boosted the research for the development of targeted therapeutic strategies. There is increasing evidence that normal MLL as well as MLL-fusion act in the form of dynamically-assembled high molecular complexes whose association is essential for MLL-mediated normal and leukemic hematopoiesis [66,67,110]. Recently genetic and structural studies suggested that interactions between LEDGF/p75, MENIN and MLL could offer interfaces for potential therapeutic targeting [100,170,171] The interaction between MLL and MENIN has been structurally characterized and small molecules targeting this interface have been identified [142,166,169,170]. These compounds bind to a central hydrophobic pocket of MENIN and thereby destabilize the LEDGF/p75-MLL-MENIN ternary complex. The small molecule MENIN inhibitor (MI-2-2) was demonstrated to specifically induce cell growth arrest and differentiation in leukemia cells with MLL-fusions [166,168]. However MENIN is also a known tumor suppressor in endocrine tissues suggested to be linked to its interaction with the JUN-D protooncogene [172]. Structural data suggested that the interaction of JUN-D with MENIN is highly similar to the MENIN-MLL interaction suggesting possible cytotoxic effects of MENIN inhibition in other contexts (e.g. MEN-syndrome) than solely MLL-rearranged leukemia. Very recent studies have shown that expression of some MLL-regulated genes might not be dependent on MENIN suggesting a LEDGF/p75-MENIN independent chromatin interaction mechanism [173]. In correlation it is known that MLL interacts directly with non-methylated CpG through its CXXC domain whose targeting has also been suggested as a potential therapeutic strategy for MLL-rearranged leukemia [98,139,140]. MLL has been shown to be a major regulator of transcription in mammalian cells influencing approximately a third of all expressed genes in a context-dependent manner [174]. MLL is essential for maintenance of adult hematopoietic stem cells but also important for formation and function of other organs [75,76,175]. Therefore, as targeting of the MENIN-LEDGF/p75-MLL interface might also affect the function of WT non-rearranged MLL in hematopoietic and non-hematopoietic tissues, the strategies need to be carefully evaluated for a beneficial therapeutic window between normal and malignant cells.

Our work aimed to functionally explore interference with MLL-MENIN-LEDGF/p75 complex as a strategy for targeted therapy of MLL-fusion leukemia. Previous studies have characterized LEDGF/p75 as a key host-cell co-factor during HIV-1 replication by tethering the viral pre-integration complex to active chromatin [176]. In addition, both, the MLL-MENIN and the HIV pre-integration complex was shown to interact with LEDGF/p75 through the so-called integrase binding domain (IBD) [100,150]. Interestingly, expression of an eGFP-fused LEDGF/p75 C-terminal fragment (eGFP-LEDGF/p75$_{325-530}$) encompassing the IBD and the MLL/MENIN interaction domain, was able to selectively impair HIV-1 replication through competition with endogenous LEDGFp75 [148,149]. These observations led us to explore this strategy for targeted interference with MLL fusion-mediated leukemogenesis (**Fig. 9**). Expression of eGFP-LEDGF/p75$_{325-530}$ selectively impaired clonogenic growth of MLL-fusion expressing human and murine AML cell lines (**Figs. 13 & 14**). Decreased self-renewal capacity upon functional interference with LEDGF/p75 was associated with downregulation of *Hoxa9* and general growth defect in murine MLL-fusion transformed cells (**Fig. 13**). Through structural-functional analysis of the LEDGF/p75-IBD, we were able to depicted two peptide-size-IBD derived fragments able to phenocopy the "anti-leukemic" effects of the full-length LEDGF/p75$_{325-530}$ in MLL-fusion expressing cells (**Figs. 15-19 & 21**). Interestingly, the two identified peptide size fragments LEDGF/p75$_{375-386}$ and LEDGF/p75$_{424-435}$ are located on either side of the IBD indicating the existence of two distinct MLL-interaction sites on LEDGF/p75 [150] (**Figs. 17C & 18**).

