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Doctorate thesis

The deregulated expression of miR-125b in acute myeloid leukemia is dependent on the transcription factor C/EBPα.

Supervisor:
Prof. Claudio Talora

Candidate:
Paula Vargas Romero

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“*It always seems impossible until it's done*”- Nelson Mandela

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ABSTRACT

MicroRNA-125b (miR-125b) is highly expressed in many cancers such as B cell lymphomas and myeloid leukemia and inflammatory disorders such as rheumatoid arthritis, atopic dermatitis, and multiple sclerosis. However, the underlying mechanism of miR-125b dysregulation remains to be explored. Relevant to myeloid leukemia, C/EBPα is frequently mutated in AML, but surprisingly, none of the observed mutations result in full ablation of the gene, indicating that its activity is required for AML. Interestingly, C/EBPα in normal hematopoiesis and in AML is able to induce the expression of some miRNAs during myeloid development and leukemia. Previously, it has been shown that the manifestation of Hailey-Hailey disease was in part dependent on Notch1 downmodulation mediated by miR-125b upregulation. Notably, while the involvement of Notch signaling as an oncogene in acute lymphoblastic leukemia (T-ALL) is well characterized, Notch signaling has been described as a tumor suppressor in myeloid malignancies, like acute myeloid leukemia (AML), where the activation of Notch1 is silenced. In this study we found that C/EBPα positively regulated miR-125b expression and contributes to the up-regulation of this microRNA in AML. We observed the binding of C/EBPα to the miR-125b promoter in cells where Notch1 is not activated, and that C/EBPα negative cells display decreased expression of miR-125b and higher activation of Notch signaling pathway. Furthermore, by transient and lentivirus transfection, we observed that miR-125b targets Notch1. We have thus identified C/EBPα as a novel key regulator of the positive control of miR-125b expression in acute myeloid leukemia. To what extent C/EBPα contributes to myeloid transformation, is unclear. Our study demonstrates the existence of C/EBPα/miR-125b oncogenic axis, further providing evidence that C/EBPα is required for AML, and we suggest that miR-125b dysregulation plays a critical role in the differential expression and activity of Notch1 between T-ALL and AML.

INTRODUCTION

1.1. Acute Myeloid Leukemia (AML).

Acute myeloid Leukemia (AML) is a clonal hematopoietic neoplasm characterized by the proliferation and accumulation of myeloid progenitors in the bone marrow due to the block in differentiation along one or more hematopoietic lineage, frequently resulting in hematopoietic insufficiency (Löwenberg *et al*., 1999; Stone *et al*., 2004). AML is the most common acute leukemia diagnosed in adults and the estimated 5 year survival rate among AML patients is approximately 30%, with mortality been largely attributed to chemotherapy resistance (Lobry *et al*., 2013; Roe and Vakoc, 2014). Research has been focused on understanding the mechanisms implicated in AML leukemogenesis, by the characterization and the study of acquired cytogenetic abnormalities such as translocation t(15;17), t(8;21), abnormalities in the long arm o chromosome 11 (11q) and inversion inv(16), which may provide prognostic information that could influence responsiveness to chemotherapy and risk of relapse (O'Donnell *et al*., 2011). But one of the most relevant hypothesis for AML leukemogenesis is the involvement of hematopoietic stem cell or progenitor cell capable of transformation, known as leukemic stem cells (LSCs). LSCs maintain the ability of self-renewal and derived from the accumulation of genomic alterations affecting proliferation, cell death and genes involved in the regulation of hematopoietic differentiation (Renneville *et al*., 2008).

Besides these major cytogenetic abnormalities, gene mutations also constitute important events in AML pathogenesis. These oncogenic events are divided in three classes, in which the class 1 of mutations (such as FLT3-ITD, c-Kit, RAS, PTPN11) confers a proliferation and survival advantage to blast cells (Grove and Vassiliou, 2014), while class II mutations blocks differentiation (AML1 or runt-related transcription factor 1 RUNX1 and CCAAT/Enhancer binding protein alpha C/EBPα) and class III mutations affects genes implicated in cell cyle and apoptosis (P53 and NMP1). The block of differentiation occur by alteration at specific stages, like mutations of CEPBA gene or the core-binding factor (CBF) complex genes, like RUNX1, which results in different subtypes of AML (Renneville *et al*., 2008)

Hence, the study of transcriptional factor specific for hematopoietic differentiation has been a subject of intense research. Mutations of these transcriptional factors, including PU.1, GATA1 and C/EBPα, have been found in AML (Nerlov, 2004). The role of C/EBPα during normal differentiation and AML will be discussed in the following sections.

1.2. C/EBPα.

C/EBPα, coded by the intronless CEBPA gene located at chromosome 19q13.1 (Pabst and Mueller, 2009), is a member of a family of leucine zipper transcription factors, also constituted by C/EBPβ, C/EBPδ and C/EBPε, and is a key myeloid transcriptional factor that plays an important role in myeloid differentiation (Keeshan *et al*., 2003; Sun *et al*., 2006). C/EBPα binds as a homodimer or heterodimer with other C/EBP proteins or other transcriptional factors, and has been shown to regulate a number of hepatic and adipocyte genes. In addition, another C/EBP family member, CHOP-10, can dimerize with C/EBP proteins to inhibit transcriptional activation and induce apoptosis (Tenen *et al*., 1997).

Both C/EBPα and C/EBPβ have single mRNAs that can encode transcriptionally active and repressive forms, depending on the usage of alternative AUG codons in the same reading frame. The shorter form, p30, contains the same carboxyl terminus as the full-length known as p42, but lacks the amino-terminal 117 amino acids. One of the main differences between these two isoforms is the ability of p42 to block cell proliferation and act as a tumor suppressor, and also to induce differentiation of adipocytes and granulocytes (Pabst *et al*., 2001; Nerlov, 2004). The 42-kDa normal protein also acts as a transcription factor with a crucial role during differentiation of various cell types including hepatocytes, enterocytes, keratinocytes, lung, mammary gland cells and hematopoietic cells (Leroy *et al*., 2005). Growth factors and nutrients promote proliferation and increase activity of translation initiation factors eIF2α/ eIF4E that leads to an increment of the p30 isoform at the expense of p42, as eIF2α and eIF4E promote the translation of a small open reading frame that bypass the initiation codon of p42. The ratio between p42 and p30 is important to be regulated by the cells due to increased levels of p30 would delay the terminal differentiation of adipocyte and neutrophil granulocyte lineages. This regulation is achieved through extracellular signaling that targets rapamycin and protein kinase R signaling (Nerlov, 2004).

The distribution of C/EBPα functions relies on its domains. The basic region leucine zipper (BR-LZ) DNA-binding domain located at the C-terminal region has been shown to mediate protein-protein interactions with other C/EBP proteins or other transcriptional factors, such as GATA1, PU.1, EST1, RUNX1, c-JUN and E2F (Paz-Priel and Friedman, 2011). The leucine zipper consists of repetitive leucine residues spaced every 7 amino acids that results in a α helical amphipathic structure, with both hydrophobic and hydrophilic faces. Just the amino terminal to the zipper is a basic region highly positive charged that interacts with DNA. This leucine zipper is directly involved in homodimerization and heterodimerization. C/EBPα,

C/EBPβ and C/EBPδ are strongly similar in their C-terminal basic region and leucine zipper domains, and diverge in the N-terminal transactivation domain. Despite this similarity of the C-terminal, C/EBPα and C/EBPβ can bind with vastly different affinities to the same promoter site (Tenen *et al*., 1997). The three trans-activation elements TE-I, TE-II and TE-III mediate interactions with the transcriptional machinery (TBP/TFIIB; CBP/p300; SWI/SNF). TE-1 and TE-II located at the N-terminal region are required for E2F active repression independent of the retinoblastoma protein (RB). E2F is an important transcriptional factor that regulates genes necessary for cell-cycle progression, and activates transcription of the c-Myc oncogene, which blocks granulocyte differentiation (Paz-Priel and Friedman, 2011). Thefore, C/EBPα is able to downregulate c-Myc expression, through repression of E2F, allowing the differentiation (Leroy *et al*., 2005).

Whereas C/EBP proteins are expressed in a number of diverse tissues, their expression in the hematopoietic system may be limited to myeloid cells where plays a pivotal role in early stages of myeloid differentiation, because it has been shown that C/EBPα is expressed in human myelomonocytic cell lines and not in human erythroid, B-cell and T-cell lines (Tenen *et al*., 1997; Leroy *et al*., 2005). During normal hematopoiesis, C/EBPα is absolutely required for the formation of granulocytic monocytic progenitors (GMPs) from the common myeloid progenitor (CMPs). C/EBPα executes this function by coupling the direct transcriptional activation of myeloid-specific genes with the arrest of cell proliferation (Roe and Vakoc, 2014). C/EBPα levels finally diminish as immature myeloid cells mature to neutrophils or monocytes. In the hematopoietic studies of murine cells and human cell lines, C/EBPα was observed highly expressed in proliferating myelomonocytic cells upon induction of differentiation and downregulated with maturation, pattern of expression diverse from the adipocytes where C/EBPα is upregulated and plays an important role during their differentiation and in fully differentiation functions (Tenen *et al*., 1997). It is specifically upregulated during granulocytic differentiation, and in C/EBPα-null mice lack of white adipose tissue, GMPs and all subsequently mature granulocytes (neutrophils and eosinophils), while all the other blood cell types are present in normal proportions (Zhang *et al*., 1997; Nerlov, 2004; Rosenbauer and Tenen, 2007).

C/EBPα regulates many myeloid-specific genes, but is also a strong promoter of cell-growth arrest by coordinating exit from the cell cycle. Multiple mechanisms by which C/EBPα acts on the cell cycle have been reported, such a stabilization of wild-type p53-activated fragment 1 (WAF1, also known as p21), recruitment of RB to C/EBP-responsive gene promoters,

repression of E2F (mentioned previously), inhibition of cyclin-dependent kinase 2 (CDK2)/CDK4 activity, and recruitment of the SWI-SnF complexes to chromatin. However, at least for granulopoiesis, repression of E2F seems to be the most important mechanism, as mice with a targeted mutation of C/EBPα that results in defective repression of E2F-dependent transcription failed to support granulocytic differentiation (Rosenbauer and Tenen, 2007).

As mentioned before, C/EBP family members can interact with other transcriptional factors, including NF-κB and Rel proteins, members of CREB/ATF family, Sp1, RB and members of the fos/Jun zipper family. The amino terminal region of C/EBPα has been shown to interact with TATA binding protein (TBP), PU.1, a master regulator of myeloid genes, and AML1 (Tenen *et al*., 1997; Friedman, 2007). These interactions are relevant during myeloid development and leukemia. During myeloid development, RUNX1 stimulates the transcription of C/EBPα and PU.1, and C/EBPα also activates PU.1 expression. C/EBPα then heterodimerizes with AP-1 proteins that cooperates with PU.1 to lead forward the monocytic lineage, whereas the homodimers of C/EBPα cooperate with NF-κB p50 to favor granulopoiesis (Friedman, 2007; Paz-Priel and Friedman, 2011). Myb can also cooperate with C/EBP factors to activate target genes in myeloid cells, possibly mediated through the p300/CBP coactivator (Tenen *et al*., 1997).

With respect to the possible role of C/EBP proteins in cancer, C/EBPα inhibits cell proliferation in fibrosarcoma lines through the cyclin-dependent kinase inhibitor p21 and can acts as a tumor suppressor of hepatoma lines and other cell types. The human C/EBPα gene is located in the chromosome 19q13.1, and C/EBPβ maps in the chromosome 20q13.1. Neither of these is frequent site of chromosomal translocations found in human myeloid leukemia (Tenen *et al*., 1997). However, mutations in CEBPA have been reported, and will be mentioned in more detail in the section below. C/EBPα mutations have been only observed in myeloid malignancies and with a high frequency in AML, but more recently, have been reported in gastric carcinoma (Leroy *et al*., 2005; Resende *et al*., 2007).

1.2.1. C/EBPα in Acute Myeloid Leukemia (AML).

Loss of C/EBPα function observed in AML contributes to leukemogenesis by blocking granulocytic differentiation. Recently, three mechanisms of C/EBPα inactivation have been reported. The first one is downregulation of C/EBPα expression by the AML1-ETO fusion

transcript in t(8;21) leukemia cells. The second mechanism is inhibition of the translation of C/EBPα mRNA by interaction with heterogeneous nuclear ribonucleoprotein E2 (hnRNPE2), induced by BCR-ABL fusion protein. Finally, inactivating C/EBPα mutations have been reported in hematological malignancies, especially in AML, and this inactivation of transcriptional properties of the C/EBPα protein could lead to leukemogenesis (Leroy *et al*., 2005).

1.2.1.1. Inhibition of C/EBPα in AML.

Inhibition of C/EBPα expression or activity occurs in different subsets of AML. Deletion of RUNX1 gene reduces the levels of CEBPA transcription. Mutation of RUNX1 leading to reduced RUNX1 levels or fusion proteins that dominantly abrogate RUNX1 activity have been reported in 30% of AML cases (Paz-Priel and Friedman, 2011). In addition, C/EBPα expression is suppressed by the leukemogenic fusion proteins AML1-ETO, AML1-MDS1-EVI1 or CBFB-SMMHC in AML patients bearing the chromosomal rearrangements t(8;21), t(3;21) or inv(16), respectively (Eyholzer *et al*., 2010).

Further, some activated signaling pathways in AML can control C/EBPα expression or activity. The activated receptor tyrosine kinase receptor mutant, FLT3-ITD, found in 30% of AML cases, can regulates CEBPA transcription and leads extracellular signal-regulated kinases (ERK) modification of C/EBPα serine S21 to reduce its activity. This phosphorylation induces a conformational change in C/EBPα, such that the transactivation domains of two C/EBPα molecules within a dimer move farther apart (Pabst and Mueller, 2009). BCR-ABL, intracellular constitutively activated tyrosine kinase, inhibits translation of C/EBPα. Trib2 is able to induce C/EBPα proteosomal degradation, dependent upon interaction with COP1 (Paz-Priel and Friedman, 2011).

1.2.1.2. C/EBPα mutations in AML.

C/EBPα is frequently mutated in AML. Approximately 5-15% of AML samples examined have C/EBPα mutated, and they are most prominently in the AML-M2 subtype where these mutations are observed in the 16-20% of the patients (Pabst *et al*., 2001; Nerlov, 2004; Leroy *et al*., 2005). Nevertheless, none of this mutations result in the full ablation of the gene, suggesting that residual activity of C/EBPα is required for leukemogenesis and its activity is necessary in AML to attain their myeloid identity (Ohlsson *et al*., 2013). C/EBPα mutations

reported by Pabst *et al*. are located at the N-terminal region and typically lead to premature termination resulting in loss of the full-length 42kD and the expression of the N-terminally-truncated C/EBPα p30 (Paz-Priel and Friedman, 2011). These N-terminal mutations act as a dominant-negative inhibitor of the full-length of C/EBPα, affecting the DNA binding and also the transactivation of the direct targets (Pabst *et al*., 2001). The mutation that cause the production of p30 results in a lack of repression of E2F activity, but retains the granulocytic lineage commitment, through the basic region that allow the protein-protein interaction, but not terminal differentiation, which is blocked by E2F activity and induces cell proliferation (Nerlov, 2004).

