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CICLO XXVII

Role of the stamness transcription factor ZNF521 in MLL-rearranged acute myeloid leukemia

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Now, here, you see, it takes all the running
you can do, to keep in the same place.
If you want to get somewhere else,
You must run at least twice as fast as that!"
(L. Carroll)

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SUMMARY

Acute myeloid leukemias (AMLs) that harbor translocations involving the *MLL* gene on chromosome 11q23 generate fusion transcripts that give rise novel fusion proteins with potent oncogenic properties and capable to destabilize the normal transcriptional activities. MLL fusion oncoproteins have been shown to initiate leukemic transformation primarily by overexpression of a specific set of genes, including *HOXA4, 5, 6, 7, 9, 10* (overall defined as "HOXA-code" genes), *MEIS1* (a cofactor of "HOXA-code" proteins) and *MYB*. It's well established that the majority of these genes are involved in normal programs of self-renewal, maintenance and proliferation of hematopoietic stem cells and early progenitors. Therefore is not surprise that the deregulation of the stemness genetic programs due to MLL fusion oncogenes is a crucial step for leukemic transformation. To identify new stemness genes involved in MLL-mediated transformation we performed gene set enrichments analysis (GSEA) using public database of geneset profiles of normal hematopoietic cells in a cohort of pediatric AML previously analyzed. These analyses identified a series of genes more highly expressed in MLL-rearranged AML including the well known HOXA9, HOXA5 and MEIS1, together with an apparently novel gene: ZNF521 or zinc finger protein 521

ZNF521 encodes for a zinc finger protein and, like HOXA9, is strongly expressed by CD34+ hematopoietic stem cells and drastically decreases during differentiation. To evaluate the importance of *ZNF521* in MLL-rearranged AML, we performed a series of functional and mechanistic studies to uncover the role of ZNF521 in *MLL*-rearranged cells. We used lentiviral vectors to silencing the ZNF521 and expression vectors to induce MLL-fusion proteins such as MLL-AF9. These studies, both *in vitro* and *ex vivo*, demonstrate that the growth inhibition, reduced clonogenicity and cell cycle arrest induced by ZNF521 depletion is mediated through enhanced myeloid differentiation. Moreover, we demonstrate that ZNF521 is a direct target of MLL-fusion oncoproteins such as MLL-AF9 and MLL-ENL.

Collectively, these findings identify ZNF521 as critical effector of MLL fusion in leukemogenesis that might be targeted to overcome the differentiation block associated

with *MLL*-rearranged AML and thus highlight ZNF521 as potential therapeutic target in treating this subtype of aggressive leukemias

RIASSUNTO

Il gene *MLL* è spesso coinvolto in traslocazioni cromosomiche che causano la formazione di nuovi trascritti di fusione in grado di codificare delle proteine chimeriche con elevate proprietà oncogeniche e di de-regolazione dell'attività trascrizionale. Le oncoproteine MLL di fusione sono capaci di iniziare la trasformazione leucemica provocando una overespressione di diversi geni, tra cui quelli più critici sono gli *HOXA4-10 (*che complessivamente costituiscono l'*HOXA-code)*, *MEIS1* (un cofattore delle proteine HOXA-code) e *MYB*. La maggior parte di questi geni sono implicati nei normali programmi di *self-renewal*, mantenimento e proliferazione della popolazione cellulare ematopoietica staminale e dei progenitori. È evidente che la deregolazione dei programmi genetici associati alla staminalità, provocata dalla presenza degli oncongeni MLL di fusione, ha un ruolo cruciale nella trasformazione leucemica delle cellule ematopoietiche.

In questo studio abbiamo identificato, tramite GSEA (Gene Set Enrichment Analysis), i profili genetici delle cellule CD133+ normali ottenuti da database pubblici e, successivamente abbiamo valutato la loro espressione in una serie di leucemie acute mieloidi (LAM) pediatriche precedentemente analizzate in altri studi di espressione genica. I risultati mostrano che tutti i target noti degli oncogeni *MLL* di fusione (*HOXA, MEIS1*) sono up-regolati esclusivamente nelle LAM con traslocazioni del gene *MLL*. Abbiamo inoltre osservato che tra i geni maggiormente up-regolati è presente anche *ZNF521,* un gene che codifica per una proteina appartenente alla famiglia delle proteine zinc-fingers. Come *HOXA9*, anche *ZNF521* è altamente espresso nelle cellule ematopoietiche staminali CD34+ e la sua espressione diminuisce rapidamente durante il differenziamento. Per valutare l'importanza di *ZNF521* nelle LAM con traslocazioni del gene *MLL* abbiamo eseguito una serie di studi funzionali e meccanicistici *in vitro* ed *ex vivo* con cellule primarie, utilizzando sia vettori lentivirali per il silenziamento del gene *ZNF521*, sia vettori di espressione di diversi oncogeni con traslocazioni di *MLL*. Questi studi hanno dimostrato che il silenziamento di *ZNF521,* che ne determina una diminuzione di espressione ed induce le cellule a differenziare, causa un'inibizione della proliferazione cellulare, una drastica riduzione della clonogenicità e l'arresto del ciclo

cellulare in fase G1. Inoltre, abbiamo dimostrato che *ZNF521* è tra i target diretti delle oncoproteine MLL di fusione, ed è quindi attivamente coinvolto nella trasformazione leucemica in seguito alle traslocazioni del gene *MLL*.

In conclusione, ZNF521 si è rivelato essere un nuovo importante effettore degli oncogeni *MLL* di fusione e un fattore cruciale nel mantenimento dello stato indifferenziato delle cellule mieloidi leucemiche che presentano riarrangiamenti del gene *MLL* e potrebbe quindi dimostrarsi un importante nuovo target terapeutico.

INTRODUCTION

1. HEMATOPOIESIS

Hematopoiesis is the process that gives rise to all blood cellular components during embryonic development, and throughout adulthood to produce and replenish the hematopoietic system. It is estimated that this process provides to produce almost 10^{10} cells every hour for replenish continuously aged or damaged blood cells (Williams, 1995). This system of generative activity is tightly regulated by progressive restriction of cell fate potentials initiating from hematopoietic stem cells (HSCs) to lineage-restricted progenitors that produce all lineages of mature blood cells (**Figure 1**).

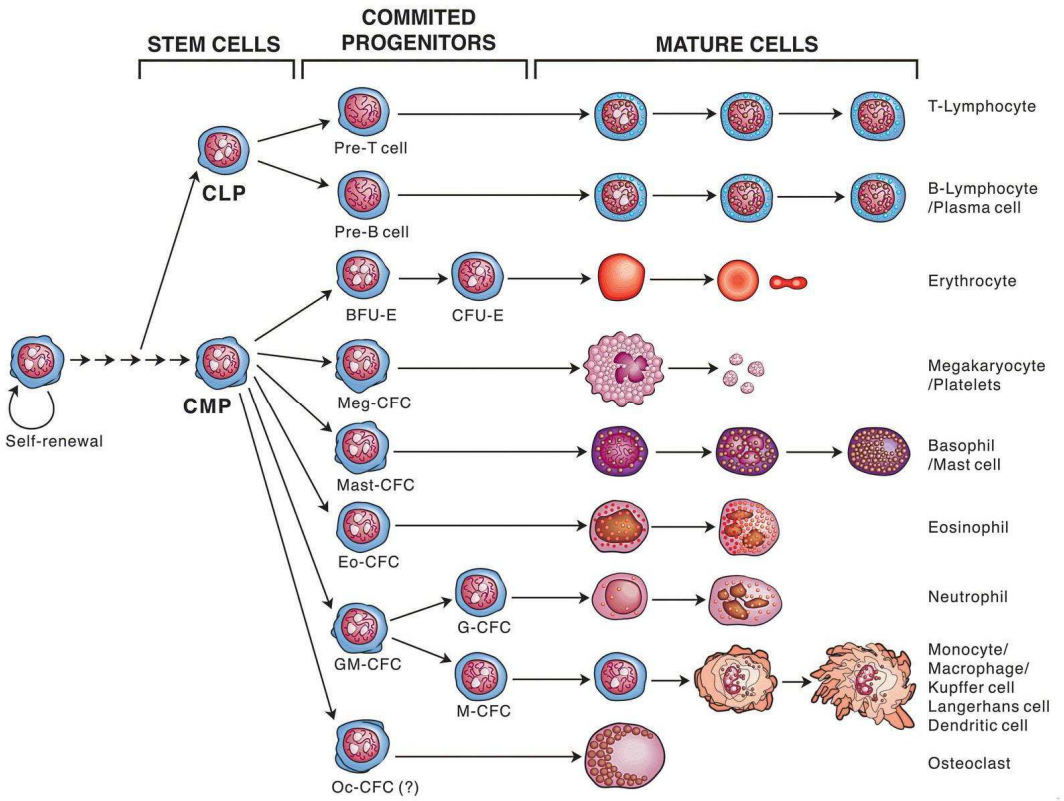

Figure 1: Hematopoiesis.

The figure shows the development of hematopoietic stem cells (HSC). All blood cells are derived from HSCs, which are at the basis of the adult blood cell differentiation hierarchy. HSCs can be subdivided into self-renewing HSCs and multipotent progenitors. These include common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). CMP further divides to give rise to more differentiated progenitors, committed to granulocytes and macrophages (GMs), and megakaryocytes and erythroid cells (MEPs). CLP produces progenitors committed to T cells and B cells. Successive division and differentiation of these progenitors give rise to fully differentiated B- and T-cells. CMPs maturation generate neutrophils, eosinophils, basophils, monocytes, platelets and erythrocytes.

Deregulation along the developmental pathway leads to various hematological disease like anemia, immunodeficiencies or leukemia. Therefore, a deeper knowledge of the mechanisms that finely regulate this balancing is pivotal for understanding of both normal hematopoietic development and pathogenesis of hematopoietic disease.

All cellular blood components are derived during fetal and adult life through a mechanism that is conserved across vertebrates and involve two waves: the primitive wave and the definitive wave (Galloway, 2003). The primitive wave, which involves an erythroid progenitor, gives rise to erythrocytes and macrophages during early embryonic development (Palis, 2001; Madhumita, 2013) and is predominantly marked by erythroid progenitor to produce red blood cells that facilitate tissue oxygenation during growing embryo (Orkin and Zon, 2008). The primitive wave is transitory, however, and these erythroid progenitors are not pluripotent and do not have renewal capability. By contrast, definitive hematopoiesis occurs later in development, notably at different time points in different species. In vertebrates, a definitive wave involves hematopoietic stem cells (HSCs) that originate in the aorta-gonad-mesonephros (AGM) region of the developing embryo. In humans, HSCs are produced in yolk sac and placenta from where they migrate to the fetal liver and then to the bone marrow, which is the location for HSCs in adults (Dzierzak, 2007; Cumano, 2007).

1.1. HSC: the paradigm of self-renewal and differentiation.

The integrity of the hematopoietic system depends on a large number of blood cell lineages being continuously replenished from a rare population of pluripotent hematopoietic stem cells (HSCs), representing a paradigm for how multi-lineage diversity can be achieved from a common stem cell through lineage commitment and subsequent differentiation (Kondo, 2003). Hematopoiesis requires a continuous production of progenitors and mature blood cells from HSCs through differentiation processes. In fact, HSCs sustain lifelong production of all blood cell types through finely balanced divisions leading to self-renewal and differentiation. Differentiation is associated with a loss of self-renewal capacity, requiring HSCs as a population to self-renew to maintain itself.

The fate choice of HSCs to either self-renew or differentiate is controlled by a complex interplay between intrinsic mechanisms and extrinsic signals from the surrounding environment or stem cell niches (Moore, 2006).

Extrinsic (environmental) signals are predominantly derived from stromal cells and their products (Wineman, 1996; Blazsek, 1995). The marrow, in particular, contains specialized environments that regulate the balance of HSC self-renewal and differentiation and comprise what has been termed the stem cell niche. In fetal and adult mammals, HSCs predominantly reside in fetal liver (FL) and bone marrow (BM), respectively. However, HSCs do not originate in FL or BM, but rather migrate from other tissues to these sites during embryonic development. In mammals, most blood cells have relatively short lifespans. For this reason, HSCs continuously differentiate into multiple lineages of different blood cell types, simultaneously replicating themselves through self-renewal to prevent depletion of the stem cell pool in the BM. Nevertheless, external environmental signals must integrate with intrinsic molecular machinery to control the fate choices of individual HSCs. Such genetic mechanisms predetermine the behavior of HSCs and thus should limit the generation of HSC heterogeneity (Muller-Sieburg, 1996; Abkowitz, 1998; Chen, 2000).

The changes in gene expression over the course of hematopoietic differentiation are profound and complex. The number of differentially expressed genes is similar within hematopoiesis and across human tissues, suggesting comparable complexity. In fact, cell

fate specification involves the action of primary lineage determinants (transcription factors, TF) that initiate and resolve mixed lineage patterns of gene expression by activating lineage appropriate genes and repressing alternate lineage genes (Laslo, 2006). Knockouts or forced expression experiments on model organisms (e.g., *mice*, *zebrafish*, *chicken*, *drosophila*, *xenopus*) were performed to understand the functions of the critical transcription factors. In details, it is possible to see in **Figure 2** the expression of the most important TF during the blood development. The knockdown of these genes leads to a block in differentiation, meaning a crucial role of the down-regulated gene, in the process of cell maturation

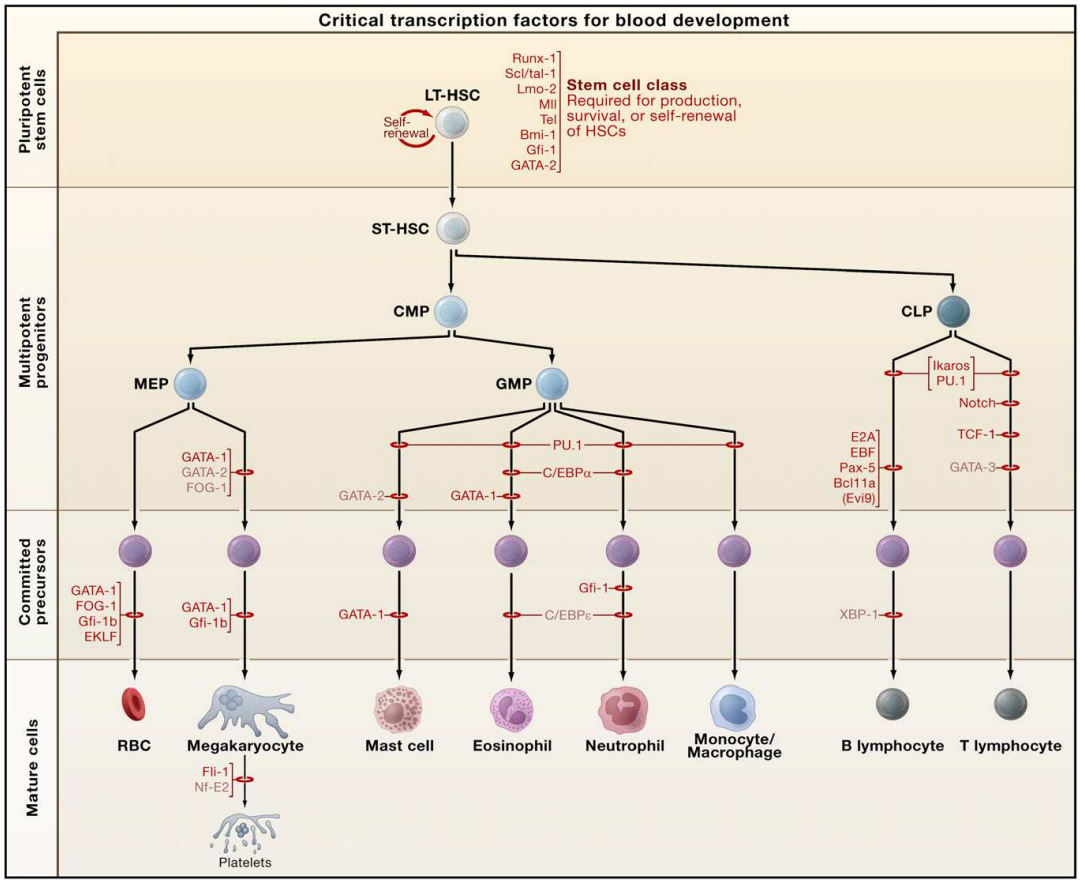

Figure 2: Transcription Factors during blood cell development.

Red bars indicate the stages at which hematopoietic development is blocked in the absence of a given transcription factor, as determined through conventional gene knockouts. Abbreviations: LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; CMP, common myeloid

progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor; RBCs, red blood cells. Figure adapted from (Orkin and Zon, 2008)

In the last years, many studies focused on the analysis of gene regulatory networks that direct cell fate decisions within the hematopoietic system. Gene disruption studies have shown that GATA-1 is necessary for erythroid and megakaryocyte development whereas PU.1 is required for the generation of myeloid (macrophage and granulocyte) and lymphoid lineages (Scott, 1994; Orkin, 1998). Based on the findings that PU.1 and GATA-1 could inhibit each other's molecular activities (Rekhtman, 1999; Zhang, 1999), it was proposed that this cross-antagonism is critical for generation of megakaryocyte/erythroid versus myeloid progenitors (Cantor, 2002).

Runx1 is a member of the runt family of transcription factors and plays an important role in definitive hematopoiesis (Wang et al., 1996). Knockout experiments in mice reveal that ablation of *Runx1* means losing definitive erythroid, myeloid and lymphoid cells, indicating its importance in definitive hematopoiesis. *Runx1* knockdown also leads to a decrease in the expression of *cmyb*, which belongs to the myb family of proto-oncogenes (Kalev-Zylinska, 2002; Burns, 2005; Gering, 2005). Further experiments on zebrafish reveal the importance of other genes during hematopoietic development. In fact in an early stage of development, cells co-expressing *tal1*, *gata2*, *lmo2*, *fli1* and *etsrp,* appear to be indispensable for both endothelial and hematopoietic differentiation (Paik, 2010).

A remarkable feature of transcription factors in the hematopoietic system is that the majority are involved in chromosomal translocations or with somatic mutations in human hematopoietic malignancies. Furthermore, experimental manipulation of the genes for such factors in mice often promotes malignancy; in fact hematopoietic cell fate is tightly associated with the origins of leukemias.

Although up to now, several studies have shown and unveiled many aspects concerning the molecular mechanisms regulating HSC self-renewal versus differentiation, but many other are still to be elucidate.

2. LEUKEMIA

Leukemia is defined as cancer of the blood forming system and characterized by proliferation of abnormal white blood cells, not fully developed and called blast or *leukemia cells* in the bone marrow.

