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Role of the MLL-AF4 chimeric protein in the molecular pathogenesis of t(4;11) acute lymphoblastic leukemia

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Abstract

Chromosomal rearrangements involving the *Mixed Lineage Leukemia* (MLL) gene are associated with very aggressive forms of acute lymphoblastic leukemia (ALL), often refractory to conventional therapies. In particular, patients carrying the translocation t(4;11)(q21;q23) have the worst prognosis among patients with other MLL-associated malignancies. Although it has been largely shown that the MLL-AF4 fusion protein has the capability to up-regulate genes involved in the self-renewal/differentiation balance of the hematopoietic stem cell, the mechanism induced by this oncoprotein is still poorly understood. Previous functional proteomic studies performed in our group identified the molecular partners of the native AF4 protein, the most common MLL translocation partner in infant ALL, and confirmed that this protein is deeply involved in a complex protein network, important for the regulation of the Pol II-dependent transcription. In this study, we cloned in an eukaryotic expression vector the complete cDNA encoding MLL-AF4 and transiently expressed the recombinant protein in Hek293 cells. In order to understand which molecules take part in the aberrant pathway induced by the MLL-AF4 oncoprotein, we aimed to identify some of its molecular interactors, starting from the proteins that are already known to interact with AF4. We showed that MLL-AF4 binds to CdK9 that by interacting with cyclin T1 forms the positive elongation factor (P-TEFb), which is involved in the activation of the Pol II elongation machinery. We also found that MLL-AF4 interacts with CRSP130 and CRSP33, two members of the so-called "Mediator Complex", thus suggesting that MLL-AF4 is involved in the regulation of the Pol II-dependent transcription. Interestingly, we found that MLL-AF4 also interacts with the tyrosine-kinase receptor FGFR2, and with a protein belonging to the family of 14-3-3s (the isoform θ), involved in diverse intracellular pathways. Moreover, we observed down-regulation in the

expression of *HoxA9*, one of the MLL target genes, in the cells co-expressing both recombinant MLL-AF4 and 14-3-3 θ, thus suggesting that this interaction could modulate transcriptional processes induced by MLL-AF4. Elucidating the role of fusion protein interactors such as FGFR2 and 14-3-3 θ is very important for identifying new molecular targets for the therapy of the MLL-AF4-dependent B-cell ALL.

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LIST OF ABBREVIATIONS

AA: aminoacids**; AEP:** AF4–ENL–P-TEFb complex; **ALL:** Acute Lymphoblastic Leukemia; **bp:** base pairs; **CdK9:** cyclin dependent kinase 9; **CRSP:** Cofactor Required for SP1; **FAB:** French-American-British; **HEK:** human embryonic kidney; **HoxA9:** homeobox A9; **FGFR2:** Fibroblast Growth Factor Receptor 2; **IP:** immunoprecipitates; **MLL:** Mixed Lineage Leukemia; **NT:** nucleotide; **POL II**: RNA-Polimerase II; **P-TEFb**: Positive-Transcription Elongation Factor b; **WCE:** whole cell extracts.

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Chapter 1

Introduction

1.1 Acute Lymphoblastic Leukemia (ALL): an overview

All different types of blood cells are produced in the bone marrow starting from a small number of hematopoietic stem cells, which go through a maturation process to give rise to terminally differentiated blood cells, namely, red cells (or erythrocytes), white blood cells (or leukocytes) and platelets (Fig.1).

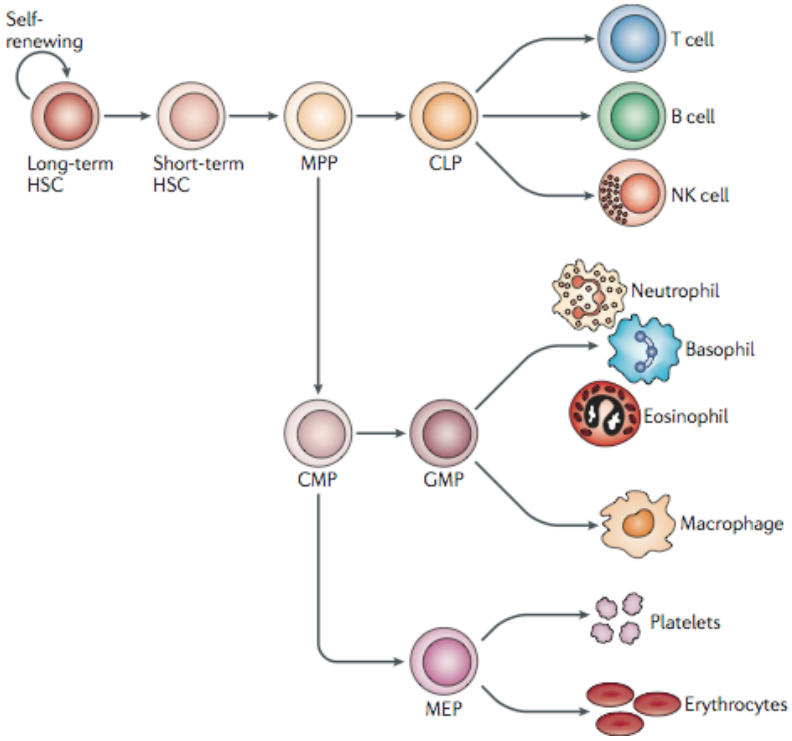


Fig.1 The hematopoietic cascade. Long-term haematopoietic stem cells (HSCs) have the capability to self-renew and also to give rise to all the cell types of the bone marrow and peripheral blood. Other pluripotent progenitors, short-term HSCs and multipotent progenitors (MPPs) have less self-renewal capacity. Together, these three cell types constitute the hematopoietic stem and progenitor cell (HSPC) population. MPPs are thought to differentiate into the two main branches of hematopoietic development that arise from the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP)(King & Goodell, 2011).

Leukemias are cancers that originate from the malignant transformation of blood-forming elements at various stages of differentiation and involve predominantly the bone marrow and peripheral blood, but can spread to other parts of the body including both lymphoid (lymph nodes, spleen) and non-lymphoid (liver, central nervous system, skin) organs.

Depending on the cell of origin, leukemias can be subdivided in acute or chronic. This distinction has also a pathological, clinical, therapeutic and prognostic significance. In acute leukemia, the malignant cells (blasts) derive from very immature counterparts and tipically do not carry out any normal functions. Usually, the proliferation rate is extremely high, and the clinical course is very aggressive. In contrast, chronic leukemia is characterized by preservation of the differentiation process and consequent expansion of mature-appearing cells that are produced at a higher rate than normal cells and accumulate over time in the blood; for this reason, the percentage of blasts in the bone marrow and peripheral blood is much lower compared to acute leukemias.

The leukemic transformation can involve either lymphoid or myeloid cells. Using these two parameters (acute vs chronic and lymphoid vs myeloid), a total of four main categories are identified, each of which is further divided into different subcategories. Acute Lymphoblastic Leukemia (ALL) is the most common type of leukemia in infants. According to the morphologic characteristics of the lymphoblasts, the French-American-British (FAB) Cooperative Working Group defines three categories of ALL:

- L1: lymphoblasts are small cells characterized by a high nucleus-to-cytoplasm ratio. The cells have distinct nucleoli and nuclear membranes (fig. 2A);

- L2: lymphoblasts are larger, often in a more heterogenous population, with a lower nucleus-to-cytoplasm ratio, prominent nucleoli and nuclear membranes that may be reniform or irregular (fig. 2B);
- L3 or Burkitt-like leukemia: lymphoblasts are morphologically heterogeneous and characterized by strongly basophilic cytoplasm and prominent cytoplasmatic vacuolization (Conter et al, 2004) (fig.2C).

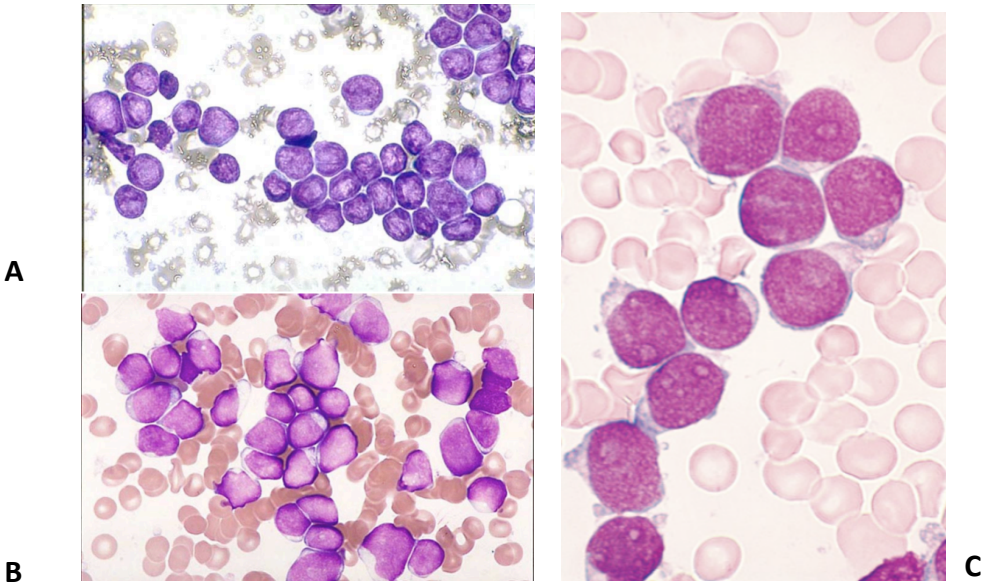


Fig.2. The FAB classification of ALL. The French-American-British Cooperative Working Group, defines three categories of lymphoblasts and, thus, three different types of ALL (L1, A; L2, B; L3, C), based on the following blast features: nuclear cytoplasmic ratio; presence, prominence and frequency of nucleoli; regularity of nuclear membrane outline, and cell size (Conter et al, 2004).

The first-line treatment currently indicated for ALL is systemic chemotherapy, with protocols differing according to patient age. Most ALL patients receive a combination of different chemotherapeutic drugs. The most active agents used in induction regimens include methotrexate, vincristine, prednisone, asparaginase, anthracycline and

dexamethasone. Mantainance chemotherapy for the responding patients consists of 6-mercaptopurine, cyclophosphamide, cytarabine, prednisone, vincristine, carmustine, daunorubicin, doxorubicin, teniposide in various combinations. Prophylactic radiation therapy to the central nervous system is also frequently used to prevent or delay the occurrence of CNS relapse.

1.2 Molecular genetics of ALL

In most cases of ALL, as in other lymphoid malignancies, a single damaged progenitor cells is capable to indefinitely expand, self-renew and give rise to malignant poorly differentiated precursors. It is not completely clear where in the normal course of differentiation the "clonal event" occurs. Nowadays, thanks to the recent development of molecular cytogenetic techniques (e.g. FISH and CGH), it is possible to recognize chromosomal abnormalities in the leukemia cells of most of the cases of pediatric ALL (Conter et al, 2004).

Translocations are the most commons structural chromosomal changes in ALL. They are assumed to play a fundamental role in the leukemogenic process and in most cases are associated with elevate risk of early treatment failure (Conter et al, 2004).

Among the most frequent translocations, t(9;22) is the most one associated with adult ALL (Lee, 2011). Moreover, chromosomal rearrangements involving *TCR* genes are recurrent and are responsible for the aberrant or ectopic expression of proto-oncogene, some of them being directly or indirectly involved in T-cell development, such as *NOTCH1*, *MYB*, *HOXA* (Graux, 2011).

The process of malignant transformation in pediatric acute leukemias is complex, requiring at least two leukemogenic hits that result in DNA damage, ranging from point-mutations to double-strand DNA breaks leading to chromosomal translocations, deletions, duplications or inversions. Investigations with single nucleotide polymorphism arrays have confirmed that leukemic blasts have multiple copy-number aberrations (Mullighan et al, 2007). The exception seems to be leukemias with translocations involving the *Mixed Lineage Leukemia gene*, *MLL* and different chromosomal partners that are rarely accompanied by other genetic lesions, suggesting that *MLL* rearrangements are oncogenic events with the potential to induce leukemia in a single hit (Szczepanski et al, 2010). Different abnormalities with *MLL* involvement can be detected in almost all types of hematological malignancies. The most common are the reciprocal translocations that fuse the first 8-11 coding exons of *MLL* in frame to a partner gene that resides on a different chromosome. The result of these rearrangements is the production of an oncogenic chimeric protein (Ayton & Cleary, 2001).

Internal tandem duplications within the MLL coding region have been described in almost 10% of adult AML cases. This feature leads to an elongated version of the MLL protein where the N-terminal amino acid sequence is duplicated and fused to itself in a tandem repeat. The presence of this kind of rearrangement in AML most likely also worsens disease outcome (Dohner et al, 2002; So et al, 1997; Yu et al 1996).

The unaltered *MLL* gene itself can be amplified in samples of AML and myelodysplastic syndrome suggesting that an increase of the MLL gene dosage might evoke similar downstream effects like the presence of an MLL fusion or PTD 11 (Dolan et al, 2002; Poppe et al, 2004).

However, most *MLL* rearrangements are due to reciprocal chromosome translocations that link *MLL* to any of more than 100 partner genes (Meyer et al, 2009).

The partner proteins most commonly found associated with MLL (AF4, AF9, ENL, AF10, ELL) are transcriptional elongation factors (Bitoun et al, 2007). Since these factors physically associate, the partner of MLL within the fusion proteins seems to recruit the other elongation factors and thereby augmenting the target genes transcription (Mueller et al, 2009). Despite the large number of its partners, all MLL fusions are invariably formed according to the same pattern: the C-terminus of MLL is replaced in frame by the protein partner. The partner proteins frequently contain activation domains in their C-terminus, and consequently, the C-terminal minimal transactivator domain suffices to convert MLL to an active oncoprotein (Slany, 2005).

The *Mixed Lineage Leukemia* (*MLL*) gene and protein

The long arm of chromosome 11 was long noted by onco-hematologists as a hotspot for genomic rearrangements. In particular the cytogenetic band 11q23 became notorious because alterations of this locus were associated with especially aggressive leukemias (Esseltine et al, 1982; Kaneko, 1982).

Nowadays we know that this is because 11q23 harbors the *Mixed Lineage Leukemia* gene (*MLL*, known also as *HRX* or *ALL-1*) and abnormalities at 11q23 frequently convert MLL to an active oncogene (Slany, 1995).

MLL is a member of an evolutionarily conserved family of proteins known as "the trithorax group" (trxG), which are positive regulators of gene expression during the development. Indeed, trxG proteins are involved in the so-called “cellular memory”. This epigenetic mechanism is necessary in development and differentiation whenever

transcriptional patterns must be heritably fixed for future cell generations, also in absence of the initially determining transcription factors (Slany, 2005).

The *MLL* genomic structure consists of 36 exons distributed over 100 kb and produces a 12-kb mRNA that encodes a 3969 amino acid protein with an estimated molecular weight of 430 kDa (Aplan, 2006). Three main domains have been implicated in the MLL function: the AT hooks motifs, which bind within the minor groove of the double helix; a region homologous to DNA methyltransferase (DNMT), which binds to non-methylated CpG dinucleotides – a feature characteristic of CpG islands in transcriptionally competent genes; and a highly conserved H3K4 methyltransferase (SET) domain, which methylates lysine 4 in histone 3 (Ayton & Cleary, 2001), (Fig. 3).

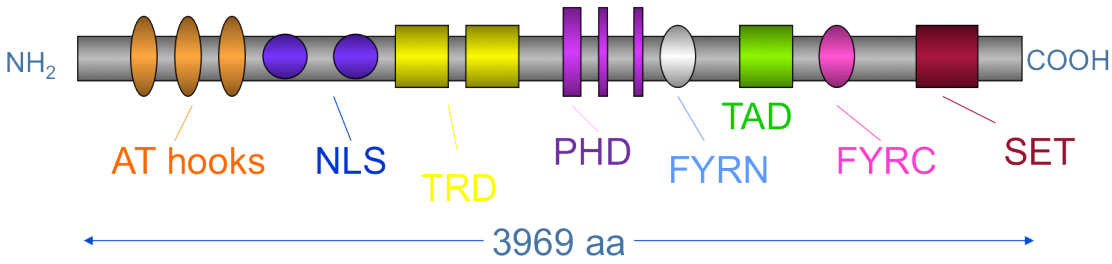


Fig. 3. Schematic representation of the most relevant functional domains in the wild-type MLL protein. Starting from the N-terminus portion of the MLL protein, there are three AT-hooks motifs, two Nuclear Localization Signals (NLS), two Transcriptional Repression domains (TRD), three Plant Homeo Domain (PHD) fingers, the FYRN region, the Transactivation Domain (TAD), the FYRC region and the SET domain.