It was also observed in great many AML cases, mutation or insertion of one or more amino acids in the basic region leucine zipper (BR-LZ) DNA-binding domain. Most of these mutations are clustered in the junction between the basic region and the leucine zipper, resulting in non-proper alignment of the DNA-binding residues with the major groove of the DNA once the LZ has dimerized. The mutations in the BR-LZ DNA-binding region of C/EBPα might have a dominant-negative effect by dimerizing with and disrupting the DNA-binding of the other two isoforms, C/EBPβ and C/EBPε, which are expressed during normal granulopoiesis (Nerlov, 2004), consequently resulting in proteins that might contribute to leukemic transformation. Indeed, although the C-terminal mutations cannot bind to DNA themselves, these oncoproteins inhibits apoptosis via induction of Bcl-2 or Flice inhibitory protein, dependent upon interaction of their basic region with NF-κB p50 who bounds to the promoter regions of these target genes. Interestingly, in two-thirds of AML cases harboring C/EBPα mutations, on allele harbors an N-terminal variant and the other allele a C-terminal mutation (Pabst and Mueller, 2009; Paz-Priel and Friedman, 2011).

1.2.2 C/EBPα expression in Acute Lymphoblastic Leukemia (ALL).

C/EBPα is restricted to the myeloid lineages and it is not expressed in lymphocytes or their progenitors or in the erythroid/megakaryocytic lineages. Overexpression of a wild type C/EBPα occurs in B precursor ALL carrying the translocation t(14;19)(q32;q13), which join C/EBPα and the Immunoglobulin heavy chain enhancer locus in B-cell precursors. In these cases no mutations for C/EBPα were found (Paz-Priel and Friedman, 2011).

1.2.3. C/EBPα and miRNAs.

Several microRNAs (or miRNAs) are induced by C/EBPα in the course of normal myeloid differentiation (Paz-Priel and Friedman, 2011). MiR-223 is preferentially expresses in myeloid cells and regulates normal granulopoiesis, by triggering neutrophil differentiation and is necessary for maintaining proper function of mature neutrophils. Its regulation is mediated through a conserved CEBP/PU.1 site upstream of the pri-miR-223 transcription start as well as through a non-conserved C/EBPα/NFIA responsive element in an intronic sequence of the pri-miR-223 (Eyholzer *et al*., 2010). During granulopoiesis, C/EBPα targets miR-34 that silence E2F3 to suppress proliferation, but in AML C/EBPα mutations revealed a lower expression of miR-34a and miR-181 family and elevated levels of E2F3 (Pulikkan *et al*., 2010). Also in granulocytic differentiation, miR-30c has been proposed as an important target of C/EBPα. In AML, miR-30c is downregulated, especially in AML patients with C/EBPα mutations (Katzerke *et al*., 2013). In myeloid leukemic Kasumi-1 cell line, has been reported that tumor-suppressive miR-29a/b1 cluster is a direct target of C/EBPα (Eyholzer *et al*., 2010).

1.3. MiRNAs

MicroRNAs (miRNAs) are small-noncoding single-stranded RNAs, approximately 22 nucleotides of length (Bartel, 2004), evolutionarily conserved that are encoded within the genomes of almost all eukaryotes. With more than 200 members per species in higher eukaryotes, miRNAs are one of the largest families, representing about 1% of the genome (Kim, 2005).

In general, mature miRNAs and argonaute (AGO) proteins form the RNA-induced silencing complex (RISC), ribobucleoprotein complex that mediates post-transciptional gene silencing (Winter *et al*., 2009) by base pairing to partially a complementary sequences in the 3' untranslated regions (3'-UTR) of targets mRNAs (Fabian and Sonenberg, 2012). Due to the ability of miRNAs to regulate gene expression, they have key roles in diverse processes, including control of development, cell differentiation, apoptosis and cell proliferation (Bartel, 2004). These miRNAs form a complex network due to their ability to regulate the target genes. A single miRNA can bind and controlled many different mRNAs and, conversely, several different miRNAs can cooperate to control a single mRNA (Kim, 2005). Even though, miRNA-binding sites are usually located in the 3'-UTR of mRNAS, in the sequence of the

miRNAs, the domain at the 5' end that comprises the nucleotides from the position 2 to 8 is important for the target recognition known as the miRNA seed. More than 60% of human protein-coding genes contain at least one conserved miRNA-binding site. Thus, it is crucial that the biogenesis and the function of miRNAs are tightly regulated, and their dysregulation is often associated with many human diseases, including cancer (Fabian and Sonenberg, 2012).

1.3.1. Biogenesis of miRNAs.

MiRNAs regulation takes place at multiple steps, including their transcription, processing and loading onto AGO proteins (Figure 1). Early observation of the genomic position of miRNAs indicated that most of them are located in the intergenic regions (>1 Kb away from the annotated/predicted gene), although a sizeable minority was found in the intronic regions of known genes in the sense and antisense orientation. Thereby, most of these miRNAs are transcribed as autonomous units. However, 50% of known miRNAs are found in close proximity to others miRNAs, and in fact, clustered miRNAs are generated as polycistronic primary transcripts (Kim, 2005; Fazi and Nervi, 2008).

MiRNAs genes are transcribed by either RNA polymerase II or, in some cases, RNA polymerase III, into primary miRNA transcripts (pri-miRNAs) that are several kilobases long and contain a local hairpin structure (Kim, 2005; Fabian and Sonenberg, 2012). A typical pri-miRNA consists of a 33-35 bp double-stranded stem, a terminal loop and single-stranded RNA segments at both the 5' and 3' sides. The nuclear RNase III Drosha initiates the maturation process by cropping the stem-loop to release a small hairpin-shaped RNA of approximately 65 nucleotides in length, precursor miRNA (pre-miRNA) (He and Hannon, 2004). Drosha, a nuclear protein of approximately 160 kDa, contains two tandem RNase III domains (RIIID) and a double-stranded RNA-binding domain (dsRBD). Drosha forms a complex called Microprocessor together with the cofactor DGCR8 (DiGeorge critical region 8, also known as Pasha in *D. megalogaster* and PASH-1 in *C. elegans*) (Filipowicz *et al*., 2008). DGCR8 is a protein of approximately 120 kDa that localizes in the nucleoplasm and the nucleolus, contains two double-stranded RNA-binding domains essentials for miRNA processing, while its conserved C terminal region interacts with Drosha. The double-stranded stem and the unpaired flanking regions of the pri-miRNA structure are critical for DGCR8 binding and Drosha cleavage. The two RNAs domains of Drosha cleave the 5' and 3' arms of

the pri-miRNA hairpin. Drosha cleaves 11 base pairs away from the single-stranded RNA/double-stranded RNA junction at the base of the hairpin stem (Winter *et al*., 2009).

Other non-canonical pathways have been described for miRNA biogenesis. Intron-derived miRNAs are released from their host transcripts after splicing. If the resulted intron has the appropriate size resembling the pre-miRNA, it bypasses Drosha cleavage and is processed in the cytoplasm by Dicer generating and miRNA called mirtron (Winter *et al*., 2009; Havens *et al*., 2012).

Following nuclear processing by Drosha, pre-miRNAs are exported to the cytoplasm, where maturation is completed. The protein exportin 5 (EXP5, encoded by *XPO5*) forms a transport complex with GTP-binding nuclear protein RAN-GTP and a pre-miRNA (He and Hannon, 2004). After the translocation through the nuclear pore complex, GTP is hydrolysed, resulting in the disassembly of the complex and the release of the pre-miRNA into the cytosol (Ha and Kim, 2014).

After their export from the nucleus, the cytoplasmatic RNase III Dicer subsequently processes pre-miRNAs into approximately 22 nucleotides miRNA duplexes. Each strand of the duplexes bears 5' monophosphate, 3' hydroxyl group and two nucleotides protruding as overhangs at each 3' end (Filipowicz *et al*., 2008). In general, Dicer cleavage sites are located at a fixed distance from the 3′ end of the terminus of dsRNAS (the 3′-counting rule). This distance is typically 21–25 nucleotides in length and depends on the species and the type of Dicer. Dicer is highly conserved protein, found in almost all eukaryotic organisms, of approximately 200 kDa. The C-terminal tandem RIIIDs of Dicer form an intramolecular dimer to create a catalytic center. The N-terminal helicase domain of Dicer allows pre-miRNA recognition by interacting with the terminal loop and increases the processing of certain pre-miRNAs (Winter *et al*., 2009). The PAZ domain of Dicer shows two basic pockets that bind to the 5′ end and 3′ end of the pre-miRNA. The pockets are spatially arranged in a way that they can be occupied simultaneously by the 5′ end and 3′ end of the pre-miRNA when the RNA has a two-nucleotide-long 3′ overhang. This domain is also found in a group of highly conserved AGO proteins (Ha and Kim, 2014). In mammals, AGO2, which has a strong RNase-like endonuclease activity, can support Dicer processing by cleaving the 3' arm of some pre-miRNAs, thus forming an additional processing intermediate called AGO2-cleaved precursor miRNA (ac-premiRNA) (Krol *et al*., 2010).

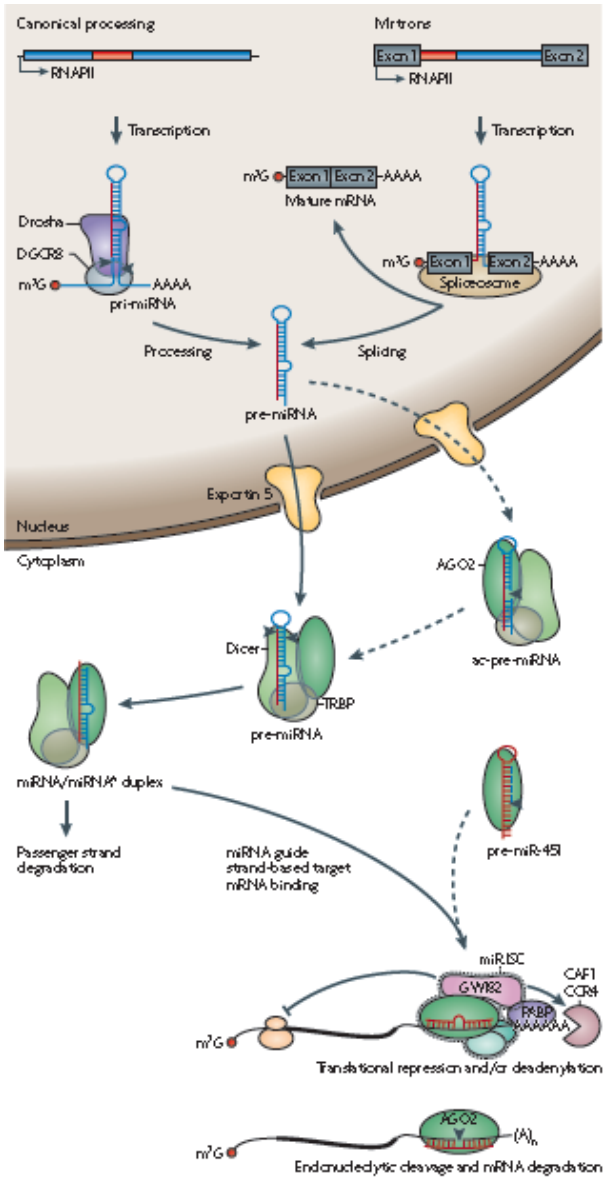

Figure 1. MiRNAs biogenesis and function. MiRNAs are processed from RNA polymerase II-specific transcripts of independent genes or from introns of protein-coding genes. In the canonical pathway, pri-miRNA processing occurs in two steps, catalyzed by two members of the RNase family III of enzymes, Drosha and Dicer, operating in complexes with dsRNA-binding proteins (dsRBPs). In the first nuclear step, the Drosha-DGCR8 complex processes pri-miRNA into an approximately 70-nucleotide precursor hairpin, pre-miRNA, which is exported to the cytoplasm. Some pre-miRNAs are produced from very short introns (mirtrons) as a result of splicing and debranching, thereby bypassing Drosha-DGCR8 step. In either case, cleavage by Dicer, assisted by TRBP, in the cytoplasm yields an approximately 20-bp miRNA/miRNA* duplex. In mammals, AGO2 can support Dicer processing by cleaving the 3' arm of some pre-miRNAs, forming the additional intermediate ac-pre-miRNA. Following processing, one strand of the duplex (the guide strand) is preferentially incorporated into the RISC complex, whereas the passenger strand is released and degraded. Mature miRNAs guide the RISC complex to the 3' UTR of the complementary mRNA targets and repress their expression by several mechanisms: repression of mRNA translation, destabilization of mRNA transcripts through cleavage, deadenylation and localization in P bodies (Krol *et al*., 2010; Fazi and Nervi, 2008).

The small RNA duplexes generated by Dicer are loaded onto AGO protein to form the effector complex RISC (Bartel, 2009). The assembly of this complex consists in two steps: the loading of the RNA duplex and its subsequently unwinding (Ha and Kim, 2014). RISC assembly is mediated by the RISC loading complex that is composed of the RNase Dicer, the double-stranded RNA-binding domain proteins TRBP, PACT (protein activator of PKR), and the core component AGO, which also mediates RISC effects on mRNA targets. TRBP and PACT facilitate the Dicer-mediated cleavage of the pre-miRNAs (Winter *et al*., 2009). The AGO proteins (four AGO proteins described in humans) are characterized by a bilobal architecture, composed of the N-terminal lobe with and N-terminal domain and a PAZ domain, and C-terminal lobe with a middle (MID) domain and a PIWI domain. The 5'monophosphate of the guide RNA is tightly anchored to the 5'-phosphate-binding pocket at the interface between MID and PIWI domains. The MID domain of AGO contacts the 5' nucleobase of the guide strand, that threads along the channel of MID-PIWI lobe to reach the PAZ domain that binds to the 3' end of the guide strand. The seed of guide miRNA is arranged in a helix conformation that facilitates the scanning of the target mRNAs. RISC loading of small RNA duplexes is an active process that requires ATP, whereas the release of the passenger strand is ATP-independent. The heat shock cognate 70 (HSC70, also known as HSPA8)-heat shock protein 90 (HSP90) chaperone complex uses ATP and mediates a conformational opening of AGO proteins, so that AGO can bind dsRNA (Ha and Kim, 2014).

To form the active RISC that performs gene silencing, the double-stranded duplex needs to be separated into the functional guide strand, which is complementary to the target, and the passenger strand, which is subsequently degraded (Winter *et al*., 2009). The thermodynamic stability of the base pairs at the two ends of the duplex determines which strand is to be selected. The strand with a relatively unstable terminus at the 5' side is typically selected as the guide strand. An additional determinant for the strand choice is the first nucleotide sequence: AGO proteins select for guide strand with a U at nucleotide position 1 (Kim, 2005). According to the "rubber band" model, structural tension is introduced to the open conformation of the AGO proteins. The release of this tension may drive the ATP-independent unwinding of the passenger strand. AGO proteins can also cleavage the passenger strand if the duplex is matched at the center. However, this mechanism is rarely used in the miRNA pathway, as most duplexes have central mismatches and human AGO1, AGO3 and AGO4 lack slicer activity (He and Hannon, 2004). Thus, miRNA duplex unwinding without cleavage is a more general process than passenger strand cleavage.

Mismatches in the guide strand at the nucleotide positions 2-8 and 12-15 can promote unwinding of miRNA duplexes in all four AGO proteins. The RISC removes the passenger strand that is quickly degraded, to generate the mature RISC (Ha and Kim, 2014).

1.3.2. MiRNAs function: gene silencing.

MiRNAs interact with their mRNA targets via base-pairing to induce mRNAs cleavage or translation repression (Bartel, 2004). The most stringent requirement is a perfect base-pairing of the miRNA 5' nucleotides 2-8, representing the seed region of interaction with its targets. In addition, an A residue across position 1 of the miRNA and A or U across position 9 improve miRNA activity; although they do not need to base-pair with mRNAs nucleotides. Complementarity of the miRNA 3' is less critical for the binding, though it stabilizes the interaction. Generally, miRNA-mRNA duplexes contain mismatches and bulges in the central region (miRNA position 10-12). Most predicted and experimentally characterized miRNA sites are positioned in the mRNA 3' UTR. However, animal miRNAs may also target 5' UTR and coding regions of mRNAs; these last ones seem to be less strong than those in the 3' UTR. Interestingly, in some cases the 5' UTR target sites appears to activate the translation. MiRNA-mediated repression can be modulated by 3' UTR-binding proteins such as HuR and Dnd1, and AGO-interacting proteins of the TRIM-NHL family (Fabian *et al*., 2010).