As a result, there is a loss of hematopoietic function due to the lack of mature granulocytes and monocytes as well as decreased red blood cell and platelet production. These abnormal precursor cells are capable of proliferation and cell division, but lack the capacity to differentiate (Borer, 1989). Leukemia can be divided into an acute and chronic form; acute leukemias are characterized by the clonal expansion of hematopoietic progenitor cells caused by a maturation arrest combined with enhanced proliferation resulting in a fast increase of immature blood cells. In contrast, in chronic leukemia, abnormal blood cells have a slower expansion of terminally differentiated cells that can still execute their normal function. A further division can be made depending on the lineage of the progenitor cell that is affected. When the leukemia originates from a lymphoid progenitor cell, it is named a lymphocytic or lymphoblastic leukemia, and when it originates from the myeloid lineage, it is called a myeloid or myelogenous leukemia. This identifies the 4 major types of leukemia: acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL). In children, chronic leukemias are very rare, ALL comprises the largest part (75-80%), and AML accounts for 15-20% of pediatric leukemias (Cordell, 1999).

2.1. Leukemia Stem Cell: an hallmark in AML

For several years uncontrolled proliferation was considered the distinguishing property of any malignant disease. The definition of a cancer stem cell (CSCs) is based on its functional properties, that is a malignant cell that has the ability to self-renewal and also to differentiate into multiple cell types to recapitulate the cell populations of the original tissue (Jondan, 2007).

The Cancer Stem Cell Model, also known as the Hierarchical Model proposes that tumors are hierarchically organized CSCs lying at the apex (Bonnet, 1997).In the cancer stem cell model, within the cancer population of the tumours there is a small subset of cancer cells, CSCs, which constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor. Considering that. it is difficult to completely eradicate them during treatment, they have become an intriguing target for future cancer therapy.

Much of the evidence about the cancer stem-cell hypothesis has come from studies in hematologic malignancies. First experimental evidence, suggesting the existence of Leukemic stem cells (LSCs), resulted from observations made almost 40 years ago: it was demonstared by Park and collaborator in the 1971 that only 1 out of 10,000-100,000 mouse myeloma cells obtained from mouse ascites were able to form colonies in semi-solid medium (Park, 1971). Similarly, human leukemia cells from AML patients also formed colonies at very low frequency suggesting the presence of a small number of LSCs within the bulk of leukemic blasts (Sabbath, 1985; Griffin, 1986).

The first conclusive evidence for CSCs came in 1997. Bonnet and Dick isolated a subpopulation of leukemia cells that expressed surface marker CD34, but not CD38 (Bonnet, 1997). The authors established that the $CD34^{+}/CD38^{-}$ subpopulation is capable of initiating tumors in NOD/SCID mice that were histologically similar to the donor. (Bonnet, 1997; Lapidot, 1994).

It has been demonstrated that only a small number of LSCs can restore themselves and maintain the disease, whereas the majority of leukemia cells are in a more mature state unable to initiate the disease. It is also known as LSCs share analogous processes of the self-renewal and somehow differentiation of normal stem cells (Krause, 2007). In particular, it was discovered that similar signaling pathways involved in the control of self-renewal of HSCs are also key elements maintaining stemness in LSCs. These pathways include the well-known PI3K/Akt/mTOR (Fransecky, 2015), Wnt/beta-catenin (Wang, 2010; Lento, 2013), Hedgehog (Mar, 2011; Irvine, 2012), NF-kB (Kagoya, 2014; Zhou, 2015), Notch (Liu, 2013) and Bcl-2 (Domen, 2000; Lagadinou, 2013).

Although LSCs have the capacity for self-renewal and differentiation, evidence has shown that a substantial number of LSCs are found in a quiescent G0 phase (Guzman, 2001).

This could provide a possible reason for the failure of chemotherapeutics to eliminate LSCs as they commonly target rapidly cycling populations.

In AML, the origin of LSCs can be the result of accumulation of genetic disorders. It has been rationally postulated that LSCs arise from HSCs as the result of accumulation of oncogenic mutations, based on the observation that stem cells persist for long periods and undergo a number of cell divisions increasing the likelihood to obtain the minimum number of mutations necessary for malignant transformation (Lapidot, 1994; Bonnet, 1997; Warner, 2004). Alternatively, LSCs may also result from more differentiated progenitor cells that have reacquired the capacity for self-renewal and accumulated additional mutations for malignant transformation (Cozzio, 2003; Warner, 2004).

The intensive molecular investigation over the past two decades has shed new light on a large number of recurrent genetic lesions that have been identified to be associated with different subtypes of leukemias.

One of these aberrations can be attribute to *MLL* translocations. It is well documented that *MLL* gene rearrangements are able to transform myeloid non-self-renewing progenitor cells into LSCs in AML (Krivtsov, 2006; Passegue, 2006). In fact, generation of knock-in mouse models, as well as model of transduced BM experiments, has demonstrated how *MLL* fusion oncogenes are able to induce leukemic transformation in normal hematopoietic cells. First, Corral demonstrates that *Mll–AF9* fusion knock-in mouse model, that constitutively expressed MLL-AF9, develops AML (Corral, 1996). Subsequently, Collins and colleagues developed a conditional knock-in model that produced *Mll-Af9* by interchromosomal translocation, similar to the mechanism for translocation formation in human cells. This mouse model also had a propensity for leukemia development (Collins, 2000). A similar approach was used to develop a conditional *Mll–Enl* knock-in model, which also led to a rapid onset of AML (Forster, 2003). Moreover, important insights into MLL fusion-mediated leukemia development came from a study that assessed leukemogenic transformation of committed myeloid progenitors by MLL–ENL (Cozzio, 2003). A particularly important aspect of this work was the demonstration that mouse myeloid leukemias can originate not only from HSCs but also from committed myeloid progenitors that have no inherent self-renewal capabilities. As GMPs (Granulocite-Macrophage Progenitors), similar to all committed myeloid progenitors, do not possess self-renewal activity (Na Nakorn, 2002), MLL–ENL

expression appears to be able to re-activate at least some aspects of hematopoietic cell self-renewal. Expression of MLL–AF9 in GMPs leads to leukemogenic transformation of GMPs to LSCs (Krivtsov, 2006; Somervaille, 2006). Intriguingly, C/EBPα is a key myeloid transcription factor, which is required for the formation of granulocytic monocytic progenitors (GMPs) during normal hematopoiesis (Zhang, 2004). Ohlsson and colleagues, comparing human *MLL*-rearranged AML and normal progenitors gene expression profiles identified C/EBPα as a putative collaborator in MLL-rearranged AML. They found that deletion of C/EBPα rendered murine hematopoietic progenitors completely resistant to MLL-ENL–induced leukemic transformation, whereas C/EBPα was dispensable in already established AMLs. This data show that C/EBPα collaborates with MLL-ENL to activate a group of genes that, together with *Hoxa9* and *Meis1*, are responsible for the early events that transform normal hematopoietic cells into malignant cancer cells. (Ohlsson, 2014).
Therefore, these observations open important questions regarding how the deregulation of some genes and transcription factors with a role in stemness may contribute to the leukemia. Furthermore, seen that the balance of self-renewal and commitment to differentiation is dramatically deregulated in leukemic cells a better understanding at the molecular mechanism will be a fundamental source for molecular-based therapies in the future.

2.2 Characteristic of MLL-rearranged AML

Leukemias harboring rearrangements that involved the *MLL* (*Mixed Lineage Leukemia*) gene on chromosome 11q23 possesses unique biological and clinical characteristics. Genetic alterations in the *MLL* gene are associated with more than 70% of infant leukemias, but are less frequent in older children leukemias. Approximately 10% of adult leukemias bears MLL-translocations and *MLL*-related translocations are also commonly observed in secondary acute leukemias after topoisomerase inhibitor treatment (Felix CA, 1998). It is a disease characterized by an extremely dismal prognosis, in part due to its poor responses to the conventional therapeutic treatment, such as chemotherapy (Balgobind BV, 2011; Slany, 2009).

These aberrations juxtapose the amino-terminus of MLL with the C-terminus of the fusion partners, destroying the normal histone methyltransferase function of MLL adding the heterologous functions of the fusion partner (**Figure 3**).

MLL rearrangements generate a large variety of oncogenic MLL fusion proteins. To date, more than 60 different fusion partners have been identified, among which the most common ones are nuclear proteins with transcriptional activating activity (Krivtsov, 2007; Monroe, 2010; Yokoyama AL, 2010).

Wild Type (WT) MLL is a very large, 431 kDa protein with many different identified domains that mediate protein-DNA, protein-protein, or protein-RNA interactions. The MLL protein is proteolytically cleaved into an N- and a C-terminal fragment by the protease TASPASE1 (Hsieh, 2003). The MLL N-terminal fragment has a Menin binding region, 3 AT hooks, a repression domain, 4 PHD fingers, an atypical bromo-domain and a FYRN (Phenylalanine and Tyrosine Rich N-terminus) domain. The MLL C-terminal fragment has the transcriptional activation domain, a FYRC (Phenylalanine and Tyrosine Rich C-terminus domain).

The chromosomal break point region is just before PHD. Hence the fusion protein contains a portion of MLL N-terminus through the repression domain fused in frame with a C-terminal partner protein fragment. The rest of the N-terminus (from the PHD finger region to the TASPASE1 cleavage site) and the whole of MLL-C fragment is deleted in the MLL fusion protein as shown in Figure x.

Therefore, MLL fusions do not conserve the TASPASE1 cleavage site, which has been shown to cause resistance to cell cycle specific degradation (Liu, 2010).

Nevertheless, the deleted regions are frequently, but not always, represented in the reciprocal translocation product. In addition, the *MLL* gene is also involved in other aberrations such as partial tandem duplications (PTD) that occur in nearly 8% of AML patients with normal cytogenetic features (**Figure 3**).

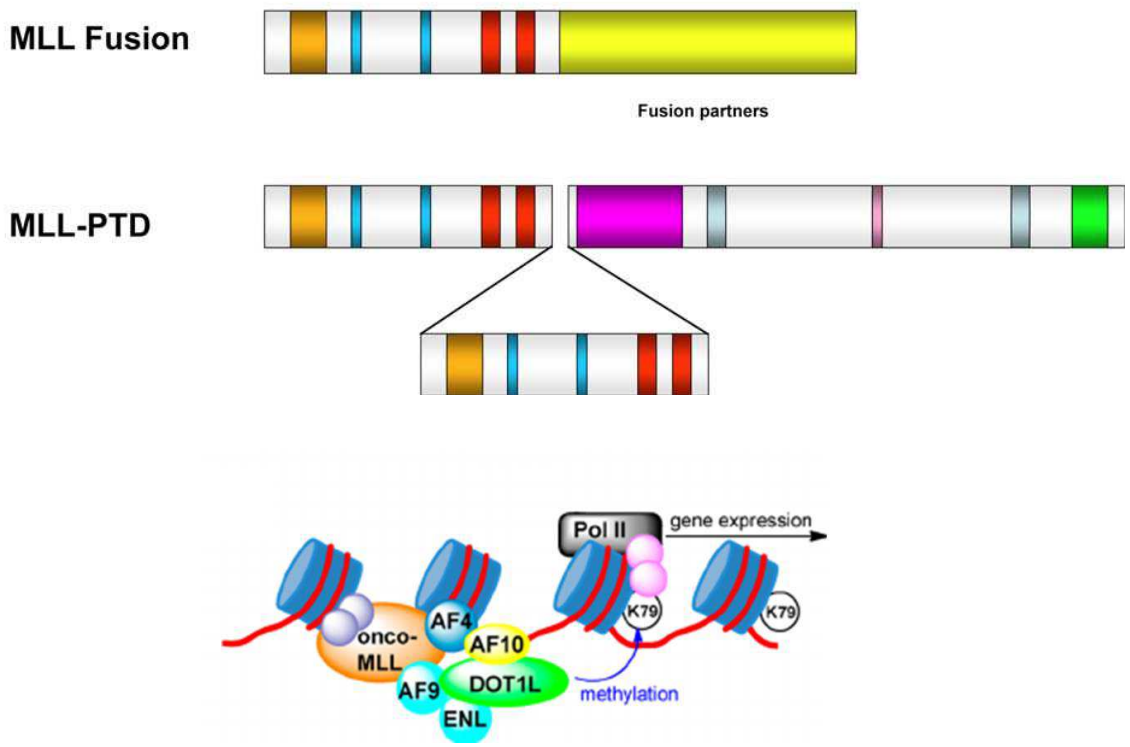

Figura 3: Structure of MLL fusions and onco-MLL complex.

Intriguingly, only six frequent partner proteins (*AF4, AF9, ENL, AF10, ELL*, *AF6*) constitute the bulk (>80%) of all clinical cases of MLL leukemia (Meyer, 2009) (**Table 1**), whereas the remaining fusions proteins were cloned each from a few isolated, mostly adult patients. These MLL rearrangements can be classified into five groups, according to differences in cellular location and putative function (**Table 1**) (Krivtsov, 2007).

	Putative function	Chr.	Fusion partner	Frequency in MLL associated leukaemia
Group 1	Nuclear, putative DNA-binding proteins	4q21 9p23 19p13.3 10p12 19p13.1	AF4 AF9 ENL AF10 ELL	>80%
Group 2	Cytoplasm, presence of coiled-coil oligomerization domain	1q32 17p13 19p13 6q27 Xq13	EPS15 GAS7 EEN AF6 AFX	>10%
Group 3	Cytoplasm, septin family, interact with cytoskeletal filaments, have a role in mitosis	Xq22 22q11 Xq24 17q25 4q21	SEPT2 SEPT5 SEPT6 SEPT9 SEPT11	>1%
Group 4	Nuclear, histone acetyltransferases	16q13 22q13	CBP P300	>1%
Group 5	MLL partial tandem duplication	11q23	N/A	4–7% of all AML with normal karyotyp

Table 1: Classification of MLL fusions. MLL rearranges with a large number of partner genes. Each rearrangement can be classified into 5 groups based on putative function and cellular location of the chimera. (Table rearranged from Krivtsov, 2007)

- The first group is characterized by fusion partner genes encoding the nuclear DNA-binding proteins AF4 (ALL1 fused gene from chromosome 4), AF9, AF10, ENL (eleven nineteen leukemia gene) and ELL (eleven nineteen lysine-rich leukemia gene). Taken all together, these aberrations account for more than 80% of *MLL* translocation in leukemia patients.
- The second group involves cytoplasmic proteins such as AF6, AFX, GAS7, EEN, AF1p and Eps15. These fusion partners are found in more than 10% MLL rearranged leukemias. The common feature of this group of protein is coiled-coil oligomerization domains that are important for their transformation potential (So, 2003).
- The third group of fusion partners includes septins (SEPT2, SEPT5, SEPT6, SEPT9 and SEPT11), which are cytoplasmic proteins playing a role in mitosis and cytoskeletal structure (Hall, 2004). This group characterize only the 2% of MLL associated leukemias.
- The fourth group is characterized by the histone acetyltransferases p300 and CBP. MLL is fused with these proteins retaining histone acetyltransferase activity,

although TA domain, which mediates interaction of p300/CBP with wildtype MLL, does not exist in MLL fusions (Ida, 1997; Hall, 2004). These MLL fusions are also observed in 2% of MLL associated leukemias.

- The fifth group contains only MLL–partial tandem duplication (MLL–PTD). MLL–PTD results from a variable number of duplication of exons 5 to 12 that are inserted before exon 11 or 12. MLL–PTD is found in 4-7% normal karyotype AML patients and also often associated with trisomy 11 abnormality (Shiah, 2002)

Approximately 50% of pediatric AML cases with an *MLL* rearrangement consist of t(9;11)(p22;q23). The other 50% predominantly include t(6;11)(q27;q23), t(10;11)(p12;q23), t(11;19)(q23;p13.1) and t(11;19)(q23;p13.3) (Raimondi, 1999) as shown in **Figure 4**.

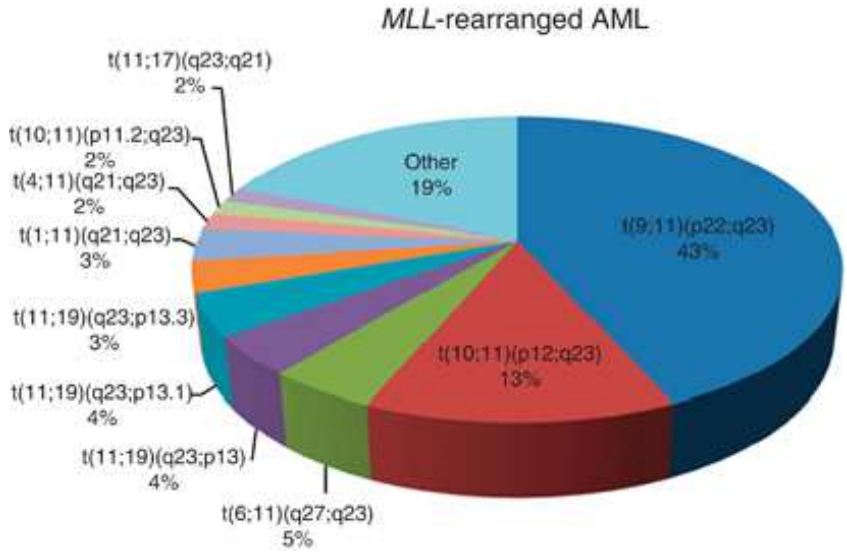

Figure 4: The distribution of translocation partners of MLL in pediatric AML. More than 60 different fusion partners of MLL have been identified, however t(9;11)(p22;q23) is the most common translocation that occurs in *MLL*-rearranged AML, accounting more than 40% of cases. Other frequent rearrangements are caused by the translocation t(10;11)(p12;q23), t(6;11)(q27;q23), t(11;19)(q23;p13) and t(1;11)(q21;q23) accounting for 13, 5, 11 and 3% of cases, respectively. Figure from (Balgobind, 2011)

Interestingly, over 80% of human AML containing *MLL*-rearrangements exhibit strong expression of

genes normally restricted to HSC including, *HoxA7, HoxA9*, *HoxA10* and *Meis1* (Afonja, 2000; Ferrando, 2003; Lawrence, 1999). Some of them, generally genes highly expressed in human AML with MLL-AF9 translocation, have been demonstrated to regulate survival and self-renewal of LSCs (Faber, 2009; Wong, 2007). Among them, *HoxA9* and *Meis1* represent the best-characterized *MLL* downstream target, which take part of a wider transcriptional program critical for self-renewal of MLL LSCs. Therefore, identification and functional characterization of target genes directly or indirectly regulated by MLL fusions, is pivotal to understand the origin of the MLL-related leukemia as well as the abnormal function of HSCs, which will be crucial in order to develop new molecular-based therapies.