Between the AT hooks and a large transcriptional repression domain (TRD1+TRD2) there are two small regions, NLS1 and NLS2, which specify the subnuclear localization of the MLL protein (Slany et al, 1998). TRD1 contain the DNA methyltransferase homology domain,

DNMT1, including the CXXC zinc finger domain (Zeleznik-Le et al, 1994) whereas TRD2 mediates transcriptional repression through recruitment of the histone deacetylases HDAC1 and HDAC2, which can also interact with part of TRD1 directly (Xia ZB et al, 2003). The mature MLL protein is cleaved by a specific protease which cuts the protein at amino acid residues 2666/2667 and 2718/2719. The enzyme responsible of this proteolitic event is called taspase 1, a threonine protease that cleaves MLL into N300/320 and C180 terminal polypeptides (Hsie et al, 2003). Both parts are associated through the FYRN domain (aa 2023-2073) and FYRC domain plus part of the SET domain (aa 3666-3876) (Daser & Rabbitts, 2007). The FYR domains are defined as 40-90 aminoacids-long sequences rich in phenylalanine (F) and tyrosine (Y) residues (Pless et al, 2011). Three PHD zinc finger domain are present in the N-terminus portion of MLL that is lost after the translocation event. The third PHD has been found to interact with the nuclear cyclophilin CYP33 and this interaction seems to affect *HOXC8* and *HOXC9* transcription (Fair et al, 2001). The transcriptional activation domain (TAD) is not found in *Drosophila* trithorax, but it is conserved in vertebrate MLL-homologues and binds directly to the co-activator CBP (CREB-binding protein) (Bannister et al, 1996).

MLL regulates the *Hox* genes expression

In mammals, MLL positively regulates the clustered *homeobox* (*Hox*) genes and the homeobox cofactor *MEIS1*, which play a key role in hematopoietic differentiation (Milne et al, 2002). Overexpression of individual *Hox* genes is leukemogenic in mice (Kroon et al, 1998). Normally, *Hox* expression is high in hematopoietic stem cells and gradually decreases during

differentiation. Failure to downregulate *Hox* expression inhibits hematopoietic maturation and can lead to leukemia (Thorsteinsdottir et al, 2002).

The H3K4 methylation status of the chromatin correlates with an active state of transcription, so it is likely the *HOX* gene promoters are H3K4 methylated through the MLL SET domain (Daser & Rabbitts, 2007). However, Milne et al (2002) demonstrated that, although the leukemogenic MLL-AF9 fusion lacks the SET domain, it can still activate *HoxC8* without H3K4 methylation activity.

Data from ChIP-seq analysis revealed that *MEIS1, HOXA7, HOXA9* and *HOXA10* are among the 226 primary targets of MLL-AF4 in a human ALL cell line (Guenther et al, 2008). Interestingly, Orlovsky et al (2011) found that downregulation of *MEIS1* and *HOXA* in *MLL*-rearranged acute leukemia impairs engraftment and reduces the proliferation of the transformed cells.

The AF4 gene and protein

The *AF4* gene, also known as *FEL*, was first described as the most frequent fusion partner with *MLL* in the infant acute lymphoblastic leukemia (Gu et al, 1992) and encodes a transcriptional activator implicated in lymphopoiesis and Purkinje cell function in the cerebellum (Bitoun et al, 2007).

The *AF4* gene localizes on 4q21 cytogenetic band, spans 134050 bp and consists of 20 exons. Its mature transcript is 9290 bp long, the length of its ORF is 3633 bp and is translated in a 1210 aa-long protein, with a molecular weight of 140 kDa.

Northern Blot experiments found that AF4 mRNAs are widely expressed in hematopoietic cells and tissues (Chen et al, 1993). Moreover, the lymphoid nuclear protein related to AF4 (*LAF4*), isolated from Burkitt's lymphoma, shares high degree of sequence homology with AF4 and it showed to possess DNA binding ability, thus hypothesizing a potential role as a transcriptional activator (Ma & Staudt, 1996). AF4 and LAF4, together with AF5q31 and FMR2 belong to the so-called "ALF" family and share three conserved regions: the N-terminal homology domain, the ALF domain, which contains a serine/proline rich region containing GTP-binding domain, and the C-terminal homology domain. Furthermore, each member of the ALF family, except FMR2, has a transactivation domain (Prasad et al, 1995). The ALF domain seems to promote the protein proteasome-dependent degradation by mediating its interaction with SIAH (seven *in absentia* homologue) ubiquitin ligase (Oliver et al, 2004; Bursen et al, 2004). As a further confirmation that AF4 works a transactivator factor, Li et al (1998) showed that AF4 localize into the nucleus, because of its nuclear targeting sequences.

A murine AF4-knockout model demonstrated that AF4 is important for normal lymphocyte development and cell growth (Isnard et al, 2000). AF4 was also identified as the disease gene in the robotic mouse, a dominant N-ethyl-N-nitrosurea mutant that, besides the defect in early T-cell maturation develops ataxia because of Purkinje cell degeneration in the cerebellum (Isaacs et al, 2003; Bitoun & Davies, 2005). Furthermore, AF4 was found to have transcriptional regulatory properties that entail elongation and chromatin remodeling involving Pol II, also through the interaction with the P-TEFb complex, ENL and/or AF9 (Erfurth et al, 2004). Notably, AF4 associate with Dot1L, the histone methyltranseferase that modify H3K79 and marks actively transcribed genes (Bitoun et al, 2007). Recently, Yokoyama

et al (2010) demonstrated that AF4 takes part to a higher-order complex, which is constituted by P-TEFb, ENL, AF5q31 (AEP complex), and that this complex is recruited by wild-type MLL on its target promoters (i.e. *HOXA9* and *MEIS1*). Thus, it seems that chimaeric proteins originating from the fusion of MLL to one of the AEP components, could constitutively form hybrid complexes, that cause sustained expression of MLL-target genes that leads to leukemic transformation of the hematopoietic cells.

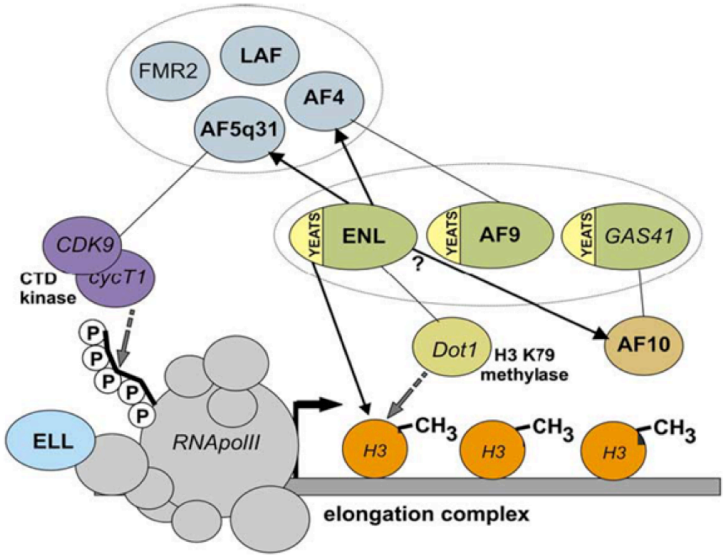


Fig.4. A complex containing AF4 and ENL family proteins with P-TEFb facilitates oncogenic and physiologic MLL-dependent transcription. Many of the nuclear MLL fusion partners are connected in a complex that is associated with chromatin modifications imposed during transcriptional elongation. Methylation of histone H3 lysine K79 and phosphorylation of the C-terminal domain of the RNA polymerase II are enzymatic events that occur during the elongation.

The t(4;11) chromosomal translocation produces an active fusion oncoprotein, MLL-AF4

The *MLL* gene participates in a large variety of different chromosomal translocations, that result in the production of chimaeric oncoproteins, which initiate critical steps of

malignant transformation and lead either to the development of AML or ALL Leukemia. However, the aberrant molecular mechanism induced by the fusion proteins remains unclear.

To date, over 90% of the leukemias diagnosed in newborns/infants (<1 year old) are pro-B stage acute lymphoblastic leukemias harboring the leukemic fusion gene MLL-AF4 (Bueno et al, 2011). This translocation is the hallmark of a high-risk acute lymphoblastic leukemia (ALL), which has a particularly poor prognosis in infants (Pui et al, 2002).

Although most evidence reported that the oncogenic properties reside in the MLL-AF4 product, there are some data demonstrating that the combined effects of the two reciprocal t(4;11) fusion proteins MLL-AF4 and AF4-MLL confer resistance to apoptosis, cell cycling capacity and growth transformation (Gaussmann et al, 2007). Moreover, the same group showed that the AF4-MLL fusion protein is capable of inducing ALL in mice without requirement of MLL-AF4 (Bursen et al, 2010). In contrast Kumar et al (2010) have recently demonstrated that the loss of AF4-MLL had no effect on the growth of either RS4;11 or SEMK2-M1 cells, which express both reciprocal fusion products. Indeed in SEMK2-M1 cells there were no changes in cell cycle or apoptosis with loss of AF4-MLL, whereas knockdown of MLL-AF4 significantly inhibited growth of both RS4;11 and SEMK2-M1.

The MLL-AF4 fusion site differs among t(4;11)-positive cell lines. Indeed, SEM cells express a transcript in which exon 9 of MLL is fused to exon 4 of AF4, whereas RS4;11 cells express an e10-e4 variant (Stong et al, 1985; Thomas et al, 2005). In our research group a hitherto unknown MLL-AF4 breakpoint was identified, in which the sequences corresponding to MLL exon 9 are fused with the sequences corresponding to exon 11 of the AF4 gene in the fusion transcript. The predicted structure of the new MLL-AF4 chimeric protein lacks the

portion of the *AF4* transactivation domain, spanning residues 480 to 560, which is encoded by exon 10 of the *AF4* gene and the first nucleotides of exon 11 (Pane et al, 2002) (Fig. 5).

Following the t(4;11) translocation, genomic sequences encoding the AT-hook and proline-rich regions of MLL are fused in-frame to the AF4 sequence that encodes the NLS and GTP-binding activity, thereby resulting in a chimeric mRNA of 12.5 kb that encodes a predicted fusion protein of 240 kDa (Li et al, 1998).

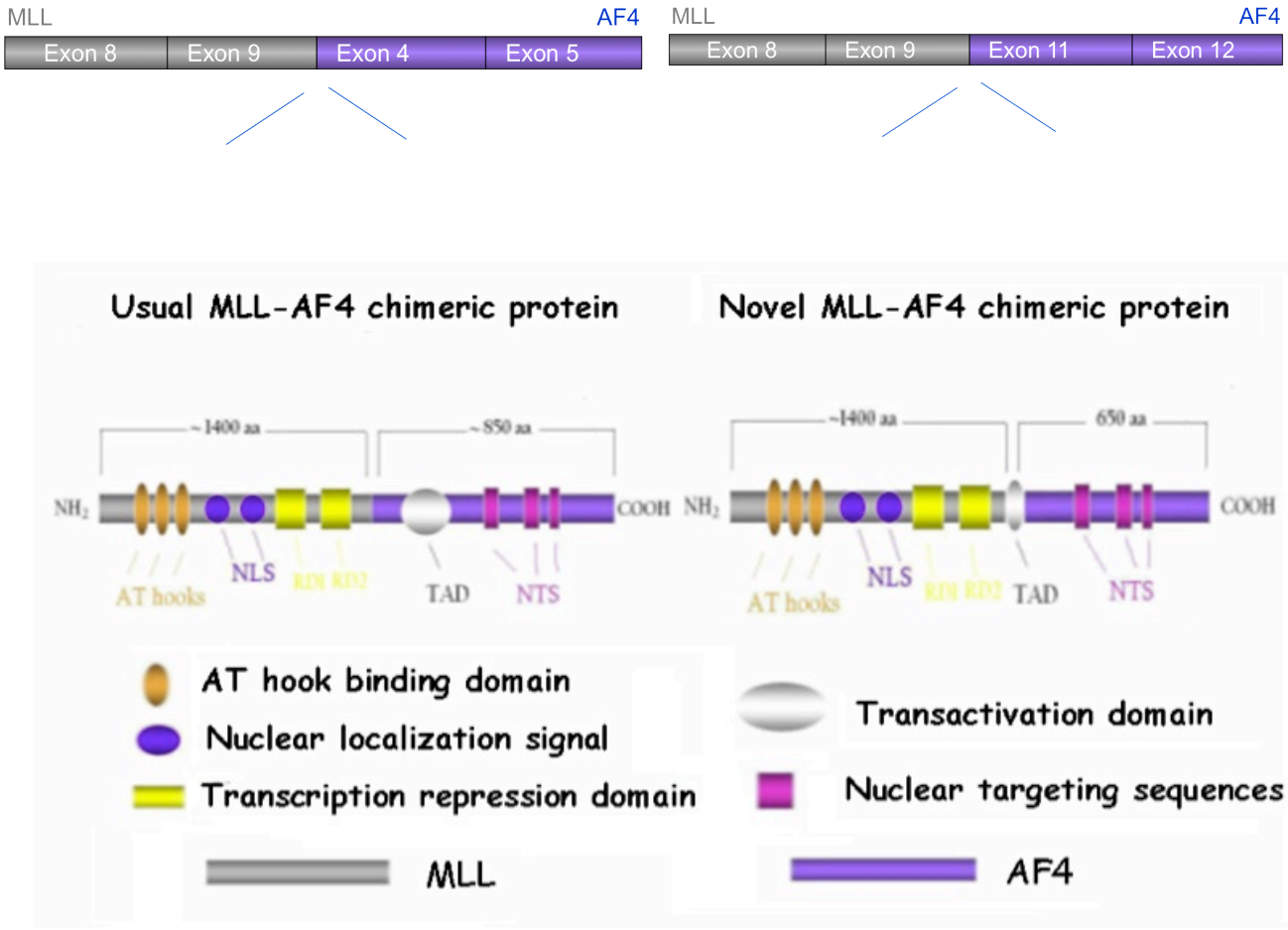


Fig. 5. Different breakpoints and different fusion proteins MLL-AF4. Although the most frequent MLL-AF4 breakpoint consists in the in-frame fusion of exon 9 of MLL and the exon 4 of AF4 ("usual"), many other different breakpoints are known and characterized in the t(4;11) ALL patients. The one we called "novel" consisted in the fusion of exon 9 of MLL and the exon 11 of AF4, thus skipping a large sequence encoding the transactivation domain of the AF4 protein.

Although the MLL fusion proteins lack the SET domain of MLL, they retain the ability to bind *Hox* genes and other promoters (Krivstov et al, 2008). The mechanisms by which MLL fusions may deregulate gene expression include the recruitment of a protein complex that could induce an abnormal activity of histone modification. For example, Bitoun et al (2007) demonstrated that AF4 belongs to a transcriptional regulatory chromatin remodeling complex, as well as AF9, ENL and AF10 proteins, which are also implicated in leukemia and human neurological dysfunctions. They provide evidence that these proteins collaboratively function as positive regulators of gene transcription by stimulating p-TEFb kinase activity. They also mediate Dot1-dependent methylation of H3-K79, thereby facilitating the transition of Pol II into productive elongation and its progression along the DNA template by maintaining the chromatin structure in an open conformation. Krivstov et al (2008) demonstrated that the H3K79 methylation is enhanced at many *loci* in leukemia cells from a murine model of MLL-AF4 and in human MLL-AF4 leukemia cells, and showed that enhanced methylation is correlated with increased gene expression. The suppression of H3K79 methylation leads to inhibition of gene expression in MLL-AF4 cells.

Previous data obtained in our research group showed that the native AF4 protein is part of a large network of molecular interactions involved in the regulation of the RNA-Polymerase II transcriptional machinery (Esposito et al, 2011).

The t(4;11) rearrangement occurs in an early hematopoietic progenitor cell

A large body of evidence suggests that high risk t(4;11) (q21; q23) ALL originates in primitive lymphoid-restricted $CD34^{+}C19^{-}$ stem cells thereby supporting the hypothesis that

the poor outcome is a consequence of the transformation of a primitive stem/progenitor cell (Hotfilder et al, 2005).

Furthermore, Montes et al (2011) showed that the enforced expression of MLL-AF4 in human cord blood-derived hematopoietic stem and progenitor cells enhances the hematopoietic repopulating cell function and clonogenic potential. However, these events seem to be not sufficient for initiating leukemogenesis *in vivo*. This inability to develop a MLL-AF4^{+} ALL disease model based on human cord-blood-CD34^{+} progenitors suggests that secondary mutations or the AF4-MLL reciprocal product might be required to develop overt ALL.