MiRNAs post-transcriptionally control protein abundance by repressing translation and/or initiating mRNA degradation through deadenylation and decapping of their targets (Filipowicz *et al*., 2008; Bartel, 2009). Published studies indicate that miRNAs regulate gene expression in six distinct pathways:

(1) Inhibition of translation initiation, either by targeting the cap recognition step (AGO proteins compete eIF4E for binding to the cap structure) or by inhibiting ribosome 80S complex assembly (AGO proteins recruit eIF6, which prevents the large ribosomal subunit from joining the small subunit) (Eulalio *et al*., 2008; Fabian *et al*., 2010).

(2) Repression postinitiation steps have also been reported, like blocking translation elongation or by promoting premature dissociation of ribosomes (ribosome drop-off) (Eulalio *et al*., 2008; Fabian *et al*., 2010).

(3) The cotranslational protein degradation model proposes that translation is not inhibited, but rather the nascent polypeptide chain is degraded contranslationally (Eulalio *et al*., 2008; Fabian *et al*., 2010).

(4) mRNA decay mediated by miRNAs triggering deadenylation and subsequent decapping of the mRNA target. These mechanisms require AGO proteins, the P body component GW182, the CAF1-CCR4-NOT deadenylase complex, decapping enzyme DCP2 and several decapping activators including DCP1, Ge-1, EDC3 and RCK/p54. GW182 proteins are another group of factors important for mRNA-induced repression. They interact directly with and act downstream of AGOs (Eulalio *et al*., 2008). GW182 directly interact with AGO proteins via the GW repeats, and AGO function to bridge the miRNA to the silencing effectors, the GW182 proteins. The C-terminal part of GW182 interacts with the poly(A) binding protein (PABP) and recruits the deadenylases CCR4 and CAF1 (Krol *et al*., 2010).

(5) When RISC, containing AGO2 encounters mRNAs bearing sites nearly perfectly complementary to miRNA, these mRNAs are cleaved endonucleolytically and degraded. Although rare in animals, this is common in plants (Krol *et al*., 2010).

(6) MiRNAs might also silence their targets by sequestering mRNA in discrete cytoplasmic foci known as mRNA processing bodies or P bodies, , where the miRNA-targeted mRNA can be sequestered from the translational machinery and degraded or stored for subsequent use (Eulalio *et al*., 2008; Fabian *et al*., 2010).

1.3.3. MiRNAs in normal Hemotopoiesis.

Hematopoiesis is a complex process controlled by the coordinated expression and function of several genes. Recent studies have emphasized the control of gene expression by several miRNAs in the hematopoietic system and the relationship between imbalance of miRNAs and leukemic phenotype. MiRNAs have been shown to be a key supporting actors in molecular control networks of hematopoiesis, including lineage decisions, stem cell progenitor transitions, niche control and other cell functions. Several miRNAs play a critical role in stem/progenitor, lymphoid, myeloid, erythroid and megakaryocytic biology, and in the immune function of these cell lineages (Dell'Aversana and Altucci, 2012).

MiRNAs are involved in a variety of biological processes including cell cycle regulation, apoptosis, differentiation, development, metabolism and aging. Hence, dysregulation of miRNAs-mediated pathways may contribute to pathological conditions such as tumors, including hematological cancers, and can be involve as tumor suppressor genes or oncogenes (Dell'Aversana and Altucci, 2012).

In different hematopoietic tissues have been shown differential expression of three miRNAS: miR-181, miR-223 and miR-142. MiR-181 was strongly enriched in B lymphocytes, miR-223 expression was restricted to cells of myeloid origin and miR-142 was highly expressed in B-lymphoid and myeloid lineages (Chen *et al*., 2004). Overexpression of miR-150 led to the blockage of B lymphocytes, and selective deletion of the miR-17-92 cluster prevents the transition from pro-B to pre-B cells, by the increase of Bim (Yendamuri and Galin, 2009).

In the myeloid development, miR-223 seems to have a role in the fate decision of the common myeloid progenitor betweem granulocyte or monocyte lineages, because in the first one, miR-223 is highly expressed, while is repressed in the monocyte component. C/EBPα, as mentioned before, regulates miR-223 expression and competes with NFIA (a CCAAT-box binding transcription factor) for the site located in the promoter of miR-223 gene. During induced-retinoic acid differentiation of the progenitor cell into the granulocytic lineage, leads to the replacement of NFIA with C/EBPα. Apart from C/EBPα, also PU.1 seems capable of activating miR-223 transcription in mice. Induction of monocyte/ macrophage differentiation with TPA of HL60 cell line leads to the overexpression of miR-424, also controlled by PU.1 (Yendamuri and Galin, 2009).

MiR-146a is a transcriptional target of NF-κB and, in the hematopoietic system, and it is expressed at relatively high levels in mature immune cells, including dendritic cells, macrophages and granulocytes, and in splenic B and T cells. It seems that miR-146a plays a role in bone marrow development because its overexpression inhibits megakaryopoiesis. In the myeloid cell development, miR-146a has a repressive effect, and in addition, downregulate NF-κB-induced inflammation, and suppress the development of autoimmunity and hematopoietic malignancies (O`Connell *et al*., 2011).

MiR-155 was among the first miRNAs identified in the hematopoietic system because of its enhanced expression levels in certain types of lymphomas. MiR-155 is expressed in low levels in most hematopoietic cells, is downregulated in developing erythrocytes and it is highly expressed in in mature immune cells promoting myeloid development. Another family of miRNAs with emerging importance in myeloid biology is miR-125 family, and its role will be discussed in more detail in the following section (O`Connell *et al*., 2011).

1.3.4. MiRNAs dysregulation in cancer.

The importance of individual miRNAs has been established in specific cancers (Mavrakis *et al*., 2011). The first evidence of the involvement of miRNAs in human cancer derived from studies in Chronic Lymphoblastic Leukemia (CLL), particularly in an attempt to identify tumor suppressors in a frequently deletion that has been reported in chromosome 13q14. In this critical region, it is contained two miRNA genes, miR15a and miR16-1, expressed in the same polycistronic RNA, providing the evidence that the loss of these two miRNAs, caused by the deletion in chromosome 13q14, could be involved in the pathogenesis of a human cancer (Iorio and Croce, 2012). Thereafter several studies provided clear evidence that alterations in the miRNAs expression are involved in cancer. Hence, the studies of miRNAs have been focused in establishing if their profiles can be used for tumor classification, diagnosis and prognosis, and therapy (Esquela-Kerscher and Slack, 2006; Iorio and Croce, 2012).

Recently, it have been showed that about 50% of reported miRNAs are located in areas of the genome, known as fragile sites, that are associated with cancer. This indicates that miRNAs might have a crucial function in cancer progression (Esquela-Kerscher and Slack, 2006). Functional studies performed in cancer cell lines or mouse models of diverse types of malignancies, through overexpression or knockdown of miRNAs have supported their role in tumorogenesis (Farazi *et al*., 2013). In this regard, some examples will be mentioned in the following sections.

1.3.4.1. MiRNAs as tumor suppressors.

Like a protein-coding gene, a miRNA can act as a tumor suppressor when its loss of function can contributes to the malignant transformation of a normal cell. MiRNA loss of function might be due of genomic deletion, mutation, epigenetic silencing and/or miRNA processing alterations. For example, the most common chromosomal abnormality observed in CLL is deletion of the 13q14.3 region, where miR-15a/miR-16-1 cluster is located. MicroRNAs endoced by the miR-15/16 locus function as a tumor suppressors. Expression of these miRNAs is downregualted in CLL, melanoma, colorectal cancer, bladder cancer and other solid tumors. MiR-15/16 targets multiple oncogenes, including BCL2 Cyclin D1 and MCL1. The most important target of miR-15/16 in CLL is considered Bcl-2, as it is an anti-apoptotic gene that is widely overexpressed in CLL (Garzon *et al*., 2009). Interestingly, it has

been shown that also miR-125b and miR-155 contibute to Bcl-2 repression in human leukemic B-cells (Willimott and Wagner, 2012). Another important miRNA family considered to function as a tumor suppressor is the let-7 family, often downregulated in many tumors, including lung, cervical and breast cancer, and members of this family inhibit well characterized oncogenes, such as RAS family, HMGA2 and c-myc, and induce cell cycle arrest and apoptosis (Garzon *et al*., 2009; Farazi *et al*., 2013). Further supporting the role of miRNAs as tumor suppressors, abnormalities located in chromosome 7q32 are frequently encountered in myelodysplasia and AML. Interestingly, *miR-29b-1/miR-29a* are located on chromosome 7q32. These *mir-29* family members have been shown to be downregulated in MDS and AML as a consequence of chromosome 7q32 deletion. Restoration of *mir-29b* in AML cell lines and primary samples induces apoptosis and dramatically reduces tumorigenicity in a xenograft leukemia model. These data support a tumor suppressor role for miR-29 that recently it has been shown to be dowregulated also in CLL, lung cancer, invasive breast cancer and cholangiocarcinoma (Garzon *et al*., 2009). These tumor suppressor effects can be explained by the direct targeting of the anti-apoptotic protein MCL-1 and the oncogene TCL-1 by miR-29 family. Interestingly, most of the miRNAs with a tumor suppressor role (like miR-15a/miR-16-1, miR-29 family, let7) have more than one genomic location, and although they are transcribed from different loci, the mature form is identical, and the different loci could be differentially regulated (Garzon *et al*., 2009). Additional studies have shown that the expression of miR-143 and miR-145 are significantly reduced in colorectal cancer. The downregulated levels of these miRNAs have been also observed in breast, prostate, cervical and lymphoid cancer cell lines (Esquela-Kerscher and Slack, 2006).

1.3.4.2. MiRNAs as oncogenes.

Several miRNAs have been described to function as oncogenes, often known as Oncomir, such as miR-155 and miR-21, both among the most overexpressed miRNA in human cancers. MiR-155 is contained in a host noncoding RNA named B cell integration cluster (BIC) that cooperates with the oncogene c-myc. Several groups have shown that miR-155 is highly expressed in pediatric Burkitt lymphoma, Hodgkin disease, primary mediastinal non-Hodgkin Lymphoma, CLL, AML, lung cancer and breast cancer. In the case of miR-21, this miRNA is upregulated in a wide variety of hematological malignancies and solid tumors, including AML, CLL, glioblastoma, and cancer of the pancreas, prostate, stomach, colon, lung, breast and liver. When overexpressed in mice, miR-21 leads to pre-B malignant lymphoid-like

phenotype, demonstrating that this gene acts as a genuine oncogene. Its overexpression blocks apoptosis by targeting tumor suppressor genes such as PTEN (phosphatase and tensing homolog), PDCD4 (programmed cell death 4) and TPM1 (tropomyosin 1). Moreover, the upregulation of another cluster, miR-17-92 cluster is frequently amplified in follicular lymphoma and diffuse large B cell lymphoma. Also, the members of this family are highly expressed in a variety of solid tumors, including breast, colon, lung, pancreas, prostate and stomach cancer (Garzon *et al*., 2009). These miRNAs promote proliferation, inhibit apoptosis, induce tumor angiogenesis, and cooperate with c-myc to cause lymphoma in mice. Interestingly, the miR-17-92 cluster is transactivated by c-myc in liver cancer (Cairo *et al*., 2010). The effects of this cluster's expression on cell cycle and proliferation are due to the regulation of E2F transcription factors. Additionally, miR-17-92 cluster targets the pro-apoptotic protein Bim and the tumor suppressors PTEN and p21. Moreover, it has been reported that miR-17-5p is downregulated in breast cancer cell lines, and its restored expression decreased the proliferation of these cells. The observations raise the question of a possible dual role, oncogenic and tumor suppressor, depending on the tissue and its transcriptome, including the miRNA targets expressed in the particular tissue (Garzon *et al*., 2009). Moreover, in T-ALL a group of miRNAs (miR-19b, miR-20a, miR-26a, miR-92 and miR-223) are responsible of promoting T-ALL development in mice because they have cooperative effects on tumor suppressor genes implicated in the pathogenesis of T-ALL, including Ikaros, PTEN, Bim, PHF6, NF1 and Fbw7 (Mavrakis *et al*., 2011).

1.3.4.3. MiRNAs dysregulation in leukemia.

Leukemogenesis is a complex process characterized by the abnormal proliferation of blood precursor cells of myeloid or lymphoid origin and by the presence of chromosomal abnormalities, such as deletions, translocations or inversions, or genetic mutations affecting the control of hematopoietic cell proliferation and differentiation (Wang *et al*., 2014). Leukemia is classified both clinically and pathologically as acute or chronic, based on differentiation state and clinical evidence, and myeloid or lymphoid, according to the cell type. In order to distinguish between these types of leukemia, biomarkers of different leukemia subtypes based on genetic, phenotypic, or molecular characteristics have been reported. For chronic lymphocytic leukemia, the expression of CD38 and ZAP-70 can be used as prognostic marker, while the mutational status of NPM1 or nucleophosmin (nucleolar phosphoprotein B23, numatrin), FLT3-ITD (FMS-like tyrosine kinase 3-internal tandem

duplications), C/EBPα (CCAAT/Enhancer binding protein alpha) and MLL (Mixed Lineage Leukemia) are associated with the outcome of treatment of patients with cytogenetically normal Acute Myeloid Leukemia (AML) (Dell'Aversana and Altucci, 2012).

MiRNAs are becoming increasingly investigated for their ability to control a wide range of physiological and pathological processes including human leukemias (Chen *et al*., 2004; Schotte *et al*., 2012). More recently, studies found that miRNAs and long non-coding RNAs (lncRNAs) can be used as biomarkers for diagnosis and prognosis of cancer, specifically of leukemia, due to their extensive deregulation that has been observed (table 1). Many studies support the role of miRNAs in altering signaling pathways stablished in Chronic Lymphocytic Leukemia (CLL), Chronic Myeloid Leukemia (CML), Acute Lymphoblastic Leukemia (ALL) and Acute Myeloid Leukemia (AML) (Lu *et al*., 2005; Zhu *et al*., 2011; Wang *et al*., 2014; Dell'Aversana and Altucci, 2012).

ALL is one of the most common malignancies present in pediatric groups, and it is characterized by clonal proliferation of early B or T-lymphocytes progenitors that results in the accumulation of leukemic cells in bone marrow and infiltration in peripheral organs. The first evidence of the involvement of miRNAs in ALL was demonstrated when an insertion of miR-125-1, a human homologue of Lin-4, was seen in a rearranged heavy-chain immunoglobulin gene locus in a patient with B-cell ALL (Yendamuri and Galin, 2009). It has been reported that in ALL cells, compared to $CD34^{+}$, 14 miRNAs are upregulated (miR-182a, miR-142-3p, miR-124-5p, miR-150, miR-181a, miR-181b, miR-181c, miR-193a, miR-196b, miR-30e-5p, miR-34b, miR-365, miR-582, miR-708) and 5 downregulated (miR-100, miR-125b, miR-151-5p, miR-99a, let-7e). The group of Mi *et al*. had identified 27 miRNAs that were differentially expressed between ALL and AML; among these miR-128a and miR-128b were overexpressed, whereas let-7b and miR-223 were strongly downregulated (Mi *et al*., 2007; Dell'Aversana and Altucci, 2012).