3. MLL GENE IS REQUIRED DURING DEVELOPMENT AND HEMATOPOIESIS

MLL is the mammalian homolog of the Drosophila protein found in trithorax and is a member of the evolutionarily conserved trithorax group (trxG) family of proteins that positively regulate gene transcription and act antagonistically to the Polycomb group (PcG) proteins (Djabali, 1992; Gu, 1992; Ringrose, 2004). It belongs to the MLL family of SET domain containing histone methyltransferases that methylates histone H3 on lysine 4 (Milne, 2002; Shilatifard, 2012). The H3K4Me3 mark at gene promoters is associated with active transcription (**Figure 5**). In fact, MLL positively regulates the expression of target genes including multiple *homeodomain* (*Hox*) genes through H3K4 methylation of gene promoters (Milne, 2002). *Hox* genes are transcription factors that participate in the development of multiple tissues, including the hematopoietic system (Abramovich, 2005). Mouse models have conclusively shown *Mll* to have a crucial role in the control of *Hox* gene expression and in the development of the axial skeleton and hematopoietic systems of mammals. In fact, homozygous deficiency for MLL results in early embryonic lethality at embryonic day 10.5 (E10.5), exhibiting multiple patterning defects, and

heterozygous deletion of MLL incurs homeotic transformation, indicating altered *Hox* gene expression (Yu, 1995; Yagi, 1998; Ayton, 2001). Other experiments on Mll-deficient mice demonstrated that ESCs without Mll were unable to differentiate into any hematopoietic cell types in adult animals or in the fetal liver (Ernst, 2004a). Moreover, Ernst and colleagues, using an *in vitro* system, have demonstrated that the block in hematopoietic development was accompanied by global reduction in *Hox* gene expression and could be rescued by the reintroduction of individual *Hox* genes (Ernst, 2004).

In other experiments conducted in adult mice, the conditional ablation of Mll demonstrated its critical role to maintain adult hematopoietic stem cells (McMahon, 2007). Despite interaction with active transcription factors, MLL interacts also with multiple proteins that suppress gene expression, such as histone deacetylase 1 (HDAC1) and HDAC2, CYP33, PcG proteins PC2 and CTBP (Xia, 2003).

Besides its known role in embryonic development and hematopoiesis, MLL also orchestrates cell cycle progression at least through regulating the expression of cyclins and CDK inhibitors (Milne, 2005-B; Xia, 2005; Takeda, 2006; Kotake, 2009).

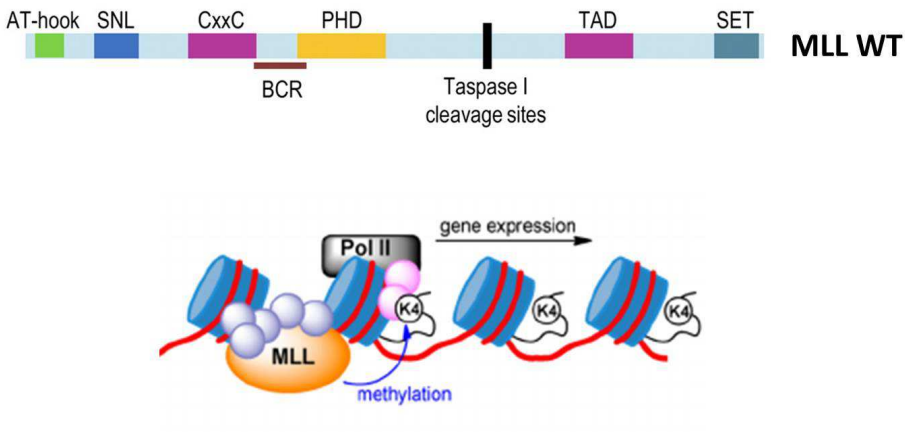

Figure 5: Structure of wild type MLL and the MLL complex. MLL is a very large, 431 kDa protein with many different identified domains that mediate protein-DNA, protein-protein, or protein-RNA interactions. MLL is part of a large chromatin modifying complex in which the SET domain of MLL has histone methyltransferase and histone acetyltransferase activity. During the formation of this complex, MLL

protein is proteolytically cleaved into an N- (MLL-N) and a C-terminal (MLL-C) fragment by the protease Taspase I. The MLL-N fragment has a Menin binding region, 3 AT hooks, a repression domain, 4 PHD fingers, and a CxxC domain. The MLL-C fragment has the transcriptional activation domain, and associates with histone acetyltransferases to ensure histone modification and methyltransferase activity. The chromosomal break point region is just before PHD.

3.1. MLL-rearranged AML is a leukemic stem cell disease

The mixed lineage leukemia is a subtype of leukemia that arises in particular in infants. It seems thus likely that the translocation occurs in uterus (Eguchi, 2006). In fact environmental factors to which the fetus is exposed in utero may have an important role in the development of MLL-rearranged AML, which seem to occur because of inappropriate non-homologous end joining of double-strand breaks (Aplan, 2006). Normal MLL associates with other factors to activate the transcription of many genes. The MLL-N is involved in target selection and is retained in the fusion proteins: for this reason it seems that MLL fusions will share many target loci with wild type MLL. This assumption has been confirmed for the clustered *HOX* homeobox genes that are under control of MLL as well as of MLL fusion proteins. Remarkably, it has been demonstrated that expression of an *MLL* fusion gene *(MLL-AF9*) GMPs induces a ‘‘HSC stem cell-like’’ signature that includes various *HOX* genes (Krivtsov, 2006). The acquisition of a stem cell signature by leukemic GMPs may contribute to self-renewal of leukemia stem cells. The *HOX* factors, together with their cofactor *Meis1*, positively regulate the pool size of HSC and lineage-specific hematopoietic progenitors by promoting cellular proliferation as well as arresting cellular differentiation (Azcoitia, 2005; Lawrence, 2005; Wang, 2005). The dysregulation of *Hox* genes and *Meis1* was directly linked to malignant hematopoiesis. In normal hematopoiesis, the expression pattern of the *Hox* genes and *Meis1* is dynamic, present at high levels in stem cells and early precursors and quickly down regulated as progenitors differentiate (Sauvageau, 1994). Therefore, a continuous ectopic *HOX* expression will block differentiation and create a rapidly proliferating pre-leukemic precursor pool (**Figure 6**). There are many other genes regulated by *MLL*-

rearrangements, but for certain, *HOX* deregulation is the most important factor for MLL fusion induced leukemogenesis (Milne, 2005; Zeisig, 2004; Horton, 2005; Ernst, 2004).

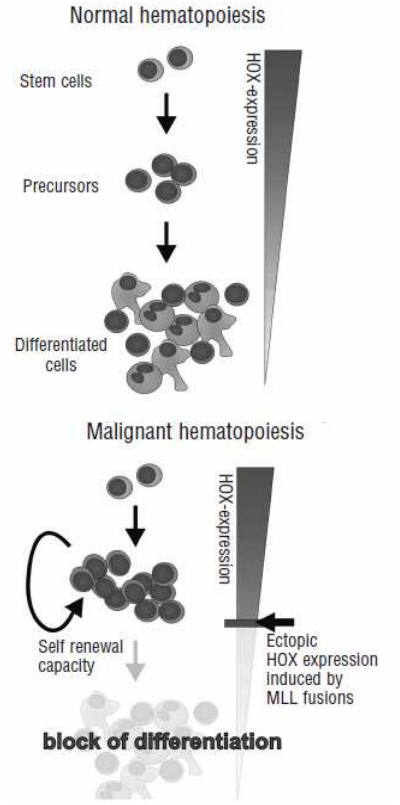

Figure 6: The role of HOX as well as Meis1 proteins in control of hematopoiesis. Transcription factors, as Hox genes and Meis1 control hematopoietic differentiation. The expression of these genes must be finely downregulated during differentiation. Therefore presence of ectopic factors, such as MLL chimeras, will deregulate expression of these genes causing a block of maturation and promoting a self-renewing of precursor cells (figure adapted from (Slany RK, 2009)).

There are accumulating evidence that suggest how the cell of origin , in which a genetic lesion occurs, can contribute to the emergence of distinct tumor subtype. In particular, Armstrong's group demonstrates that there is a difference in MLL-AF9-mediated leukemia, depending if the cells of origin are HSCs or GMPs (Krivtsov, 2013). In particular,

they demonstrate how MLL-AF9 transformed HSCs are more aggressive than MLL-AF9 transformed GMPs. Functionally defined LSCs are immunophenotypically similar, but they have differences in gene expression and DNA methylation that are determined by the cell of origin. The Gene expression profile resulting from this study show how leukemia that originates from HSCs retains enhanced expression of a set of "stem cell associated" genes. Therefore, the expression of MLL-AF9 in GMPs activates a stem cell associated program, but the expression of MLL-AF9 in HSCs allows maintenance of a more extensive stem cell-derived program that influences the LSCs behavior. All these findings indicate that many genes involved in self-renewal process in normal HSCs are shared in LSCs and allow the maintenance of a stem cell profile in MLL-related leukemia. Thus, investigating genes involved in the differentiation block is a fundamental in order to understand the basis of the aggressiveness of this disease.

4. ZNF521

The Zinc Finger Protein (ZNF521) (also known as early hematopoietic zinc finger protein (EHZF); EVI3 or Zfp521 in mouse) is a transcription factor with 30 Krüppel-like zinc finger (ZF) domains and contains an N-terminal 12-amino acid motif that interacts with the nucleosome remodelling and histone deacetylation (NuRD) complex, which is conserved among other ZF transcriptional repressors, including FOG-1, FOG2, BCL11A and SALL family members (Bond, 2004; Lin, 2004), Initially, ZNF521 was identified in a comparative analysis of the transcriptional profile between human $CD34^+$ hematopoietic progenitors and mature peripheral blood leukocytes (Bond HM, 2004). This analysis has revealed that *ZNF521* is abundantly expressed in human CD34+ progenitors and then declines rapidly during cytokine-driven differentiation (Bond, 2004). However, it has been shown that ZNF521 is also widely expressed all along the body including brain, muscle, heart, kidney, spleen, lymph nodes, placenta, thymus, fetal liver and bones (Bond, 2004). In particular, it is highly expressed in mesenchymal condensations, in prehypertrophic chondrocytes in the growth plate as well as in osteoblasts and osteocytes during endochondral bone development (Hesse, 2010; Liu TM, 2013). In all these cell types, it

appears to control cell differentiation, as well as the function of mature cells by modulating the activity of specific transcription factors. Previous studies in hematopoietic cells have shown that ZNF521 negatively regulates Early-B Cell Factor 1 (EBF1) activity via its C-terminal domain, thus influencing B cell differentiation (Mega, 2011). It has been also shown that ZNF521 through its NuRD interaction suppresses the GATA-1 activity during erythropoiesis (Hong, 2005). Being that GATA-1 a master regulator of erythroid cell maturation by activating erythroid-specific genes and repressing genes associated with the undifferentiated state, the activity of ZNF521 may be critical for the differentiation of hematopoietic progenitors.

Recently studies, have also demonstrated that other transcription factors such as PU.1 and HOXC13, can synergistically cooperate to regulate *ZNF521* expression (Yu, 2016). Yu and colleagues demonstrate that transgenic mice over-expressing *Hoxc13* and *Pu.1* also have increased *Zfp521* expression in the fetal liver, the site of B-cell differentiation during development.

Thus, in a hematopoietic context these observations lead to speculate that ZNF521 could play a critical and specific role in human hematopoiesis. This latter, it is also corroborate by the observations that ZNF521 expression is frequently deregulated in hematopoietic malignant cells. Early studies have shown that *ZNF521* mRNA is highly expressed in several AML samples (FAB M2, M3 and M4) as well as CMLs (Bullinger, 2004; Bond, 2004). Intriguingly, in a study conducted on 363 adult acute leukemia was found that *MLL* rearrangements associate with high levels of ZNF521 (Kohlmann, 2005). Moreover, it has been described in a case of B cell-progenitor ALL, a translocation resulting in the fusion of *PAX5* gene with *ZNF521* gene (Mullighan, 2007) and most recently was identified ZNF521/Zfp521 as a partner to develop B-lineage ALL positive for both E2A-PBX1 and E2A-HLF chimeric gene product (Sera, 2016).

Collectively, these findings demonstrate that a deregulated expression of *ZNF521* may contribute to leukemic transformation

AIM OF THE THESIS

MLL-fusion proteins are potent inducers of oncogenic transformation, and their expression is considered to be the main oncogenic driving force in approximately 10% of AML patients. These oncogenic fusion proteins activate specific set of genes, of which the overexpression of *hoxa9* and *MEIS1* has demonstrated to play a synergistic causative role in *MLL* leukemogenesis. Since that both *HOXA9* and *MEIS1* play an important role in the HSC self-renewal/proliferation, we asked whether others transcriptional regulators of HSCs might have relevance in MLL-induced leukemia.

By gene set enrichment analysis (GSEA) we compared the publically datasets for genes normally upregulated in HSCs (CD133+) to our pediatric AML patients. The use of GSEA analysis allowed us to identify ZNF521 as a new gene among the well-known genes such as HOXA9 and MEIS1 in AML patients carrying MLL rearrangements. ZNF521 encodes for a transcription factor that in normal and malignant hematopoiesis has initiate to be studied and its misexpression expression have been causally linked to acute leukemia.

The aim of the present study was to establish whether the identified *ZNF521* gene is required in *MLL* leukemogenesis. In order to achieve this, we investigated the role of ZNF521 in *MLL*-rearranged AML, through the study of the knockdown of *ZNF521* in a series of human *MLL*-rearranged cell lines and patient-derived xenograft cells. We analyzed the contribution of *ZNF521* to leukemogenesis by a series of functional and mechanistic studies and investigated the *ZNF521*-dependent molecular pathway using as model THP-1 AML cell line.

MATHERIALS AND METHODS

1. PATIENT SAMPLES AND CELL LINES

All of the pediatric AML patient samples were obtained at the time of diagnosis from the University-Hospital of Padua and stratified according to the AIEOP AML 2002/01 protocol AML 2002/01 (Pession, 2013). Patient characteristics are listed in **Table 1**.

Non-*MLL*-rearranged (*n* = 34)		***MLL*-rearranged (*n* = 16)**	
normal karyotype	13 (38%)	t(10;11)(*MLL-AF10*)	5 (31%)
t(15;17)(*PML-RARα*)	6 (18%)	t(9;11)(*MLL-AF9*)	5 (31%)
inv16(*CBFB-MYH11*)	3 (9%)	t(6;11)(*MLL-AF6*)	3 (19%)
t(8;21)(*AML1-ETO*)	8 (23%)	11q23 others *	3 (19%)
FLT3-ITD +	4 (12%)		

Table 1: Genotype features of the 50 childhood AML patients analyzed for *ZNF521* expression by qRT-PCR analysis.

*t(11;19)(*MLL-ENL*) *n*=1; t(11;19)(*MLL-ELL*) *n*=1; t(X;11)(*MLL-SEPTIN6*) *n*=1

Seven BM samples from healthy donors were obtained as control. All human myeloid cell lines (THP-1, NOMO-1, OCI-AML4, ML2, HL60, K562, Kasumi-1, NB4, U-937, SEM and REH) were obtained from DSMZ (Braunschweig, Germany) and 293T cells were obtained from ATCC (Manassas, VA, USA). All cell lines were maintained under standard conditions suggested by the manufacturer.

2. QUANTITATIVE REAL TIME PCR

Total RNA was extracted with Trizol reagent (Invitrogen) and reverse transcribed into cDNA using the Superscript III First-Strand Synthesis System (Life Technology). The mRNA levels of *ZNF521*, *PU.1*, *C/EBPα*, *HOXA9* and *MEIS1* were measured by quantitative RT-PCR (qRT-PCR) with SYBR green on an AB 7900HT real time system (Applied Biosystem) using the comparative C_t method and the *GAPDH* gene expression as internal control (Schmittgen, 2008). The primer sequences for quantitative qRT-PCR are listed in **Table 2**.

Sequence of the primers used in this study

Primers for qRT-PCR

Gene name	Forward 5' → 3'	Reverse 5'→ 3'
GAPDH	AGGGCTGCTTTTAACTCTGGT	CCCCACTTGATTTTGGAGGGA
ZNF521	ACTGAAGTTTGGCAGGAGAG	TGGGATATTCAGGTTCATGTT
PU.1	AGAAGACCTGGTGCCCTA	CCAGTAATGGTCGTCATGGC
C/EBPα	AACATCGCGGTGCGCAAGAG	TTCGCGCTCAGCTGTTCCA
HOXA9	AAAACAATGCTGAGAATGAGAGC	TATAGGGGCACCGCTTTTT
MEIS1	TGATCAGCAGGCAAAGATTG	CATACTCCCTGGCATACTTTG

Primers for ZNF521 promoter^

Gene name	Forward 5' → 3'	Reverse 5'→ 3'
ZNF521 P1	AGCTGCTAGCACATTAAACTATACCAAAGA AATCCA	AGCTAAGCTTAAAACTATACTCAGTTCCCAGT TCC
ZNF521 P2	AGCTGCTAGCTCCATGTGACGTTCTTAAATG C	AGCTGCTAGCTTAGCCACTGCAGAAAGGTAA A
ZNF521 P3	AGCTGCTAGCTGACGTTCTCATTGTAGCTG GT	AGCTAAGCTTATGAAGCCAAAGCCATCATC
ZNF521 P4	AGCTGCTAGCTCCAGGCAGTTTACAGGTTA GA	AGCTGCTAGCCTGTACGTAATCACTGAGGAA ATCAT
ZNF521 P3.1	AGCTGCTAGCTGACGTTCTCATTGTAGCTG GT	AGCTAAGCTTATGAAGCCAAAGCCATCATC
ZNF521 P3.2	AGCTGCTAGCAAGTTGCTGCATTCTGCTCA	AGCTAAGCTTTTCCTTTCGTGTGGTAGCC
ZNF521 P3.3	AGCTGCTAGCTATCACACATAACTTGGGAC CAC	AGCTCTCGAGGTGGAAATTAAGAGATTCAGA ATACG

Primers for ChIP analysis

Gene name	Forward 5' → 3'	Reverse 5'→ 3'
HOXA9	AATGCGATTTGGCTGCTTTTTTATGGC	TCAAATCTGGCCTTGCCTCTG
ZNF521 p3.3	ACACATAACTTGGGACCACAC	GTCTAACCTGTAAACTGCCTGGA

shRNA sequences in Mission shRNA pLKO.1-puro-CMV-TurboGFP

shZNF521_9004	GTACCGGATCACTTGAAGATCCACTTAACTCGAGTTAAGTGGATCTTCAAGTGATTTTTTTG
shZNF521_9710	CCGGACAAGTTGCAGCAGCATATTTCTCGAGAAATATGCTGCTGCAACTTGTTTTTTG
shScram	GGACAAGTTGCAGCAGCATATTTCTCGAGAAATATGCTGCTGCAACTTGTTTTTT

Table 2: List of Primers used in this study. ^The restriction enzyme sites are underlined.