Chapter 2

Materials and Methods

Cloning procedure of cDNAs encoding MLL-AF4

Because of the wide length of the usualMLL-AF4 fusion cDNA (6.681 kb), we decided to amplify the cDNA in 3 different overlapping fragments, by using primer pairs, each specific for the following fragments: “MAF1-2-3”, containing nt 1-3725 of MLL; “usualMLL-AF4 breakpoint”, from nt 3726 to 5137 of MLL; “AF4”, the portion of AF4 that takes part in the chimaera, nt 1040-3635 of AF4. The MAF1-2-3 fragment was subdivided in 3 further fragments: MAF1, MAF2 and MAF3.

RNA extraction

Total RNA was extracted from RS4;11 cells with TRIzol (Invitrogen), according to the manufacturer’s instructions. The RNA was resuspended in H_20 and stored at -80 °C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out by using “SuperScript™ III Reverse Transcriptase” (Invitrogen) kit, with “Random Primers” (Invitrogen), following the manufacturer’s instructions. 5 μg of RNA was used as template for the RT reaction. The cDNA was amplified by using primer pairs specific for: MAF1, MAF2, MAF3 and usualMLL-AF4 breakpoint. 100 ng of cDNA were used as template for the amplification, by using “Expand High Fidelity PCR System” (Roche).

Topo-Cloning

Each PCR product was cloned in the pCRII-TOPO entry vector (Invitrogen), according to the manufacturer's procedure. This vector provides the direct insertion of an amplified PCR product without digestion and ligation procedures. This procedure bases on the *Taq* terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The pCRII-TOPO has single overhanging 3' deoxythymidine (T) residue, which allows PCR inserts to ligate efficiently with the vector.

Cloning of MLL-AF4 in p3XFlag

For the cloning of MLL-AF4, we chose the p3XFlag (Sigma) as eukaryotic expression vector. This plasmid provides three adjacent FLAG epitopes upstream of the multiple cloning region. The presence of this epitope, which is easily recognized by a specific antibody anti-FLAG, allows to facilitate the detection and purification procedures of the protein of interest.

In order to introduce the fusion MLL-AF4 cDNA in the p3XFlag, we first amplified the single MAF1, MAF2 and MAF3 sequences from the "pCRII-TOPO+MAF1", "pCRII-TOPO+MAF2", "pCRII-TOPO+MAF3" constructs, respectively, by using primer pairs, containing sequences recognized by specific enzymes, at their 5' end. Then, by performing series of digestion and cloning of the three overlapping fragments, we obtained the product MAF1-2-3 in the p3XFlag.

The usualMLL-AF4 breakpoint fragment was first amplified from the cDNA with specific primer pairs, containing the specific sites recognized by EcoRI and XbaI at their 5' ends, from the pCRII-TOPO+usualMLL-AF4 breakpoint. Then, both insert and p3XFlag were digested with

EcoRI and XbaI. The digestion products were loaded on 0.8% agarose gel and purified by "QIAquick gel extraction kit" (Qiagen). The ligation reaction was performed at a molar concentration of 1:2 (vector : insert), by following the Ligafast Protocol (Promega).

100 μl of TOP10 strain of *E. coli* cells were transformed in sterile conditions with 10 μl of the ligation products and kept on ice for 20'. Then the suspension was incubated at 42°C for 45" and then at 37°C for 45', following the adding of 250 μl of Luria-Bertani (LB) broth. Then, the suspension was spread on a LB+agar plate, containing the antibiotic ampicillin [100 mg/ml]. Indeed, thanks to the ampicillin resistance gene, encoded by the p3XFlag, it was possible select only the cloning positive colonies, after the O.N. incubation of the plate at 37°C. Some colonies were picked and cultured in LB+ampicillin at 37°C O.N., in order to obtain a high amount of plasmids. The plasmidic DNA was purified from the bacterial suspension, by using the "Plasmid/Cosmid purification kit" (Qiagen). The plasmids were tested by restriction digestion and sequenced at the Core Facility for DNA Sequencing of Ceinge-Biotecnologie Avanzate.

Reproduction of the novelMLL-AF4 breakpoint

The novel MLL-AF4 breakpoint was obtained from the usualMLL-AF4 cDNA, by a series of amplification and ligation procedures, as described below.

Two primer pairs (A and B) were designed both upstream and downstream to the known different portion among the usual and the novel breakpoint (the blue portion in fig.6). The two regions were amplified by PCR and then, the PCR products were blunt-ligated. The Forward A and the Reverse B were used for the amplification of the whole region "novelMLL-

AF4" breakpoint. The PCR product was then digested with EcoRI and XbaI, and cloned in p3XFlag, as already done with the usual breakpoint. All the procedure is shown in fig. 6.

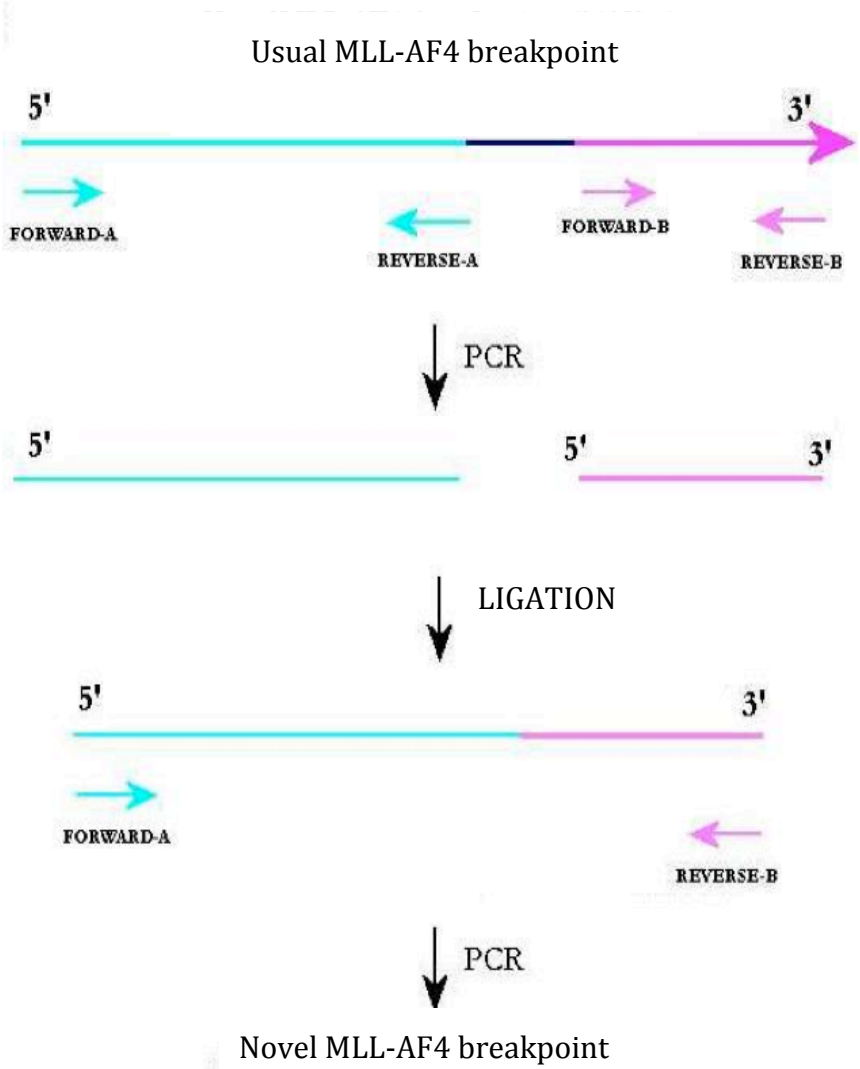


Fig. 6. Reproduction of the cDNA encoding "*novel*MLL-AF4 breakpoint". The "novel" breakpoint is ~ 700 bp smaller than the usual one. The procedure for reproduction of the novel breakpoint consisted in the amplification of the regions immediately upstream and downstream to the different sequence between the two breakpoints (the blue region), by using specific oligonucleotide sequences (forward A and reverse A for the upstream region; forward B and reverse B for the downstream sequence). The two amplicons (A and B) obtained from the "usual" breakpoint were than undergone to ligation reaction and amplified by using forward A and reverse B primers.

The p3XFlag+AF4 full length, already obtained in our lab for previous experiments, was used for obtaining the whole usual/novel MLL-AF4 clones. 5 μg of this plasmid was used as destination vector and digested with NotI and PstI (Roche), thus removing all the N-terminus AF4 portion not-included in the MLL-AF4 fusion. Then, both usualMLL-AF4 and novelMLL-AF4 breakpoint were excised by digesting the p3XFlag+usual/novelMLL-AF4 constructs with the same enzymes and inserted in the destination vector, by ligation reaction, as already described.

Finally, 6 μg of both p3XFlag+MAF1-2-3 and p3XFlag+usual/novelMLL-AF4 were digested with NotI and BamHI. The linear fragment of ~3 kb originating from the latter reaction, was ligated with MAF1-2-3, originated from the first digestion, according with the same Ligation protocol (Promega), already described. Some colonies "p3XFlag+MAF1-2-3+usual/novelMLL-AF4" were tested by restriction digestion and sequenced at the Core Facility for DNA Sequencing of Ceinge-Biotecnologie Avanzate.

Cloning of cDNA encoding MLL-AF4 in a tet-on system

In order to obtain a regulated expression of MLL-AF4, the tetO/mEPO plasmid was used as destination vector for cloning the fusion FLAG+usual/novelMLL-AF4 cDNAs. The contransfection of the tetO vector and another vector (Bs/IRES-M2), encoding the reverse-tetracyclin transactivator (rtTA), allows the transcription of the cloned cDNA only when the rtTA binds to the tetO *cis*-element, in presence of doxycycline, an analogue of the tetracycline.

Following the manipulation procedures of the tetO/mEPO (kind gift from Prof. Pastore's Lab, Ceinge-Biotecnologie Avanzate), aimed to excise the sequence encoding the murine erythropoietin, the vector tetO and both the p3XFlag+usual/novelMLL-AF4 full length were digested with SnaBI and BstXI endonucleases. Both vector and insert were loaded on 0.8 % agarose gel and purified, as already described above. The ligation reaction was performed at a molar concentration of 2:1 (vector : insert), by following the Ligafast Protocol (Promega).

Some colonies were tested by restriction digestion and sequenced at the Core Facility for DNA Sequencing of Ceinge-Biotecnologie Avanzate.

Cell Cultures

Hek293 and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin.

RS4;11 is a cell line established from the bone marrow of a patient in relapse with an acute leukemia that was characterized by the t(4;11) chromosomal abnormality (Stong et al, 1985). We used this cells to obtain the "usual" MLL-AF4 breakpoint.

697 cell line is a cloned human pre-B leukemic cell line derived from childhood acute lymphoblastic leukemia that carries the t(1;19) translocation (Williams DL et al, 1984; Tsujimoto Y et al, 1985).

Both RS4;11 and 697 grow in suspension and were cultured in Roswell Park Memorial Institute (RPMI) medium, with 10% FBS.

All the cell cultures were incubated at 37°C in humidified atmosphere with 5 % CO_2.

Enrichment and flow cytometric analysis of $CD34^+$ cells

Human hematopoietic stem and progenitor cells were collected from donors' cord blood of the Biological Sample BioBank of Ceinge-Biotecnologie Avanzate. $CD34^+$ cells were isolated in high purity, by using MACS magnetic separation system (Miltenyi Biotech), according to manifacturer's instructions. Both positive and negative fractions were collected, in order to analyze the purity degree of the eluted fractions by flow citometry. Cells from positive elution, flow through and washes, respectively, were concentrated in 100 μl and stained with the appropriate amount of anti-CD34 phycoeritrin (PE)-conjugated (Becton Dickinson) and anti-CD45 Peridinin Chlorophyll Protein (PerCP)-conjugated (Becton Dickinson) for 30' at 4°C. Then, the cells were washed and resuspended in 200 μl of PBS and analized with a FacsARIAII cytometer of the Core Facility "Experimental and Clinical Flow Citometry" of Ceinge-Biotecnologie Avanzate. Cells gating and debris exclusion were performed by a FSC/SSC dot plot; the frequency of $CD34^+$ cells was evaluated on a CD45-PerCP versus CD34-PE dot plot of stained cells. The positive fraction was cultured in a 24-wells plate at a concentration of 5 X 10^5 cells/well, in 1 ml of Iscove Modified Dulbecco's Medium (IMDM) with 1% penicillin/streptomycin and 10% FBS.

Cell transfection

Hek293 and HeLA cells were transfected with either Lipofectamine (Invitrogen) or Polyfect (Qiagen), according to the manufacturers' instructions. For the tet-on system, Hek293 cells were contransfected with the same amount of both plasmid tetO+usual/novelMLL-AF4 and Bs/IRES-M2 and cultured with medium containing either 0.1 μl/ml or 1 μl/ml doxycycline.

$CD34^+$ cells were transfected with Superfect (Qiagen) (Teixeira et al, 2001). The transfection efficiency was evaluated by Real-Time PCR, detecting the MLL-AF4 transcript in both transfected and mock control cells.

Protein extraction, Western Blot and antibodies

48 hours after the transfection, cells were spun down and resuspended in lysis buffer (50 mM pH 8 Tris-HCl, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 10% Glycerol) with 1:100 Protease Inhibitor Cocktail (Sigma) and 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and incubated on ice for 20'. The whole cell extracts (WCE) were clarified by centrifugation at 12,000 rpm for 15' at 4° C. The supernatants were collected and their concentration was measured with the "Biorad Protein Assay" (BioRad). For the Western Blot analysis, either 30 μg of WCE or 20 μl of immunocomplexes were denatured by heating in an equal volume of Laemmli buffer (BioRad). The samples were loaded on Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE), with a polyacrylamide mix percentage spanning from 8 to 12%, depending on the molecular weight of the protein of interest. A molecular weight marker ''Precision Plus ProteinTM Standards Dual Colors" (Biorad), as well, was loaded on the gel, in order to test the precise molecular weight of the protein of interest. The buffer used for the electrophoretic separation was Tris-Glycine-SDS. Then, the proteins on the gel were transferred by elettroblotting to a PVDF membrane, which was first incubated with Tween-Tris-Buffered-Saline-(TTBS)-5% Milk for the blocking step, and then probed with the following antibodies: anti-FLAG (Sigma), anti-CRSP33 (Santa Cruz Biotechnology, Inc), anti-CRSP130 (Santa Cruz Biotechnology, Inc), anti-Cdk9 (Santa Cruz

Biotechnology, Inc), anti-FGFR2 (Santa Cruz Biotechnology, Inc), anti-14-3-3 θ (Santa Cruz Biotechnology, Inc) and anti-α tubulin (Sigma). The secondary antibodies were conjugated to the HorseRadish Peroxidase (HRP) and the proteins were detected on the filter, by using "ECL plus" kit (GE Healthcare) for the immunoprecipitates and "ECL" kit (GE Healthcare) in the other cases.

Semiquantitative-PCR

For the Semiquantitative-PCR analysis on HoxA9 transcript, total RNA was extracted from RS4;11, Hek293 transfected with p3XFlag+usual/novelMLL-AF4, control lymphocytes, and mock control, respectively, by using the "RNAspin mini RNA isolation" kit (GE Healthcare), which also provided the treatment with DNase, in order to degradate the DNA. 5 μg of each RNA were retrotranscribed as already described above (see RT-PCR in this section). 3 μl of cDNA were used as template for the semiquantitative PCR. For this amplification reaction, *Taq* DNA polymerase (Invitrogen) and 1 μl of *HoxA9* and *GAPDH*-specific forward and reverse primer (10 μM) were added to the reaction mix. The reaction was carried out at 32 cycles, then the PCR products were loaded on a 1.5 % agarose gel, containing ethidium bromide (0.05 μl/ml) in buffer 4 mM Tris-Acetate-EDTA (TAE). The bands were visualized at Gel Doc with Quantity One (BioRad) software. The oligonucleotides used for the semiquantitative-PCR analysis of HoxA9 and glyceraldehyde-3 phosphate-dehydrogenase (GAPDH) are contained in table 1.