Based on the recently published partial structure of the MLL-MENIN-LEDGF/p75 complex the LEDGF/p75$_{424-435}$ peptide directly targets a critical interface between the αE helix of the LEDGF/p75 IBD, α4 of MENIN and α2 of MLL, explaining its inhibitory effect [166] (**Fig. 27**). The contacts of the LEDGF/p75$_{375-386}$ peptide with the MLL-MENIN complex were however not resolved in the published crystal structure. However, our collaborators have structurally and biochemically shown that a direct interaction exists between MLL and LEDGF/p75 in the absence of MENIN [177]. This is at odds with the co-IP data presented in our paper and in previous work that suggested MENIN to be required for the interaction [100,177]. It is possible that co-IP experiments might be not sensitive enough to detect the interaction or that the interaction is modulated by posttranslational modifications. However, their results strongly support the existence of another interaction site for MLL in the LEDGF/p75 IBD that can be disrupted through overexpression of the fragment LEDGF/p75$_{375-386}$.

Based on this work our collaborators (J. De Rijck *et al*., KU Leuven) went further on the characterization of the MLL-LEDGF/p75 interface and managed to depict another direct interaction. This interface involves MLL helixes α1- α2 anchored via F148 and F151 between two LEDGF/p75 IBD inter-helical loops and forming targetable hydrophobic pockets [167] (**Fig. 27**). In more details MLL F148 occupies a hydrophobic pocket on the LEDGF/p75-IBD surface formed by L363, L368, I403, F406, K407 and V408, while MLL F151 is buried on a pocked formed by the LEDGF/p75-IBD amino acid residues I359, K360, L363, T399, K402 and I403. Interestingly this interaction seemed to be independent of MENIN recruitment showing the potency of targeting selectively MLL-LEDGF/p75 interaction without interfering with MENIN. In addition, there seems to be a partial but not integral overlap between the LEDGF/p75-MLL and LEDGF/p75–HIV integrase interfaces. In this regard, we showed that overexpression of LEDGF/p75 IBD interacting cyclic peptides known to disrupt HIV-Intergrase interaction were also able to impair the clonogenic potential and *Hoxa9* expression of murine MLL-AF9 expressing bone marrow cells [152,167] (**Figs. 26 & 27**). Collectively, these results suggested two distinct potential druggable interfaces in the LEDGF/p75-MENIN-MLL complex and one of them seems to be not only essential for the leukemic activity but also for HIV-replication. Therefore currently ongoing studies now aim to develop small molecules that are able to imitate the activities of the peptides used for defining the critical interfaces. A similar strategy led to a novel class of small molecules that target the HIV-integrase that are currently tested in clinical trials [151].

Previous studies demonstrated that small molecules inhibitors targeting the MLL-MENIN interaction or the DOT1L histone methyltransferase associated with the MLL-fusions results in differentiation and apoptosis of MLL fusion gene-driven leukemic cells [124,126,133,169,178]. However, despite a significant defect on growth and self-renewal of MLL-fusion expressing cells, we did not observe any significant induction of apoptosis or differentiation upon expression of the LEDGF/p75 fragments (**Figs. 20 & 23**). This controversial observation might result from the retroviral expression approach we used. Indeed we showed that MLL-fusion protein is very highly expressed in leukemic cells extracted from a retrovirally MLL-AF9 diseased AML mouse (**Fig. 11**). Therefore, it is likely that by using a competition approach through retroviral expression of the LEDGF/p75 fragments, we were not able to reach enough expression to totally outcompete the MLL-fusion complex necessary to induce terminal differentiation and apoptosis. Nevertheless using this strategy, we were able to show that expression of the eGFP-LEDGF/p75$_{325\text{-}530}$

or eGFP-LEDGF/p75$_{375-386}$ fragments moderately delayed leukemia induction in a mouse bone marrow transplantation model. Our collaborators (KU Leuven) further proved that LEDGF/p75$_{325-530}$ disrupts the MLL/MENIN-LEDGF/p75 complex in co-IP experiments and an AlphaScreen protein-protein interaction assays (**Fig. 25**). These results are in agreement with the hypothesis that LEDGF/p75$_{325-530}$ competes with endogenous LEDGF/p75. Importantly, like LEDGF/p75$_{325-530}$, the tagged peptide fragments disrupted the LEDGF/p75-MLL complex in a dose-dependent manner (**Fig. 25**)**.** Surprisingly, the untagged LEDGF/p75$_{375-386}$ peptide inhibited the MLL-LEDGF/p75 interaction, whereas the LEDGF/p75$_{424-435}$ peptide did not (**Fig. 25**). Possibly, steric hindrance of the tag helps LEDGF/p75$_{424-435}$ to disrupt the complex.