3. LENTIVIRAL SHRNA VECTOR, TRANSDUCTION AND FACS-SORTING

For knockdown studies, two shRNAs against *ZNF521* and a control scrambled shRNA (shScram) were used (Mission pLKO.1-puro-CMV-TurboGFP system, Cat Number TCRN0000229710 and TCRN0000229004, Sigma-Aldrich) (see **Table M2** for shRNA sequences). Lentiviral cell transduction was performed as described previously (Indraccolo, 2002). After culture in fresh medium and 96 hours after infection, GFP-positive cells were sorted using a MoFlo XDP cell sorter (Beckman Coulter) and used for further experiments. Alternatively, cells were gated for GFP expression and subjected to flow cytometry analyses. *ZNF521* knockdown efficiency was measured by qRT-PCR and western blot analyses.

4. PLASMIDS CONSTRUCTS, TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

pMSCV-neo-Flag-MLL-AF9, MSCV-PML-RARA-IRES-GFP, MSCV-AML1-ETO-GFP, pCMVMLL-3xFlag and pCMVMLL-ENL-3xFlag have been previously described (Abdul-Nabi, 2010; Liu, 2007; Tan, 2011). Flag-tagged proteins were previously verified by Western blot with anti-Flag M2 antibody (Sigma), as well as the GFP-tagged proteins by expression of green fluorescence protein (GFP) *in vitro*.

Wild-type *ZNF521* promoter from -4493 to + 65 relative to the transcription start site (TSS) was divided in four genome fragments [P1 (-3810 to 4993), P2 (-2560 to 3970), P3 (-1060 to 2729) and P4, +62 to -1260)] and each one was isolated from a genomic DNA obtained from a pool of buffy coat by PCR amplification using primer pairs containing specific restriction sites. All PCR products were purified, sequenced and cloned in TOPO TA cloning kit (Invitrogen). P1 and P3 plasmids were *Xho*I/*Hind*III-digested and the fragments were recloned into *Xho*I/*Hind*III sites downstream of the luciferase gene of the pGL42.8 luciferase vector (Promega) to generate *ZNF521P1-luc* and *ZNF521P3-luc* constructs, respectively. Similarly, P2 and P4 plasmids were *Nhe*I/*Xho*I-digested and the fragments inserted into *Nhe*I/*Xho*I sites of pGL42.8 vector to generate *ZNF521P2-luc* and *ZNF521P4-luc* constructs, respectively. The genome fragment P3 (-1060 to 2729) was subdivided in three smaller parts [P3.1 (-2101 to -2729), P3.2 (-1533 to -2143) and P3.3 (-1060 to -1610)] and each part was PCR amplified from genomic DNA using primers containing specific restriction sites and cloned in TOPO TA vector. P3.1 and P3.2 plasmids were *Xho*I/*Hind*III-digested and the fragments were recloned into *Xho*I/*Hind*III sites of pGL42.8 vector to generate *ZNF521P3.1-luc* and *ZNF521P3.2-luc* constructs. P3.3 plasmid was *Nhe*I/*Xho*I-digested and the fragment recloned into *Nhe*I/*Xho*I-digested pGL42.8 to generate *ZNF521P3.3* construct. (Primers used for genomic DNA amplification are listed in **Table M2**).

For luciferase assay, 293T cells were cotransfected with 0.5 µg of the reporter plasmid, 1 µg of expression plasmid or empty vector and 0.5 µg of *Renilla* luciferase reporter vector (Promega) as internal control for normalization of transfection efficiency, for a total of 2 µg of combined plasmids per well. The cells were then harvested at 48 hours after

transfection using a Dual-Luciferase reporter assay system (Promega) and the Victor3 TM 1420 Multilabel Counter (PerkinElmer). Data are presented as the mean ratio for triplicate experiments.

5. CHROMATIN IMMUNOPRECIPITATION (CHIP) AND PCR DETECTION

ChIP assay was performed using the Imprint Chromatin Immunoprecipitation kit (Sigma), according to the manufacturer's protocol with minor modifications. Briefly, 293T cells (3.5×10^6 cells) were transfected with 10 μg of Flag-MLL-AF9 or Flag-MLL-ENL expression plasmids. 48 hours post transfection, were cross-linked with 1% formaldehyde (Sigma) for 15 minute at room temperature. Subsequently, the lysed cells were isolated and sonicated on ice to shear DNA into fragments of 200 bp to 1 kb. Then, the chromatin complexes were incubated into pre-treated Stripwells (Sigma) with anti-Flag M2 monoclonal antibody (Sigma), or normal mouse IgG (Sigma) as indicated. The input DNA was isolated from sonicated lysates before immunoprecipitation as a positive control. Purified DNA was then resuspended in TE buffer (10 mM Tris-HCL and 1 mM EDTA, pH 8.0) for PCR. ChIP assay from 2 $x10^6$ of *MLL-AF9*-expressing NOMO-1 cells or HL60 cells was performed as above reported using a N-terminal MLL monoclonal antibody (Santa Cruz Biotechnology) or a C-terminal MLL polyclonal antibody (Sigma) or a mouse IgG (Sigma) as indicated. Purified ChIP DNA was amplified by regular PCR. Primers amplifying the ZNF521 promoter region and the HOXA9 promoter region used for the ChIP PCR are listed in **Table 2**.

6. MICROARRAY ANALYSIS

Total RNA from sorted THP-1 cells transduced with shRNAs was isolated using Trizol as above reported and processed for microarray analysis using the Affymetrix GeneChip

3'IVT express Kit (Affimetrix) after RNA quality control using Agilent 2100 Bioanalyzer (Agilent). Gene expression profile was performed using a Human Genome U133 2.0 Plus chip (Affymetrix), as previously described (Bresolin, 2010). The data were RMA-normalized using R software (http://www.r-project.org/) with BioConductor package (www.bioconductor.org). Shrinkage t test was used to identify differentially expressed genes between shScram and shRNA ZNF521 THP-1 cells selected with a local FDR <0.05 (FDR). Hierarchical clustering analyses were performed using Euclidian distance and Ward's methods. Gene set enrichment analysis (GSEA) was performed using GSEA version 2.0 software (Broad Institute; http://www.broadinstitute.org/gsea) with genes ranked by difference of class and statistical significance by 1000 gene set permutations. Gene set permutation was used to enable direct comparisons between shScram and shRNA ZNF521 results (<7 replicates). Median of probes was used to collapse multiple probe sets to a single value per gene for each sample. Gene sets with a FDR <0.05 were declared to be statistically significant. The microarray gene expression data have been submitted in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) under accession GSE79110

7. WESTERN BLOT AND IMMUNOFLUORESCENCE STAINING

Western blot and immunofluorescence were performed using standard procedures. The antibody against ZNF521 was from Novus Biologicals (72009). Gamma-Tubulin (T6557), Actin (A5316), Flag M2 (F3165) antibodies were from Sigma Aldrich. For immunofluorescence analysis, antibody against CDKN1A/p21 (2947S) was from Cell Signaling and antibody against CDKN1B/p27 (610241) was from BD Biosciences.

For immunofluorescence analysis, 5×10^4 FACS-sorted cells were harvested 7 days after transduction and cytocentrifuged onto slides at 500 rpm for 5 minutes (Cytospin 4 cytocentrifuge, Thermo Scientific). Cells were fixed in 4% formaldehyde for 15 minutes, permeabilized with PBS containing 0.1% Triton X-100 (Sigma) for 10 minutes, and blocked with 5% BSA in PBS for 30 minutes at room temperature, followed by incubation

with primary antibodies in 1% BSA-PBS overnight at 4°C. The following day, slides were washed with PBS and incubated with the secondary antibody Alexa Fluor 594 Goat Anti-mouse IgG (H+L) (1:2000, Life Technologies) for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:10000; Sigma) for 13 minutes. A minimum of 50 cells/sample was scored in three different fields in at least three independent cytospin preparations. Cells were visualized and counted by a confocal microscope (Vico, Eclipse Ti80, Nikon) equipped with a digital camera. Images were captured with ImageProPlus software (Media Cybernetics).

8. CELL FUNCTION ANALYSIS

8.1 Cell viability and clonogenic assay

Viability assay was performed using the colormetric diphenyltetrazolium bromide (MTT) Cell Proliferation Kit I (Sigma) and measured via Victor 3 Microplate reader (PerkinElmer). Briefly, FACS-sorted GFP positive cells (5×10^4) from cell lines and/or *ex vivo* primary patient-derived cells were collected 4 days after transduction and seeded (in triplicates) in 96-well culture plate. At a designated time point, cell viability was measured by adding 10 µl of MTT solution (5 mg/ml) to each well. After 3 hours of incubation, the absorbance (OD) of each well was measured at 570 nm using a microplate reader (Victor 3; PerkinElmer). Cell proliferation was calculated by the following formula: (mean OD ZNF521-shRNA wells)/(mean OD shScram-control wells) × 100%. For the clonogenic assay, 2×10^3 cells at day 4 of transduction were FACS-sorted and then resuspended and seeded in methylcellulose medium (MethoCult H4534; Stem Cell Technologies). After 14 days, colonies were counted under 20x magnification with a stereoscope after exposure to MTT (Sigma) for 3 hours at 37° C.

8.2. Cell cycle, apoptosis assay

Cell-cycle analysis was performed by flow cytometric analysis of propidium iodide-stained cells at 7 days after shRNA transduction. Briefly, 2.5×10^5 cells were washed in PBS and then fixed in 70% ethanol and put at -20°C overnight. The samples were rehydrated in cold PBS, treated with lysis buffer containing RNase and 0.1% Triton X-100 (Sigma) and stained with propidium iodide (PI). Cell cycle distribution was measured on gated GFP-positive cells. Analyses were performed using the Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA) and MultiCycle Cell Cycle Analysis Software (Phoenix Flow Systems, San Diego, CA). A minimum total of 10.000 gated events were collected for each sample. Cell death was measured at day 4 and day 7 after transduction using AnnexinV-Fluos staining kit (Invitrogen) and analyzed by flow cytometry. Briefly, 2.5×10^5 cells were resuspended in 100 µl of 1x annexinV-binding buffer and incubated with 1 µl of allophycocyanin-conjugated annexinV (Invitrogen) and 1 µl of DAPI (1:10000, Sigma) for 15 minutes at room temperature. The apoptotic cells ($AnnexinV^+/DAPI^-$) were determined on gated GFP-positive cells as described above.

8.3. Expression of CD11b and CD14, morphological analysis and cell differentiation induction

PE-conjugated anti-CD11b (BD Biosciences) and Phycoerythrin-Cyanin (PC7) anti-CD14 (Beckman Coulter) were used to analyze myeloid differentiation. Briefly, on day 7, 2×10^5 transduced cells were harvested, washed and labeled with conjugated antibodies in PBS for 15 minutes in the dark. Then, cells were washed and analyzed by flow cytometry of gated GFP-expressing cells as previously reported. The expression of the cell surface markers was analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA). Cell morphology was determined on cytospin preparations. Four days after transduction, GFP positive cells were FACS-sorted and placed in culture. Three days later (7 days post-transduction), 0.5×10^5 cells were harvested and washed in PBS buffer and

spun onto slides for 5 minutes at 500 rpm using a Shandon CytoSpin4 cytocentrifuge and the slides were stained with a Wright-Giemsa stain. Images were taken at 40x magnification using a Nikon microscope (Vico, Eclipse Ti80, Nikon) and acquired with ImageProPlus software (Media Cybernetics). For the differentiation induction with ATRA (1μM; Sigma) or Securinine (15 μM; Sigma), after 3 days of treatment cells were collected and analyzed for cell morphology, level of ZNF521 transcripts and protein expression as previously described.

9. STUDIES WITH AML PATIENT-DERIVED XENOGRAFT CELLS

Primary *MLL-AF9*-expressing cells were obtained from BM samples of diagnosed AML pediatric patients stored in the BioBank of the laboratory of Pediatric Hematology of the University Hospital of Padua, (Italy) according to the guidelines of the local ethics committee. Initial AML xenografts were established by tail vein injection with 8×10^6 primary cells suspended in 300 μl of PBS in 6- to 8-week-old NSG mice, which were purchased from Charles River (Wilmington, MA, USA). All animal experiments were performed in accordance with institutional guidelines and established protocols (Agnusdei, 2014). Engraftment was monitored by weekly blood collections and flow cytometry analysis with antihuman CD45 (BD Biosciences). The engraftment rate was defined by the number of days required for the transplanted human CD45+ cells to reach at least 20% in the peripheral blood. Human leukemic cells from the spleens of engrafted mice were collected and cultivated in RPMI supplemented with 10% Human serum (Euroclone), antibiotics, and cytokines SCF, FLT-3L and TPO (40 ng/ml for each), IL-3 and IL-6 (20 ng/ml for each). (All cytokines were obtained from Inalco, Milan, Italy). For *ex vivo* experiments, two independent biological replicates were performed.

10. DATA ANALYSIS

Data are presented as mean ± SD. Each experiment was performed at least 3 times, except where stated otherwise. The differences were examined using 2-tailed *t* test, Mann–Whitney *U*-test or kruskal-Wallis one-way analysis of variance followed Dunn's test as appropriate (GraphPad Prism; GraphPad). Results were considered significant at $P<0.05$.

RESULTS

1. GSEA analysis of upregulated stemness genes in pediatric AML

One of the most important features of AML carrying *MLL* rearrangements is that to show an up-regulation of stem cell-associated genes including *HOXA* and *MEIS1*. In order to identify other uncharacterized stemness genes, we interrogated public signatures based on CD133+ cell populations isolated from normal/healthy versus gene expression profiles of 82 pediatric AML samples (61 with normal MLL and 21 with MLL rearrangements) previously analyzed. As shown in Figure X, among the canonical upregulated genes, whose overexpression are hallmarks of *MLL*-rearranged AML, we observed the *ZNF521* gene. Interestingly, ZNF521, which encodes a transcription factor, is a marker of HSC (Matsubara, 2009) with potential role in the regulation of HSC homeostasis, but with unknown function in *MLL*-mediated leukemogenesis.

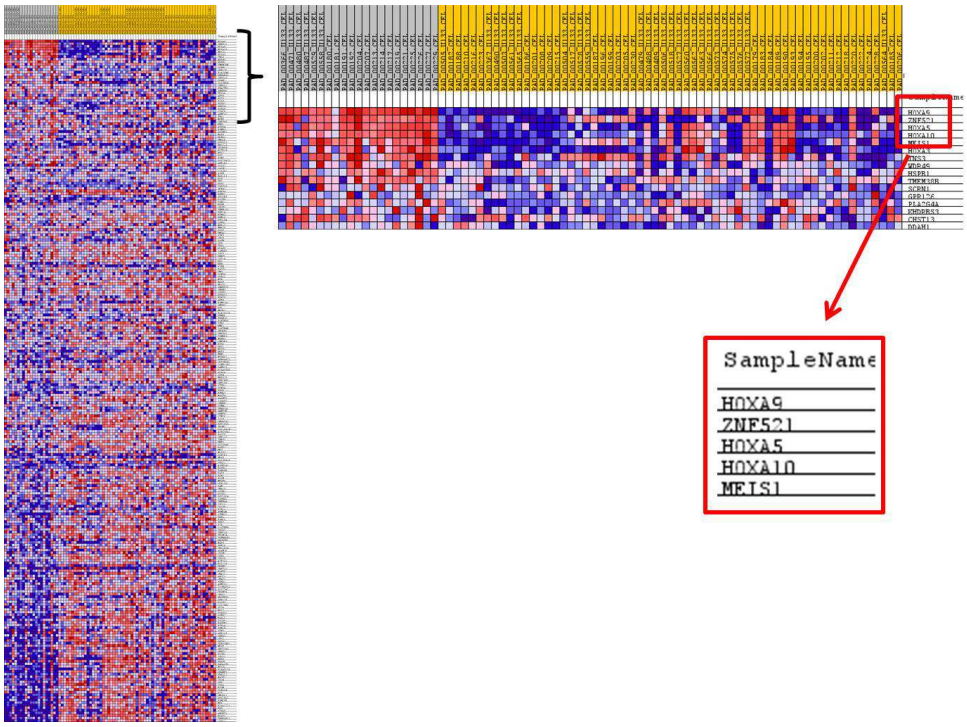

Figure 7. Gene Set Enrichment Analysis (GSEA). Heat map displaying the Jaatinen hematopoietic stem cell_upregulated gene in CD133+ versus CD133- normal cells in 82 AML pediatric samples including 21 MLL-rearranged. among the top five most differentially expressed genes (red box), ZNF521 appears among the genes most upregulated in aml samples with MLL rearrangements (gray lanes) than AML sample without MLL rearrangements (yellow lanes).

2. ZNF521 *IS ABERRANTLY OVEREXPRESSED IN PEDIATRIC* MLL-*REARRANGED AML*

Previously, by use of microarray analysis, we found a frequent ZNF521 overexpression in pediatric AML with MLL rearrangements (Pigazzi, 2011). To validate these data and analyze the relationship between ZNF521 expression and distinct *MLL*-fusion genes, we performed quantitative real-time PCR (qRT-PCR) in an independent cohort of 50 pediatric AML patients (16 *MLL*-rearranged and 34 non-*MLL*-rearranged; **Table 1**) and 7 normal bone marrow (BM) controls. We found that *ZNF521* was expressed at significantly higher

level in AML patients with *MLL* rearrangements compared to non-rearranged AML and normal controls (P<0.001, **Figure 8**).

Figure

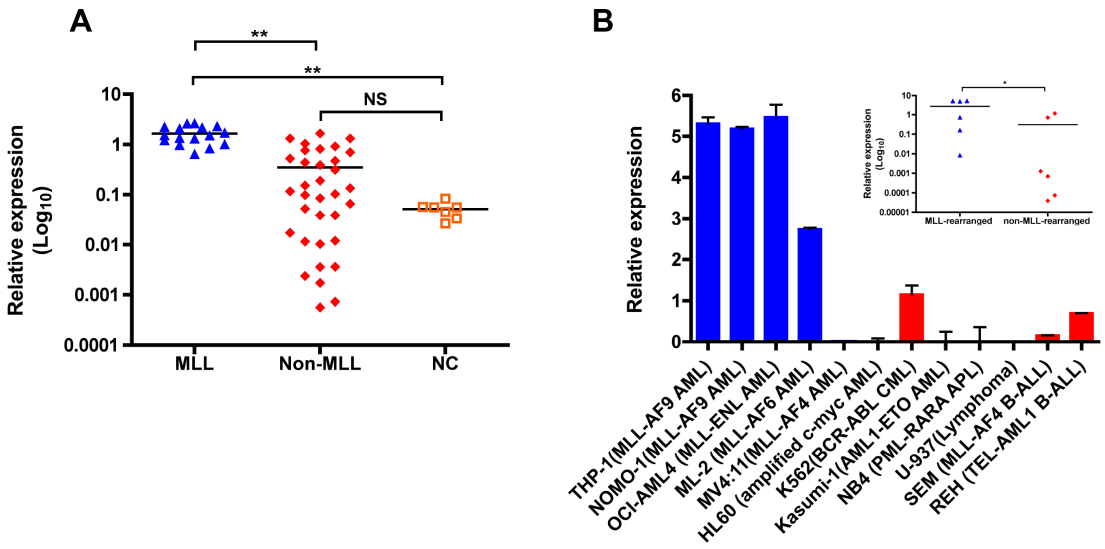

Figure 8. *ZNF521* is aberrantly overexpressed in *MLL*-rearranged AML. (A) qRT-PCR for the expression of *ZNF521* in 16 *MLL*-rearranged AML (MLL), 34 non-*MLL*-rearranged AML (Non-MLL) and 7 normal control (NC). The results are normalized to *GAPDH* and analyzed by 2−ΔCt method. NS, not significant, ***P*<0.001, kruskal-Wallis test. (B) qRT-PCR analysis of *ZNF521* expression in a representative panel of human leukemic cell lines normalized to *GAPDH* and analyzed by 2−ΔCt method. Data are represented as mean ± SD of three independent experiments. y axis is linear. Inset, dot plots of mean *ZNF521* mRNA levels in *MLL*15 rearranged and non-*MLL*-rearranged cell lines from data presented in (B). **P*<0.05, Mann–Whitney *U*-test.