RT-Real Time PCR

The Real-Time PCR was perfomed in triplicate for each sample. RNA was extracted as described in the "Semiquantitative PCR" subparagraph and retrotranscribed with "SuperScript^{TM} III Reverse Transcriptase" (Invitrogen) kit, with "Random Primers" (Invitrogen). We used the iQ^{TM} SYBR Green Supermix (Bio-Rad), in which dNTPs, *Taq* DNA Polimerase, buffers $MgCl_2$, and the intercalant probe SYBR GREEN are already premixed. For each replicate, 7.5 μl of iQ^{TM} SYBR Green Supermix, 1 μl of each forward and reverse gene-specific primer (10 μM) were mixed with sterile ddH_2O, to a final volume of 14 μl. As template of the reaction, either 1 μl of cDNA, retrotranscribed from 1 μg of RNA extracted from Hek293 or 697 cells, respectively, or 2 μl of cDNA, retrotranscribed from 500 ng of $CD34^+$ cells-derived RNA was used for each replicate. The Real-Time protocol consisted in an initial denaturation at 95°C, followed by 40 cycles, each providing a step at 95°C for 15" and another one at 60°C for 1'. The last cycle of the reaction consisted in a series of steps in temperature gradient. From one step to the next one there was an increase of 0.2°C, until 90°C. During this gradient the fluorescence emissed by the SYBR GREEN probe was detected by the iCycler Real-Time-PCR software (BioRad), at a wavelength of 490. The fold increase of transcript expression was calculated with the formula: $2^{-\Delta\Delta Ct}$, where ΔCt is the difference between the Ct of the gene of interest and the housekeeping gene in each cell population. The primers used for the Real-time PCR of HoxA9, FGFR2, β-actin and β-glucuronidase (GUS) are contained in table 1.

Primer	Sequence
HOXA9 F	TGTGGTTCTCCTCCAGTTGATAGAG
HOXA9 R	TCGGTGAGGTTGAGCAGTCGAG
GAPDH F	GGTCGTATTGGGCGCCTGGTCA
GAPDH R	CCAACCCATGACGAACATGGGGGC
β-ACTIN F	CGCGACAGGATGCAGAAGGAGA
β-ACTIN R	CGTCATACTCCTGCTTGCTG
FGFR2 F	CTCAAGCACTCGGGGATAAA
FGFR2 R	TGTTTTGGCAGGACAGTGAG
GUS F	GAAAATATGTGGTTGGAGAGCTCATT
GUS R	CCGAGTGAAGATCCCCTTTTTA

Table 1. Oligonucleotides used for the semiquantitative- and Real-Time PCR.

Immunofluorescence

2 X 10^4 HeLa cells were seeded in a chamber-slide (Nalgene-Nunc) and cultured in 300 μl of fresh medium at 37°C. After 24 h, the cells were transfected (Polyfect, Qiagen) with the recombinant constructs and cultured for 48 hours at 37°C. Then, the cells were fixed in 4% PBS paraformaldheyde solution for 5' at room temperature (RT) and permeabilized with 0.2 % Triton in PBS for 30' at RT. After the permeabilization, the cells were first incubated for 1 h at RT with 2% Bovine Serum Albumin (BSA), 10 % FBS, 0.1 % Triton in PBS for the blocking and then with the primary antibody solution: 2% BSA, 10 % FBS, 0.1 % Triton in PBS, containing a dilution of 1:500 α-Flag, for 3 h at RT. Then, a second incubation in dark was performed with the same solution, containing the fluoresceine isotyocyanate (FITC)-conjugated α-mouse in a 1:100 dilution. Between each step, cells were washed with PBS to discard the reagents in excess.

After the last wash, each well was incubated for 5' at RT with 200 μl of 4',6-diamidino-2-phenylindole (DAPI) solution (0.05 μg/mL in PBS). The chamber-slide was then left to air dry and analized at a fluorescence microscope (Zeiss). The digital images were taken by an Axio Vision (Zeiss) software.

Immunoprecipitation Assay

The total proteins extracted from the transfected cells were immunoprecipitated, by incubation with anti-Flag agarose microbeads (Anti-FLAG M2 affinity gel, Sigma). 40 μl of microbeads were used for every 10 mg of WCE. Before starting the immunoprecipitation, the

microbeads were spun down and the supernatant discarded. The beads were first washed 3 times with a buffer containing 50 mM Tris, 150 mM pH8 NaCl, 1mM pH8 EDTA and then incubated with the appropriate amount of WCE for 3 h at 4°C on a wheeling device. Then, the microbeads were spun down at 3,000 rpm for 5' and washed 3 times with with a buffer containing 50 mM Tris, 300 mM pH 8 NaCl, 10% Glycerol, 0.5 PMSF, 1mM EDTA, 0.1 % NP-40, 1: 100 Protease inhibitor Cocktail (PIC). Then, the microbeads were washed 3 more times with 50 mM Tris, 150 mM pH 8 NaCl, 10% Glycerol, 0.5 mM PMSF, 1 mM EDTA, 0.1 % NP-40, 1:100 PIC and, then, transferred on a microcolumn. The last wash buffer was discarded by spinning the column. The elution of the proteins from the microbeads was carried out by incubating the column for 15' at 4 °C with buffer containing 50 mM TRIS, 150 mM pH 8 NaCl, 10% Glycerol, 0.5mM PMSF, 1mM EDTA, 0,1% NP-40, 1:100 PIC, and 200 µg/ml peptide FLAG (Sigma). The eluted protein fraction was collected, by centrifugation at 3,000 rpm for 2' and stored at -80°C.

Chapter 3

Results

3.1 Cloning of cDNA encoding MLL-AF4 in the p3XFLAG vector

Most of the AF4 molecular partners that were identified in our group by the proteomics experiments are involved in the regulation of the Pol II-mediated transcription, thus suggesting a direct role of AF4 in this process. Here, we asked whether the MLL-AF4 fusion shares one or more partners with the native AF4, and how these interactions could influence and regulate the aberrant activity of the MLL-AF4 chimaera. Thus, the first aim of the present project was to create a cell system that exogenously expressed the MLL-AF4 chimaeric protein.

We cloned the cDNA of the MLL-AF4 fusion protein to characterize their interaction with other members of the complex in which the native AF4 protein is involved.

To clone the cDNA encoding MLL-AF4 fusion protein, we chose two different variants of MLL-AF4: the most common one found in ALL patients carrying t(4;11), which we called "*usual*MLL-AF4" and a more rare and smaller one, detected by our group (Pane et al, 2002) in an infant ALL patient, that we called "*novel*MLL-AF4". The reason why we cloned both isoforms was that the *novel* breakpoint had a smaller AF4 portion and we could eventually expect differences in the interactome of the two different isoforms.

We cloned the *usual* isoform by reverse transcription of the RNA from RS4;11 cell line. The MLL-AF4 breakpoint carried by these cells is an in-frame fusion between the exon 9 of MLL and the exon 4 of AF4.

Because of the wide length of this fusion cDNA (6.681 kb), we decided to amplify the cDNA in 3 three different overlapping fragments, by using primer pairs, each specific for the following fragments: “MAF1-2-3”, “usualMLL-AF4 breakpoint” and “AF4” (fig 7). Then, we subcloned all the three PCR products in the entry vector pCRIITOPO. We chose the p3XFLAG7.1 as final destination vector because it usually allows high levels of transcription of cloned genes, thanks to the upstream strong eukaryotic CMV (Cytomegalovirus) promoter. Furthermore, this plasmid allows an N-terminal in-frame fusion of the cDNA of MLL-AF4 with a sequence (triple epitope FLAG), which is easily recognized by a specific monoclonal antibody (anti-FLAG), thereby facilitating the detection and/or purification of the recombinant proteins.

We engineered the *novel* construct by specific amplification of the regions immediately upstream and downstream of the sequences lacking in the novel fusion breakpoint, followed by a ligation reaction (see fig. 6 in the Materials&Methods section). Fragments “MAF1-2-3” and “*usual/novel*MLL-AF4breakpoint” were respectively cloned in the p3XFLAG (Fig. 8). Then, we digested the subclone “*usual*MLL-AF4 breakpoint” by restriction enzyme and inserted it into the p3XFLAG+AF4 construct (fig. 9A). Lastly, we inserted the fragment “MAF1-2-3” from p3XFLAG+MAF1-2-3, upstream to the sequence “usualMLL-AF4breakpoint/AF4” in the p3XFLAG (fig. 9B).

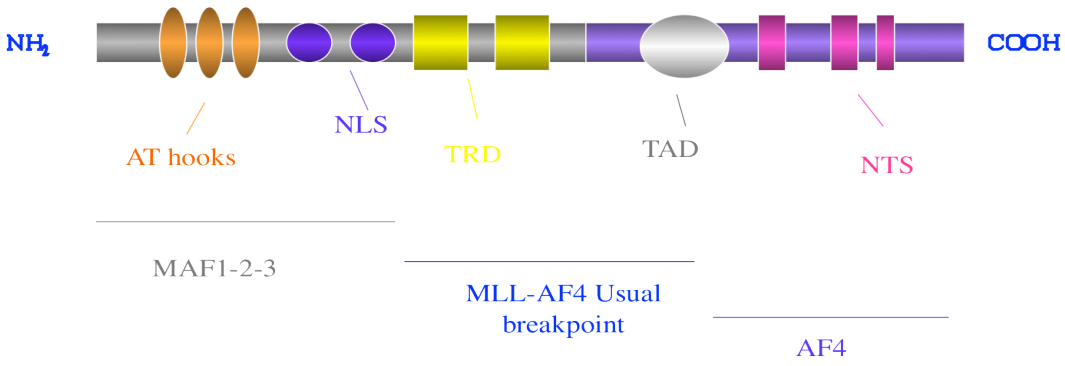


Fig. 7. Subdivision of MLL-AF4 cDNA in 3 overlapping fragments. The first fragment "MAF1-2-3" includes the sequence encoding all the MLL N-terminal domains; the "MLL-AF4" breakpoint is the sequence corresponding to the "usual" junction region between MLL and AF4; the third fragment, "AF4", encodes all the C-terminal domains of the AF4 protein.

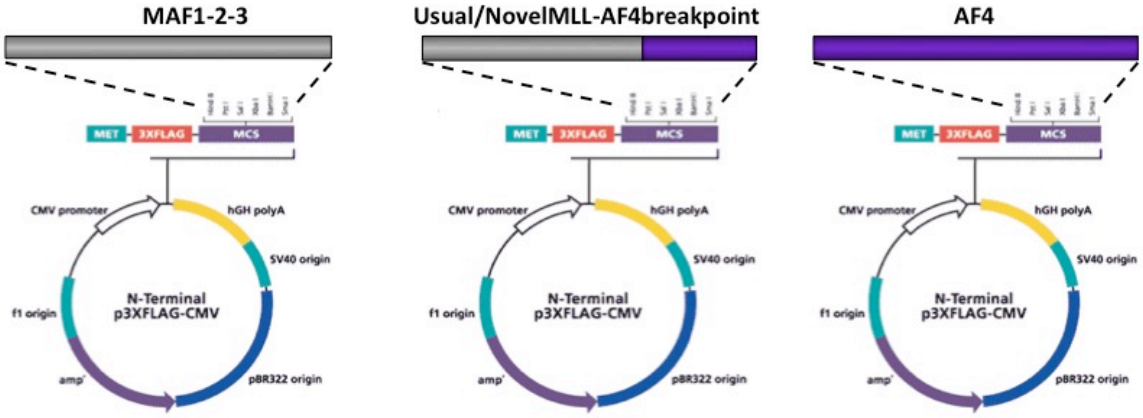


Fig. 8. Cloning of the 4 cDNA fragments covering the whole MLL-AF4 sequence. The single fragments, "MAF1-2-3", "usualMLL-AF4 breakpoint", "novelMLL-AF4 breakpoint" and "AF4", each containing its own open reading frame, has been cloned in the p3Xflag eukaryotic expression vector.

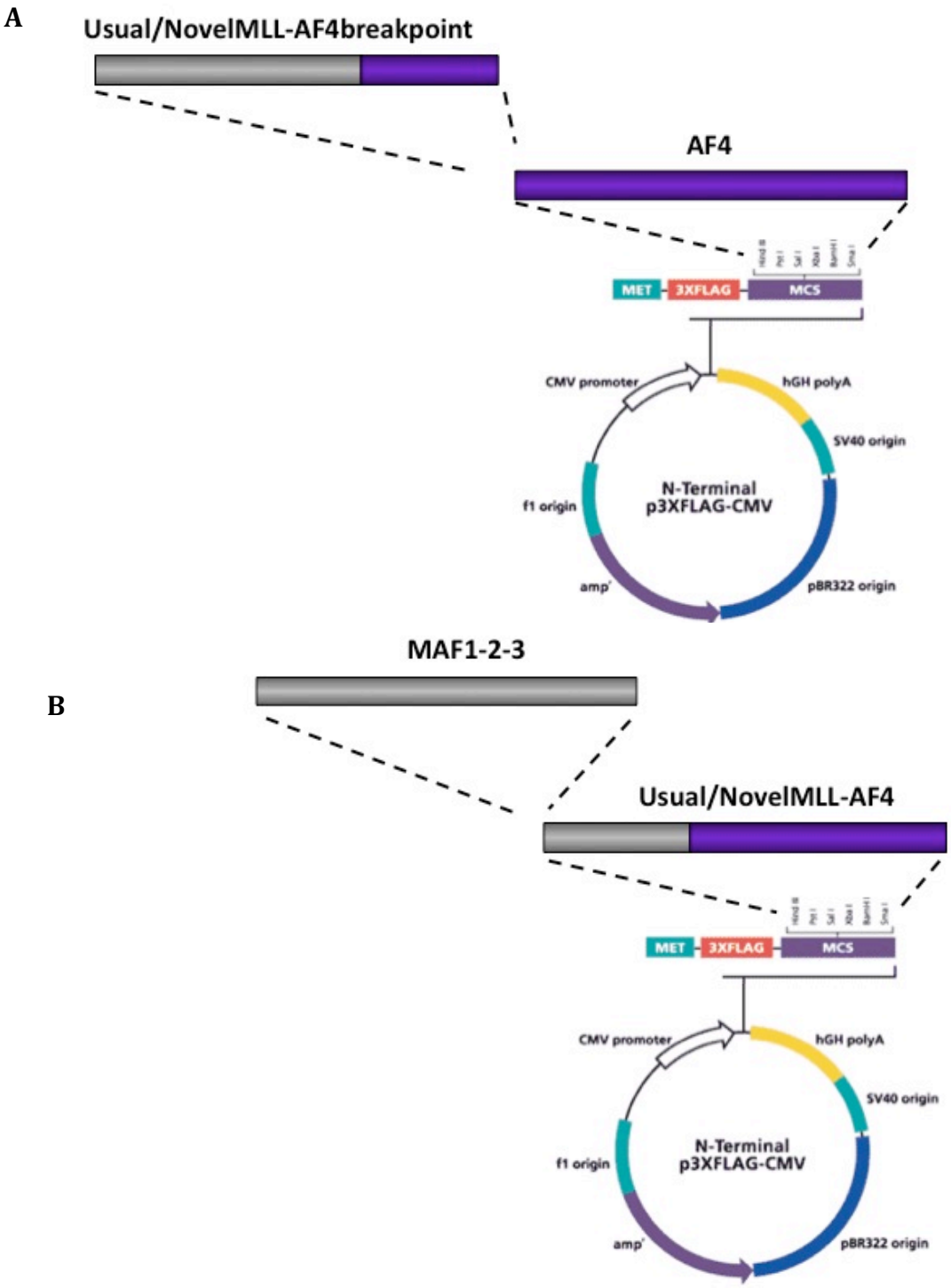


Fig. 9. Cloning procedure of the full length MLL-AF4. Both usual and novel MLL-AF4 breakpoint have been excised from the original p3XFlag constructs by restriction digestion and cloned in the p3XFlag+AF4, digested by the same enzymes (A). Then, the "MAF1-2-3" portion was added upstream to the usual/novelMLL-AF4 constructs, thus obtaining both the usual and the novel MLL-AF4 full length (B).

Positive clones were selected by RE digestion. Correct size of DNA bands was detected by gel electrophoresis. Integrity of one positive clone was verified by sequencing.

3.2 Expression of recombinant MLL-AF4 in human cell lines

We transiently transfected HEK293 and HeLa cells with the plasmids p3XFLAG+*usual*MLL-AF4, p3XFLAG+*novel*MLL-AF4, p3XFLAG+MAF1-2-3, p3XFLAG+*usual*MLL-AF4 full length and p3XFLAG+*novel*MLL-AF4 full length (fig. 10), by liposome methodology. After 48 hours, we extracted total proteins from both transfected and non-transfected cells and detected the fusion protein by using recombinant specific anti-FLAG antibody in Western blot experiment. As shown in fig. 11, Western Blot revealed an efficient expression of all the recombinant proteins: *usual*MLL-AF4 breakpoint (110 kDa), *novel*MLL-AF4 breakpoint (90 kDa), *usual*MLL-AF4 full length (230 KDa), *novel*MLL-AF4 full length (210 KDa), MAF1-2-3 (128 KDa), in Hek293 cells.