Table 1. MiRNAs as diagnostic and prognostic biomarkers in leukemia (Wang *et al*., 2014).

miRNA	**Expression**	**Biomarker**
miR-128a, miR128b	Upregulated in ALL vs. AML	Diagnostic
let7b, miR-223	Downregulated in ALL vs. AML	
miR-181a	Upregulated in M1/M2 vs. M4/M5	Diagnostic
miR-29a, miR-142-3p	Downregulated in AML	Diagnostic
miR-424	Downregulated in AML with NPM1 mutA	Diagnostic
miR-155	Upregulated in FLT3-ITD mutation positive AML	
miR-181, miR-30d, let-7a, miR-125b	Downregulated in CLL	Diagnostic
miR-326	Downregulated in CML	Diagnostic
miR-148, miR-424	Upregulated in T-lineage vs. B-lineage ALL	Diagnostic
miR-151	Downregulated in T-lineage vs. B-lineage ALL	
miR-191, miR-199a, miR-181a, miR-181b	Upregulated in AML	Prognostic
miR-155	Upregulated in FLT3-ITD mutation positive AML	Prognostic
miR-375, miR-378, miR-212, miR-9	Upregulated in AML	Prognostic
miR-29b	Downregulated in AML	Prognostic
miR-181b, miR-223	Downregulated in CLL	Prognostic
miR-21	Upregulated in CLL	Prognostic
miR-196b	Upregulated in MLL-associated AML	Prognostic
miR-150, miR-146a	Upregulated in CML	Prognostic
miR-142-3p, miR-199b-5p	Downregulated in CML	
Circulating miR-92a	Downregulated in acute leukemia	Diagnostic
Circulating miR-150, miR-342	Downregulated in AML	Diagnostic
Circulating miR-181b-5p	Upregulated in AML	Prognostic
Circulating miR-195, miR-29a, miR-222, miR-20a, miR-155	Upregulated in B-cell CLL	Diagnostic
Circulating miR-155	Upregulated in T-cell leukemia	Prognostic

AML is characterized by an accumulation of granulocytic monocyte precursors in bone marrow and peripheral blood. MiRNA patterns have been correlated with molecular abnormalities like t(11q23), t(15;17), trisomy 8 and FLT3-ITD mutations and molecular subtypes of AML (Garzon *et al*., 2008). MiRNA expression has also been investigated in some AML associated with rare translocations, like in the case of miR-125b overexpression observed in AML carrying the t(2;11)(p21;q23) translocation (Bousquet *et al*., 2008). Specific alterations were correlated with prognosis; patients with high expression of miR-191 and miR-199a had worse outcome and miR-212 expression is associated with prolonged overall survival and relapse-free survival. Apart from distinct miRNA signatures for diagnosis and prognosis, the functional effects of some alterations have been shown. Mice develop an expansion of the granulocyte/monocyte population with pathological features characteristic of myeloid neoplasia when miR-155 is overexpressed (Yendamuri and Galin, 2009). In addition, it has been shown that several novel miRNAs are located in leukemia-associated genomic alterations, like miR-145 and miR-146a are found in the deleted region del (5q) of myeloid malignancies and are also downregulated in cell lines with chromosome 5q deletion or diploid at this locus. In the case of miR-481, located within a deleted region on chromosome 7q, is able to target meningioma 1 and its high expression is correlated with poor outcome of AML patients (Dell'Aversana and Altucci, 2012).

1.4. MiR-125 family.

MiR-125 family is composed of three homologs hsa-miR-125a, hsa-miR-125b-1 and hsa-miR-125b-2. MiR-125a is located at 19q13, while miR-125b has been verified to be transcribed from two loci located on chromosomes 11q23 (has-miR-125b-1) and 21q21 (has-miR-125b-2). Among the most important families, miR-125 family has been reported to be implicated in a variety of carcinomas and other diseases, and it could act as either repressors or promoters. MiR-125 family play important roles in many different cellular processes such as cell, differentiation, proliferation and apoptosis because could targets many different proteins involved in these processes (Sun *et al*., 2013).

1.4.1. MiR-125b.

MiR-125b is highly conserved among mammals, vertebrates and nematodes. In humans, there are two paralogs (as mentioned before, hsa-miR-125b-1 on chromosome 11 and hsa-

miR-125b- on chromosome 21), coding for the same mature sequence. In humans, these paralogs are organized in clusters. MiR-125b-2 is part of the cluster with miR-99a and let-7c, and it is encoded approximately 50kb downstream of hsa-miR-99a and hsa-let-7c, which are located about 650 bp from each other. MiR-125b-1 is organized in a cluster with let-7a-2 and miR-100. Although the distance of 50kb is greater than the standard for a cluster, these regions are considered cluster because from the analysis of various genomes shows that each region are highly conserved in its organization. Due to these organizations of the miR-125b paralogs, miR-125b may contain an alternative promoter, apart from the one encoding for the other members of the cluster, making possible the transcription of miR-125b solely (Figure 2) (Shaham *et al*., 2012).

MiRNAs are located in introns of protein-coding genes (approximately 60%) contain putative promoters regulating transcription independent of their host gene, and this is supported by the observed discrepancy in expression between the miRNAs and their host genes. Most of the studies agree on hsa-miR-99a, hsa-let-7c and hsa-miR-125b-2 being intragenic, whereas hsa-miR-100, hsa-let-7a-2 and hsa-miR-125b-1 on being intergenic (Shaham *et al*., 2012).

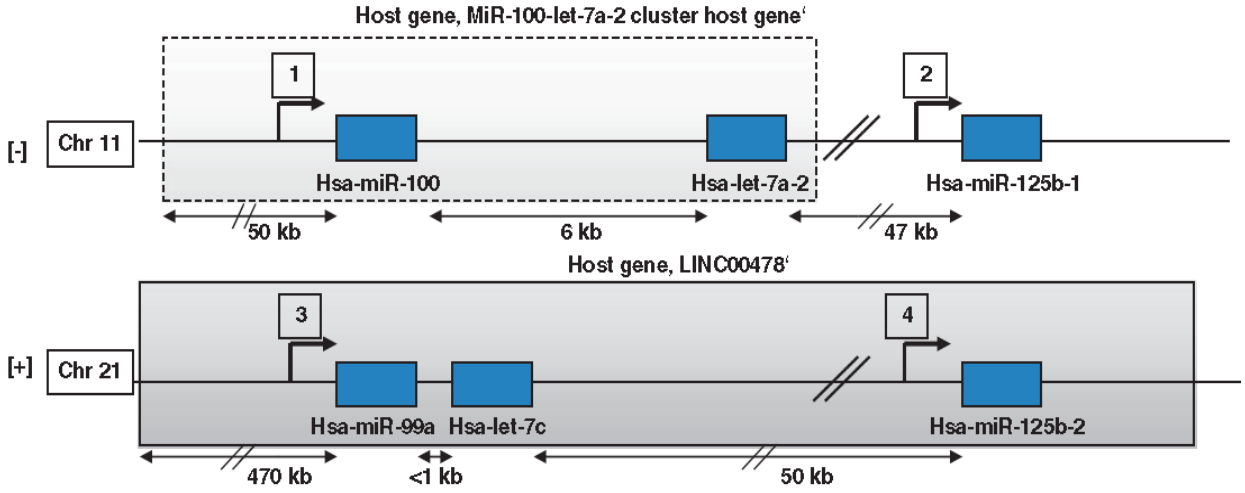

Figure 2. Genomic organization of miR-125b clusters. Schematic diagrams of host genes containing miR-125b loci and potential promoter regions 1-4 (Sun *et al*., 2013).

1.4.2. Regulators of miR-125b expression.

Relatively little is known about the regulation of miR-125b expression, due to the contradictory data existing regarding this matter. It has been reported that miR-125b can be induced by the caudal type homeobox 2 (CDX2) (Lin *et al*., 2011), induced or repressed by the nuclear factor kappa B (NF-κB) and P53, and to repressed by myelocytomatosis viral oncogene homolog (MYC) and v-akt murine thymoma viral oncogene homolog 1 (AKT1). Also has been proposed that in prostate cancer, miR-125b can be directly regulated by androgens (Shaham *et al*., 2012).

1.4.3. MiR-125b in normal Hematopoiesis.

MiRNA expression profiles in multiple hematopoietic subpopulations demonstrated that miR-125b was one of the most expressed miRNAs in hematopoietic stem cells (HSC), compared with all the other progenitor populations. Interestingly, whereas the levels of miR-125b drop significantly in committed progenitors, the levels in common progenitors are higher (Shaham *et al*., 2012). MiR-125 directly targets and downregulates proapoptotic factors, like Bak1, KLF13 and BMF, increasing the survival of immature hematopoietic cell populations (O'Connell *et al*., 2011). There are contradictory findings regarding the lineage that is expanded because of the overexpression of miR-125b. Ooi *et al*. found preferential expansion of the lymphoid lineage, whereas O'Connell *et al*. reported a preferential expansion of the myeloid lineage (Ooi *et al*., 2010; Shaham *et al*., 2012). Recent evidence has also uncovered a role for miR-125b in the development of plasma cells and effector T cells, suggesting that miR-125b regulates immune cell development. Overexpression of miR-125b alone in mice display some changes in the hematopoietic compartment, showing myeloid cell numbers dramatically increased and B-cell numbers severely diminished. Investigating the mechanism by which miR-125b regulates hematopoiesis, was found that the mRNA for LIN28a, an induced pluripotent stem cell gene, was most repressed by mi-125b in mouse hematopoietic stem and progenitor cells (Chaudhuria *et al*., 2012).

1.4.4. MiR-125b in hematological malignancies.

Dysregulation of miRNAs is linked to hematological malignancies, especially leukemia. Given its expression pattern and the putative role of miR-125b in HSCs, has been

hypothesized that its dysregulation of expression may be associated with hematopoietic malignancies and solid tumors (Shaham *et al*., 2012).

1.4.4.1. MiR-125b in myeloid malignancies.

The miR-125b is up-regulated in many neoplastic blood disorders, including AML (Tili *et al*., 2013). MiR-125b has been also reported to be overexpressed in megakaryoblastic leukemia and in pediatric acute promyelocytic leukemia (APL), this last one represents approximately 10% of pediatric AML cases (Marcucci *et al*., 2011; Zhang *et al*., 2011). Interestingly, miR-125b was found to be reduced to normal levels in complete remission APL patients (Zhang *et al*., 2011). Its overexpression has been associated with several chromosomal translocations including TEL-AML1 in ALL, PML-RARA in APL and BCR-ABL in CML and B-ALL, AML1/ETO in AML, and other chromosomal abnormalities like myelodysplasia syndrome (MDS) involving the del(5q) and AML associated with the FLT3 mutation (Shaham *et al*., 2012). Furthermore, miR-125b-1 is implicated in some chromosomal translocations like t(11;14)(q24;q32) and t(2;11)(p21;q23) which leads to upregulation of miR-125b in B-cell acute lymphoid leukemia (B-ALL) or MDS and AML, respectively (Tili *et al*., 2013; Sun *et al*., 2013). Moreover, miR-125b-1 upregulation in AML with t(2;11)(p21;q23) inhibits myeloid differentiation, whereas miR-125b-2 cooperates with the mutated transcriptional factor GATA1 during leukemogenesis of Down Syndrome (DS)-associated acute megakaryoblastic leukemia (AMKL) (Klusmann *et al*., 2010; Marcucci *et al*., 2011; Bousquet *et al*., 2012).

Overexpression of both miR-125b-1 and miR-125b-2 causes a dose dependent myeloproliferative disorder that progressed into a lethal myeloid leukemia in mice (Marcucci *et al*., 2011; So *et al*., 2013). Also in nude mice, it has been observed that transplanted fetal live cells which ectopic expression of miR-125b had increased in white blood cell count, and among these mice, half died of B-cell acute lymphoblastic leukemia, T-cell acute lymphoblastic leukemia or a myeloproliferative neoplasm, indicating an important role of miR-125b in early hematopoiesis (Bousquet *et al*., 2010). Transduction of miR-125b-1 in bone marrow cells accelerated myeloid tumors induced by a C-terminal mutant of C/EBPα, suggesting that overexpression of miR-125b collaborates with other genetic alterations in the pathogenesis of myeloid malignancies (Enamoto *et al*., 2012).

1.4.4.2. MiR-125b in Acute Lymphoblastic Leukemia (ALL).

MiR-125b in ALL is upregulated by the insertion of miR-125b-1 into the Immunoglobulin heavy chain locus (IgH) enhancer locus in the samples that harbor the translocation t(11;14)(q24;q32) (Sonoki *et al*., 2005; Chapiro *et al*., 2010). The increased expression of miR-125b was associated with TEL-AML1 t(12;21) in precursor B-cells ALL. A similar increased expression of this miRNA was also described in BCR-ABL ALL (Yendamuri and Galin, 2009; Shaham *et al*., 2012).

1.4.5. Targets of miR-125b.

As previously reported, miR-125 family plays an important role in different cellular processes like normal cell homeostasis, cell survival, proliferation, differentiation, cell metastasis and many diseases (Sun *et al*., 2013). In Figure 3, it is summarized the targets of miR-125 family involved in different types of diseases pathogenesis.

Members of the Bcl-2 antiapoptotic family, such as Bcl-w, Bcl-2, Mcl-1 and Bak1, acting as Bcl-2 homologous antagonist, have been reported as miR-125 direct targets. Other molecules involved in apoptosis like P53, TP53INP1, TNFAIP3 and p38α also are described as miR-125 targets (Sun *et al*., 2013).

Furthermore, other targets involved in proliferation, differentiation, migration and metastasis have been verified for miR-125 family. MiR-125-induced downregulation of ERB2 and ERB3, which enhance kinase-mediated activation of downstream signaling pathways like MAPK, reduced cell motility and invasiveness breast cancer and endometrial cancer cells. Among other targets of miR-125 associated with these processes are HuR, Rock-1, PDPN, STAT3 and STARD13, genes identified as promoters of cell metastasis and migration. Also associated with metastasis have been described as targets of miR-125 matrix metalloprotease MMP11 and MMP13, c-Jun, ARID3B and the growth factor VEGF-A. ABTB1, an antiproliferative factor targeted by miR-125b, contributes to block proliferation in leukemia. On the other hand, this miRNA family can target CBFβ, ARID3A, LIN28A and the growth factor IGF-II, genes involved in hematopoiesis and differentiation (Sun *et al*., 2013, and the references in).