The analysis of *ZNF521* expression between the most frequent *MLL* rearrangements detected in pediatric AML did not reveal significant difference based on *MLL* fusion partners (**data not shown**). In addition, we analyzed the expression of *ZNF521* in 6 *MLL*-rearranged and 6 non-*MLL*-rearranged human leukemic cell lines. Similarly, leukemic cell lines with *MLL* rearrangements, with the exception of those carrying *MLL-AF4* fusion

transcripts, showed significantly higher *ZNF521* mRNA levels compared to cell lines with other abnormalities (P<0.05, **Figure RB**). Thus, our data indicate that ZNF521 is likely involved in *MLL*-mediated transformation in AML.

3. ZNF521 *DEPLETION REDUCES CELL VIABILITY AND CAUSES CELL CYCLE ARREST WITHOUT INDUCING APOPTOSIS OF* MLL*-REARRANGED AML CELL LINES.*

To determine whether *ZNF521* is functionally important in *MLL*-rearranged AML, we first examined the effects of *ZNF521* knockdown on the cell proliferation using a panel of human *MLL*-rearranged AML cell lines, including, THP-1, NOMO-1 (both expressing *MLL-AF9*), ML-2 (expressing *MLL-AF6*) and OCI-AML4 (expressing *MLL-ENL*). To suppress *ZNF521*, we used GFP-tagged lentiviral vectors expressing anti-ZNF521 shRNAs (ZNF004 and ZNF710) or a non-targeting shRNA sequence (shScram). After assessing transduction efficiency by flow cytometry (range 30-80%) (**Figure 9** and data not shown), GFP-positive cells were sorted and maintained under standard cell culture conditions for subsequent analysis.

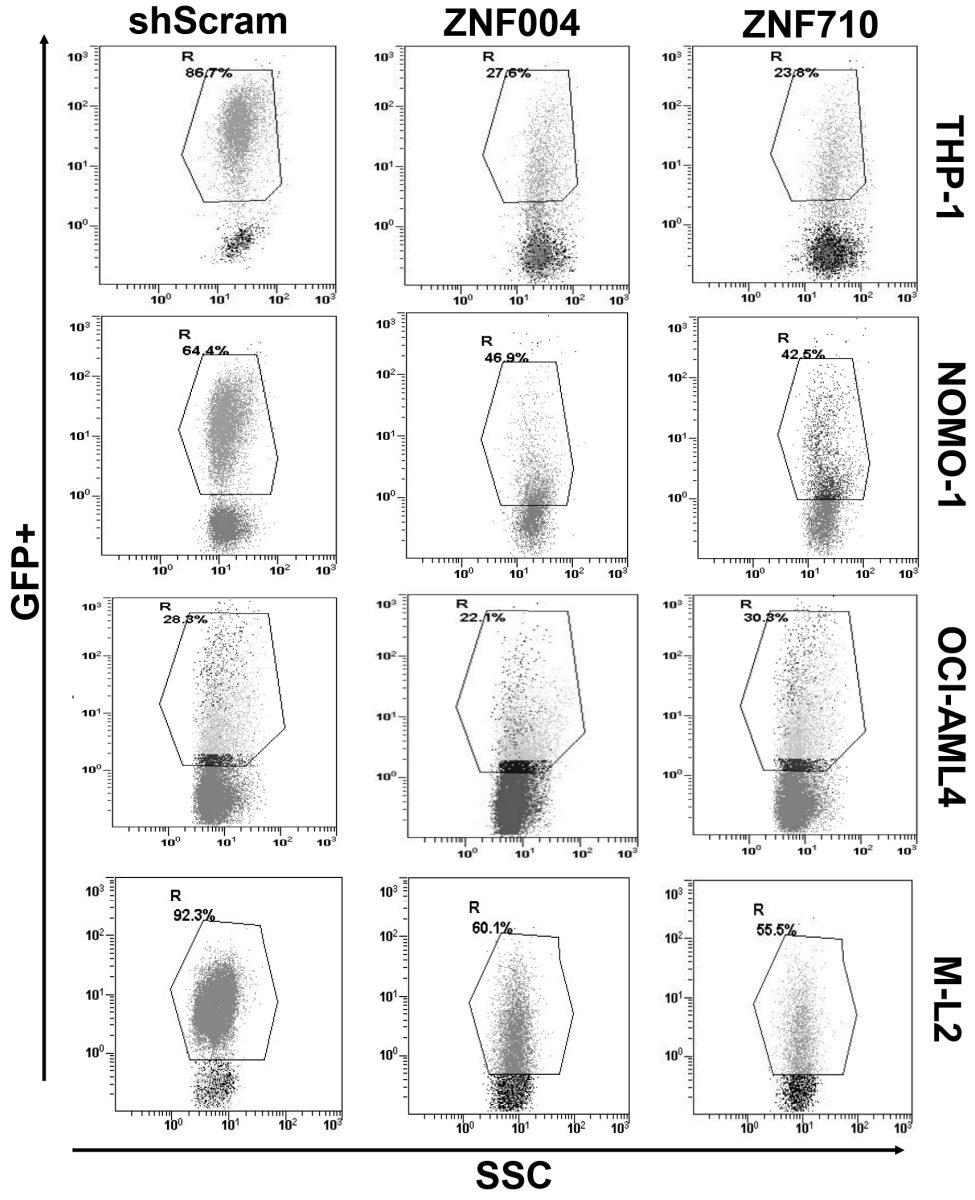

Figure 9. Flow cytometry analysis and gating strategy of shRNA transduced human *MLL*-rearranged AML cell lines. Transduced cell lines were GFP-sorted at day 4 and analyzed or maintained in culture for further evaluation. Otherwise transduced cells were analyzed after being gated for the GFP+ cells. Representative flow cytometry dot plots of gated GFP+ cells after transduction with GFP lentiviral expressing shRNA against *ZNF521* (ZNF004 and ZNF710) or expressing non-targeting shRNA sequence (shScram) are presented. The selected area indicates the sorting gates and includes the percentage of cells in each sorting gate. Gates were set to collect GFP high and GFP low-expressing cells. SSC, side scatter.

As expected, in all four cell lines downregulation of *ZNF521* varied between 60% and 75% compared to *ZNF521* mRNA expression in shScram-transduced cells, and this correlated with a decrease in ZNF521 protein amount **(Figure 10)**.

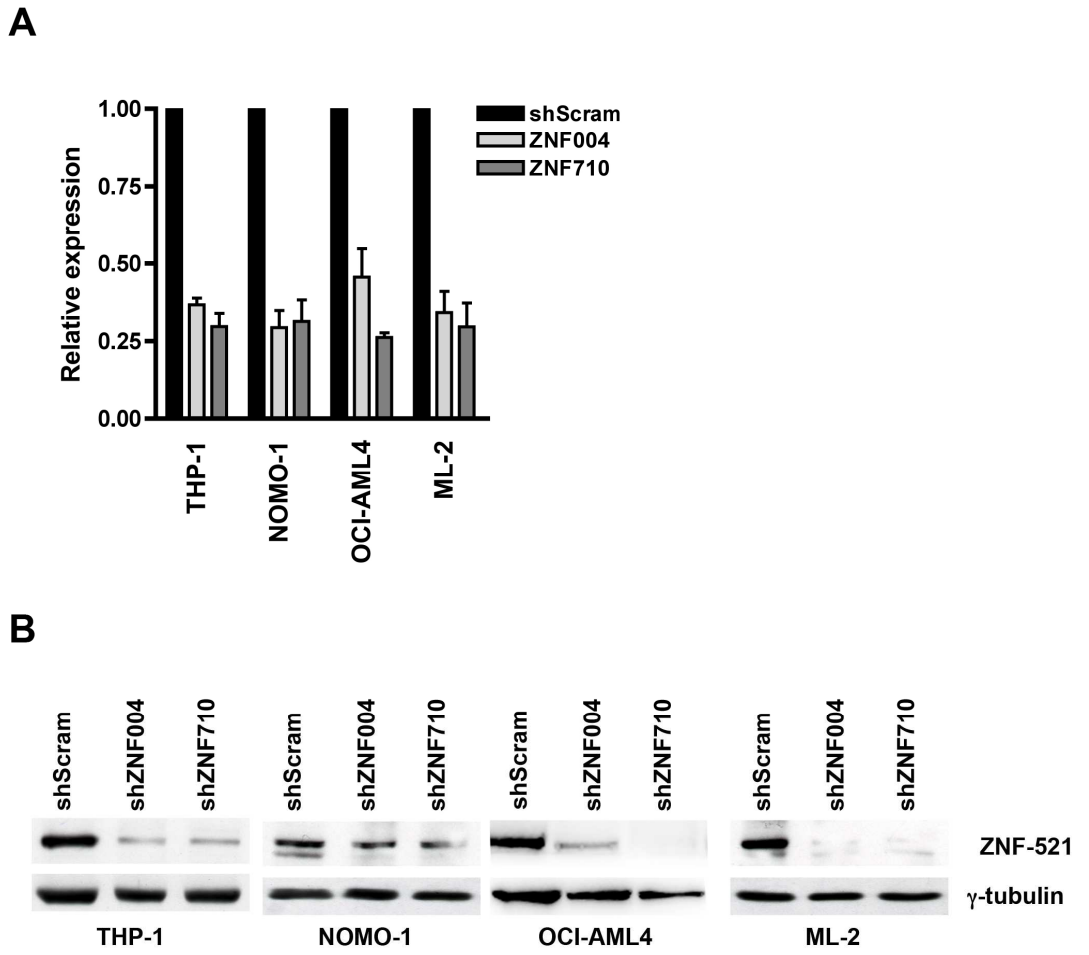

Figure 10. shRNA-mediated knockdown of *ZNF521* in *MLL*-rearranged AML cell lines. (A) *ZNF521* mRNA levels evaluated by qRT-PCR on GFP+ sorted cells after 4 days of transduction with shScram and *ZNF521* shRNAs (ZNF004 and ZNF710). The results are relative to shScram-transduced cells, normalized to *GAPDH* and analyzed by $2^{-\Delta\Delta Ct}$ method. Data are represented as mean ± SD of three independent experiments. (B) Western blot analysis for ZNF521 of cells used in (A). ϒ-tubulin was used as loading control.

In addition, *ZNF521* knockdown progressively reduced viability of all the transduced cell lines (**Figure 11A**), and it inhibited colony formation ability of *MLL*-rearranged cells, measured 2 weeks after transduction (**Figure 11B**). In order to get a deeper insight, cell cycle analysis and apoptosis induction were assessed in GFP-positive *MLL*-rearranged cells. At day 7, we observed an accumulation of cells in G1 phase (17%-77%) in three out of four cell lines (THP-1, NOMO-1 and ML-2) expressing anti-ZNF521 shRNAs. This was most likely due to S phase reduction (from 29% to 65%) rather than G2/M alterations (**Figure 11C**). However, annexin V/DAPI assay measured at day 4 and day 7 demonstrated that *ZNF521* knockdown did not caused increased apoptosis (**Figure 11D**), suggesting that ZNF521 may be involved in proliferation and differentiation of *MLL*-rearranged cells rather than in cell survival.

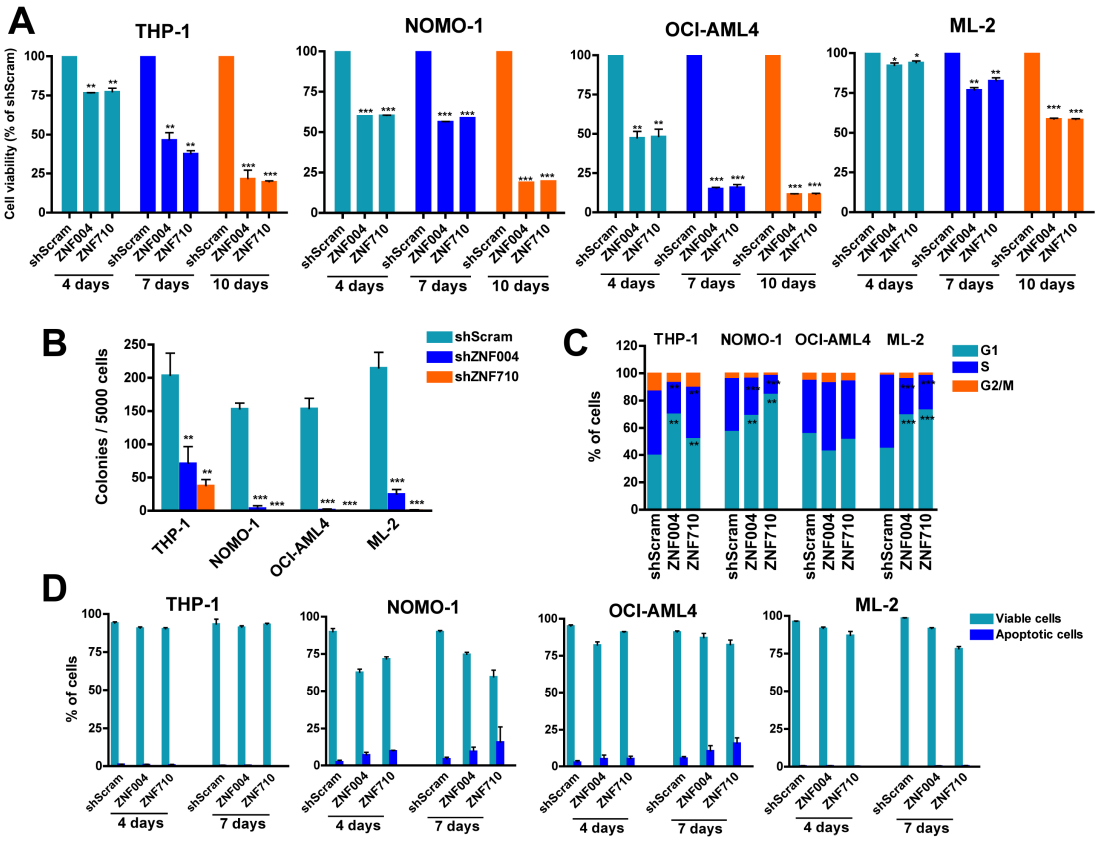

FIGURE 11: *ZNF521* depletion impairs cell proliferation, induces cell cycle arrest but not apoptosis in *MLL*-rearranged cell lines.

(A) MTT cell viability assay in the MLL leukemic cells THP-1, NOMO-1, OCI-AML4 and ML2 transduced with *ZNF521* shRNAs (ZNF004 or ZNF710) or non-targeting scramble control (shScram). GFP+ cells were sorted 4 days after transduction and placed in appropriate medium. Graphs show percentage of GFP+ cells measured at day 4, day 7 and day 10, normalized to the percentage of shScram cells. Data are represented as mean ± SD of at least three independent experiments. **P*<0.05, ***P*<0.001, ****P*<0.0001, *t*-test. (B) Colony formation of GFP+ cells transduced with *ZNF521* shRNAs or shScram. Error bars represent mean ± S.D. of three independent experiments. ***P*<0.001, ****P*<0.0001, *t*-test. (C) Cell cycle distribution at day 7 of *ZNF521* knockdown cells and control shScram of gated GFP+ cells. Data are represented as mean ± SD of three independent experiments. ***P*<0.001, ****P*<0.0001, *t*-test. (D) Percentage of apoptotic cells (Annexin V+/DAPI- and Annexin V+/DAPI+) measured after 4 and 7 days post-transduction of gated GFP+ cell population. Data are represented as mean ± SD of three independent experiments.

To substantiate this hypothesis, GFP-sorted transduced THP-1 and ML-2 cells were collected on glass slides by cytospin and stained with antibodies against p21 (CDKN1A) and p27 (CDKN1B) cell cycle inhibitors (Roy, 2015). At day 7, we observed an increase of both p21 and p27 protein expression in *ZNF521* knockdown cells, suggesting a prolonged G1/S transition as the main reason for the aforementioned cell cycle arrest (**Figure 12**). Taken together, these findings indicate that *ZNF521* expression is essential in the growth potential of *MLL*-rearranged AML cell lines.