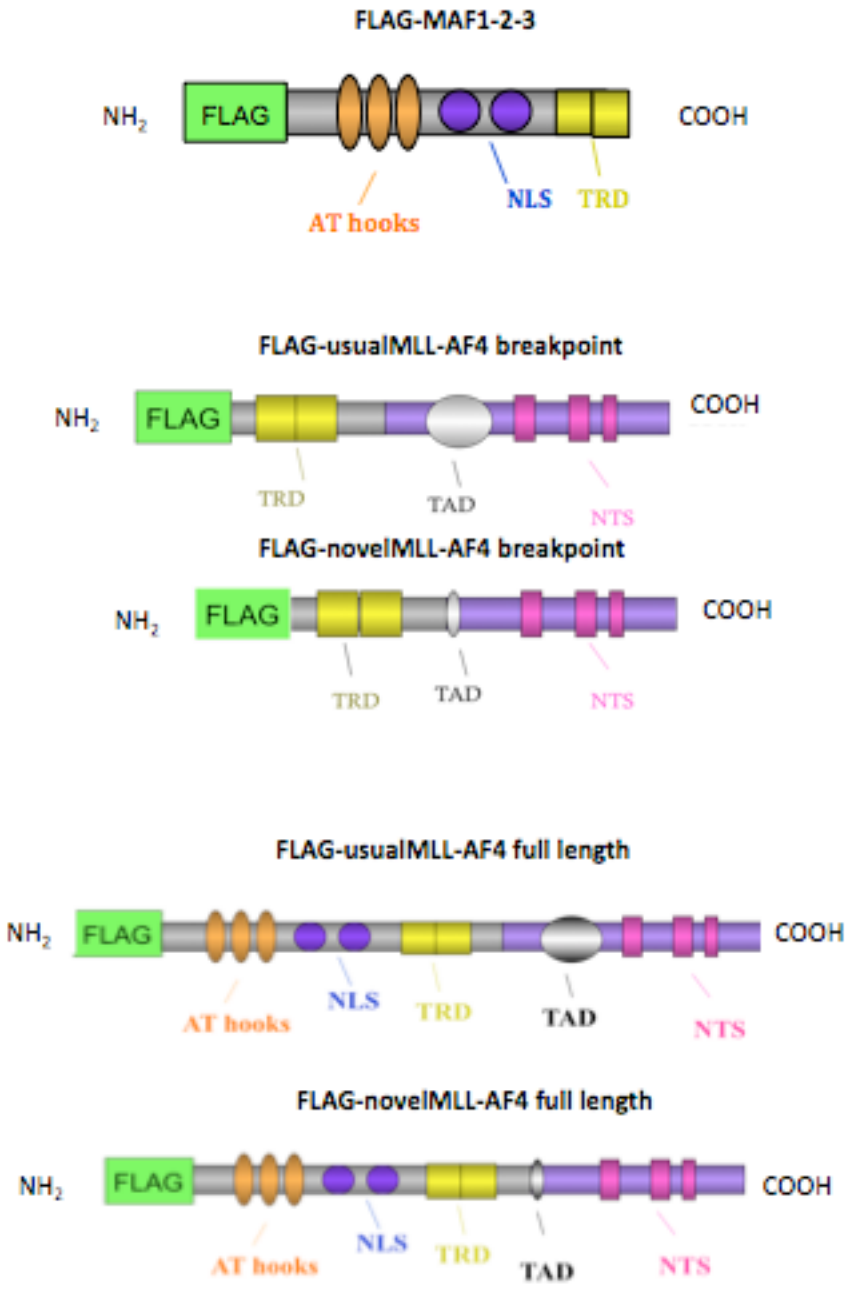


Fig 10. Schematic representation of the FLAG-protein constructs obtained. All the five constructs cloned in the p3XFlag had their own ORF, in-frame with the sequence encoding the upstream triple epitope FLAG. Each one of these five cDNA produced a different protein, fused with the triple-epitope FLAG at the N-terminus.

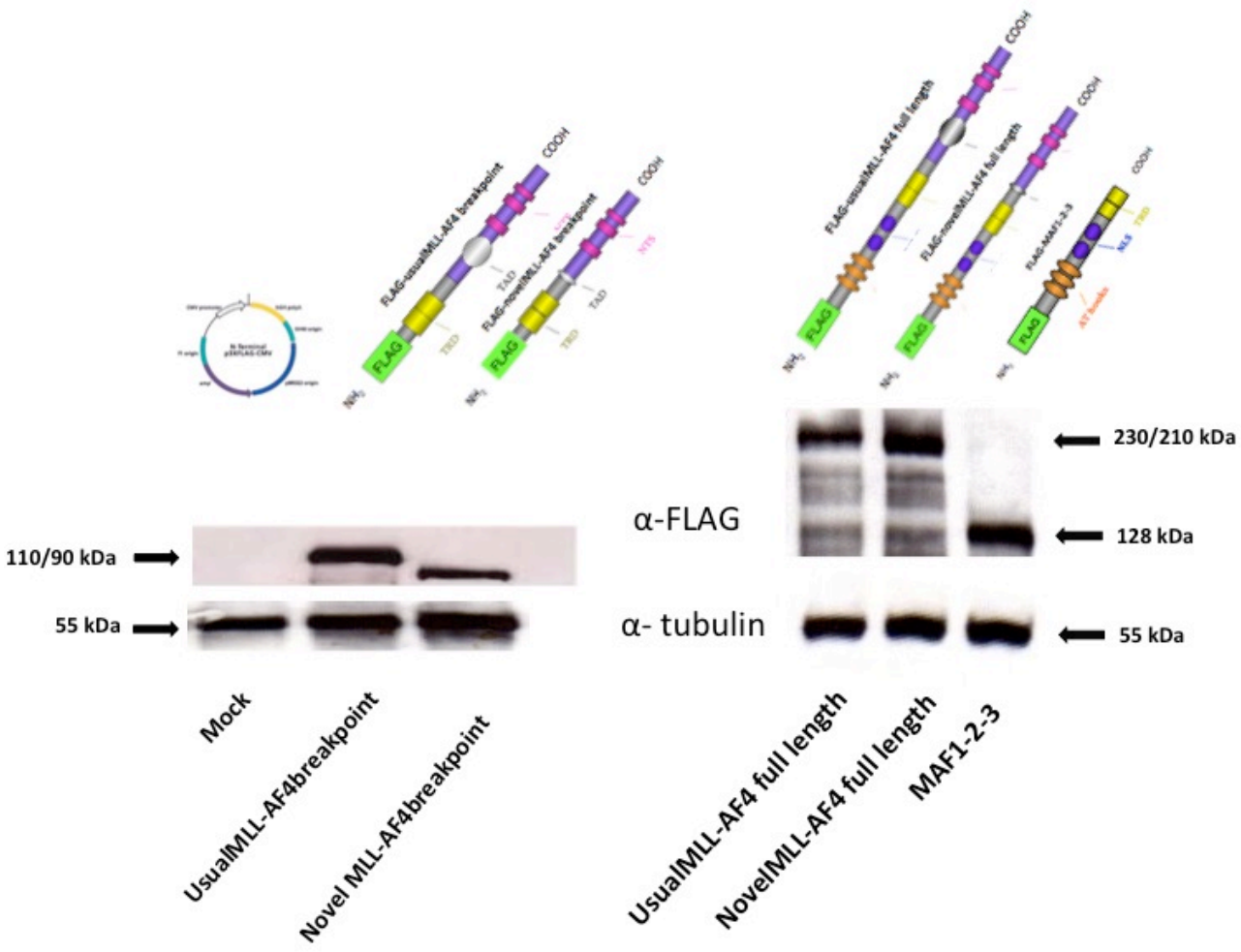


Fig 11. Transient expression analysis of the FLAG-recombinant proteins. 50 μg of whole protein extracts from HEK293 cells transfected respectively with empty p3XFLAG vector, FLAG-*usual*MLL-AF4 breakpoint, FLAG-*novel*MLL-AF4 breakpoint, FLAG-*usual*MLL-AF4 full length, FLAG-*novel*MLL-AF4 full length, FLAG-MAF1-2-3, were resolved on 8% SDS-PAGE and analyzed by western blot. The α-Flag antibody, used at a dilution of 1:5000, recognized specific bands at the expected molecular weight: FLAG-*usual*MLL-AF4 breakpoint (110 kDa), FLAG-*novel*MLL-AF4 breakpoint (90 kDa), FLAG-*usual*MLL-AF4 full length (230 kDa) and FLAG-*novel*MLL-AF4 full length (210 KDa), FLAG-MAF1-2-3 (128 KDa). No band was detected in the mock control lane. The membrane was also probed with α-tubulin (55 kDa) as loading control.

3.3 Inducible expression system of MLL-AF4 chimaeras

In order to obtain an inducible expression system of MLL-AF4 chimeric protein, detectable by α-flag antibody, we cloned cDNA encoding FLAG-*usual/novel*MLL-AF4 full length in the TetO-mEPO vector, which we manipulated to remove the insert, e.g. the cDNA encoding the murine erythropoietin (mEPO), and replace this sequence with the cDNAs of our interest (Fig. 12). Then we cotransfected Hek293 cells with tetO+*usual/novel*MLL-AF4 and the BS/IRES-M2 vector, containing the sequence encoding the reverse tetracycline transactivator 2S-M2 (rtTA2S-M2). In presence of doxycycline, this chimaerical transcriptional factor specifically binds the target promoter (tetO) (Koponen et al, 2003; Salucci et al, 2002), and activates the transcription of the downstream cDNA.

We tested the expression of FLAG-chimaeras in the cotransfected Hek293 cells, cultured in *medium* either with or without doxycyclin, by Western Blot analysis. As shown in the fig.13, we had the expression of recombinant proteins only after adding doxycyclin into the medium (either 0.1 or 1 μg/mL) (Fig. 13).

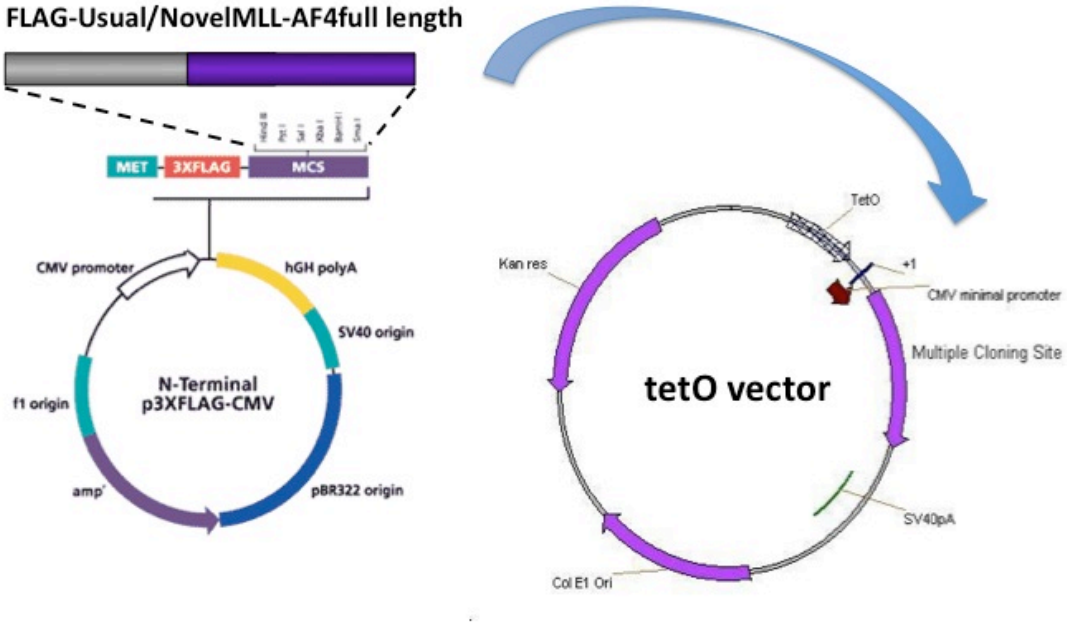


Fig. 12. Cloning of the cDNAs encoding FLAG-usual/novelMLL-AF4 full length in a tet-on vector. Following the removal of the insert cDNA encoding the murine erythropoietin from the tetO/mEPO vector, the cDNAs encoding the FLAG-usual/novelMLL-AF4 full length have been excised from the p3XFLAG constructs and insert in the Multiple Cloning Site of the tetO vector. This plasmid contains a tetO *cis*-element fused to a CMV minimal promoter, thus allowing the regulated expression of the cDNA of interest, in presence of doxycyclin and the reverse-tetracycline transactivator.

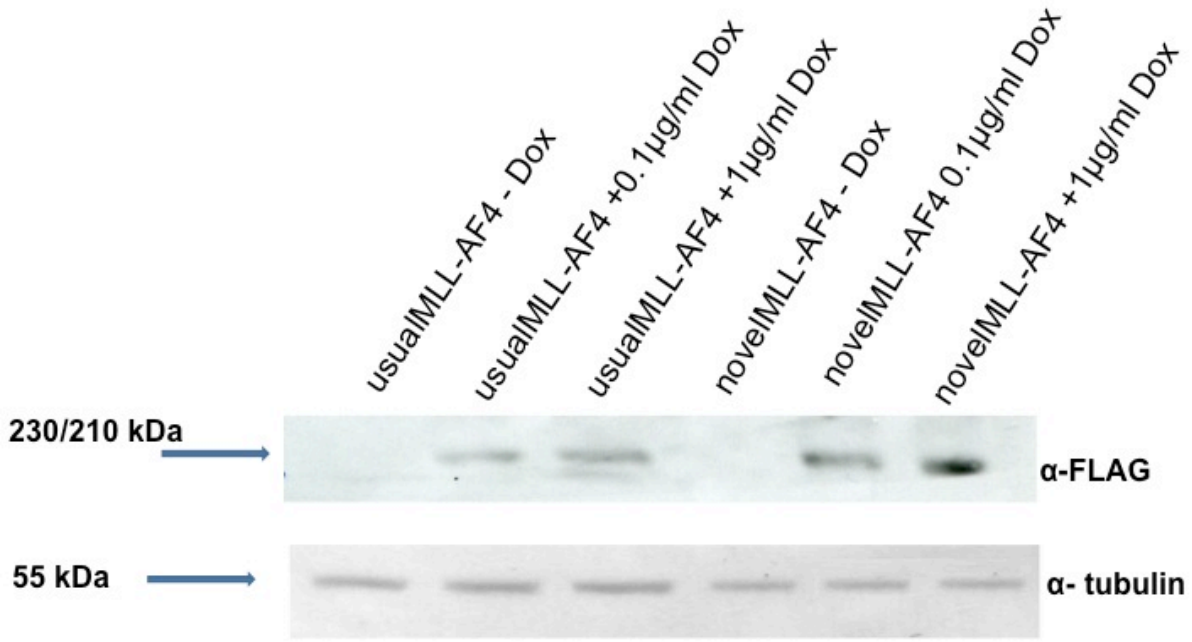


Fig. 13. Analysis of FLAG-recombinant proteins expression in a system inducible by doxycycline. 50 μg of whole protein extracts from HEK293 cells cotransfected in either absence or presence of doxycycline in the medium, with either tetO+FLAG-*usual*MLL-AF4 full length or tetO+FLAG-*novel*MLL-AF4 full length plasmids, respectively, and the Bs/IRES-M2 vector, were loaded and resolved on 8% SDS-PAGE and analyzed by western blot. The α-Flag antibody (1:5000 diluted) recognized specific bands at the molecular weight of 210/230 KDa for both FLAG-*usual*MLL-AF4 full length and FLAG-*novel*MLL-AF4 full length only in the lanes corresponding to the cells treated with either 0.1 or 1 μg/mL of doxycyclin. No FLAG-MLL-AF4 protein is detectable in the untreated cells, because of the expression of the tTS repressor, which is encoded by the Bs/IRES-M2 vector and, in absence of doxycycline, binds to tetO, blocking the basal expression of the cloned cDNAs. The membrane was also probed with α- tubulin (55 kDa) as loading control.

3.4 The recombinant FLAG-MLL-AF4 chimaeras localize into the nucleus

Although our doxycycline-inducible system allowed us to have a suitable expression level of the MLL-AF4 chimaeras, we used the non-regulated expression system with p3XFlag for the following experiments.

We first investigated the subcellular localization of our recombinant proteins, by immunofluorescence. We transiently transfected HeLa and Hek293 cells and stained them with the anti-FLAG primary antibody and then with fluorochrome (FITC)-conjugated secondary antibody. The fixed cells were also stained with DAPI, in order to detect the nuclei of the cells. In the merged section, the FITC signals, corresponding to the recombinant proteins, colocalized with DAPI, thereby indicating that both chimaeras migrate into the nucleus, as expected because of the Nuclear Localization/Targeting Sequence of both MLL and AF4 proteins (NLS).