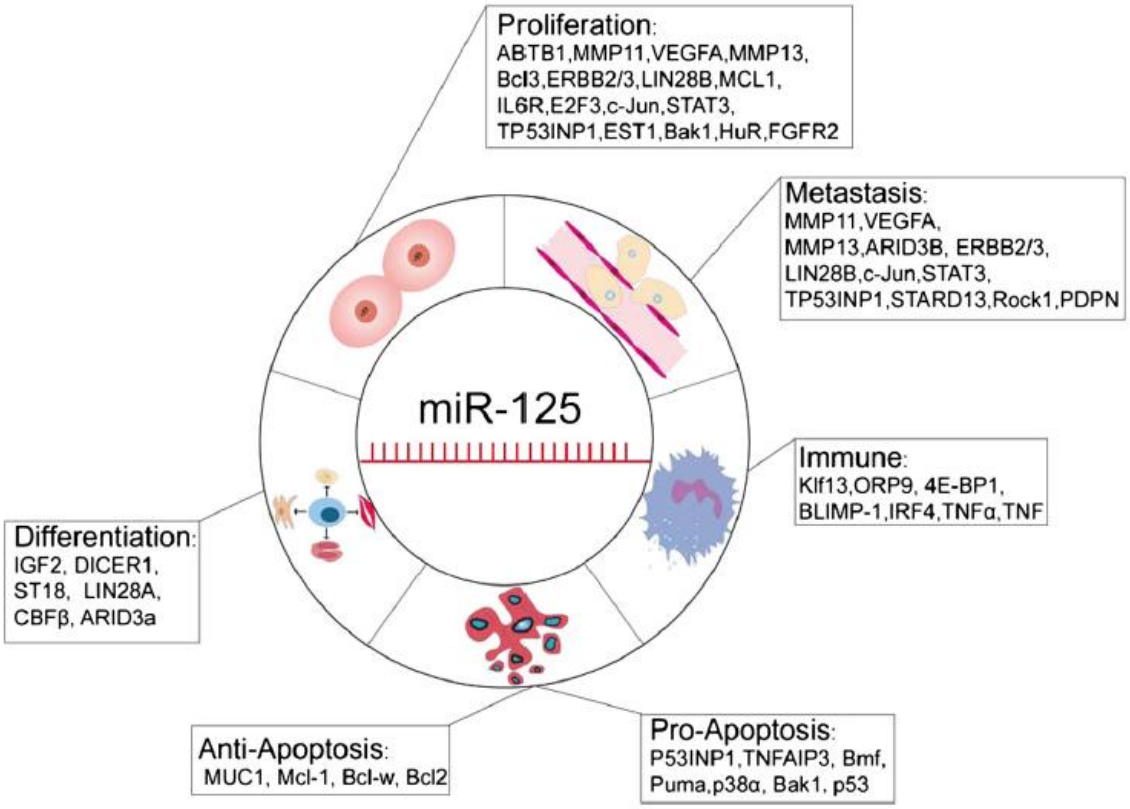

Figure 3. Schematic diagram of miR-125 family targets involved in different types of disease pathogenesis. (Sun *et al*., 2013).

MiR-125 can act as a cancer promoter or tumor suppressor depending on the cell context (Figure 3). Due to the relevance on the miR-125 in many different cellular processes, further research of target genes and regulation pathways that could be controlled by this miRNA is necessary, also to determine its potential use as therapeutic strategy (Sun *et al*., 2013). In some tumors miR-125b was overexpressed, like in prostate and colorectal cancer. Interestingly, miR-125b was found to be downregulated in breast and oral cancers, and melanoma and carcinomas (Xu *et al*., 2012; Wang *et al*., 2012). Thus, miR-125b seems to have a dual role, and this could depend on the cellular context. MiR-125b was found to act as oncomiR in hematologic malignancies by targeting tumor suppressors. On the other hand, in the breast cancer cells this particular miRNA can function as a tumor suppressor by blocking the translation of oncogenes (Bousquet *et al*., 2012).

P53 transcriptional factor is a key tumor suppressor and controls multiple cellular pathways in response to stress. MiR-125b downregulates p53 expression (Le *et al*., 2009) and it directly targets other components of p53 pro-apoptotic network, including Bak1, PUMA, BMF,

TRP53INP1 and Kruppel-like factor 13 (KLF13), thus induces the blockage of apoptosis (Bousquet *et al*., 2012; Shaham *et al*., 2012).

Inhibition of the three targets, Dicer1, suppression of tumorigenicity 18, zinc finger (ST18) and the histone methyltransferase SUV39H1 was suggested to be associated with the proliferation phenotype. MiR-125b overexpression seems to favors this inhibition. The downregulation of SUV39H1 by miR-125b may induce proliferation in hematopoietic cells, through the repression of key inflammatory chemokines and cytokine genes such as interleukin 6 (IL-6), that drives hematopoietic proliferation, and monocyte chemoattractant protein-1 (MCP-1), by the decrease of the repressive H3K9me3 chromatin mark at their promoter regions (Shaham *et al*., 2012).

MiR-125b has been demonstrated to inhibit cell differentiation of granulocytes and monocytes in the myeloid lineage, and of premature plasma cells (PCs) differentiation and naïve T cells in the lymphoid lineage. MiR-125b was found to regulate a network molecules involved in T-cell differentiation into effector cells, including IFNG, IL2RB, IL10RA and PRDM1 (Rossi *et al*., 2011). The inhibition of B-cell differentiation into mature PCs occurs by targeting BLIMP-1 and IRF-4 In the case of multiple myeloma (MM); transduced miR-125b exhibited an exaggerated death rate by downregulation of its targets BLIMP-1 and IRF-4 (Shaham *et al*., 2012).

1.4.6. MiR-125b and Notch1.

In Hailey-Hailey disease (HHD), a rare autosomal dominantly disease characterized by suprabasal cell separation (acantholysis) of the epidermis, lesion skin-derived keratinocytes are distinguished by a specific miRNA profile in which miR-125b is overexpressed. In this context, miR-125b is involved through modulation of Notch1 and p63 expression (Manca *et al*., 2011), both relevant roles in keratinocyte proliferation and differentiation (Cialfi *et al*., 2010). Due to the importance of Notch signaling in the leukemia context, the following sections will be focus in this particular pathway.

1.5. Notch signaling pathway.

Notch signaling has been implicated in the regulation of proliferation, self-renewal, embryonic development, cell fate specification and stem cell maintenance in several tissues

and cell types (Artavanis-Tsakonas *et al*., 1999; Radtke *et al*., 2004). Dysregulation or loss of Notch signaling underlies a wide range of human disorders and cancer (Kopan and Ilagan, 2009). There is abundant evidence to support the importance of deregulated Notch activity in ovarian cancer, breast cancer, medulloblastoma, T cell leukemia, anaplastic large cell lymphoma and Hodgkin disease, gliomas, lung carcinomas, porstate and pancreas cancer. Therefore, blocking the intramembrane cleavage with γ-secreatse inhibitor (GSI) is an especially attractive strategy of targeted therapy (O'Neil *et al*., 2007).

The Notch receptor was first described almost 90 years ago by Morgan and colleagues in Drosophila, who observed a Drosophila strain with notched wings caused by X-linked dominant mutation. The importance of Notch in lymphocyte development and oncogenic transformation is well characterized. Furthermore, there is growing evidence that components of this oncogenic signaling pathway in lymphocytes might have a suppressive function of myeloid cell growth, as previously described in epithelial or head and neck squamous cell carcinoma (SCC). Some of the controversy may give into consideration functional redundancy between receptors, differences between ligands or the dependence of the Notch signaling to the cellular context (Lobry *et al*., 2014).

1.5.1. Components of the Notch signaling pathway.

Although Notch receptors are highly conserved between species, mammals possess 4 distinct receptors compared to Drosophila or nematodes that express Notch1 or Notch2 receptors, respectively, and five ligands, three Delta-like and two Jagged ligands (Figure 4A) (Kopan and Ilagan, 2009).

Notch receptors are single-pass type I transmembrane receptors, containing conserved protein subunits: extracellular, transmembrane and intracellular, synthetized as a single precursor that subsequently gets cleaved during the transport into the Golgi by a furin-like convertase (S1 cleavage) and exit as a noncovalentlty linked heterodimer at the cell surface. The heterodimer receptor generated by this first cleavage consists of the Nocth extracellular subunit that is noncovalently linked to a second subunit containing the extracellular heterodimerization domain (HD) and the transmembrane domain followed by the cytoplasmatic region of the Notch receptor. The extracellular portion of Notch receptors have epidermal growth factor (EGF)-like repeats, but Notch1 and Notch2 have 36 while Nocth3 and Nocth4 contains 29. These repeats are fucosylated on specific serine and threonine

residues by *O*-fucosyltransferase, required for the efficient binding to the ligands. These *O*-modifications can be elongated by the addition of *N*-acetylglucosamine by the Fringe family of 1,3 *N*-acetylglucosaminyltransferases and regulate the affinity of Notch receptors for certain ligands (Lobry *et al*., 2014). The extracellular subunit also contains three cysteine-rich LIN12/Notch repeats (LNR) that prevent ligand-independent activation and a hydrophobic stretch of amino acids mediating heterodimerization between the extracellular portion and intracellular subunit at the HD. The intracellular portion of Notch (ICN) includes domains that mediate the signal transduction, such as the RBP-Jk associated molecule (RAM) domain, a nuclear localization signal (NLS), a transactivation domain (TAD) and six Ankyrin repeats, that interact with downstream effector proteins and nuclear localization sequences (Maillard *et al*., 2005). Notch3 and Notch4 lack the classical TAD. The experimental evidence demonstrates that the high-affinity interaction between ICN and RBP-Jk occurs through the RAM domain, but for the formation of the transcriptional activation complex and the recruitment of mastermind (MAML1) are necessary the Ankyrin repeats (Lobry *et al*., 2014). This intracellular portion also contains a C-terminal proline-glutamate-serine-threonine-rich (PEST) domain involve in the regulation of the protein stability (Maillard *et al*., 2005).

The other components of the Notch pathway are the ligands, which include Delta-like and Jagged, known as Serrate in invertebrates. In mammals, the Delta-like ligands are Dll1, Dll3 and Dll4, while the Jagged ligands contains two members, Jag1 and Jag2 (Lobry *et al*., 2014). Structurally, the ligands share many characteristics with Notch receptors and are prone to similar modifications (Bray, 2006). These ligands are also transmembrane proteins with an extracellular domain that contains EGF-like repeats and an N-terminal DSL domain (Maillard *et al*., 2005).

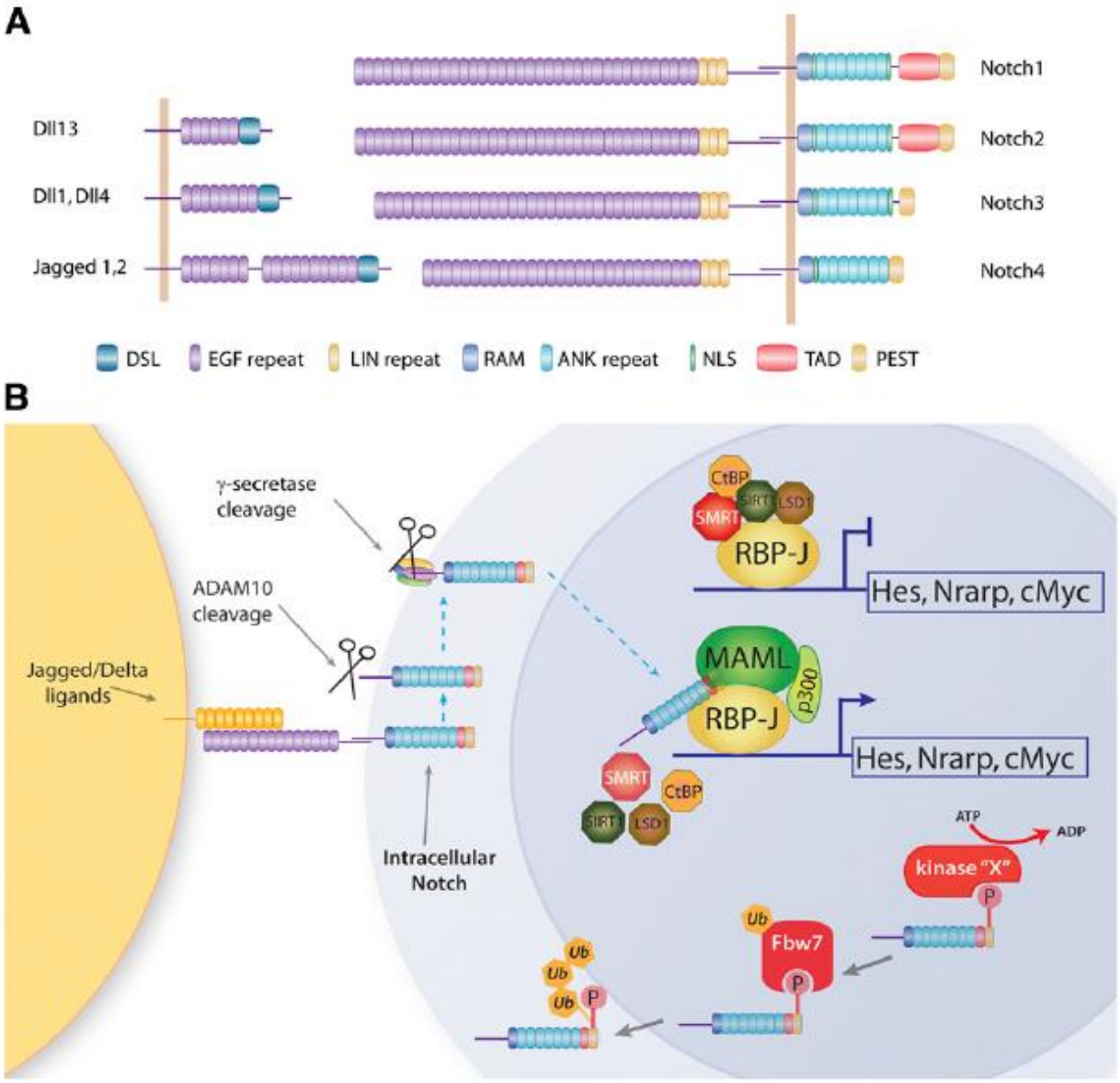

Figure 4. Mammal Notch receptors, ligands and canonical activation pathway. (A) Structure of Notch ligands and receptors. There are 5 Notch ligands in mammals: Jagged1, Jagged2, Dll1, Dll3 and Dll4. All ligands have an extracellular domain called DSL (Delta, Serrate and Lag-2) involved in the receptor binding associated with EGF-like repeats. In the case of the receptors, there are 4: Notch1, Notch2, Notch3 and Notch4. All of them have an extracellular domain with 3 negative regulatory LIN repeats and 29-36 EGF-like repeats. The cytoplasmic portion contains a RAM domain, a NLS domain, ANK repeats and a PEST domain. Only Notch1 and Notch2 have TAD domain. (B) Main features of Notch signaling. The interaction between Notch receptors and Notch ligands triggers 2 consecutive proteolytic cleavages by the ADAM10 metalloprotease and the γ-secretase complex. This cleavages produce ICN, which enters into the nucleus and displaces co-repressors (SMRT and CtBP1) to bind to RBP-Jk and recruits the coactivator MAML1 and the acetyltransferase p300. ICN activating complex is short-lived, ICN gets phosphorylated on its PEST domain and subsequently ubiquitinated by Fbw7 to be targeted for degradation by the proteasome (Lobry *et al*., 2014).

1.5.2. Mechanisms of Notch signaling pathway.

Both Notch receptors and Notch ligands are membrane proteins, and thus activation of Notch singaling is dependent on direct cell-cell contact (Fortini, 2009). When membrane receptors interact with the Notch ligand on an adjacent cell, two consecutive proteolytic cleavages on the receptor are initiated that release the ICN from the membrane. The first cleavage is ADAM10-dependent and occurs extracellularly close to the transmembrane domain (S2) generating a short-lived membrane-bound form of the transmembrane subunit (Mallaird *et al*., 2005). A second cleavage (S3) happens within the transmembrane domain by the γ-secretase complex, which is composed of Presenilin, APH1, PEN2 and Nicastrin (NCSTN). NCSTN serves as a substrate receptor for the γ-secretase complex by recognizing the amino terminal region of Notch membrane-bound form (Lobry *et al*., 2014).

After S3, ICN is release and translocates into the nucleus, where it binds to the helix-loop-helix transcription factor CSL/RBP-Jk through its RAM and Ankyrin repeat domains. RBP-Jk binds DNA in a sequence-specific manner acting as a transcriptional repressor, creating a complex with corepressors like SMRT/NcoR, SHARP (or MINT), CtBP1 and SIRT1, which recruit histone deacetylases and the histone demethylase LDS1. ICN displaces corepressors bound to RBP-Jk and allows the recruitment of coactivators such MAML1 and histone acetyltransferase p300 to generate a short-lived complex leading to transcriptional activation of target genes (Figure 4B) (Mallaird *et al*., 2005; Lobry *et al*., 2014).