Structural functional studies also suggested that LEDGF/p75 might be able to thether the MLL-complex to its target genes like the *HOX* cluster by its PWWP chromatin-binding domain but independent of the MENIN interaction [100,179]. Importantly replacement of the LEDGF/p75-MENIN binding domain by the PWWP domain of LEDGF/p75 was sufficient to rescue leukemic transformation activity of MLL-fusion in the absence of MENIN [100]. Very recently the same researchers suggested that only the CXXC domain of MLL and the PWWP domain of LEDGF/p75 were essential for DNA binding to target promoter genes and for transforming capacity of the MLL-ENL fusion *in vitro* and *in vivo* [179]. Based on these results and to strengthen the specificity of our IBD-interfering strategies for MLL-LEDGF/p75 interfaces, we engineered a mutated MLL-AF9 fusion containing the PWWP domain of LEDGF/p75 but lacking the putative MENIN-LEDGF/p75-interaction site. Briefly, the PWWP domain of LEDGF/p75 was fused to MLL-AF9 whereas the MENIN binding sites were removed and a MLL F129A abrogating point mutation of the LEDGF/p75 binding domain was inserted. However, in several attempts, we were not able to obtain stable transformation by retroviral expression of this MLL-AF9 mutant in mouse bone marrow cells *in vitro* or leukemia induction *in vivo* (data not shown). This observation goes along with our finding that the F129A mutation was not sufficient to totally abrogate LEDGF/p75-MLL interaction [177]. In addition, according to the structure established by our collaborators, addition of MENIN in the MLL-LEDGFp75 *in vitro* interaction assay induces a 4-fold stimulation of the interaction [167]. These observations suggest that that, the PWWP-F129A MLL-AF9 is not able to form a stable MLL-LEDGF/p75-MENIN interaction essential for malignant transformation of the cells.

Importantly, we observed that expression of the eGFP-LEDGF/p75$_{325-530}$ or LEDGF/p75 IBD-interacting cyclic peptide (CP65-eGFP) had strong anti-leukemic activity but did not impair normal bone marrow stem and progenitor cells (**Figs. 13 & 26**) suggesting a possible therapeutic window between normal and leukemic cells through functional inhibition of LEDGF/p75-MLL interactions. In addition, according to our results functional interference with LEDGF/p75 through overexpression of the IBD-derived fragment seemed to be rather selective for MLL-rearranged leukemic cells, as murine BAF3 cells immortalized by overexpression of a constitutively active kinase (FLT3-ITD) or other human AML cells without MLL-fusion (e.g. HL60, JURKAT, KASUMI) were not affected (**Figs. 13, 14, 21 & 22**). Therapeutic interference with the MLL-LEDGF/p75-MENIN interactions represents an indeed targeted and rather specific approach unlike other strategies that interfere with other, more functionally associated mediators of the MLL-complex such as BRD4, PAFc or pTEFb that are more general effectors of transcription, and despite the efficient anti-leukemic effects, are associated with significant toxicity [122,180,181].

Advances in understanding of MLL fusion leukemia have led to the identification of several potential therapeutic strategies. Currently, inhibition of H3K79me DOT1L histone methyltransferase associated with the MLL-fusion complex is the most clinically advanced concept. Recently developed small molecule inhibitors of DOT1L, such as EPZ004777, SGC0946, and EPZ-5676, have been shown to efficiently block H3K79 methylation, inhibit expression of MLL fusion target genes and selectively kill cells with MLL translocations[178,182,183]. However, DOT1L inhibitors seem to act slowly and inefficiently in many MLL-rearranged cell lines and only xenografts of a particularly sensitive human cell line (MV4;11) have successfully been treated *in vivo* [178,182]. Nonetheless, EPZ-5676 has been recently promoted into phase 1 clinical studies as a targeted therapy for patients with *MLL*-rearranged leukemias and represents the first reported histone methyltransferase inhibitor to enter human clinical trials. In this context our collaborator at Novartis (R. Tiedt and V. Craig, NIBR Oncology, Basel) aimed to identify synergistic modulators of DOT1L inhibition. Using a deep coverage shRNA screening platform ("DECODER")[184] they identified several candidate sensitizer genes whose depletion strengthens the action of DOT1L inhibition in human AML cells harboring MLL fusions. Notably, they observed a strong enrichment for genes that are implicated in the MLL complex including *MLL* and *LEDGF/p75.* In absence of any available pharmacological active agents that are able to target LEDGF/p75, they set out to test the EPZ004777 DOT1L inhibitor in combination