Interestingly, we observed that, although the "*usual/novel*MLL-AF4 breakpoint" constructs lacked the Nuclear Localization Signal of MLL, they could still reach the nucleus, suggesting that the NLS of AF4 are sufficient to allow the transport of the MLL-AF4 proteins into the nucleus (Fig. 14).

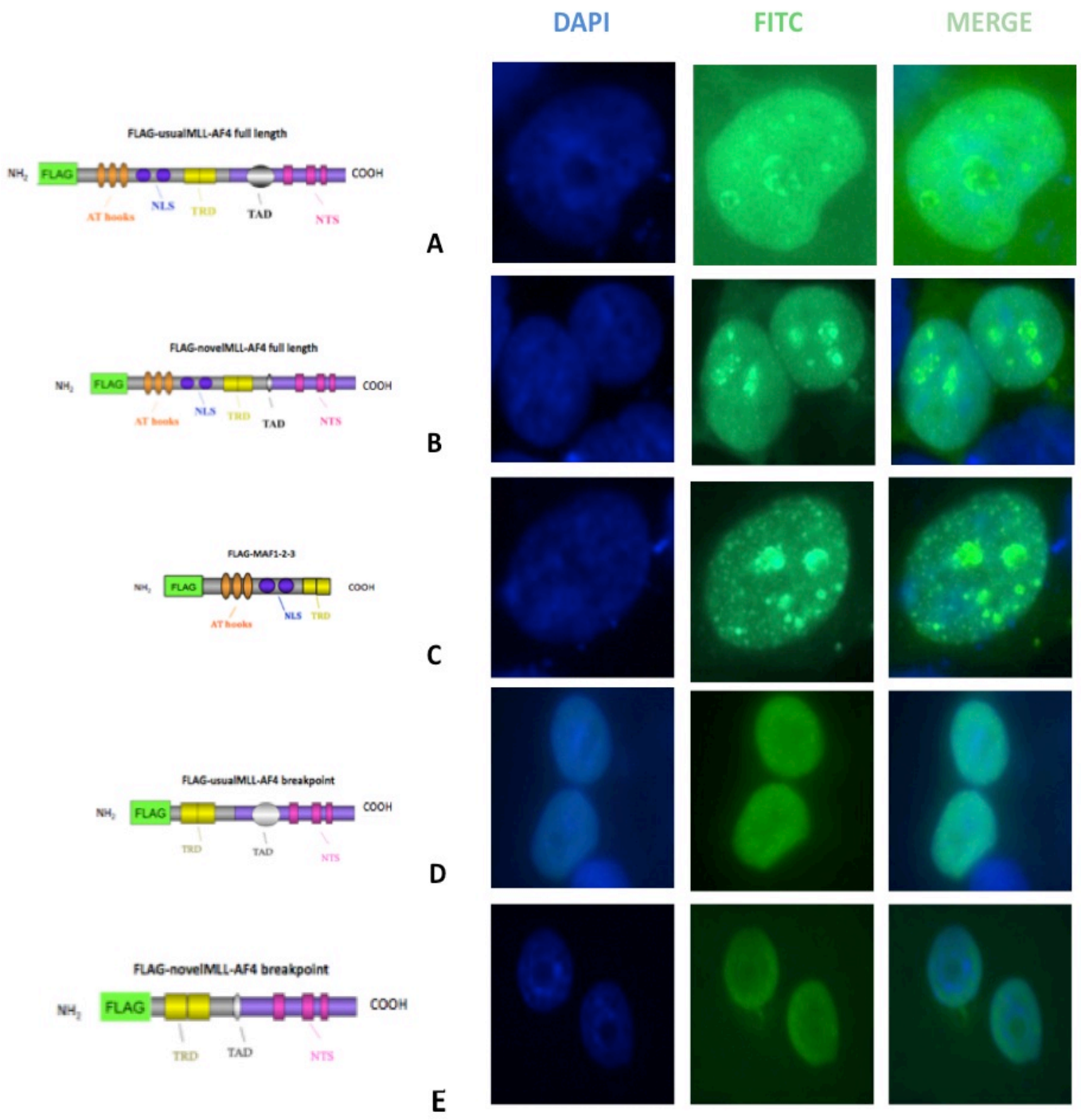


Fig. 14. The FLAG-usual/novelMLL-AF4 chimaeras localize into the nucleus. HeLa cells were transfected with "p3XFLAG+*usual*MLL-AF4 full length" (A), "p3XFLAG+*novel*MLL-AF4 full length" (B) and "p3XFLAG+MAF1-2-3" (C), "p3XFLAG+*usual*MLL-AF4 breakpoint" (D) and "p3XFLAG+*novel*MLL-AF4 breakpoint" (E). Cells were stained with FITC and DAPI and analyzed by immunofluorescence microscopy. The DAPI channel shows the nuclear staining, whereas the FITC channel detects the signal of the specific FLAG-MLL-AF4 construct. The left lane is the merged image of DAPI and FITC.

3.5 The recombinant FLAG-MLL-AF4 proteins up-regulate the HoxA9 gene

The *MLL* gene positively regulates the homeobox gene HoxA9 and the homeobox cofactor Meis1 (Milne et al, 2002). Furthermore, Meis1 and HoxA9 are among the most common targets of MLL-AF4 chimaera by ChIP-seq (Guenther et al, 2008).

Therefore, we investigated the expression level of HoxA9 gene by semi-quantitative PCR. Below is a list of the cell lines we used:

- RS4;11, the ALL lymphoblastoid cell line that endogenously express the *usual* MLL-AF4 protein;
- HEK293 cells, transfected respectively with p3XFLAG+MAF1-2-3, "*usual*MLL-AF4" and "*novel*MLL-AF4";
- Immortalized lymphocytes, as a control for the basal expression of HoxA9 gene in the blood cells;
- non-transfected HEK293 cells, as a negative control (MOCK).

We extracted total RNA from these cells and reverse transcribed it into cDNAs. Then, we amplified the cDNA by PCR using HoxA9- and GAPDH (glycerladeyde-phosphate dehydrogenase)-specific primers. As shown in fig. 15, the amplification product is a clear band at the expected molecular size in the RS4;11, in the Hek293 cells expressing both *usual/novel* MLL-AF4, thus demonstrating that our recombinant chimaeras were able to up-regulate the HoxA9 gene.

Notably, *HOXA9* expression was also detected in Hek293 overexpressing MAF1-2-3. This data confirmed the findings from Martin et al (2003), showing that also truncated forms of MLL are sufficient to work as potent transcriptional activators.

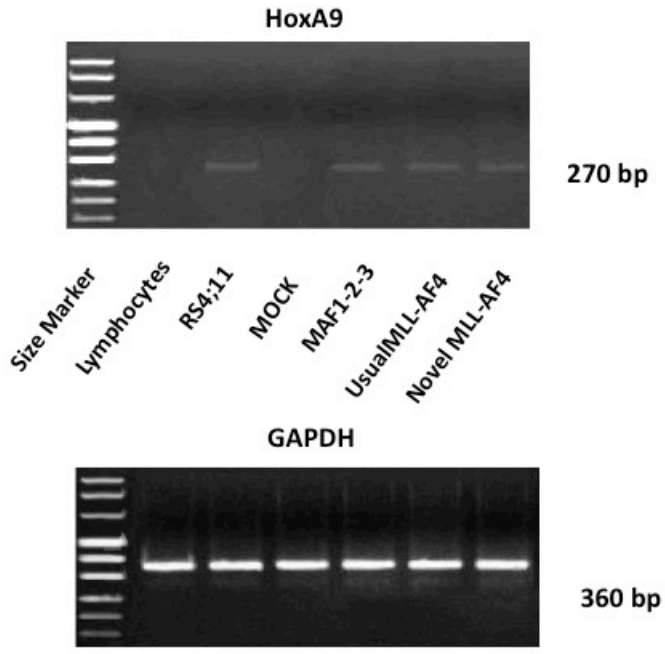


Fig 15. Recombinant FLAG-MLL-AF4 induces the expression of *HOXA9*. cDNAs derived from RS4;11 cells, HEK293 cells transfected with p3XFlag+MAF1-2-3, p3XFlag+usualMLL-AF4 full length, p3XFlag+novelMLL-AF4 full length, empty p3XFlag, respectively, and control lymphocytes were amplified in semi-quantitative conditions with oligonucleotides specific for the HoxA9 transcript. The PCR products were loaded on 0.8% agarose gel and visualized at the UV GelDoc (BioRad). A specific band at 270 bp is visible in the RS4;11, MAF1-2-3, usualMLL-AF4 and novelMLL-AF4 lanes, thus indicating the expression of HoxA9 induced by the chimaera full length and the N-terminal portion in the endogenous system of RS4;11 cells as well as in the exogenous system of the transfected HEK293.

3.6 MLL-AF4 interacts with Cyclin-dependent-Kinase 9 (CdK9) and with members of the "Mediator Complex"

Bitoun et al (2007) showed that AF4 associates with the Positive-Transcription elongation Factor b (P-TEFb), a dimeric complex formed by Cyclin T1 and CdK9, which is involved in the activation of the RNA-pol II-mediated transcription. They demonstrated that AF4 coimmunoprecipitates with Cyclin T1 and CdK9 and that AF4 is a positive regulator of P-TEFb kinase activity and elongation function. Therefore, AF4 is a positive regulator of the Pol II transcriptional elongation.

Here, we evaluated the possible interaction between MLL-AF4 and CdK9. We transfected HEK293 cells with p3XFLAG+*usual/novel*MLL-AF4 and extracted total proteins. Then we immunoprecipitated the FLAG-*usual*/*novel*MLL-AF4 recombinant proteins by using anti-FLAG agarose beads and analyzed the immunocomplexes by Western Blot with anti-CdK9 antibody (Fig.16). As shown in Fig 16, both MLL-AF4 chimaeras interact with CdK9, whereas the MAF1-2-3 does not, thus confirming that the portion of AF4 present in the chimaera is involved in this binding. We also detected the CdK9 protein in the whole cell extracts (WCE), as a control for all the input samples.

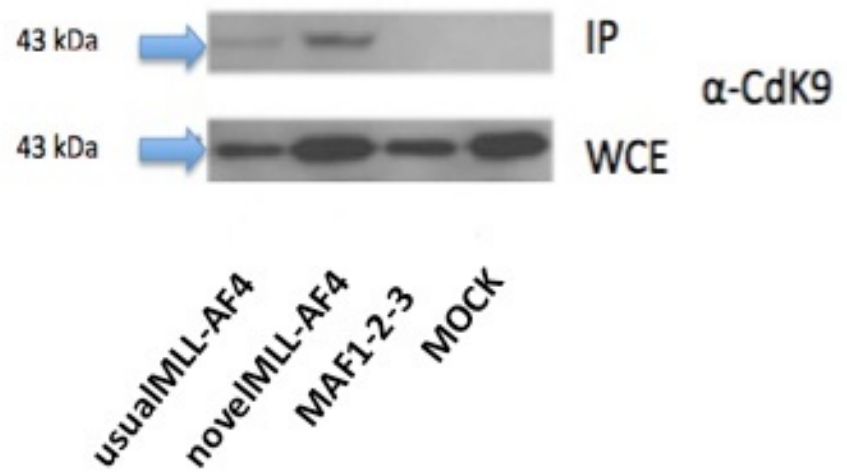


Fig 16. Western blot analysis reveals the interaction of both usual and novel MLL-AF4 with CdK9. 48 hours after the transfection of the HEK293 cells with the usualMLL-AF4 full length, novelMLL-AF4 full length, MAF1-2-3 and empty vector (mock), respectively, the whole cell proteins were collected. 10 mg of whole cell extracts (WCE) were used as input for the immunoprecipitation assay, by using agarose anti-FLAG beads. 20 μl of the immunocomplexes and 50 μg of WCEs were loaded and resolved on 12% SDS-PAGE and analyzed by western blot. The α-CdK9 antibody, used at a dilution of 1:1000, recognized a specific band at 43 kDa in immunoprecipitates (IP) of both usual and novel MLL-AF4-expressing cells, but not in the MAF1-2-3 and mock corresponding lanes. The 43 kDa band was also detected in all the WCEs used as input of the immunoprecipitation assays.

A recent study from our group of the interactome of the AF4 protein showed that some of the AF4 interactors belong to the so-called “Mediator Complex”, which is involved in the regulation of the Pol II-mediated transcription. Since the MLL-AF4 chimeric protein includes the C-terminus and the transactivaction domain of AF4, we also tested some Mediator’s members as partners of MLL-AF4, too. To this aim, we expressed the flagged MLL-

AF4 proteins in HEK293 cells and immunoprecipitated the protein complexes by anti-FLAG agarose beads. The immunocomplexes were resolved by SDS-PAGE and analyzed by Western Blot, using anti-CRSP33 and CRSP130 antibodies. The Western Blot results showed that while both chimaeras interact with CRSP130 (Fig. 17A), only the "*usual*" chimera interacts with CRSP33 (Fig. 17B). This suggests that the binding domain of AF4 and MLL-AF4 to CRSP33 is localized in the portion of the AF4 that is lost in the *novel* isoform.

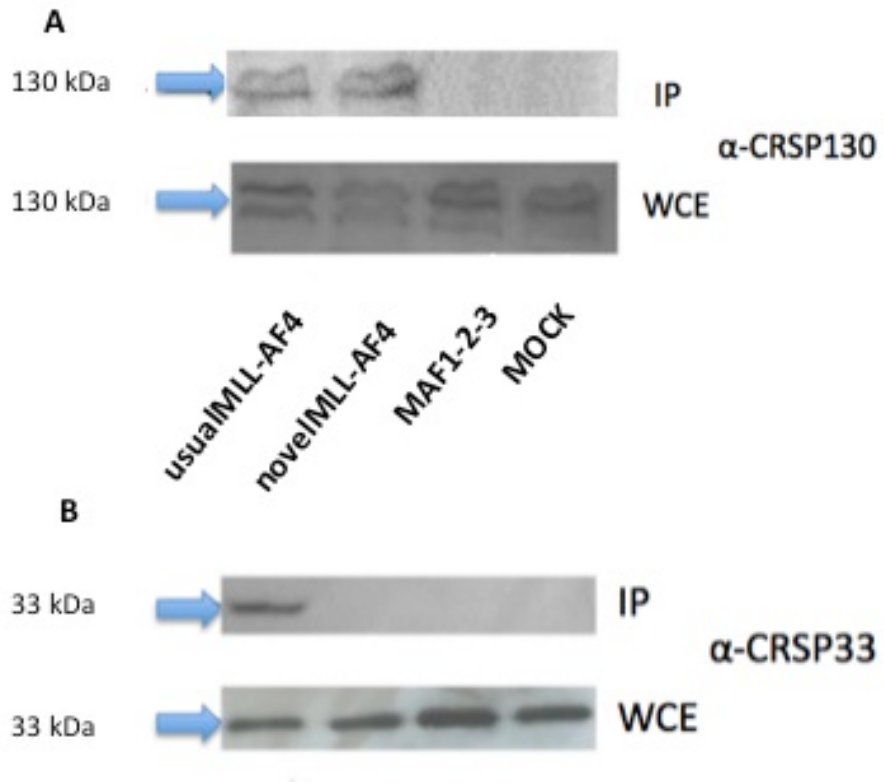


Fig 17. MLL-AF4 interacts with CRSP130 and CRSP33, members of the "Mediator Complex". The immunoprecipitation assays were performed as already reported above. 20 μl of immunocomplexes and 50 μg of WCEs were loaded and resolved on 10% SDS-PAGE and analyzed by western blot. The α-CRSP130 antibody detected specific band at 130 in the IP of both usual and novel MLL-AF4-expressing cells, whereas the α-CRSP33 revealed a specific band at 33 kDa only in the IP from the cells that express the usual chimaera. Both 33 kDa and 130 kDa bands were also detected in all the WCEs of transfected cells, used as input of the immunoprecipitation assays, but no band was detected in the IP of FLAG-MAF1-2-3 transfected cells and in the mock transfection control. Both α-CRSP-130 and α-CRSP-33 primary antibodies were used at a dilution of 1:200.