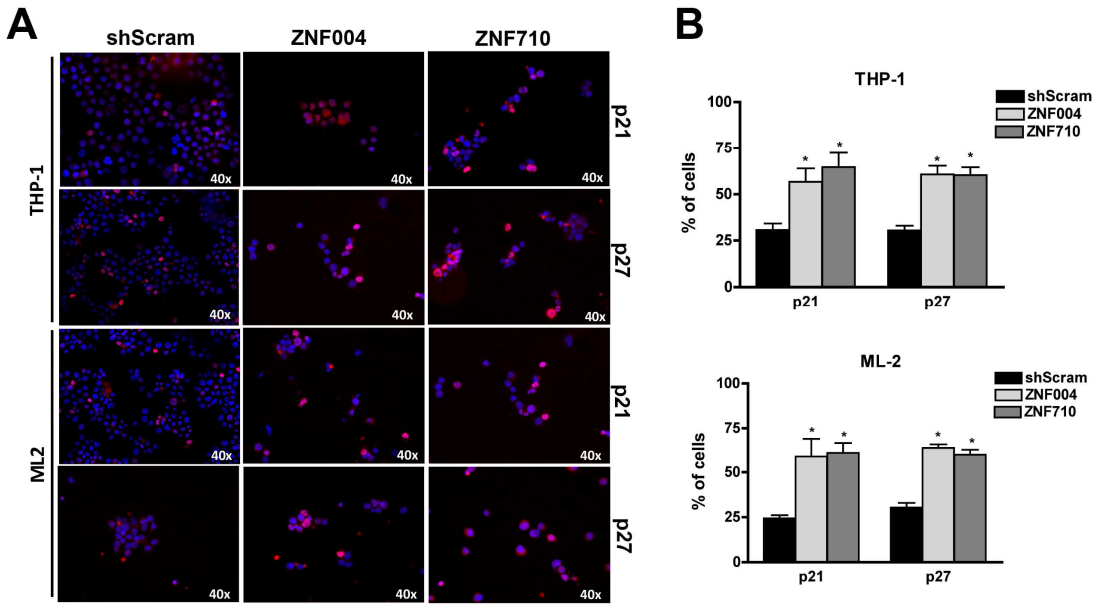

Figure 12. *ZNF521* depletion increases the expression signal of p21 and p27. (A) Representative confocal imaging on cytospin preparations of THP-1 and ML-2 cell lines analyzed for p21 and p27 expression by immunofluorescence assay. Transduced cells with shScram or ZNF521 shRNAs (ZNF004 or ZNF710) at day 7 were stained with anti-p21 and anti-p27 antibodies and Alexa Fluor 594-conjugated goat anti-mouse IgG (red). Corresponding nuclei were counterstained with DAPI (blue). Original magnification, x 40. Images were collected by confocal microscope (Vico, Eclipse Ti80, Nikon) and processed with ImageProPlus software (Media Cybernetics). Results are representative of at least three independent experiments for each cell line. (B) Percentage of p21 and p27 positive cells was quantified respect to total nuclei (see supplemental method). Data are mean ± SD of three cytospin preparations for each cell line of three independent experiments. * $P<0.05$, *t*-test

4. DEPLETION OF ZNF521 *INDUCES MYELOID DIFFERENTIATION OF* MLL-*REARRANGED AML CELL LINES.*

Given that ZNF521 can regulate lineage progression of different cell types, including hematopoietic cells (Bond, 2004; Matsubara, 2009; Mega, 2011), we analyzed whether *ZNF521* depletion might influence differentiation in *MLL*-rearranged leukemic cells. Flow cytometry analysis of CD11b and CD14 myeloid markers was then performed on GFP-positive cells and revealed a change of these markers in 3 out of 4 cell lines transduced

with *ZNF521* shRNAs (**Figure 13A**). The phenotypic changes were also sustained by a more mature macrophage-like morphology observed in all these cell lines upon *ZNF521* depletion as compared with transduced control cells (**Figure 13B**). Additionally, maturation induced by *ZNF521* depletion was also supported by upregulation of *C/EBPA* and *PU.1* mRNA levels, two myeloid differentiation markers (**Figure 13C**).

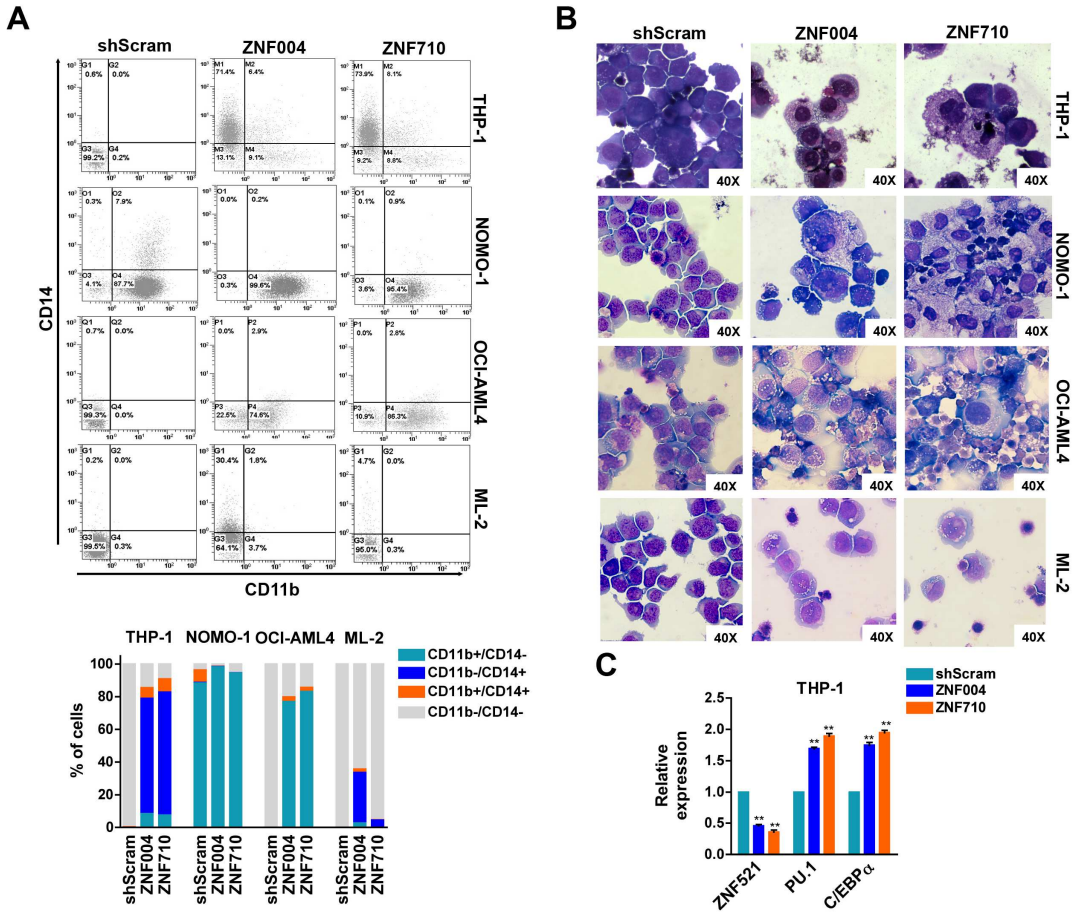

Figure 13. *ZNF521* depletion induces myelomonocytic differentiation in *MLL*-rearranged cell lines.

(A) Representative flow cytometry dot plots of gated GFP+ cells analyzed for CD11b and CD14 expression after 7 days of transduction. The mean percentage of CD11b+/CD14-, CD11b-/CD14+, CD11b+/CD14+ and CD11b-/CD14- cells of three biological replicates are shown below. (B) Representative Wright-Giemsa staining of cytospin preparations at day 7 of THP-1, NOMO-1, OCI-AML4 and ML2 GFP+ cells transduced with *ZNF521* shRNAs or shScram. Original magnification, x 40. (C) qRT-PCR on THP-1 GFP+ cells for the expression of *ZNF521*, *PU.1* and *C/EBPα* at day 7 post transduction with *ZNF521* shRNAs or shScram. The results are relative to shScram-transduced cells, normalized to *GAPDH* and analyzed by $2^{-\Delta\Delta Ct}$ method. Data are represented as mean ± SD of three independent experiments. ** $P<0.01$, *t*-test.

Furthermore, a downregulation of *ZNF521* expression occurred in response to treatment with all-*trans* retinoid acid (ATRA) and with Securinine, two differentiation agents administered to THP-1 and NOMO-1 AML cells, respectively (**Figure 14**).

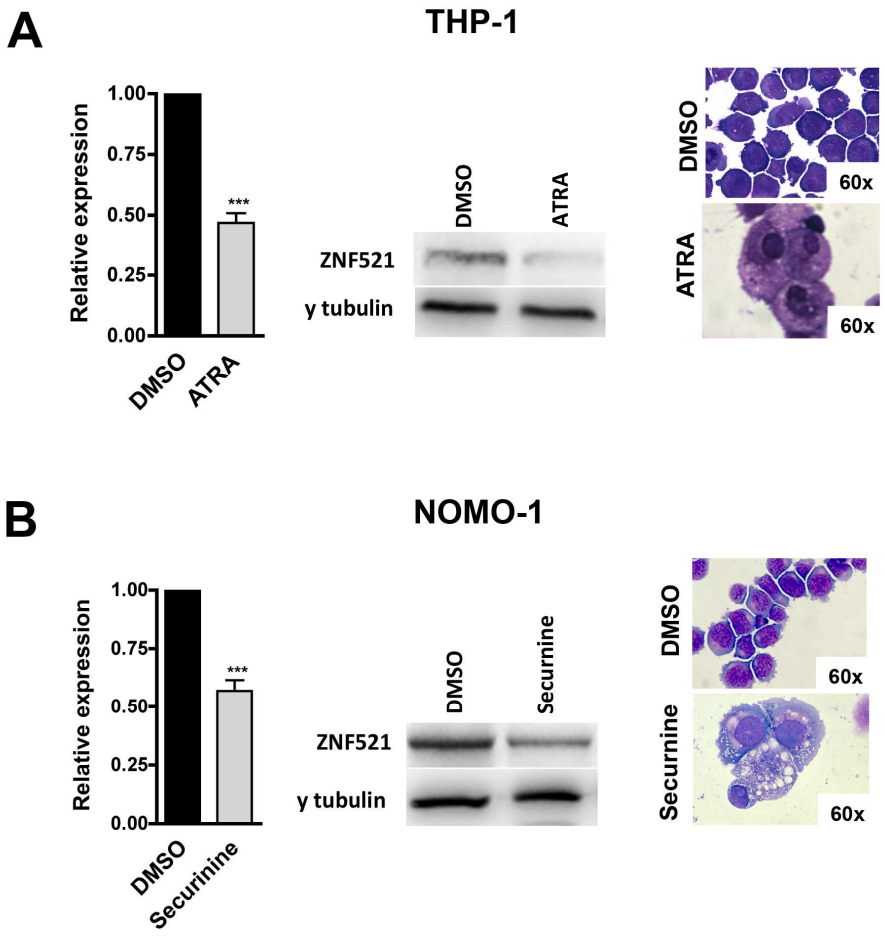

Figure 14. Effect of differentiation-induced agents on *ZNF521* expression in human *MLL*-rearranged cell lines. (A) qRT-PCR (left panel) and Western blot (middle panel) analyses of ZNF521 in THP-1 cell line after treatment with 1μM ATRA for 72 hours compared with vehicle control (0.1% DMSO). For qRT-PCR data the *ZNF521* mRNA expression is shown relative to vehicle control normalized to *GAPDH* and analyzed by $2^{-\Delta\Delta Ct}$ method. Data are represented as mean ± SD of three independent experiments. *** P<0.001, *t*-test. Representative Wright-Giemsa-stained cytospin preparations of cells treated with 1μM ATRA for 72 hours (right panel). Original magnification, x 60. (B) Same experiments as in (A) were performed in NOMO-1 cell line after 72 hours of treatment with 15μM Securinine. qRT-PCR (left panel), Western blot (middle panel) analyses of ZNF521 expression and representative Wright-Giemsa-stained cytospin preparations of NOMO-1 cell lines after 72 hours of treatment (right panel) are shown.

In particular, ATRA and Securinine, previously tested on these cell lines by others (Niitsu, 2001; Gupta, 2011), were able to reduce ZNF521 mRNA and protein expression, and stimulate *MLL*-rearranged cell differentiation, supporting the hypothesis that ZNF521 is required to maintain those cells in an undifferentiated state.

5. EFFECTS OF ZNF521 *DEPLETION IN PATIENT-DERIVED AML XENOGRAFT CELLS.*

To extend our findings to primary cells containing MLL-AF9 oncogene, we transduced *ZNF521* shRNAs in *ex vivo* cells obtained from patient-derived xenografts (**Figure 15A**). Two out of four patients harboring *MLL-AF9* fusion protein (**Table 4**) resulted in successful engraftment into NSG mice.

ID	WBC,x10^9/L	FAB	% BM blasts at diagnosis	Disease status at biopsy	karyotype
1426	37.5	NA	90	Diagnosis	46,XY,t(9;11)(p22;q23)
726*	NA	M5	80	Diagnosis	46,XY,t(9;11)(p22;q23)
1315*	85.2	NA	80	Secondary	46,XY,7p,t(9;11)(p22;q23)
1368	222.4	NA	80	Diagnosis	46,XY,t(9;11)(p22;q23)

Table 4. Clinical features of patients for xenotransplantation into NOD/SCID mice.

* Sample-patient successfully engrafted; NA not available.

The kinetics of such engraftment, measured by percentage of human CD45+ cells in the peripheral blood varying between 22.3% to 42.2%, ranged from 47 to 67 days and led to expansion of leukemic cells with the same immunophenotype and cytogenetic features of the original patient sample (**data not shown**). *Ex vivo* experiments demonstrated that *ZNF521* depletion strongly impaired viability and colony formation of mononuclear cells obtained from two primary MLL-AF9 AML patient-derived xenografts (**Figure 15B,C**). Most importantly, an increased expression of myeloid differentiation markers CD11b and

CD14 (**Figure 15D**) and morphological features of mature monocytes/macrophage blast-like was observed (**Figure 15E**). These findings suggest that *ZNF521* overexpression is critical to maintain an immature phenotype consistent with the *MLL*-rearranged cell lines results.

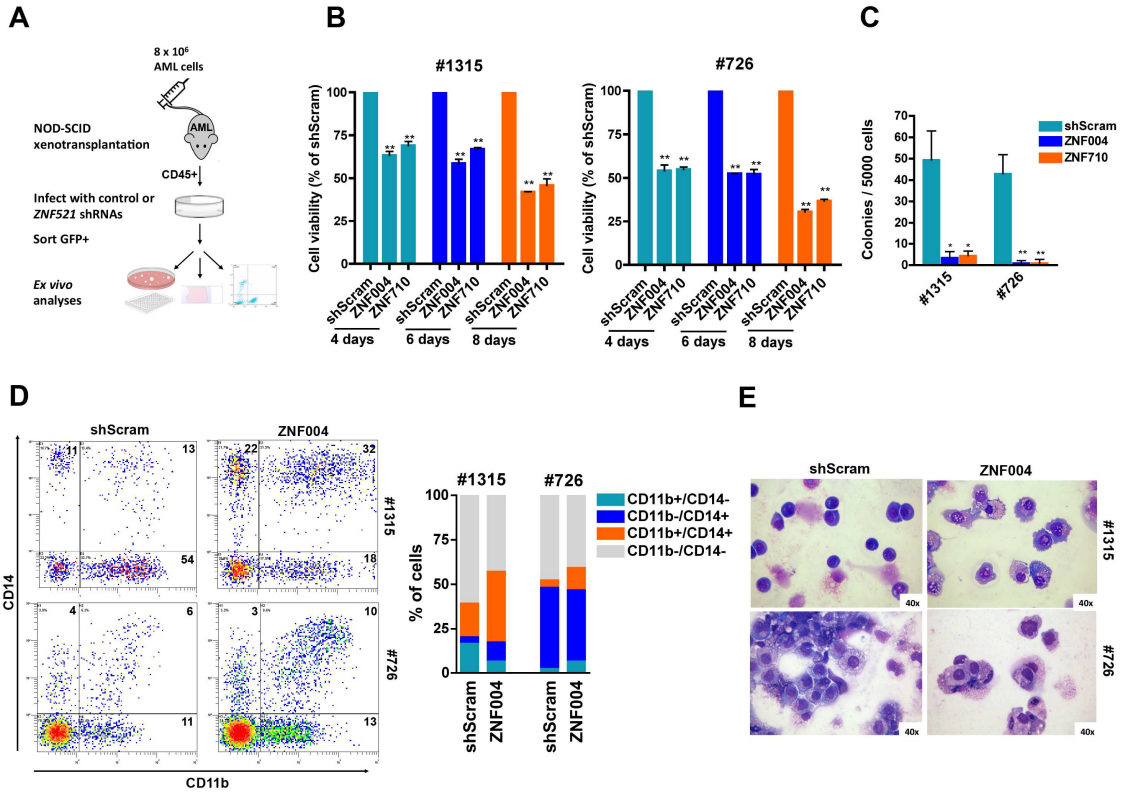

Figure 15. *ZNF521* depletion impairs cell growth and induces differentiation on primary MLL-AF9 AML patient-derived xenograft cells. (A) Flow chart of experimental procedure for analyzing the role of *ZNF521* in *ex vivo* cells obtained from patient-derived xenografts. Leukemic cells from patient #726 or patient #1315 were isolated from primary AML mice and infected with lentivirus encoding an shRNA to *ZNF521* or shScram. Four days after transduction cells were FACS-sorted for GFP expression and cultured. (B) MTT cell viability assay in *ex vivo* cells. Data are represented as mean ± SD of three independent experiments. ***P*<0.001, *t*-test. (C) Clonogenic growth of transduced *ex vivo* GFP+ cells following 14 days in methylcellulose culture. Data are shown as the means ± SD for triplicate analyses. **P*<0.05, ***P*<0.001, *t*-test. (D) Representative flow cytometry dot plots showing expression of CD11b and CD14 in human CD45+ cells in the GFP+ cells population. Numbers indicate percentage of the four populations. The mean

percentage of CD11b+/CD14-, CD11b-/CD14+, CD11b+/CD14+ and CD11b-/CD14- populations of three biological replicates are shown in the right panel. (E) Representative Wright-Giemsa-stained cytospins of *ex vivo* GFP+ cells at day 4 post transduction with *ZNF521* shRNA (ZNF004) or shScram. Original magnification, x 40.

6. GENE EXPRESSION CHANGES AFTER ZNF521 *DEPLETION IN THP-1 CELLS.*

To investigate the gene expression pattern in MLL-AF9 AML cells expressing high levels of *ZNF521*, we performed microarray analysis of shZNF521- or shScram-transduced THP-1 cells. Since that the differentiation was overt after 7 days of transduction as reported above, we performed gene expression profiling at day 4 after transduction. A total of 158 genes showed a significant change of expression (>1.5-fold change, FDR<0.05), 58 were upregulated while 100 were downregulated (**Figure 16A and Table 5**).