It have been identified a large number of genes target by Notch, many could be cell type-dependent but a few are well-characterized including Hairy/Enhancer of Split (Hes) of transcriptional repressors, the Notch-related Ankyrin repeat protein (Nrarp), cyclin D, p21, NF-κB, c-myc, Deltex and many others (Cheng *et al*., 2013; Lobry *et al*., 2014).

Notch signaling is regulated by ubiquitination of ICN through its C-terminal PEST domain. So far, only E3 ligase Fbw7, an F-box protein serves as a substrate-recognizing component of the Skp1/Cul1/F-box ubiquitination complex, has been demonstrated to target Notch for degradation by the proteasome. Fbw7 binds to a degron sequence –COOH-terminal end of the Nocth1 PEST domain leading to the ubiquitination of ICN and degradation. For the recognition of Fbw7 to this sequence on the target protein must be first phosphosrylated at a core threonine residue. CDK8, ILK and GSK3 kinases were shown to phosphorylate ICN and trigger Fbw7-dependent degradation (Lobry *et al*., 2014).

1.5.3. The oncogenic role of Notch in the lymphoid compartment.

Notch signaling plays an important role in lymphocyte development and oncogenic transformation. Notch signaling has emerged as a specific therapeutic target for T cell lymphoblastic leukemia and colon cancer (Kopan and Ilagan, 2009). In the hematopoietic system, Notch1 is involve in multiple stages of T-cell development because its function ablation in hematopoietic progenitors results in a failure in the specification of the T-cell lineage at the expense of B-cell development. Notch signaling is continuously required at different stages of T-cell development and leukemogenesis, regulating the progression through the early DN1, DN2 and DN3 stages of thymocyte maturation, the rearrangement of the TCRβ gene and the lineage decisions between the αβ and γδ lineages, and in some systems, between CD4 and CD8 lineages (Bellavia *et al*., 2002; Campese *et al*., 2003; Ferrando, 2009).

Deregulated Notch signaling during T-cell development results in malignant transformation, leading to the development of T-ALL, which represents approximately 15% and 25% of ALLs seen in children and adults, respectively (Aster, 2005; Grabher *et al*., 2006). The oncogenic potential of Notch1 in T-cell leukemia was identified by the observation that a rare translocation t(7;9)(q34;q34.4) in T-ALL patients resulted in overexpression of the active form of Notch1, due to the fusion of a truncated Notch1 gene (missing the EGF-like, LNR and HD domains) into the TCRβ locus. The oncogenic activity of this constitutively active form was demonstrated by the rapid development of T-ALL in mice transplanted with hematopoietic progenitors infected with a retrovirus that drove the expression of ICN1 (Ferrando, 2009).

Moreover, Notch1 has been found mutated in about 56% of T-ALL cases (Weng et al., 2004; Mansour *et al*., 2006). Two major "hotspots" of mutations were characterized: mutations in the HD that generate ligand-independent activation and approximately found in 40% of human T-ALL cases, and in the PEST carboxyl-terminal domain that induce stability of the ICN1 (Van Vlierberghe and Ferrando, 2012). In addition, inactivating mutations or deletions in Fbw7 were identified in about 15% of T-ALL cases, and in these cases Fbw7 mutant cannot bind to the ICN and lead it to degradation, and also contributes to GSI resistance (O'Neil *et al*., 2007; Park *et al*., 2009; Wang *et al*., 2011). Furthermore, T-cell leukemia can be modeled in mice by enforced expression of ICN1 or ICN3 in hematopoietic progenitors (Pear *et al*., 1996; Bellavia *et al*., 2000; Lobry *et al*., 2014). In the case of Notch3, important in T-cell differentiation, it has been associated with NF-κB activation and T-cell

tumorogenesis in mice (Bellavia *et al*., 2000), and miR-150 was reported to targeted Notch3, suggesting an important role in leukemogenesis (Ghisi *et al*., 2011).

Mutated Notch1 has also been identified in CLL. These mutations impair Fbw7-dependent Notch degradation and an overall frequency of 8-12%, mainly in patients with more aggressive CLL. Another role of Notch signaling in the lymphoid compartment is in marginal zone B-cell differentiation. Deletion of Notch2 or RBP-Jk showed lack of marginal zone B cells, and in lymphoma patients have been described Notch2 mutations in splenic marginal zone with a frequency of 21-25%. Most of these mutations affected the PEST domain (Lobry *et al*., 2014).

1.5.4. The tumor suppressor role of Notch in the granulocyte/monocyte compartment.

Available data suggest an important role of Notch signaling in myeloid cell differentiation. However, the Notch effects in granulocyte/monocyte development or whether Notch is oncogenic or tumor-suppressive in AML are still controversial (Tohda, 2014). In vitro and in vivo evidence suggest that Notch is relevant in maintenance of progenitor cells and suppresses myeloid terminal differentiation, whereas other studies showed that Notch activation is required for granulocytic differentiation (Cheng *et al*., 2013; Lobry *et al*., 2014). Despite the expression of Notch1 receptor and ligands in primary AML patient samples, cell-autonomous activation is not observed in the majority of AML patients, also its target Hes1 are in low level (Chiaramonte *et al*., 2005; Kannan *et al*., 2013; Tohda, 2014), and reactivation of Notch pathway, both in vivo and in vitro, induced rapid cell cycle arrest, aberrant differentiation and apoptosis of AML cells. Moreover, as Notch has been suggested to play a role of tumor suppressor in several solid tumors, Notch receptor-specific activation could therefore constitute a therapeutic target (Lobry *et al*., 2013).

Aifantis group showed that Notch loss of function through deletion of NCSTN or compound deletion of Notch1 or Notch2 resulted in myeloproliferative syndrome with common features of chronic myelomonocytic leukemia (CMML), that in a high rate progress to AML. In this study, whole-transcriptome analysis showed that Notch signaling inhibited monocytic/granulocytic differentiation in an early multipotent progenitor mediated by Hes1 direct repression of PU.1 and C/EBPα, essential factors of myeloid development. Enforced expression of Hes1 in hematopoietic stem and progenitor cells induced differentiation toward an erythrocytic/megakaryocytic fate at expenses of granulocytic/monocytic fate. These results

suggested that Notch signaling might prevent uncontrolled proliferation and transformation of myeloid cells during the hematopoietic development acting as a tumor suppressor in myeloproliferative disorder. Nevertheless, few studies have implicated Notch signaling in AML (Lobry *et al*., 2014). In AML have been reported activating mutations of Notch1 but in few primary samples and cell lines. These cell lines were derived from AML relapse patient initially diagnosed with T-ALL. In this case, was demonstrated that this type of mutations are mostly restricted to T-ALL and in rare AML cases (Palomero *et al*., 2006).

AIM OF THE WORK

MiR-125b is upregulated in many neoplastic blood disorders, including acute myeloid leukemia (AML) (Bousquet *et al*., 2010; Shaham *et al*., 2012; Sun et al., 2013). However, the underlying mechanism of miR-125b dysregulation remains to be explored. Relevant to myeloid leukemia, C/EBPα is frequently mutated in AML, but surprisingly, none of the observed mutations result in full ablation of the gene indicating that activity of C/EBPα is required for AMLs (Nerlov, 2004; Ohlsson *et al*., 2013). Number of works revealed that miRNAs are regulated by C/EBPα in the course of normal myeloid differentiation (Paz-Priel and Friedman, 2011) and in myeloid leukemia (Eyholzer *et al*., 2010; Katkerze *et al*., 2013). MiR-125b targets Notch1 in the skin disorder context (Manca *et al*., 2011). Notably, while the involvement of Notch signaling as an oncogene in T-ALL is well characterized, Notch signaling has been described as a possible tumor suppressor in myeloid malignancies. In this regard, while T-ALL cells express Notch1 receptor, its activation is silenced in AML (Lobry *et al*., 2014 and references therein). Therefore, based on the importance of C/EBPα and the dysregulation of miR-125b expression in AML, in this work we investigated the mechanism of miR-125b regulation mediated by C/EBPα. Given the evidence that Nocth1 may be targeted by miR-125b, we studied whether miR-125b overexpression might account for the differential Notch1 expression between T-ALL and AML.

MATERIALS AND METHODS.

3.1. Human cell samples.

AML patient samples were obtained from Division of Hematology, Department of Cellular Biotechnologies and Hematology, Sapienza University of Rome (Rome, Italy), and their characteristics are summarized in table 2. T-ALL patient samples were previously described (Kumar *et al*., 2014). All patients provided written informed consent in accordance with the Declaration of Helsinki. Hematopoietic $CD34^+$ cells were isolated from healthy donor using a CD34 selection kit (Miltenyi Biotec. Caldero di Reno, BO, Italy) as previously described (Zardo *et al*., 2012). The percentage of cells positive for the CD34 antigen was analyzed by fluorescence activated cell sorting (FACS) analysis using PE-conjugated mouse anti-human CD34 antibody (BD Bioscience, Milano, Italy) and was found to be around 95%.

Table 2. Patient characteristics.

Patient sample	Karyotype
1	46, XX
2	47, XX, +8
3	46, XY
4	47, XX, +11
5	46, XY
6	46, XY
7	46, XY
8	46, XY
9	46, XY
10	46, XY

3.2. Cell cultures and treatments.

Jurkat, DND41, HL60, NB-4 ME-1 and AML193 were purchased from DSMZ (DMSZ Braunschweig, Germany) cultured as indicated in the DMSZ catalogue. Molt3 cell line was kindly provided by Prof. Indraccolo S. All cell lines, except AML-193, were cultured in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1mmol/L L-glutamine and 1% penicillin-streptomycin (Gibco) at 37ºC in 5% CO_2. AML-193 cell line was cultured in Iscove's Modified Dulbecco's Medium (IMDM, Euroclone, Milan, Italy) complemented with 10% FBS, 1mmol/L L-glutamine, 1% penicillin-streptomycin,

insulin 5 mg/L and transferrin 5mg/L and incubated as described previously. HEK293T cells for lentivirus production were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco) complemented with 10% FBS, 1mmol/L L-glutamine and 1% penicillin-streptomycin. Jurkat/miR-125b (PRMIH125bPA/AA-1), HL60-Antago-miR125b (MZIP125bPA/AA-1) (System Biosciences, CA. USA) cells were maintained in RPMI 1640, supplemented as mentioned before. Retionic Acid (RA, Sigma-Aldrich, Italy) was used at a concentration of 1 μM in $0{,}25x10^6$ HL60 or NB4 cells per 1 mL cultures for 48 hours.

3.3. RNA isolation.

Total RNA from cells was extracted using TRIzol Reagent (Invitrogen Monza, Italy) according to manufacturer's instructions. Briefly, in each sample was added TRIzol reagent and incubated at room temperature for 5 min, then 200 μL of chloroform per 1 mL of TRizol were incorporated mixing vigorously 15 s by hand and incubated for 2-3 min. at room temperature. Samples were centrifuged at 12,000 x g for 15 min at 4ºC. The upper aqueous phase was transferred carefully into a new tube and equal volume of Isopropyl alcohol was added mixing well and incubating for 10 min at room temperature. Samples were centrifuged at 12,000 x g for 10 min at 4ºC. The supernatant was removed and the pellet was washed with 75% ethanol per 1 mL of TRIzol used by centrifuging at 7500 x g for 5 min at 4ºC. The wash was discarded and the samples were air dried for 5-10 min before re-suspended in RNase free water.

3.4. MiRNA and mRNA detection by quantitative Real Time PCR (q-PCR).

Reverse transcription (RT) for human for U6snRNA and miR-125b were carried out with TaqMan MicroRNA Assay kit (Applied Biosystems, Foster City, CA, U.S.A.) using 20 ng of total RNA sample and the specific stem-loop primer according to manufacturer's protocols. High capacity cDNA Reverse transcription kit (Applied Biosystems, Foster City, CA, U.S.A.) was used for reverse transcription of 1 μg of total DNA for pri-miR-125b-1 and 2, human Notch1, Hes1, Deltex1 and C/EBPα expression analysis and using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression for normalization. Quantitative Real Time PCR (q-PCR) analysis was performed on a StepOne Real Time System (Applied Biosystems, Foster City, CA, U.S.A.) machine using Taqman gene expression assays. Primer sequences (Applied Biosystems) are provided in Table 3. PCR reactions were run using manufacturer's

recommended cycling conditions: 50ºC for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. hGAPDH and U6snRNA were used as endogenous controls to normalize sample data. Each sample was run in triplicate and 2-ΔΔCT method was used to calculate the relative mRNA or miRNA abundance in the different samples compared to the mean of all control samples represented as unitary value.

Table 3. Taqman Gene expression, miRNA and Pri-miRNA Assays.

Taqman expression Assay	Catalog
hGAPDH	Cat. 4351370 assay Hs99999905_m1
U6snRNA	Cat. 4427975 assay 001973
hsa-miR125b	Cat. 4440887 assay 000449
hsa-miR125b-1	Cat.4427012 assay Hs03303095_pri
hsa-miR125b-2	Cat.4427012 assay Hs03303224_pri
hNotch1	Cat. 4331182 Hs01062014_m1
hHes1	Cat. 4331182 Hs00172878_m1
hDeltex1	Cat. 4331182 Hs01092201_m1
hC/EBPα	Cat. 4331182 Hs00269972_s1

3.5. Immunoblot analyses.

Whole-cell lysates were prepared in RIPA buffer. Protein concentration was determined by Bradford (Biorad, Hercules, CA, U.S.A.) and 40 μg of protein were boiled for 5 min in Laemmli sample buffer (Biorad) and loaded on 8% or 12% polyacrylamide gel (SDS-PAGE). After electrophoretic separation, proteins were electrotransferred into an Immun-Blot-PVDF membrane (Biorad). The membrane was then blocked at room temperature with blocking agent 5% in phosphate-buffered saline (PBS)/Tween 0,05%. The primary antibodies for Notch1 (C-20) and Tubulin, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Notch1 Val1744 and Anti-C/EBPα antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Blots were incubated for 2 h at room temperature or with an anti-Notch1 Val1744 and C/EBPα overnight at 4°C according Cell Signaling Manufacture's instruction for detection of phosphor-ERK. Then were revealed with a secondary antibodies HRP conjugated (Santa Cruz) and ECL Advance system 0(Cyanagen).

3.6. Lentiviral infection.

HEK293T cells were cotransfected using LIPOFECTAMINE 2000 protocol (Invitrogen Monza, Italy) with either an empty vector or miR-125b/miRZIP125b, along with the packaging plasmids pVSVG, REV, and GAG. Virus-containing supernatants were collected at 24 and 48 hours after transfection, filtered through a 0.45-mm filter and centrifuged at 35 000 rpm for 2 hours at 4°C in a Sorvall WX ultracentrifuge using a TH-641 rotor. Pellets were resuspended in phosphate-buffered saline and stored at -80°C. Lentiviral transduction of indicated cells was performed by spininfection. Each cell pellet were resuspended in 2 mL of media by gently pippeting up and down, and then transferred into their own 6-well plate tissue culture plate. The negative selection of the transduced cells was done by puromycin treatment.

3.7. Luciferase reporter assay.

Transfection of wild type C/EBPα-expressing construct (C/EBPa-pcDNA3), gently provided by Prof. Gianluca Canettieri, Sapienza University of Rome) in HEK293T cells was performed with the Lipofectamine 2000 protocol (Invitrogen Monza Italy) following the manufacturer's instructions. Cells were transiently transfected with 0,25 μg of miR-125b-luciferase responsive promoter construct (Manca *et al*., 2011), 0,001 μg of Renilla construct, plus the increase amounts of pcDNA3.1-C/EBPα (0,15, 0,3 and 0,5 μg) or control plasmid. Firefly luciferase activities from the promoter constructs and Renilla luciferase activity from the internal control plasmid were determined 24 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega, Milan, Italy). Values were normalized by using the Renilla luciferase.