3.7 14-3-3 θ binds MLL-AF4 and this interaction affects the expression of MLL target gene

The 14-3-3s belong to a family of proteins that influence a wide range of cellular mechanisms, such as cell-cycle control and apoptosis by binding to specific phosphorylated sites on diverse proteins such as tumor suppressor proteins, and regulators of cell survival, proliferation and growth (Mackintosh, 2004). Since we found that AF4 interacts with 14-3-3 θ (Esposito et al, 2011), we tested whether the MLL-AF4 chimaeras interact with 14-3-3 θ. The flagged MLL-AF4 recombinant proteins were precipitated from whole cell extracts using an antibody anti-FLAG and the immunocomplexes analyzed by Western Blot, using an antibody specific for 14-3-3 θ. The results show that both the *usual* and *novel* MLL-AF4 chimera interact with 14-3-3 θ, suggesting that the MLL-AF4 proteins retains the binding domain of AF4 to this protein (fig. 18).

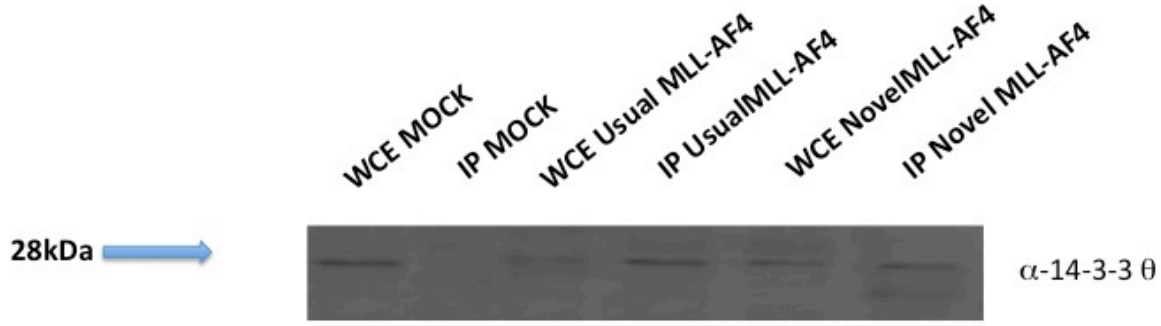


Fig 18. The 14-3-3 θ protein interacts with MLL-AF4 chimaera. 20 μl of FLAG-IP and 50 μl of WCE of cells transfected with empty vector (mock) and p3XFLAG+usual/novelMLL-AF4 full length were loaded on 12% SDS-PAGE and analyzed by western blot. The antibody directed against the 14-3-3 θ detected a band at 28 kDa in all the input samples and in the IP of both usual and novel MLL-AF4. For this analysis the α-14-3-3 θ was used at a dilution of 1:500.

Since we found that 14-3-3 θ interacts with MLL-AF4 and as the 14-3-3s are known to bind and modulate the function of their target proteins, we investigated the effects of the over-expression of 14-3-3 θ on the aberrant transcriptional activity induced by MLL-AF4. We cotransfected the Hek293 cells with both p3XFLAG-*usual*MLL-AF4 and pCDNA3+14-3-3 θ, and analyzed the expression levels of HoxA9. The Real Time-PCR results showed that overexpression of both MLL-AF4 and 14-3-3 θ rescued HoxA9 expression in normal level in the HEK293 cells, compared to the cells transfected with p3XFLAG+usualMLL-AF4. Therefore, the data suggests that 14-3-3 not only interacts with MLL-AF4, but also modulates its transactivation activity (fig. 19).

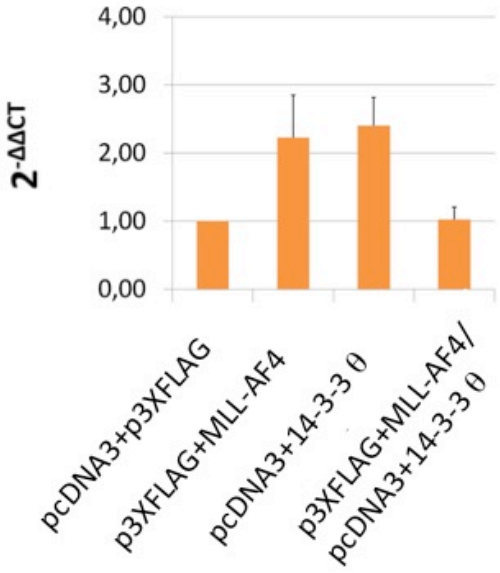


Fig. 19. The interaction between 14-3-3 θ and MLL-AF4 affects the MLL-AF4 transcriptional activity. Hek293 cells were transfected with both empty pcDNA3 and p3XFLAG vectors, p3XFLAG+*usual*MLL-AF4 full length, pcDNA3+14-3-3 θ, respectively, and cotransfected with both p3XFLAG+*usual*MLL-AF4 full length and pcDNA3+14-3-3 θ. Expression level of HoxA9 gene in the transfected cells was evaluated by Real-Time PCR. In the single-transfected cells, the level of HoxA9 is increased (~2 fold) compared to the double mock control whereas in the cotransfected cells, the level of HoxA9 transcript is rescued at basal level. The fold increase has been evaluated by the formula $2^{-\Delta\Delta Ct}$, using β-actin and β-glucuronidase as house-keeping genes (mean ± SEM).

3.8 MLL-AF4 interacts with the Fibroblast Growth Factor Receptor 2 (FGFR2)

Surprisingly, the proteomics experiments revealed that the Fibroblast Growth Factor Receptor 2 as an interactor of the native AF4 protein (Esposito et al, 2011). We found this data particularly interesting, since both AF4 and MLL-AF4 are nuclear proteins. Therefore, we tested whether the MLL-fusion proteins interact with this AF4 interactor, by immunoprecipitation and Western Blot experiments in Hek293 cells transfected with p3XFlag+MLL-AF4. As shown in fig. 20, a band has been found at the FGFR2 expected molecular weight (~120 kDa), confirming that even both forms of MLL-AF4, *usual* and *novel*, interact with this receptor.

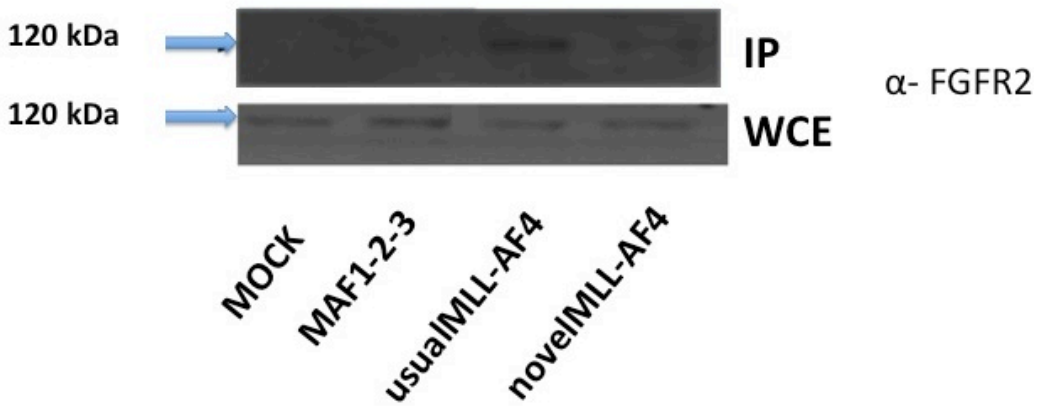


Fig 20. MLL-AF4 oncoproteins interact with the Fibroblast Growth Factor Receptor 2 (FGFR2). FLAG-IP and WCE of cells transfected with empty vector (mock) and p3XFLAG+usual/novelMLL-AF4 full length were loaded on 8% SDS-PAGE and analysed by western blot with a FGFR2-specific antibody. A band is detected at 120 kDa in all the input samples and in the IP of both FLAG-usualMLL-AF4 and FLAG-novelMLL-AF4.

3.9 FGFR2 gene expression is higher in RS4;11 than in 697

The finding that the MLL-AF4 proteins interact with FGFR2 lead us to hypothesize that this receptor could have a role in the molecular pathogenesis of t(4;11) ALL. Therefore, we investigated the expression of the FGFR2 transcript in two different human hematopoietic cell lines, i.e. the 697, a human pre-B leukemic cell line carrying the t(1;19) rearrangement (Planey et al, 2002), and the RS4;11, carrying the translocation of our interest.

As shown in fig. 21, RT-Real-Time PCR results indicated that the FGFR2 transcript is more expressed in the cells expressing MLL-AF4, compared to leukemic cells that do not have this MLL rearrangement.

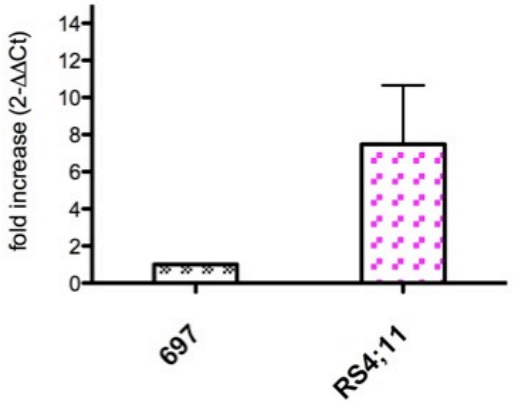
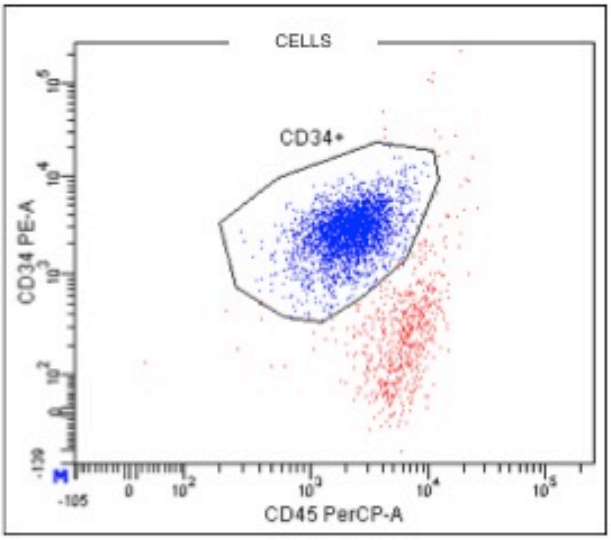


Fig. 21. FGFR2 gene expression is higher in RS4;11 than in 697 cells. Expression level of FGFR2 gene was evaluated by Real-Time PCR in leukemic cell lines. The fold increase has been calculated by the formula $2^{-\Delta\Delta Ct}$, using β-actin as housekeeping gene (mean ± SEM).

3.10 A new cell model for MLL-AF4: the human hematopoietic stem and progenitor cells?

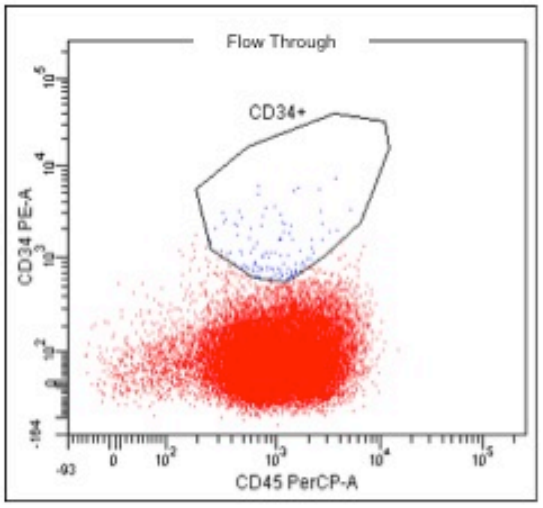
The CD34 antigen is a highly glycosylated transmembrane protein member of the sialomucinfamily of surface antigens. It is strongly expressed on human hematopoietic stem/progenitor cells and its expression shows a progressive and rapid decline as HSCs undergo differentiation (Caux et al, 1989). Whereas the natural ligand for CD34 has not yet been identified in the hematopoietic microenvironment, it is expressed on murine high-endothelial venule cells in the lymph nodes and binds to the lymphocyte homing receptor L-selectin (Baumheter et al, 1993). Hotfilder et al (2005) showed that the MLL-AF4-positive cells in ALL patients are restricted to a $CD34^{+}CD19^{-}$ compartment, suggesting that the chromosomal aberration takes place in a very undifferentiated cell that gives rise to the leukemia.

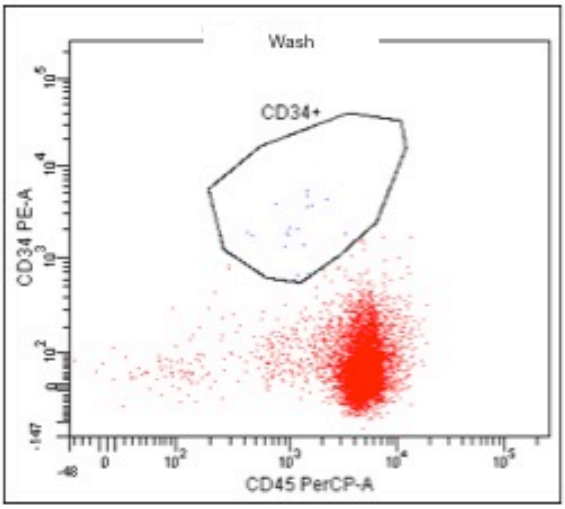
We wanted to study the effects induced by the MLL-AF4 oncoprotein in human hematopoietic stem cells (HSC). Methods used for HSCs isolation include fluorescence activated cell sorting (FACS) and magnetic activated cell separation sorting (MACS), both using either fluorescently or magnetically labeled specific antibodies (Sousa et al, 2011). We opted for $CD34^{+}$ immunomagnetic selection and collected from the Ceinge-BioBank samples of human cord blood that were not suitable for therapeutic purpose. We also evaluated the purity and the yield of the procedure by flow cytometry (Fig. 22). As expected, the average yield of $CD34^{+}$ cells purified from fresh total cord blood-derived cells by the immunomagnetic procedure was ~0.3-0.5%. Flow cytometry of the immuno-separated, eluted fraction of $CD34^{+}$ revealed a purity of 83%. Only 0.3% of cells in the flow-through and in the wash were $CD34^{+}$.



Tube: CELLS

Population	#Events	%Parent	%Total
All Events	5,049	####	100.0
CELLS	3,611	71.5	71.5
CD34+	2,996	83.0	59.3





Tube: Flow Through

Population	#Events	%Parent	%Total
All Events	50,000	###	100.0
CELLS	38,520	77.0	77.0
CD34+	111	0.3	0.2

Tube: Wash

Population	#Events	%Parent	%Total
All Events	10,000	###	100.0
CELLS	7,242	72.4	72.4
CD34+	20	0.3	0.2

Fig. 22. Purity analysis of immunoseparated cell fractions. A little aliquot of positive cell fraction was analysed by flow citometry in order to estimate the purity degree of the MACS enrichment. The 83% of eluted cells were $CD34^+$, whereas only 0.3% of cells in flow-through or wash fractions were $CD34^+$ (thanks to the kind collaboration with Dr Marica Gemei, Facility of "Experimental and clinical citometry", Ceinge Biotecnologie Avanzate s.c.a r.l.).

We cultured the purified $CD34^+$ cells in IMDM with penicillin and streptomycin and 10% fetal bovine serum. 24 h after purification, cells were transfected with the usualMLL-AF4 cDNA, cloned in p3xFLAG expression vector, and with the empty p3xFLAG vector (mock control), respectively. Total RNA was extracted from MLL-AF4-transfected and mock cells for real-time RT-PCR analysis, to first evaluate the expression of the MLL-AF4 chimaera and verify the efficiency of cell transfection at mRNA level. As expected, the chimaera transcript was detected only in the cells transfected with p3XFLAG+MLL-AF4, thus confirming the exogenous expression of MLL-AF4 in the HSPCs. Then, the real-time RT-PCR of FGFR2 was performed to verify the expression of this gene in the HSPCs enforced to express MLL-AF4.

Our preliminary results evidenced that the exogenous expression of MLL-AF4 induced increased FGFR2 transcript levels in $CD34^+$ cells, indicating that the FGFR2 gene and the related pathway could be early target/marker of the MLL-AF4 oncogenic activity (Fig. 23). Further analysis needs to better evaluate the range of increased expression of FGFR2, due to its very low expression in the hematopoietic precursors.