Probe set	Gene Symbol	IFDR	Means_shZNF004	Means_shScram
1555340_x_at	*RAP1A*	7.29E-14	3.272817153	11.69366146
1555339_at	*RAP1A*	7.29E-14	3.331697417	11.72192487
203032_s_at	*FH*	7.29E-14	2.593654642	7.004073844
230659_at	*NA*	7.29E-14	3.364707971	9.071419646
209811_at	*CASP2*	7.29E-14	3.498066564	6.977897606
1555830_s_at	*ESYT2*	7.29E-14	3.157621165	6.233288774
228854_at	*NA*	7.29E-14	4.507943084	8.700411129
1554451_s_at	*DNAJC14*	7.29E-14	3.443528107	6.599509088
222611_s_at	*PSPC1*	7.29E-14	3.892862189	6.940868082
216125_s_at	*RANBP9*	7.29E-14	4.214434937	6.786602432
227762_at	*NA*	7.29E-14	4.760415465	7.656360326
242900_at	*NA*	7.29E-14	3.972371741	6.289884015
210465_s_at	*SNAPC3*	7.29E-14	5.260781091	8.320937398
209052_s_at	*WHSC1*	7.29E-14	4.011707509	6.321660403
224577_at	*ERGIC1*	7.29E-14	5.026229524	7.551517562
219209_at	*IFIH1*	7.29E-14	9.661284664	6.360666226
227834_at	*TXLNB*	7.29E-14	5.734833216	3.702313322
219243_at	*GIMAP4*	7.29E-14	5.377588503	3.461148283
216598_s_at	*CCL2*	7.29E-14	8.428115374	5.381391593
203596_s_at	*IFIT5*	7.29E-14	7.756051699	4.938882008

227265_at	*FGL2*	7.29E-14	6.933841791	4.350212792
210889_s_at	*FCGR2B*	7.29E-14	9.3858295	5.882368351
223551_at	*PKIB*	7.29E-14	9.164302426	5.661218939
204475_at	*MMP1*	7.29E-14	7.330288469	4.387222797
229450_at	*IFIT3*	7.29E-14	11.66175734	6.924802655
206488_s_at	*CD36*	7.29E-14	8.995345742	5.33583655
228766_at	*CD36*	7.29E-14	7.475487386	4.405886495
205686_s_at	*CD86*	7.29E-14	5.675449214	3.327806002
210895_s_at	*CD86*	7.29E-14	7.799936929	4.402895353
202973_x_at	*FAM13A*	7.29E-14	7.555883194	4.23511804
231120_x_at	*PKIB*	7.29E-14	6.821698244	3.738890628
204972_at	*OAS2*	7.29E-14	7.437693218	3.962123467
209555_s_at	*CD36*	7.29E-14	7.849275842	4.152806423
206584_at	*LY96*	7.29E-14	7.396080064	3.888310801
226757_at	*IFIT2*	7.29E-14	10.04575707	5.214422189
206637_at	*P2RY14*	7.29E-14	6.443625151	3.174919145
212956_at	*TBC1D9*	7.29E-14	8.199774221	3.989995117
227609_at	*EPSTI1*	7.29E-14	10.61497537	4.856721475
202086_at	*MX1*	7.29E-14	11.59476291	5.268245207
203153_at	*IFIT1*	7.29E-14	10.51899342	4.701311253
235276_at	*EPSTI1*	7.29E-14	8.531841866	3.790497541
214453_s_at	*IFI44*	7.29E-14	10.18360475	4.365661325
204439_at	*IFI44L*	7.29E-14	9.343279892	2.623412862
208450_at	*LGALS2*	2.72E-12	6.376452482	4.13836002
230520_at	*AIG1*	3.62E-12	5.421414771	8.42334648
225237_s_at	*MSI2*	3.62E-12	4.302027085	6.63526392
209875_s_at	*SPP1*	3.62E-12	8.939636755	5.245139693
239512_at	*SRSF4*	5.43E-12	3.424633107	6.122115045
215099_s_at	*RXRB*	5.43E-12	3.360896669	5.182917746
214022_s_at	*IFITM1*	1.09E-11	9.639456366	5.780716705
227242_s_at	*EBF3*	1.40E-11	3.198777829	6.501337123
209160_at	*AKR1C3*	1.40E-11	6.237174611	3.467544531
209409_at	*GRB10*	1.52E-11	5.53032405	8.592046019
226841_at	*MPEG1*	1.52E-11	5.439137025	3.463256631
206291_at	*NTS*	8.04E-11	5.202834617	3.37912861
235625_at	*VPS41*	1.12E-10	4.019442688	6.888053529
219352_at	*HERC6*	1.12E-10	8.512887407	5.127299556
242625_at	*RSAD2*	1.12E-10	7.759363805	3.535304072
227260_at	*NA*	1.68E-10	3.468106472	5.812574528
229128_s_at	*ANP32E*	1.92E-10	3.065627289	8.041154478
211825_s_at	*FLI1*	2.56E-10	4.505153042	7.70808605
204273_at	*EDNRB*	1.21E-09	4.644985144	3.081504207
220059_at	*STAP1*	1.21E-09	6.948836147	3.9587154
34449_at	*CASP2*	2.22E-09	3.371752104	5.343900903
202869_at	*OAS1*	2.40E-09	8.708784226	3.999158316
209925_at	*OCLN*	2.53E-09	3.66215453	5.709879327
213562_s_at	*SQLE*	2.70E-09	3.41534405	6.727026189
219895_at	*TMEM255A*	3.97E-09	5.912972966	2.952282219
212681_at	*EPB41L3*	6.35E-09	6.875338979	4.226037005
1552658_a_at	*NAV3*	2.21E-08	4.719195295	7.76328231
210139_s_at	*PMP22*	2.21E-08	6.202295096	3.699815143
229167_at	*PURA*	4.17E-08	4.433892969	6.889873925
217403_s_at	*ZNF227*	5.67E-08	3.767873968	6.526712195

211559_s_at	*CCNG2*	6.59E-08	4.422448321	8.428323945
219196_at	*SCG3*	6.59E-08	5.429712116	2.812053741
213872_at	*NA*	1.09E-07	3.010296172	7.791453316
201693_s_at	*EGR1*	1.09E-07	4.539918709	7.633098497
210875_s_at	*ZEB1*	1.09E-07	2.896438208	4.421607261
222614_at	*RWDD2B*	1.98E-07	3.450423478	5.86072941
1553117_a_at	*STK38*	2.10E-07	3.665817621	6.939115251
244774_at	*PHACTR2*	2.10E-07	3.853094162	6.851288289
233292_s_at	*NA*	3.41E-07	3.184355172	4.827088859
214539_at	*SERPINB10*	4.15E-07	4.10912414	7.005074609
224046_s_at	*PDE7A*	6.57E-07	3.65311398	6.146994073
227803_at	*ENPP5*	6.57E-07	4.717081416	3.098961165
216917_s_at	*SYCP1*	6.57E-07	5.727581378	3.442402712
209723_at	*SERPINB9*	6.57E-07	6.525317888	3.87000973
206336_at	*CXCL6*	6.57E-07	5.61551641	3.22831843
1554614_a_at	*PTBP2*	9.72E-07	2.851477818	6.01832904
211450_s_at	*MSH6*	9.72E-07	3.945522181	7.983327736
1558028_x_at	*LINC00657*	9.72E-07	3.881579348	6.677504769
205408_at	*MLLT10*	9.72E-07	4.396444854	7.251866597
243835_at	*ZDHHC21*	1.29E-06	3.131394065	4.933095838
212009_s_at	*STIP1*	1.29E-06	4.231623821	6.593862637
235287_at	*CDK6*	2.42E-06	2.962197505	5.106066397
226134_s_at	*MSI2*	2.42E-06	4.910426996	7.85923352
242691_at	*NA*	3.29E-06	3.255871284	5.303849989
225864_at	*FAM84B*	3.29E-06	3.477901748	5.450077376
223220_s_at	*PARP9*	3.29E-06	10.1340089	6.754157491
205139_s_at	*UST*	3.29E-06	6.260118791	4.052990312
205997_at	*ADAM28*	3.29E-06	6.747742756	4.12818658
207723_s_at	*KLRC3*	3.29E-06	6.848721055	3.928225706
222863_at	*ZBTB10*	5.30E-06	3.003991336	4.715064116
228834_at	*TOB1*	6.78E-06	3.10919356	6.508586179
237346_at	*TGDS*	6.78E-06	2.659434222	4.423719391
239245_at	*NA*	7.78E-06	2.830149135	4.556059038
234023_s_at	*CENPJ*	8.03E-06	2.838765479	5.393589327
213470_s_at	*HNRNPH1*	8.03E-06	4.388092403	7.884930732
228746_s_at	*NA*	1.37E-05	3.420014404	7.3567208
222719_s_at	*PDGFC*	1.37E-05	3.132188837	5.747502104
226302_at	*ATP8B1*	1.37E-05	4.729303393	2.953088663
205992_s_at	*IL15*	1.37E-05	5.108587768	3.02186665
202411_at	*IFI27*	1.37E-05	10.32098553	4.155674395
205660_at	*OASL*	2.00E-05	8.816232559	5.043755348
201601_x_at	*NA*	3.82E-05	9.514157211	6.172838679
1553105_s_at	*DSG2*	8.06E-05	3.716787789	7.380013058
1555745_a_at	*LYZ*	8.65E-05	3.657795152	8.966469996
204156_at	*SIK3*	0.000191614	3.014616564	5.596974796
205883_at	*ZBTB16*	0.000191614	3.9323087	6.382959729
220773_s_at	*GPHN*	0.000191614	3.965163502	6.331618458
231955_s_at	*HIBADH*	0.000191614	4.994812536	7.532474333
205003_at	*DOCK4*	0.000191614	6.547858789	4.336486424
231504_at	*CCDC148*	0.000191614	5.515141165	3.546226457
228607_at	*OAS2*	0.000191614	6.369024331	3.246325238
208055_s_at	*HERC4*	0.000210734	3.689389873	6.041702168
209535_s_at	*NA*	0.000283867	3.889135907	6.781673843

1555154_a_at	*QKI*	0.000283867	4.751795874	7.783719526
215991_s_at	*EMC1*	0.000283867	2.249362269	3.683270007
231274_s_at	*NA*	0.000283867	3.425324062	5.435462999
204698_at	*ISG20*	0.000283867	8.165708071	5.327347603
1554411_at	*CTNNB1*	0.000376125	3.572852599	6.635933907
219599_at	*EIF4B*	0.000376125	4.228875606	7.030587927
209055_s_at	*CDC5L*	0.000376125	5.005916825	8.112529596
220241_at	*TMCO3*	0.000376125	3.662690418	5.589867462
229787_s_at	*OGT*	0.000598768	2.377767023	4.198765755
221423_s_at	*YIPF5*	0.000598768	4.764427984	7.983886289
218031_s_at	*FOXN3*	0.000598768	5.358068359	8.479315641
227978_s_at	*ZADH2*	0.000598768	4.560782669	6.978366066
239827_at	*RGCC*	0.000737413	2.922811434	4.531183441
205552_s_at	*OAS1*	0.000994295	8.52556634	4.199854718
239131_at	*NA*	0.001104595	2.869233639	4.766296652
241699_at	*NA*	0.001104595	2.524064046	3.903572868
230265_at	*NA*	0.001251697	3.971358386	7.268866264
206420_at	*IGSF6*	0.001251697	5.105353547	3.145928394
213797_at	*RSAD2*	0.001251697	7.200654765	4.091812827
233878_s_at	*XRN2*	0.001283386	4.250416536	8.047670115
208047_s_at	*NAB1*	0.001289249	2.984541409	4.570452219
201295_s_at	*WSB1*	0.001327936	3.992678087	7.276172187
216015_s_at	*NLRP3*	0.001658291	3.957640333	6.742987942
205996_s_at	*AK2*	0.001955832	6.117002334	9.595158229
1552275_s_at	*PXK*	0.002059935	4.965871756	8.855475104
1569362_at	*ALCAM*	0.002059935	2.854703141	4.974867187
204426_at	*TMED2*	0.002059935	4.787864769	8.339465929
1555996_s_at	*NA*	0.002059935	3.442752363	5.602525425
215109_at	*RC3H1*	0.002059935	3.362155971	5.244661583
235306_at	*GIMAP8*	0.002059935	4.411824265	2.656029179
206544_x_at	*SMARCA2*	0.00258171	4.255382491	7.791534959
1555526_a_at	*6-Sep*	0.00258171	3.226981218	5.427391893
243751_at	*CHD2*	0.002932455	2.862859638	4.892477052
224455_s_at	*ADPGK*	0.002932455	4.545940524	7.377039775
227404_s_at	*EGR1*	0.002932455	6.017690069	9.554573831
242277_at	*NA*	0.004064267	2.948818284	4.971712234
1554433_a_at	*ZNF146*	0.004064267	4.586934708	7.705354771
225742_at	*MDM4*	0.004064267	3.634920337	5.721579115
201075_s_at	*SMARCC1*	0.004064267	5.677039327	8.919860071
220220_at	*NA*	0.004064267	2.721459192	4.259068431
205746_s_at	*ADAM17*	0.004064267	3.986940892	6.111574752
232412_at	*FBXL20*	0.004064267	3.011811652	4.544990573
221039_s_at	*ASAP1*	0.004609795	5.191490691	8.244705505
224582_s_at	*NUCKS1*	0.004609795	4.953985417	7.709158094
239511_s_at	*SRSF4*	0.004609795	2.671862785	4.037735602
205321_at	*EIF2S3*	0.009657355	4.505151046	9.214797982
211089_s_at	*NEK3*	0.009657355	3.153835382	5.417381302
205123_s_at	*NA*	0.009657355	3.148629261	5.292748903
202269_x_at	*GBP1*	0.009657355	5.830633782	3.51352209
240771_at	*C1orf101*	0.009657355	5.03064457	2.797264367
203331_s_at	*INPP5D*	0.009973264	4.808554468	7.500169639
1570552_at	*NA*	0.009973264	2.861867372	4.310545092
239979_at	*NA*	0.009973264	6.28946785	4.050437594

238511_at	*UBL7-AS1*	0.014726225	3.881133701	6.361624354
206785_s_at	*NA*	0.014726225	4.160456492	2.549445938
209754_s_at	*TMPO*	0.017790649	3.93715112	6.449468774
238846_at	*TNFRSF11A*	0.017790649	4.856236144	3.122126895
220735_s_at	*SENP7*	0.02170125	2.719227978	4.817388541
214908_s_at	*TRRAP*	0.02170125	3.580343454	5.589555234
233303_at	*NA*	0.023432571	3.961680025	7.556922502
231918_s_at	*GFM2*	0.023432571	4.303872868	7.352626308
210786_s_at	*FLI1*	0.03509244	5.492847526	9.107798461
216593_s_at	*PIGC*	0.03509244	4.955690919	7.569466728
229540_at	*RBPJ*	0.03509244	3.648570198	5.510346371
201971_s_at	*ATP6V1A*	0.041216993	3.606919921	6.108703746
227364_at	*NA*	0.041216993	6.426258024	10.80594044
222922_at	*KCNE3*	0.041216993	2.993656568	4.937417089
234977_at	*ZADH2*	0.041216993	3.844053383	5.942264376
227299_at	*CCNI*	0.041216993	4.038234393	6.224050281
207782_s_at	*PSEN1*	0.041216993	4.654952249	7.164965461
1553685_s_at	*SP1*	0.041216993	3.317092537	5.001010317

Table 5. THP-1 microarray (genes deregulated with >1.5-fold change, FDR<0.05)

Gene Set Enrichment Analysis (GSEA) confirmed that *ZNF521* depletion affected cell cycle progression and cell fate differentiation related genes (Brown, 2006) (**Figure 16B,C**). These results showed also positive enrichment of genes downregulated in CD133+ HSCs when compared with the CD133- cell (Jaatinen, 2006), and negative enrichment of embryonic stem cells (ESC) associated genes (Wong, 2008) (**Figure 16D,E**). The enrichment of stemness-related genes found by our analysis is in line with proposed role of ZNF521 in the regulation of hematopoietic stem cell homeostasis (Bond, 2008). Furthermore, GSEA revealed a negative enrichment with genes that are upregulated in *MLL*-rearranged pediatric AML compared with non-*MLL*-rearranged AML (Mullighan, 2012) (**Figure 16F,G**). Interestingly, the *ZNF521* depletion gene set revealed positive enrichment with genes that are upregulated in hematopoietic precursors conditionally expressing *HOXA9* and *MEIS1*, including *HOXA9* target genes upregulated in hematopoietic stem cells (Dorsam, 2004; Hess, 2006) (**Figure 16H,I).** In addition, genes up-regulated or downregulated upon knockdown of *HOXA9* (Faber, 2009) were also similarly regulated in *ZNF521*-transduced THP-1 cells (**Figure 16J,K**).

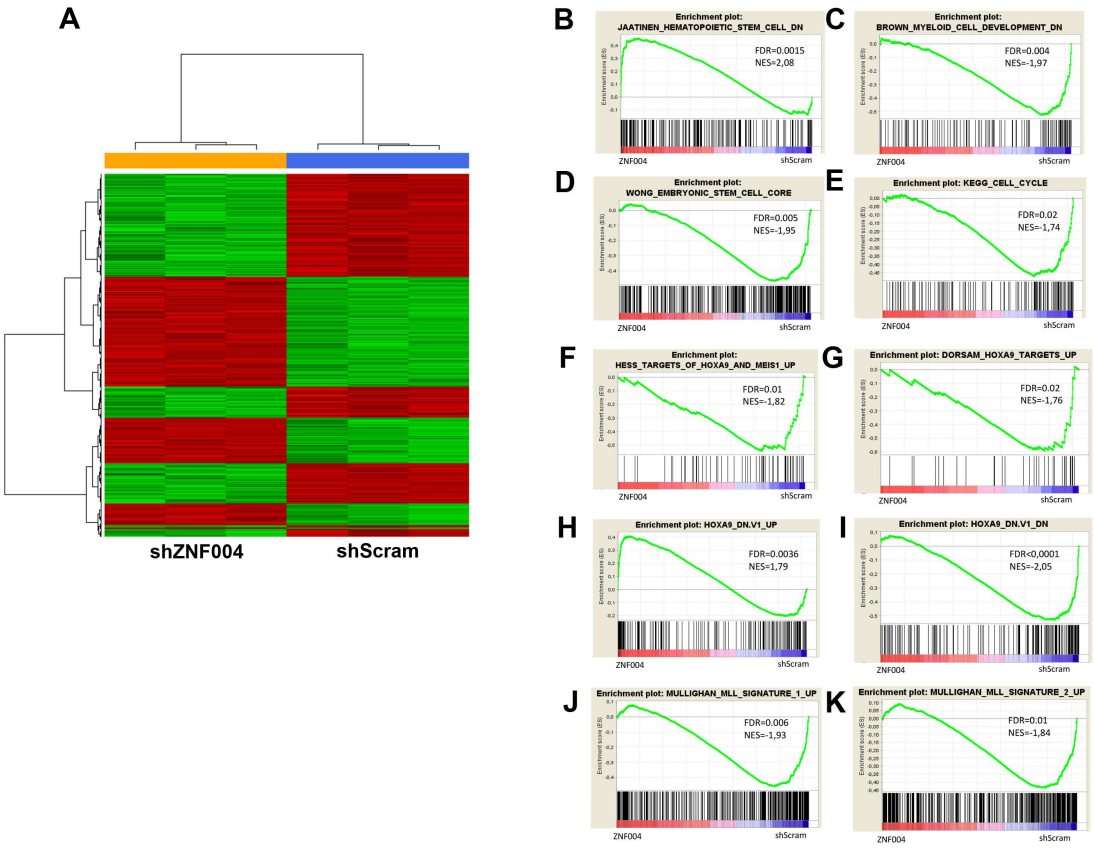

FIGURE 16. Microarray results of *ZNF521* depletion in THP-1 cells. (A) Hierarchical clustering analysis of differently gene expression profiles associated with transduced THP-1 cells with *ZNF521* shRNA (ZNF004) or control shScram after 4 days of transduction. Each column represents a sample and each row represent a gene. Relative levels of gene expression are depicted with a color scale where red represents the highest level of expression and green represents the lowest level. (B-E) GSEA plot showing gene expression signature of (B) negative enrichment of cell cycle signature, (C) negative enrichment of downregulated genes in myeloid cell development signature, (D) positive enrichment of downregulated genes in HSCs signature and (E) negative enrichment of embryonic stem cell core signature. (F,G) GSEA plot showing negative enrichment of *MLL* signature up-regulated genes in pediatric AML. (H-K) GSEA showing enrichment of upregulated genes in *HOXA9* up-regulated (H) and down-regulated (I) signatures in *HOXA9* knockdown cells, (J) positive enrichment of *HOXA9* targets up-regulated and (K) negative enrichment of *HOXA9* targets down-regulated in hematopoietic stem cells. The normalization enrichment score (NES) and the false discovery rate (FDR) values are indicated in each panel. Red and blue color bars indicated the positive and negative enrichment, respectively.