with the MENIN inhibitor MI-2-2 targeting the MLL-MENIN-LEDGF/p75 interaction [168,169]. Therefore, we focused on characterizing between potential synergisms between DOT1L and MENIN inhibition as a robust therapeutic strategy using our MLL-AF9 AML mouse model. Hereby we observed a strong decrease of self-renewal capacity *in vitro* CFU assay upon combination treatment of murine MLL-AF9 expressing cells, which was not the case when the cells were exposed only to the DOT1L or the MENIN inhibitor (**Fig. 28**). Second, we found a complete lack of AML formation *in vivo* after transplantation of *ex vivo* pre-treated MLL-AF9 cells for only 4 days provides strong evidence that short exposure to the combination treatment is sufficient to initiate an irreversible differentiation that efficiently abrogate leukemia initiation (**Figs. 29 & 30**). In contrast, we found no dramatic effect of the combination treatment on the clonogenic growth of normal bone marrow hematopoietic stem and progenitor cells (**Fig. 28**). Our collaborators from Novartis also tested the DOT1L/MENIN inhibitor combination on a subset of human non MLL-rearranged leukemic cells (K562, a chronic myelogenous leukemia cell line and two B-ALL cell lines, REH and NALM6) and observed only very limited effects on proliferation and viability *in vitro*. Similarly no significant effect of the combination was observed on clonogenic growth of human peripheral blood derived CD34+ progenitors (data not shown). All together, these results suggest therapeutic selectivity of the DOT1L/MENIN inhibitor combination for MLL-rearranged leukemia. Eventually this combination might help to reduce the potential development of resistance towards enzymatic inhibitor. Several examples highlight the success of combining targeted agents in clinical trials as a means to overcome the rapid onset of resistance to monotherapy. For instance, combining BRAF and MEK inhibitors has been shown to effectively treat Melanoma patients with BRAF V600 mutations [185]. Similarly BRAF V600E mutant colon cancers have been shown to benefit from combination therapy consisting of BRAF and EGFR inhibitors [186,187]. Both examples emphasize that targeting the same pathway at two different nodes results in a superior outcome. Evidently, the large number of possible combinations of targeted agents that could be considered for treatment remains a challenge. These results therefore highlight the power by which RNAi sensitizer screens utilizing high coverage shRNA libraries can be translated into enhanced therapeutic strategies. Following similar objectives, Klaus *et al*. recently reported that the DOT1L inhibitor EPZ-5676 displayed strong synergy with standard of care chemotherapies and DNA hypomethylating drugs in MLL-rearranged leukemia cells [188]. Likewise, Liu *et al.* demonstrated that treatment with an alternative DOT1L inhibitor significantly increased the sensitivity of MLL-rearranged leukemia cells to chemotherapeutics [189]. However, as of yet, no targeted therapeutic partners for DOT1L

inhibition have been proposed. In this regard, our study ultimately provides a rationale for combining DOT1L and MENIN inhibitors in MLL patients, which typically have a very poor clinical outcome.