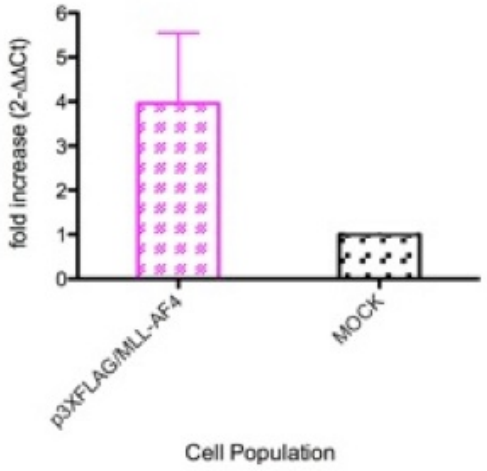


Fig. 23. MLL-AF4 up-regulates the FGFR2 transcript in hematopoietic progenitors. $CD34^+$ cells were transfected with empty p3XFLAG vector (MOCK) and p3XFLAG+*usual*MLL-AF4 full

length, respectively. Expression level of FGFR2 gene in the transfected cells was evaluated by Real-Time PCR. Preliminary results showed that in the MLL-AF4-transfected cells, the level of FGFR2 transcript is increased compared to the mock control. The fold increase has been evaluated by the formula $2^{-\Delta\Delta Ct}$, using β-actin as house-keeping gene (mean ± SEM).

Chapter 4

Discussion

The t(4;11) reciprocal chromosomal translocation is associated with a B-cells acute lymphoblastic leukemia characterized by a very poor prognosis. Following the t(4;11) chromosomal translocation, two chimeric protein take place in the leukemic clone: MLL-AF4 on chromosome 11 and AF4-MLL on chromosome 4. This PhD project aimed to clarify some aspects of the function of the MLL-AF4 fusion protein, which has the capability of MLL to bind DNA and the strong transcriptional activation domain of AF4. Many data support the hypothesis that this fusion protein sustains growth and proliferation of the malignant clone, by mis-regulating the expression of genes, such as *HoxA9* and *Meis1*, which are involved in self-renewal/differentiation balance of hematopoietic stem cells. Notably, down-regulation of these genes can somehow revert the leukemic phenotype. However, the aberrant molecular mechanism induced by this oncoprotein remains poorly understood.

Functional proteomic experiments previously performed in our research group identified many molecular interactors of the native AF4 protein (Esposito et al, 2011). Based on the cell function, the identified molecular partners can be divided into different groups. Most of them are involved in the regulation of the Pol II-mediated transcription, suggesting a direct role of AF4 in this process. Thus, our first aim was to investigate whether these proteins also interact with MLL-AF4 chimeric proteins. Therefore, we first cloned the cDNAs encoding two different isoforms of MLL-AF4: an “usual” form (*usual*MLL-AF4), which is the most common in t(4;11) ALL patients and a “novel” one (*novel*MLL-AF4), previously found in an infant ALL-patient, that lacks part of the transactivation domain of AF4. We obtained the cDNA that encodes the usualMLL-AF4 breakpoint, by using RNA extracted from RS4;11 cells, whereas the novelMLL-AF4 cDNA was created from the

usualMLL-AF4 cDNA, by a series of amplification and ligation procedures. We cloned both cDNAs in the eukaryotic p3XFlag expression vector and used these constructs to transiently transfect HEK293 cells. After confirming that our MLL-AF4 recombinant proteins were properly expressed in our system, we evaluated the expression levels of *HoxA9* gene, a well-known target of the oncogenic activity of both wild-type MLL and rearranged form MLL-AF4, by semiquantitative PCR. As expected, we found that in our expression cell systems, the expression level of the *HoxA9* gene was increased. We also found by immunofluorescence assay that both forms of MLL-AF4 localized in the nucleus, thanks to the NLS of both MLL and AF4. Furthermore, we found that the chimaera's portion lacking the NLS of MLL still localize into the nucleus, thus validating the hypothesis that the NLS of AF4 alone is able to address the fusion proteins into the nucleus.

Starting from our previous proteomic data, we investigated the interaction of the chimaera with the molecular partners of AF4, such as CRSP130, CRSP33, CdK9, 14-3-3 θ and FGFR2, by co-immunoprecipitation assay.

The CRSP130 and CRSP33 proteins, also known as Med7 and Med23 respectively, belong to the so-called Mediator Complex. This complex is able to bind directly to Pol II and plays important roles at each stage of transcription, from the recruitment of Pol II to genes in response to many signals to controlling Pol II activity during transcription initiation and elongation (Conaway & Conaway, 2011). We found that both chimaeras interact with CRSP130 subunit, but only the *usual* chimaera binds to CRSP33, suggesting not only that the binding domain of AF4 and MLL-AF4 to CRSP33 localized in the portion of the AF4 that is lost in the *novel* isoform, but also that CRSP33 is not crucial for the transforming potential of the chimaeras.

We also found that MLL-AF4 interacts with CdK9, the kinase activity of P-TEFb. Bitoun et al (2007) already showed that AF4 enhances the transcription, by promoting the kinase activity of CdK9 on the C-terminal domain of Pol II. It is possible that the MLL-AF4 chimaeras bind to the promoters of MLL target genes and enhance their transcription, according to the transcriptional activation mechanisms induced by AF4. The mis-regulated transcription of MLL target genes could contribute to explain the leukemic phenotype of the hematopoietic cells with the t(4;11) rearrangement. Our findings about this interaction seem to also fit the hypothesis formulated by Yokoyama et al (2010), i.e. that the MLL-fusion proteins are able to constitutively recruit at MLL *loci* all the members of the AEP complex that are involved in the transcriptional machinery of RNA pol II. Then, aberrant recruitment of the transcriptional complex at MLL *loci* leads to improper activation of the MLL target genes, which alters differentiation of the hematopoietic cell affected by the *MLL* gene molecular aberration (Fig. 24). Furthermore, it is now clear that the MLL–AF4 chimaera, irrespective of the transactivation domain, is still able to recruit most of the protein components that are necessary for the transcription of genes that enhance and sustain cell transformation. Thus, these results corroborate what we previously reported that even when MLL-AF4 chimaeras lack part of the AF4 transactivation domain, they can still give rise to malignant phenotype of ALL (Pane et al, 2002).

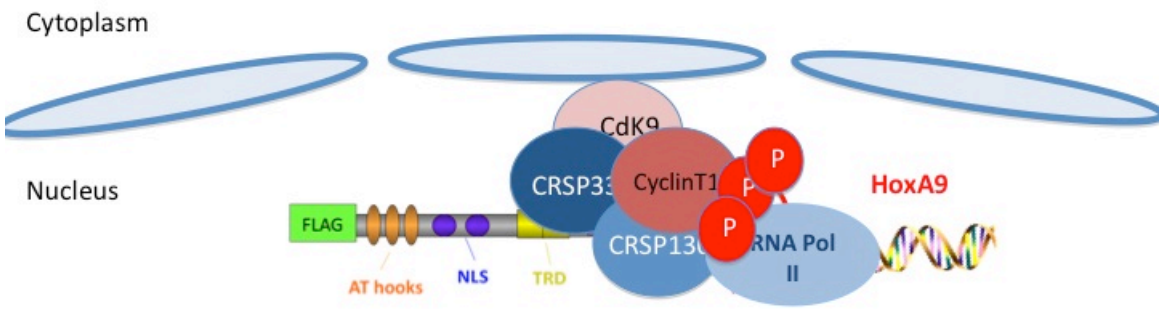


Fig. 24. Model of the protein network in which MLL-AF4 takes part in the nucleus. Thanks to the Nuclear Localization Signals of both MLL and AF4 proteins, the chimaera MLL-AF4 migrates into the nucleus. Here, the oncoprotein is able to recruit many components necessary for the Pol II-mediated gene transcription and activates the expression of HoxA9 and other target genes that enhance and sustain the oncogenic transformation in the hematopoietic cell.

Since the functional proteomics assay interestingly revealed the interaction between AF4 and 14-3-3 θ, the latter involved in many cellular functions, including signal transduction, cell-cycle control and apoptosis, we investigate the possible interaction between this protein and chimaera. The immunoprecipitation assays confirmed that both the MLL-AF4 isoforms bind to 14-3-3 θ. Therefore, since the 14-3-3s are known to modulate the function of their target proteins, we analyzed the effects of the co-expression of 14-3-3 θ and MLL-AF4 on the expression of *HoxA9,* the main MLL-AF4 target gene. We observed a lower *HOXA9* gene expression in the co-transfected cells compared to the cells transfected with the chimera cDNA alone.

According to the evidence that 14-3-3s bind many nuclear phosphorylated proteins and control their rate of nuclear import/export, thereby modulating various transcriptional processes (Mackintosh, 2004). Because MLL-AF4 preserves the binding site to 14-3-3 θ, we may suppose that 14-3-3 θ could regulate the nuclear transfer of the chimaera and consequently its activity on the target gene expression (Fig. 25). Further experiments aimed to investigate the significance of the interaction between 14-3-3 θ and MLL-AF4 are currently ongoing in our laboratories.

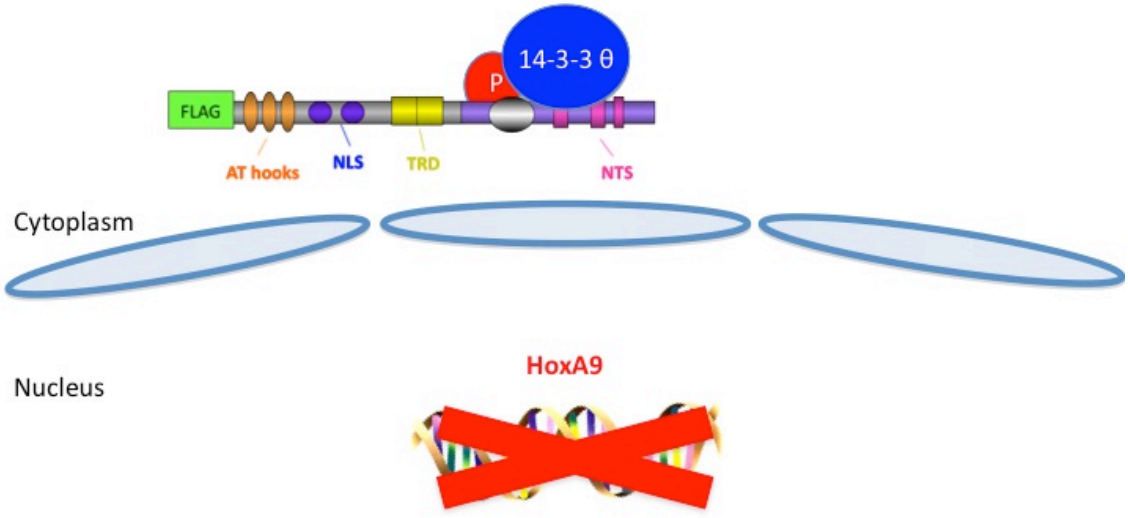


Fig. 25. Model of the likely modulation on HoxA9 expression by 14-3-3 θ. A phosphorilation residue of MLL-AF4 in the AF4 portion, could work as a docking site for the recruitment of 14-3-3 θ in the cytosol. This binding could sequester the chimaera in the cytosol and prevent its migration into the nucleus, thus inhibiting the activation of HoxA9 gene expression.

Surprisingly, we have also found that MLL-AF4, as well as AF4, interacts with FGFR2. Unpublished data from our group found demonstrated that this binding occurs in the nucleus.

Moreover, in this study, we found a higher expression of the FGFR2 transcript in the RS4;11 hematopoietic cells than in 697, a non-t(4;11) leukemia-cell line, thus indicating that FGFR2 could be overexpressed in the hematopoietic cells carrying t(4;11).

MLL-AF4 has been previously associated to a membrane receptor, the Insulin-like Growth Factor-1 receptor (IGF1R), which is down-regulated as a result of the HoxA9 knockdown in RS4;11 cells. In contrast, *HoxA9* overexpression induces IGF1R expression, suggesting that the receptor-associated pathway could yield new potential targets for the treatment of leukemias or other cancers that show the overexpression of *Hox* genes (Whelan JT et al, 2008).

Our findings strongly indicate that FGFR2 could be implicated in the molecular pathway triggered by the MLL-AF4 oncoprotein. However, we do not know yet how the transduction pathway triggered by FGFR2 (Fig. 26) could fit in the molecular pathogenesis of the t(4;11) ALL. Our perspective will be to clarify the role of this transmembrane receptor in the pathway induced by MLL-AF4, thereby opening the possibility to interfere this pathway, by designing specific inhibitors that could be used in targeted therapy. Nowadays, other types of ALL are successfully treated with drugs that specifically target the molecules directly involved in the pathomechanism induced by the aberrant oncoprotein. As well as the Philadelphia Chromosome-Positive ALL is currently treated with the last generation TKIs (Tyrosine-Kinase Inhibitor) in successful combination with established antileukemic agents (Lee et al, 2011), targeting the FGFR2 pathway at the different steps could be used as coadiuvant to the standard treatment of the t(4;11)-positive ALL, avoiding relevant therapy-related issues such as cytotoxicity and chemioresistance.

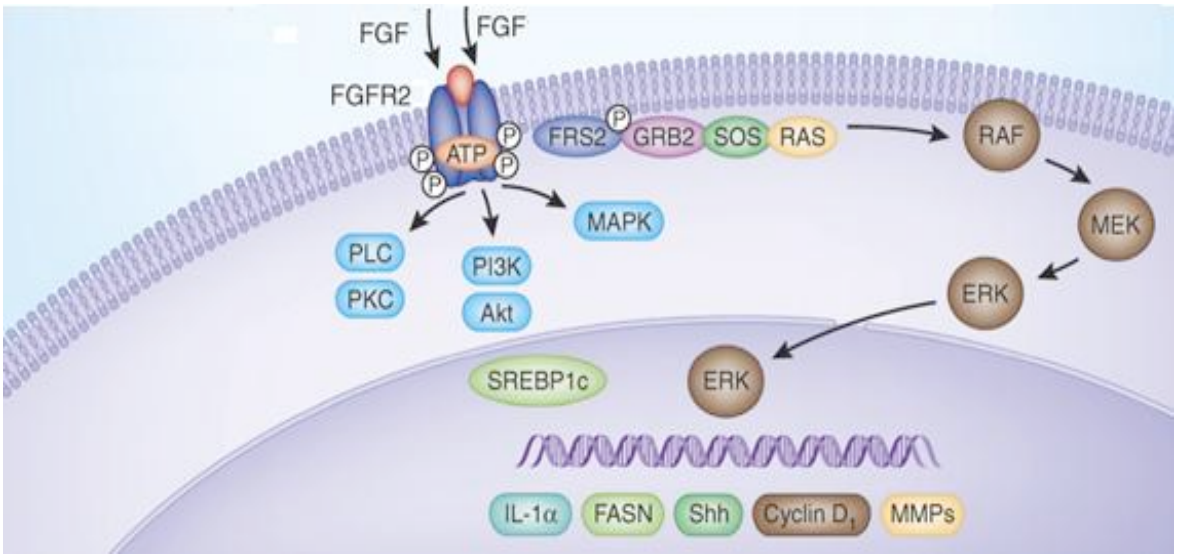


Fig. 26. The signalling pathway of the tyrosine-kinase receptor FGFR2. After the substrate (FGF) binding, the receptor dimerizes thereby leading to autophosphorylation of intracellular tyrosine residues that serve to recruit SH2 domain-containing proteins and initiate downstream signalling. The recruitment of the Ras activator SOS through the Grb2/FRS2 complex activates the extracellular signal-regulated protein kinase (ERK) pathway. Phosphorylated forms of ERKs translocate to the nucleus where they activate transcription factors involved in cell cycle control. The FGFR2 transduction also activates the phospholipase Cγ/protein kinase C and the phosphoinositide-3-kinase (PI3K)/Akt-signaling cascades (Melnic et al, 2009).

We also started to study the aberrant pathway induced by MLL-AF4 in the human hematopoietic stem and progenitor cells. Therefore we enriched the CD34+ cell from human umbilical cord blood-derived cells. In the meantime we were looking for the best conditions for studying the effects of the ectopic expression of MLL-AF4 in the human hematopoietic stem and progenitor cells, Montes et al (2011) published a paper, showing that the enforced expression of MLL-AF4 in human cord blood-derived cells enhances the hematopoietic repopulating cell function and clonogenic potential. However, these events seem to be not sufficient for initiating leukemogenesis *in vivo*. This inability to develop a MLL-AF4^{+} ALL

disease model based on human cord-blood-$CD34^+$ progenitors suggests either that secondary mutations or the AF4-MLL reciprocal product might be required to develop ALL or that the $CD34^+$ HSPCs from umbilical cord blood are not an appropriate model to study this pathway. It has to be considered that earlier hematopoietic progenitors (fetal $CD34^+$ cells or human ESC-derived $CD34^+$ cells) could represent a more suitable system to study the MLL-AF4 oncogenic pathway and could be used as a model to analyze aberrant pathway induced by the MLL-AF4 oncoprotein which could lead the discovery of novel, early molecular markers of t(4;11) leukemia.

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