Considering individual genes, we found deregulated genes with known relevance in *MLL*-fusion-mediated AML (*TET1*, *CDK6* and *Musashi2*) (Huang, 2013; Placke, 2014; Park, 2015) and in myeloid progenitors differentiation (CD14 and MEF2A) (Zheng, 2015) (**Figure 17**).

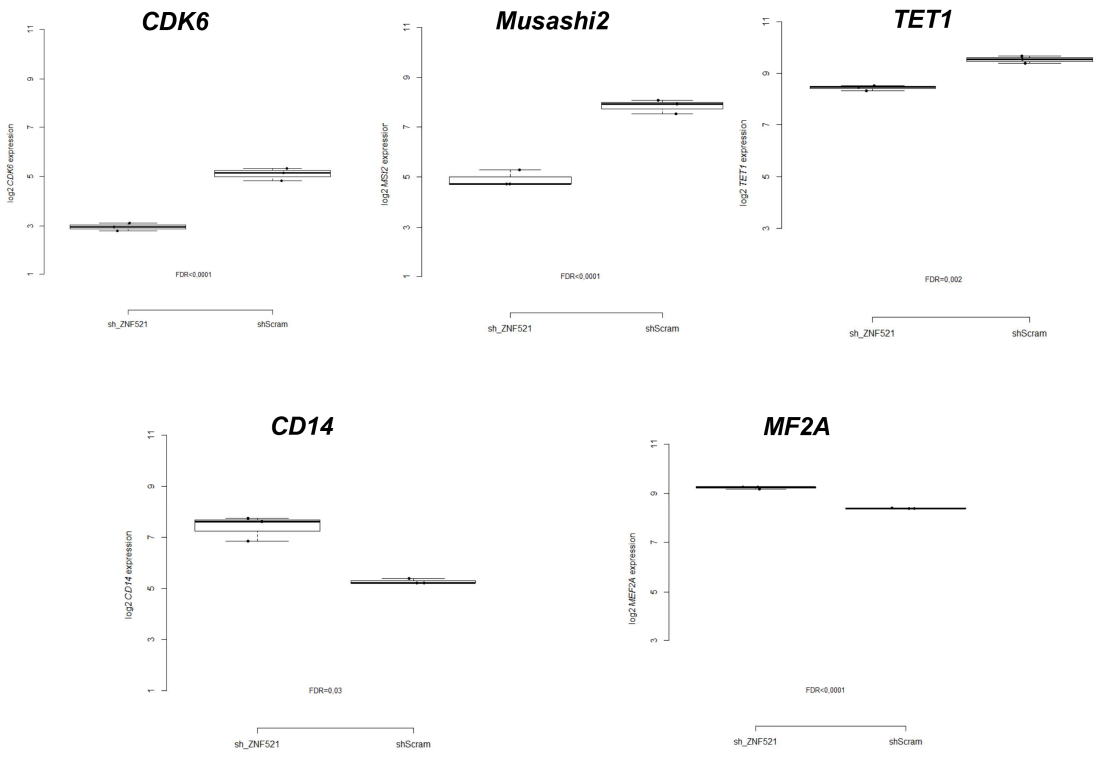

Figure 17. Dot-plots of expression of *CDK6*, *Msi2*, *TET1*, *CD44* and *MEF2A* genes selected from the microarray data of transduced THP-1 cells with ZNF521 shRNA (ZNF004) or shScram. FDR, false discovery rate.

Taken together, these results indicate that *ZNF521* expression negatively modulates genes involved in myeloid differentiation, and is required to maintain expression programs associated with *MLL*-induced transformation.

7. ZNF521 *GENE PROMOTER IS ACTIVATES BY MLL FUSION PROTEINS.*

Finally, to investigate the molecular mechanism that upregulates *ZNF521* in *MLL*-rearranged AML, we performed luciferase reporter and ChIP assays using the Flag-tagged *MLL-AF9* expression plasmid. To this end, we generated a series of constructs in which 5.0 kb of the genomic region upstream of the *ZNF521* transcription start site (TSS) was subdivided in 4 fragments (*ZNF521P1*, *ZNF521P2*, *ZNF521P3* and *ZNF521P4*) and inserted into a pGL4-basic reporter plasmid (**Figure 18A**). Luciferase assays in 293T cells showed that MLL-AF9 strongly activated the promoter region that lay between -1.3 to -3 kb (ZNF521P3) of the TSS (**Figure 18A**). To further confirm the region of ZNF521 activated by MLL-AF9, we generated 3 constructs (*ZNF521P3.1*, *ZNF521P3.2* and *ZNF521P3.3*) spanning the *ZNF521P3* fragment (**Figure 18B**). We found that the *pGL4-ZNF521P3.3* construct showed the highest luciferase activity (**Figure 18B**), indicating that the *MLL-AF9* responsive elements likely reside between -1.0 and -1.6 kb upstream of the ZNF521 TSS. Furthermore, to determine whether *ZNF521* activation was MLL fusion-dependent, we performed *ZNF521*-driven luciferase reporter assay in another *MLL* fusion gene (*MLL-ENL*) and in two non-*MLL*-associated fusion genes such as *AML1-ETO* and *PML-RARA*α. We observed that both *AML1-ETO* and *PML-RARA*α yielded only a minimal luciferase activity compared with MLL-ENL that showed even a higher promoter binding affinity than MLL-AF9 (>2.5 fold) (**Figure 18C**). Besides, wild-type (WT) MLL did not affect luciferase activity under the same settings, providing evidence that only MLL-fusion proteins likely activate *ZNF521* expression (**Figure 18C**). Consistent with these results, ChIP analyses showed that both MLL-AF9 and MLL-ENL bind to *ZNF521* promoter region in transfected 293T cells (**Figure 18D**). In order to validate MLL-AF9 binding to the *ZNF521* promoter in AML cells, we performed ChIP with lysate from NOMO-1 and HL60 cell lines that endogenously expressing MLL-AF9 and WT MLL, respectively. Since that MLL-AF9 lacks the MLL-C portion of WT MLL, an anti-MLL N-terminal (MLLN) and an anti-MLL C-terminal (MLLC) antibodies were used for this experiment. ChIP assays showed that MLLN bound specifically to the *ZNF521* promoter region in NOMO-1 but not in HL60 (**Figure 18E; upper panel**). By contrast, there was not apparently association with MLLC and *ZNF521* in both NOMO-1 and HL60 cells (**Figure 18E; lower panel**). Together, these

findings demonstrated that *ZNF521* promoter is specifically bound by MLL-AF9, and provide further evidence that MLL fusion oncoproteins may drive aberrant expression of ZNF521, which may in turn lead to a block in differentiation.

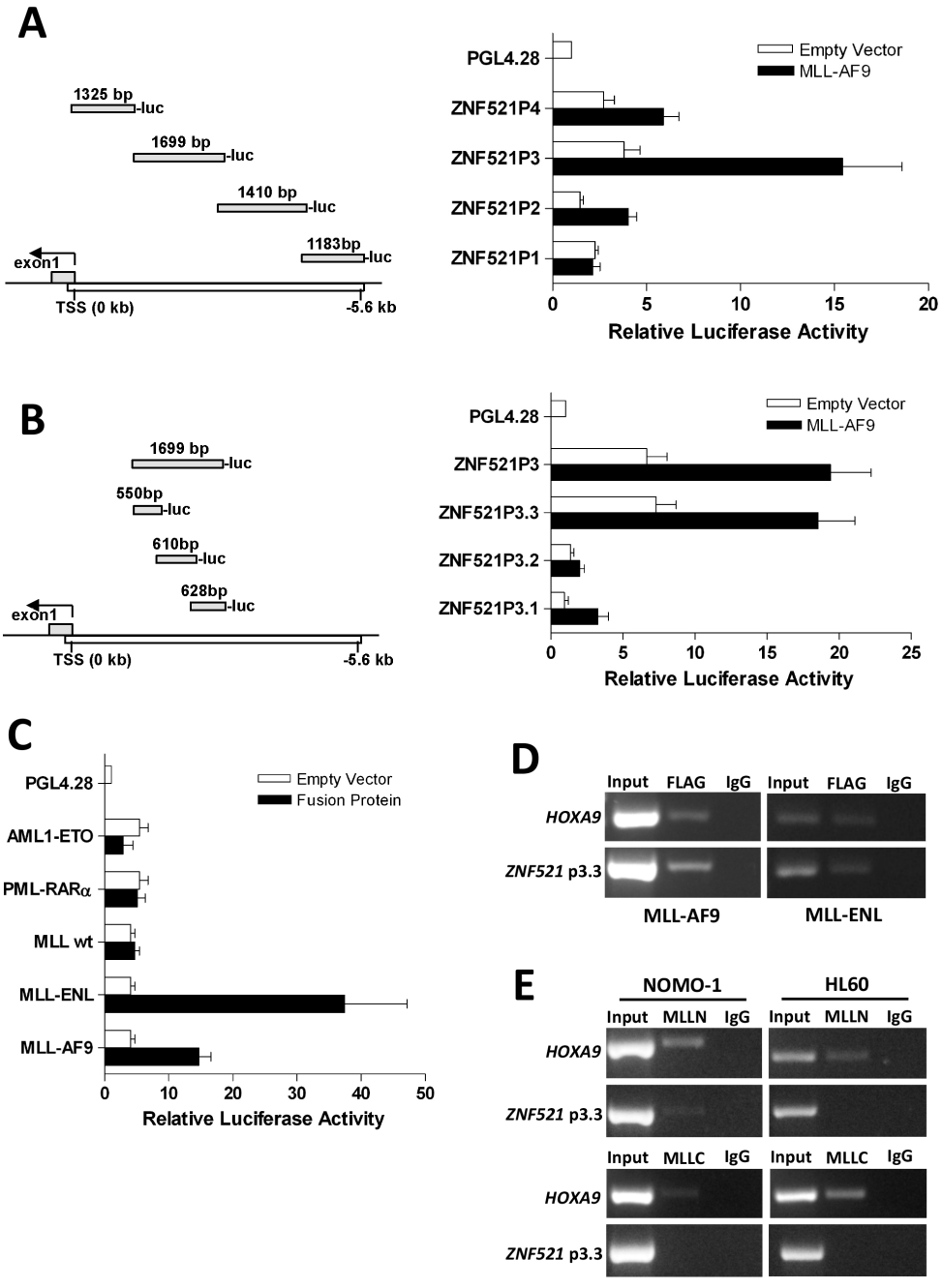

FIGURE 18. MLL-AF9 and MLL-ENL fusion oncoproteins bind to *ZNF521* promoter. (A) An illustration of the 4 fragments representing 5058 bp of *ZNF521* promoter and their positions are indicated in the left panel. The numbers above each part are referred to the length (bp) of the genomic fragment that was PCR amplified and then cloned upstream of the luciferase coding sequence (luc) of PGL42.8 plasmid. In the right panel, horizontal bars represent the luciferase activity generate by each construct following transient transfection in 293T cells with *MLL-AF9* plasmid. (B) An illustration of the 3 fragments of Z*NF521P3* and their respective positions are shown in the left panel. The numbers above each part are referred to the length (bp) of the genomic fragment cloned into PGL42.8 plasmid. In the right panel, horizontal bars represent the luciferase activity generate by each construct as described in A. (C) Luciferase activity of the *ZNF521P3*.3 fragment after transient transfection in 293T cells with *AML1-ETO*, *PML-RARα*, wild-type (WT) *MLL*, *MLL-ENL* or *MLL-AF9* is shown. For each panel (A, B and C), luciferase activity is expressed relative to the empty vector of each expression plasmid (white bars) and normalizes to Firefly/Renilla luciferase activities considering the empty pGL42.8 vector as 1. Data are represented as mean ± SD of three independent experiments. (D) Both MLL-AF9 and MLL-ENL fusion oncogenes associates with the *ZNF521P3.3* promoter region. ChIP assays were performed with the crossed-linked genomic DNA isolated from 293T cells transfected with either *Flag-MLL-AF9* or *Flag-MLL-ENL* and using anti-Flag and anti-IgG antibodies. Normal IgG was used as a negative control. Input DNA from sonicated chromatin and immunoprecipitated DNA were subjected to PCR amplification with primers spanning the *ZNF521P3.3* promoter region. PCR amplification with primers specific to the *HOXA9* promoter region was used as positive control. Data from a representative of three replicate experiments are shown. (E) ChIP analysis of the *ZNF521P3.3* promoter in NOMO-1 cells, which express MLL-AF9 and HL60 cells, which express WT MLL but not MLL-AF9, using antibodies directed to the N-terminus of MLL (MLLN; upper panel), C-terminus of MLL (MLLC; lower panel) or IgG. Immunoprecipitated chromatin samples were analyzed by PCR using primers corresponding to promoter *ZNF521P3.3* region. Note that no PCR product for *ZNF521* promoter was obtained when anti-MLLC, which recognizes only the WT MLL but not MLL-AF9, was used for immunoprecipitation.

DISCUSSION

ZNF521 is a gene that showed a significant higher level of expression in MLL-related AML so as in HSCs and can be used as a marker. In this work, I present data showing that pediatric AML patients carrying *MLL* translocations have a significantly upregulation of *ZNF521* expression independently of the fusion partner involved in the translocation with *MLL*. The overexpression of ZNF521 is a robust transcriptional feature of *MLL*-rearranged AML, consistent across independent adult and pediatric microarray datasets (Kohlmann, 2009; Jo, 2009; Pigazzi, 2011). From these data, I started my study with the aim to decrypt the ZNF521 function as transcription factor in *MLL*-rearranged AML, and understand if it might deserve attention as potential therapeutic target.

A major hallmark of leukemia and a consequence of MLL fusion proteins expression is a block in hematopoietic differentiation (Huntly, 2005). On this way, my data show that the most relevant effect of *ZNF521* depletion was to enhance myeloid differentiation of leukemia cells as evidenced by changes in cell morphology, immunophenotype and increase of a myeloid-specific gene expression in *MLL*-rearranged cell lines and primary cells. The requirement of ZNF521 in the maintenance of an undifferentiated status associated with *MLL*-rearranged AML was also supported by the fact that *ZNF521* expression drastically decreased upon treatment with specific differentiation-induced agents, such as ATRA. The observed growth defect, cell cycle arrest and reduced colony formation upon *ZNF521* depletion were secondary to cells entering into a differentiation program. Thus, MLL fusion proteins might promote leukemogenesis not only by *HOXA9* and *MEIS1* upregulation, but also by keeping the *ZNF521* overexpressed, which in turn contributes to a block of differentiation or to the maintenance of an undifferentiated state of leukemia cells.

Consistent with this finding, others have reported that loss of *ZNF521* enhanced erythroid differentiation and increased B-lineage maturation in cell lines and primary hematopoietic progenitor cell, respectively (Matsubara, 2009; Mega, 2011). Moreover, it is well established that Zfp521, the mouse counterpart of human ZNF521, in other

cellular contexts including, embryonic stem cells (ESCs), neural cells, osteoblasts and chondrocytes mainly function to control cell differentiation of primitive or mature cells by modulating the activity of specific transcription factors [46-49]. Consistent with these findings, our GSEAs in THP-1 cells depleted for *ZNF521* showed enrichment of hematopoietic stem cells (HSCs)- and ESC-associated downregulated genes and sets of genes associated with differentiation program (Brown, 2006; Jaatinen, 2006). Based on these findings, we assumed that ZNF521 has not only a role in promoting self-renewal and maintenance of HSCs but it also acts in MLL rearranged AML. Furthermore, the results support the direct activation of ZNF521 by MLL fusion proteins increasing the importance of this transcription factor in the transformation of the leukemia cells. In fact, it is showed enrichment of set of genes related to MLL fusion-dependent transformation signatures as well as to HOXA9-mediated gene expression program (Dorsam, 2004; Faber, 2009; Mullighan, 2015). Thus, the events documented after *ZNF521* depletion, which in part resemble what has been previously observed in *MLL*-rearranged cells after loss of *HOXA9*, gave a further support that ZNF521 plays a critical role in *MLL*-fusion-mediated leukemia. Interestingly, the expression of either *HOXA9,* a canonical downstream target for *MLL*-rearranged leukemia (Ayton, 2003; Argiropoulos 2007) or *ZNF521* have been shown to be restricted to CD34+ progenitor cells (Dorsam, 2004; Bond, 2008; Mullighan, 2012). Nevertheless, in gene-expression analysis, loss of *ZNF521* does not affect *HOXA9* expression, implying that both are MLL-dependent but might act in a non-mutually exclusive and additive manner. Supporting the idea that ZNF521 is particularly required for MLL-mediated leukemia, the data of luciferase reporter and ChIP assays revealed that *ZNF521* is a direct target of both MLL-AF9 and MLL-ENL fusion proteins. We defined a genomic region of 555 bp in 5' ZNF521 promoter that is thought to be crucial for ZNF521 activation by MLL fusion proteins.

This finding is consistent with prior observations that showed how the modulation of *MLL-AF9* levels resulted in concordant changes in *ZNF521* expression in different human *in vitro* models (Abdul-Nabi, 2010; Fleischmann, 2014). Surprisingly, the inspection of ChIP-seq data from Bernt et al, (Bernt, 2011) did not show peak in the vicinity of the *Zfp521* gene in an MLL-AF9 mouse leukemia model. Of note, this is also observed for other well-known targets of MLL fusion proteins such as *EVI1* and *PLZF* (Arai, 2011; Ono, 2013). About ZNF521, this can be explained by the different approaches used, and the

fact that in mouse BM *Zfp521* is primarily expressed in the HSC fraction and significantly reduced in granulocyte-monocyte-progenitor cells (GMPs) (http://servers.binf.ku.dk/bloodspot/?gene=ZFP521&dataset=nl_mouse_data), in which the analysis has been done. Future ChIP-seq experiments on human transformed HSC will likely shed further light on *ZNF521-MLL-AF9* target gene specificity.

In summary, this study unravels the anti-differentiation function of ZNF521 in MLL-rearranged cells and showed the mechanism by which ZNF521 participates in MLL-fusion mediated transformation. This data also indicate that ZNF521 is highly expressed in the majority of MLL-rearranged AML pediatric patients, and thus ZNF521 could be a potential molecular target for this subtype of aggressive leukemia.

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