3.8. MiRNA transfection and siRNA interference.

To analyze the effect of miR-125b overexpression on Notch1 expression, DND41 were transfected with either 200nM miR-Control or miR-125b (Applied Biosystem, Monza Italy) using the NEON-transfection system (Applied Biosystems, Foster City, CA, U.S.A.) following the manufacturer's protocol. Proteins were extracted after 48 hours of transfection as described in Immunoblot analysis. HL60 were transfected with 200 nM small interfering RNAs (siRNAs) for validated C/EBPα (SC-37047; Santa Cruz Biotechnology; Santa Cruz, CA, USA) and corresponding control scrambled siRNAs using Neon Transfection System

following the manufacturer's recommendations. Cells were analyzed 48 h after transfection by q-PCR using SYBR green fluorescence C/EBPα Fw1 5'-AACACGAAGCACGATCAGTCC-3' and Rev1 5'-CTCATTTTGGCAAGTATCCGA-3' normalizing respect to hGAPDH Fw 5'-TGCACCACCAACTGCTTAG-3' and hGAPDH Rev 5'-GAGGCAGGGATGATGTTC-3'(Pan *et al*., 2014).

3.9. Analysis of CEBPA coding region.

Genomic DNA was isolated form mononuclear cell preparation using the DNA TRIzol reagent (Life Technologies, Monza Italy). The entire CEBPA coding region was amplified using the following primers: F1- 5'-AGAACTCTAACTCCCCCATG-3' and R-1 5'-AGCTCAGCCCCAAGAATTCTC-3' (ENST00000498907- reference sequence). The total reaction volume of 50μL containing 100ng of DNA was amplified using Q5-High-Fidelity DNA Polymerase (New England-BioLabs EuroClones Milano Italy) following the manufacturer's instructions. PCR products were cloned into pCR-BLUNT II-Topo vector (Invitrogen Monza Italy). Plasmid was prepared using the plasmid Mini Kit (Qiagen Milano Italy). 3 clones for each samples were sequenced with the following primers: M13 Forward; M13 Revers (Contained in the pCR-BLUNTII-TOPO plasmid), F2-5'-CCGGCTACCTGGACGGCAGG-3'; R2-5'-CCCCGACGCGCTCGTACAGG-3'.

3.10. Chromatin Immunoprecipitation.

The chromatin immunoprecipitation assay was performed cross-linking to DNA in living nuclei by adding formaldehyde (252549, Sigma Aldrich, St Louis, MO, USA) of HL60 cells and AML primary samples at a final concentration of 1%. Cross-linking was proceed by 10 min at 37ºC and then was stopped by adding glycine to a final concentration of 0,125 M. Cross-linked cells were wash with PBS and the nuclei were extracted with a buffer containing Tris-HCl pH 8,0, 0,25% TritonX-100, Na-EDTA 10 mM, Na-EGTA 0,5mM and protease inhibitors; then pelleted by microcentrifugation and lysed by incubation in SDS lysis buffer (Tris-HCl pH 8,0 50mM, Sodium-Dodecil-Sulfate 0,5%, EDTA 5mM and protease inhibitors). Then resulting chromatin solutions were sonicated for 15 cycles of 15 s to generate 400-600 bp DNA fragments. After microcentrifugation, the supernatants were diluted 1:5 with dilution buffer (Tris pH 8,0 20 mM, NaCl 150 mM, TritonX-100 1%, SDS 0,01% and EDTA 1 mM). After preclearing with Salmon Sperm DNA/Protein A agarose (16-

157, Merck KGaA, Darmstadt, Germany), 5 μg of anti-C/EBPα (Cell Signaling) or normal rabbit IgG (Santa Cruz Biotechnology Inc.) were incubated on a rotating platform overnight at 4ºC. The complex antibody-protein-DNA were isolated with Salmon Sperm DNA/Protein A agarose an extensively washed (with High salt buffer, Low salt Buffer, LiCl buffer and TE 1X solution), then the bound DNA fragments were eluted and analyzed by q-PCR. q-PCR was carried out with an intercalating fluorescent dye (SYBR Green I Sensi Fast SYBR HI-ROX kit Bioline) in STEP ONE plus Thermocycler. Primer sequences designed for the consensus binding site of C/EBPα on miR-125b promoter region were Fw 5'-TGGGCATTTCTGAGTCTGTG-3' and Rev 5'-TATCTGGGGGCGCATATACA-3' and using as Negative control for ChIP analysis: Qiagen –Epitech ChIP qPCR Assay Human IGX1A (cat. 334001).

3.11. Statistical analysis.

Each experiment was repeated three times independently. All results were expressed as means SD, and *P* value is indicated. One-way ANOVA analysis for independent samples was used to determine statistical significance.

RESULTS

4.1. Comparison of miR-125b expression between AML and T-ALL.

Our group, previously showed that the manifestation of Hailey-Hailey disease, a rare skin disorder, was in part dependent on Notch1 downregulation mediated by miR-125b upregulation (Cialfi *et al*., 2010; Manca *et al*., 2011). Notably, while the involvement of Notch signaling as an oncogene in T-ALL is well characterized, Notch signaling has been described as a tumor suppressor in myeloid malignancies. It has been previously shown that miR-125b is overexpressed in AML (Bousquet *et al*., 2010, Shaham *et al*., 2012; Sun *et al*., 2013); thus, we investigated whether miR-125b overexpression might account for the differential Notch1 expression between T-ALL and AML.

As first, the expression of the mature miR-125b and the pri-miR-125b-1 and 2 were evaluated in the cell lines and primary samples on both types of leukemias. In the leukemia derived cell lines, we compared T-ALL cell lines (Jurkat, Molt3 and DND41) respect to AML cell lines (HL60, ME-1 and AML-193). The levels of this miRNA were determined by quantitative reverse transcriptase polymerase chain reaction (q-PCR) in bone marrow cells derived from AML patient samples (n=10 and healthy donors $CD34^-$ cells) and in PBMC cells derived from T-ALL patient samples (n=7 and healthy donors $CD4^+$ cells). Both primary AML samples (about 70% of the cases) and AML derived cell lines demonstrated to have higher expression of miR-125b compared to the control (Figure 5A and B), conversely, both primary and T-ALL cell lines showed lower levels of this miRNA (Figure 5A and C).

In order to investigate if the deregulation of miR-125b expression occurs either at the transcriptional or processing level, primary miR-125b expression levels were analyzed in both Jurkat and HL60 cell lines as well as in primary AML samples (Figure 5D and E). Specifically, q-PCR was performed to compare the levels of pri- miRNA. The pri-miRNAs levels of the miR-125b were found to follow the same trend of mature miR-125b expression in both cell lines examined (Figure 5A and D). In most primary AMLs, we found that miR-125b expression was transcriptionally upregulated (Figure 5E). Nevertheless, we observed that in some samples pri-miRNAs basal transcription efficiency was associated with a low abundance of the mature miRNA (Figure 5A and E, AMLs 8, 9 and 10).

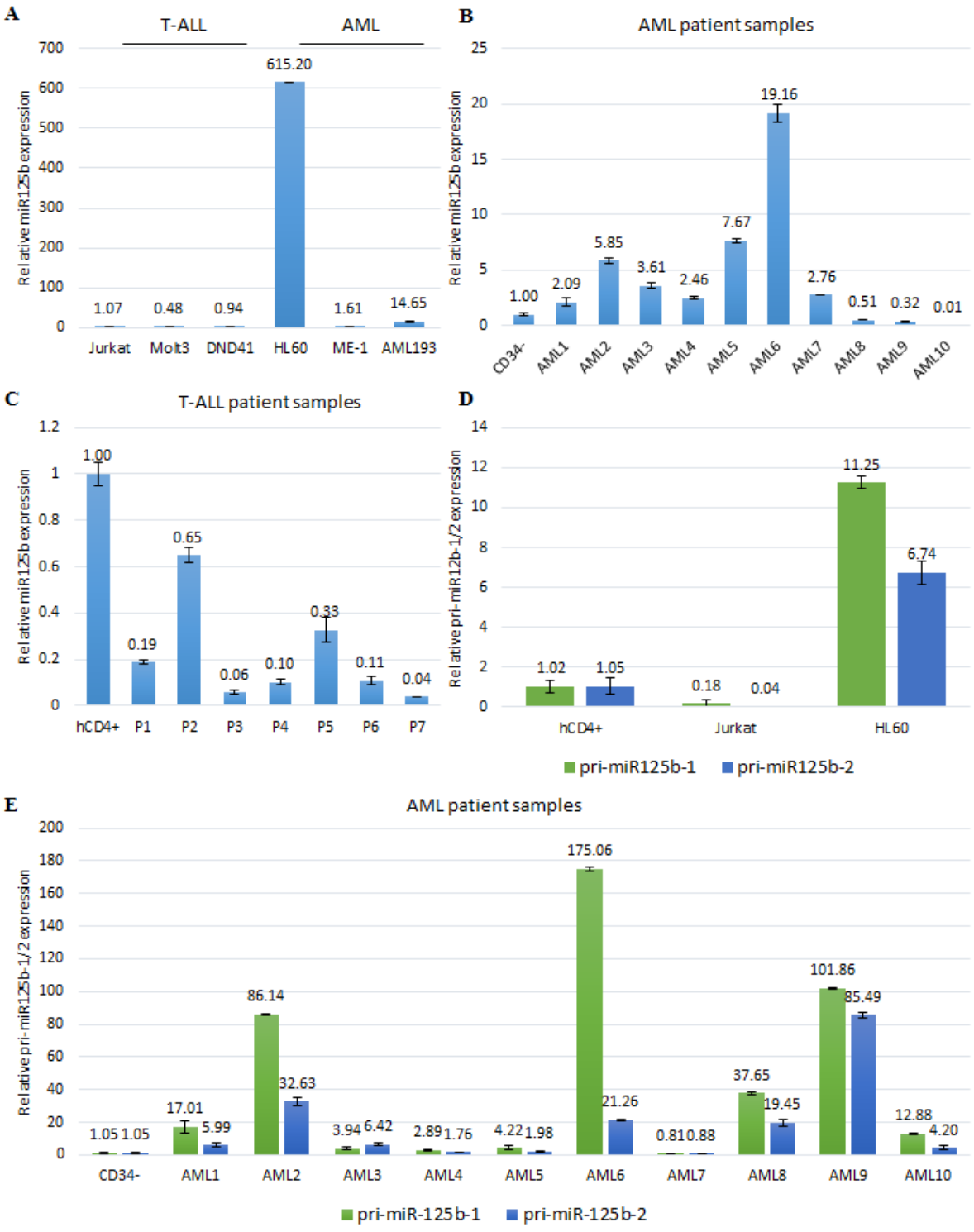

Figure 5. MiR-125b is upregulated in AML and downregulated in T-ALL. (A) MiR-125b expression was determined using AML derived cell lines, values are expressed as fold-increase over Jurkat cells. (B) MiR-125b expression was carried out using bone marrow cells derived from AML patient samples and (C) from T-ALL patient samples. All q-PCR values were normalized to U6, and in the case of patient samples, further to the expression level of 3 healthy donors. (D) pri-miR125b-1 and 2 transcripts were determined in the indicated cell lines and (E) AML patient samples. These q-PCR values were normalized to GAPDH, and further to the expression level of 3 healthy donors. All results were expressed as means ±SD.

4.2. Notch signaling pathway between AML and T-ALL.

We found an inverse correlation of miR-125b expression and Notch1 levels in both T-ALL and AML cell lines as well as in primary AML samples (Figure 5A-B and Figure 6A-B). Furthermore, the expression of miR-125b, Hes1 and Deltex1 were compared between bone marrow cells derived from AML patient samples (n=9) and PBMC cells derived from T-ALL patient samples (n=7). We observed higher level of miR-125b expression in AML when we compared with T-ALL samples (Figure 6C). Importantly, the Notch1 target genes, Hes1 and Deltex1, were significantly higher in T-ALL when compared with AML (Figure 6C). Interstingly, this is correlated with the level of activated Notch1 signaling as we observed a higher level of Notch1-Val1744 in T-ALL, when compared to AML (Figure 6A). MiR-125b was correlated with low level expression of Notch1 target genes, supporting the finding that Notch1 signaling is downregulated in AML (Figure 6C).

4.3. MiR-125b targets Notch1.

Recently, we found that Notch1 is a target of miR-125b (Manca *et al*., 2011); therefore, we analyzed the potential involvement of miR-125b in regulating the differential expression of Notch1 between T-ALL and AML cells. We analyzed Notch1 protein expression after overexpression of either miR-125b or AntagomiR-125b in T-ALL and AML cell lines, respectively.

DND41 cells, but not Jurkat and HL60 cells, are highly transfectable. To overcome this limitations DND41 cells were analyzed by transient transfection and both Jurkat and HL60 cells were transduced by lentiviral infection. We found that deregulated miR-125b expression controlled Notch1 levels in DND41 (Figure 7A) and, although with a lower effect, also in Jurkat and HL60 cell lines (Figure 7B). ME-1 cells devoid of miR-125b expression have undetectable level of Notch1 expression (Figure 5A and 6A). Additionally, in the T-ALL derived cell line, Molt3, miR-125b enforced expression did not affect Notch1 expression (data not shown).

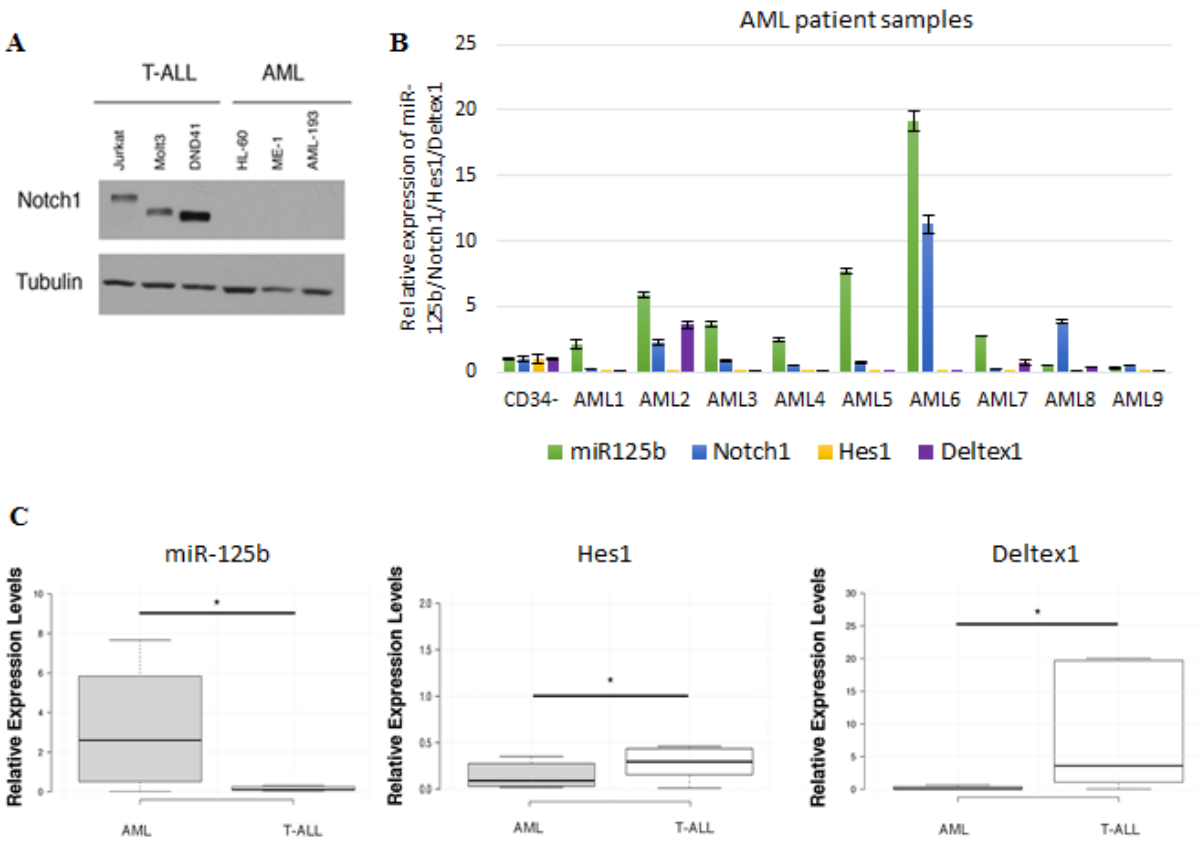

Figure 6. Notch1 signaling pathway T-ALL vs. AML. (A) Western blot analysis of Notch1 Val1744 expression in whole cell extract in the indicated cell lines. Tubulin is shown as a loading control. (B) MiR-125b, Notch1, Hes1 and Deltex1 expression were determined by q-PCR using bone marrow cells derived from AML patients (n=9 and healthy donors $CD34^-$ cells). Values are expressed as fold-increase over $CD34^-$ cells. (C) Box plot of CT values from data in AML and T-ALL patient samples. Student's *t* tests reveal significant differences between AML (n=9) and T-ALL (n=7) samples in miR-125b and Notch target genes (Hes1 and Deltex1). All q-PCR values were normalized to U6 and GAPDH; bars indicate $p< 0.05$.