Interestingly, the DECODER screening led by Novartis on MLL-fusion cells highlighted additional MLL related factors (e.g. WDR5 and BRD4) as potential therapeutic combination partners with DOTIL inhibitors. Several studies suggested that the normal non-rearranged wildtype MLL protein might play a critical role in MLL-driven leukemogenesis [116]. Going along with these studies, there is this observation that leukemic blasts with MLL rearrangements normally maintain a normal copy of the gene. In addition, some AML cases have been reported harboring amplifications of the MLL genes furthermore suggesting that the activity of un-rearranged protein can contribute to a malignant phenotype [190,191]. Indeed, recent studies suggest that targeting WDR5 an essential co-factor for the MLL-mediated histone methyltransferase activity by small molecule inhibitors or genetic knockdown impairs the leukemogenic activity of MLL fusion genes [135–138]. Interestingly WDR5 has been previously shown to act as adapter between the MLL histone methyltransferase and the MOF acetyltransferase, that seem to act closely together to allow proper activation of critical target genes [192]. Very recent work suggested that the transcriptional program directed by the MLL complex is mainly relies on MOF-mediated H4K16 acetylation [118]. Some MLL fusions involve histone acetyltransferase (HAT) such as CBP and p300 strengthen the potential of targeting the associated HAT activity in acute leukemia [83,84]. Interestingly the CBP/p300 co-activator complex has been newly shown to be critical for MLL-fusion but also for AML1-ETO driven leukemogenesis in the mouse [193]. CBP/p300 seem not only to modify chromatin of critical downstream targets but also affect the activity of oncogenic fusion proteins by direct acetylation [193]. Interestingly, acetylated lysine-residues of activated chromatin are recognized by bromodomains (BRDs). Bromodomains are understood as “epigenetic reader” protein interaction modules. Structural characterization of the BRDs interaction resulted in the discovery of potent and highly specific inhibitors of the BET (Bromodomain and Extra-Terminal) protein family [180]. Blocking of the BRD4 BET protein severely impairs transcriptional activation, particularly of the so-called “super-enhancers” that are critical regulators of key oncogenes like *c-myc* [194,195]. Multiple studies have demonstrated anti-cancer activity of small molecule BET-family inhibitors resulting in potently reduced proliferation and survival of MLL-fusion driven leukemic blasts [124,126,127]. Overall 61 BRDs have been found in 46 human proteins including many transcriptional co-regulators and

chromatin-modifying enzymes that are involved in the pathogenesis of AML suggesting additional opportunities for therapeutic targeting. Interestingly, the CBP/p300 HATs seem to recognize acetylated chromatin through their BRD, and both the HAT and the BRD seem to be crucial for MLL-CBP mediated leukemogenesis [196]. This observation suggests a possible synergistic "anti-leukemic" effect by combining HAT and BRD domain inhibition of CBP. A major advantage of this combination would be to modulate the dose of potential compounds to avoid unselective cytotoxic effects linked with either BRD or HAT targeting. Although there are currently no clinically relevant selective inhibitors that target HAT, in collaboration with the Structural Genomic Consortium (SGC, S. Knapp and co-workers) our laboratory has recently characterized a potent anti-leukemic activity of a novel small molecule that selectively targets the BRD of CBP/p300. Interestingly, this compound seems to primarily affect self-renewal of leukemic cells without significant cytotoxicity. Importantly, this compound seems to act synergistically with BET family BRD inhibitor (Picaud et al., submitted). Previous studies have shown that, although not really selective, histone deacetylase inhibitors (HDACi) have potent "anti-leukemic" activity in acute leukemia[197]. This effect was mainly associated to the release of fusion gene directed transcriptional silencing of genes that are normally regulating differentiation and apoptosis. However, HDACIs treatment applied to AML patients seem to have modest activity even in combination with the conventional chemotherapy and limited by progressive constitutional symptoms [198]. In this context, it would also be an interesting option to address the potential synergistic effects between HDAC and BRD inhibitors.

MLL fusion gene driven acute leukemia represent a clinical challenge. The experimental work of my PhD project helped to define critical protein-protein interactions particularly of LEDGF/p75 to MLL and MENIN that are essential for the leukemogenic activity of MLL fusion proteins. Most importantly, our structure functional studies revealed a MENIN-independent LEDGF/p75-MLL interface that is currently followed for design of small molecules selectively targeting this interaction. In addition, we were able to demonstrate strong synergism between DOT1L and MENIN-MLL interaction inhibitors in the context of most common MLL-fusion leukemia. We are convinced that such strategies will be necessary to overcome early resistance and relapse leading to an improved outcome for patients suffering from MLL-rearranged leukemia.

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