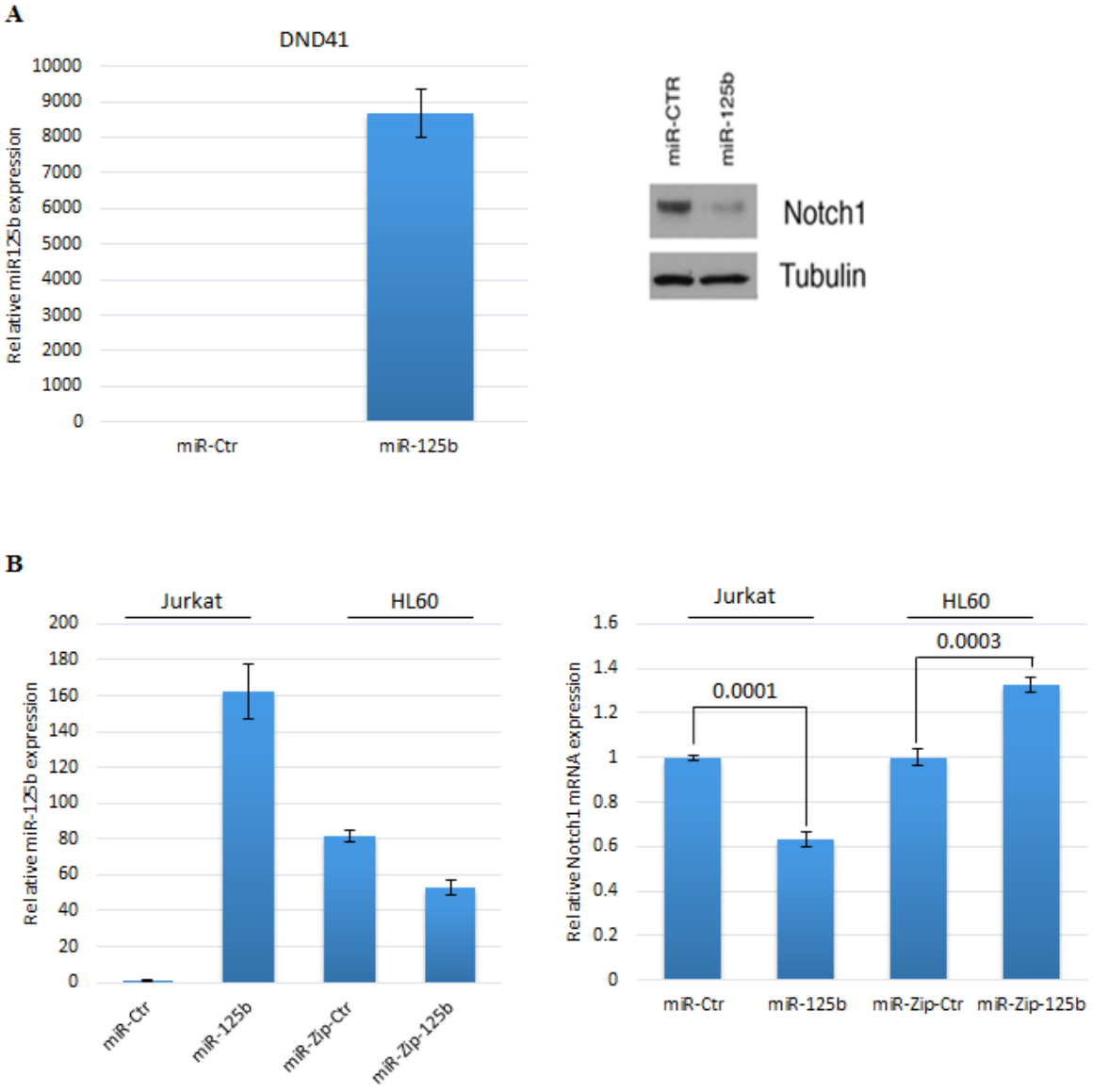

Figure 7. Notch1 is targeted by miR-125b. (A) DND41 cell line was transfected with either miR-Ctr or mature miR-125b, and miRNA expression was analyzed by q-PCR (left) and western blot was perfomed with the indicated antibodies. Tubulin is shown as a loading control (right). (B) Right panel, T-ALL (Jurkat) and AML (HL60) derived cell lines were infected with either miR-Ctr/miR-125b or miRZip-Ctr/miRZip-125b, respectively, and Notch1 mRNA expression were analyzed by q-PCR. Left panel, miR-125b expression was evaluated in the samples shown in the right panel by q-PCR. All results were expressed as means ±SD, and *P*-values are indicated. All q-PCR values were normalized to U6 and GAPDH.

4.4. MiR-125b is a direct target of C/EBPα.

To explore the mechanism regulating miR-125b expression, we first characterized the miR-125b promoter region using the Genomatix MatInspector software package, focusing on those transcription factors that have been shown to play a role in either T-ALL or AML. A scan of 2 kb of genomic sequence located upstream of the predicted pre-miR-125b start site identified putative Hes1, GATA3 and one C/EBPα consensus binding sites (Figure 8) suggesting a possible involvement of those factors in the regulation of miR-125b expression. Thus, in protein extracts from AML and T-ALL derived cell lines were first analyzed the expression of C/EBPα by western blot analysis, and in Figure 9A was observed a differential expression comparing T-ALL and AML derived cell lines. Interestingly, C/EBPα expression was associated with higher levels of miR-125b (Figure 5A and 9A).

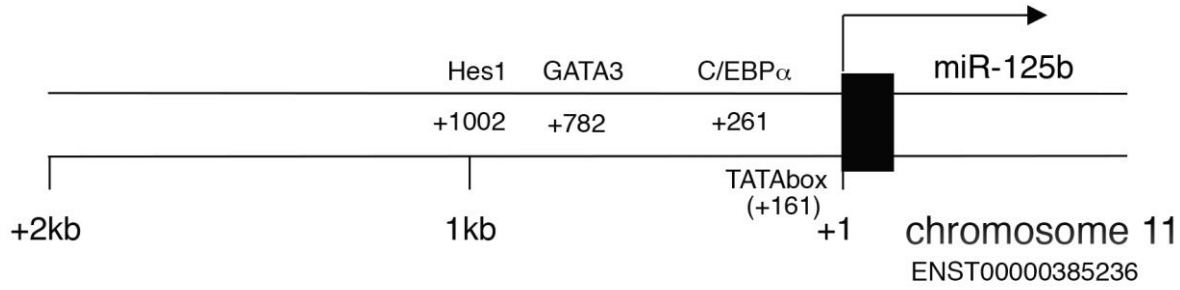

Figure 8. MiR-125b promoter region. Schematic representation of examined putative C/EBPα binding sites in the promoter regions of miR-125b.

We next examined the role of C/EBPα in the regulation of miR-125b expression by testing a miR-125b-luciferase responsive promoter construct (Manca *et al*., 2011) with increased amount of C/EBPα in a luciferase reporter assay. As shown in the Figure 9C, we found induction of miR-125b promoter activity by C/EBPα transfection in a dose-dependent fashion, indicating that C/EBPα might be a transcriptional regulator of miR-125b expression. Additionally, siRNA against C/EBPα in HL60 abrogated the basal level of miR-125b expression (Figure 9B). Next, we investigated whether C/EBPα directly regulates miR-125b promoter. For this purpose, we performed chromatin immunoprecipitation experiments in both HL60 cells and primary AML samples. The chromatin fragments were immunoprecipitated with an anti-C/EBPα antibody. The DNA fragments were analyzed with

specific primers for the indicated regions of the miR-125b regulatory region (Figure 8). In HL60, we were able to observe an enrichment of DNA from the predicted C/EBPα binding sites when compared with the immunoglobulinG (IgG) control (Figure 9D). Additionally, we observed an increased recruitment of C/EBPα onto the miR-125b promoter in AML primary samples highly expressing miR-125b primary transcript (Figure 9E).

4.5. C/EBPα and miR-125b during differentiation in AML.

The myeloid cell lines provide an important in vitro model system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells of the granulocyte/monocyte/macrophage lineage. Both HL60 and NB4 pro-myelocytic leukemia cell lines have the potential to differentiate toward granulocytic lineage by exposure to retinoic acid (RA). Thus, to explore further the role of C/EBPα in the induction of miR-125b expression, we compared C/EBPα and miR-125b expression after RA treatment. Induce RA differentiation in HL60 and NB4 strongly decreased both protein and mRNA expression of C/EBPα (Figure 10A, E and F), simultaneaously with induction of granulocytic differentiation determined in HL60 by morphology changes with Wright-Giemsa stain (Figure 10B) and gain of CD11b expression (Figure 10C). Notably, in both cell lines HL60 and NB4, the downregulation of C/EBPα expression by RA parallels the one of miR-125b (Figure 10E and F). Interestingly a similar trend was observed in $CD34^{+}$ and $CD34^{-}$ primary cells (Figure 10D).

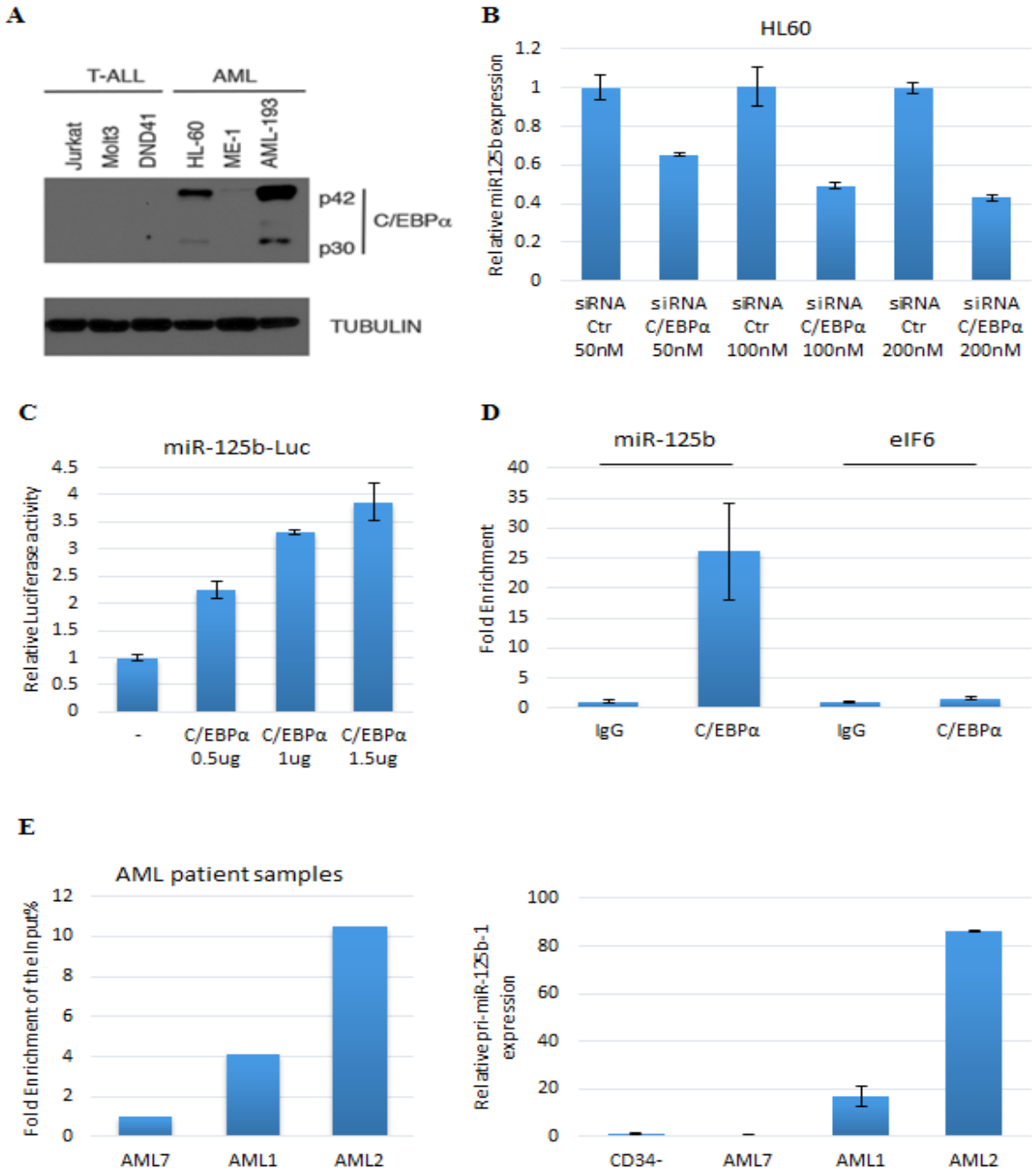

Figure 9. MiR-125b is a direct target of C/EBPα. (A) C/EBPα protein expression by western blotting in the indicated cell lines. Tubulin is shown as a loading control. (B) MiR-125b expression of C/EBPα-silenced HL-60 cells, assessed by q-PCR. (C) HEK293T cells were transfected with miR-125b-luciferase responsive promoter construct, 0.25 μg/well in 24-well dishes, and treated with the indicated amount of C/EBPα; cells were harvested 24h after transfection for luciferase assay. All conditions were tested in triplicate samples, and SD is indicated. (D) Chromatin derived from HL60-C/EBPα positive cells and (E) primary AML samples (left panel) were immunoprecipitated with anti-C/EBPα or IgG antibodies. Recovered DNA was PCR amplified with primers specific for C/EBPα-binding amplificon. Immunoprecipitation was performed 3 times using different chromatin samples, and the occupancy was calculated by using the ChIP-qPCR Human IGX1A Negative Control Assay (Qiagen). As additional control, DNA was amplified with primers specific for Hes1-binding amplificon in the eIF6 promoter, lacking of C/EBPα-binding site. (Right panel) Pri-miR-125b-1 expression of the primary AML samples determined by q-PCR.

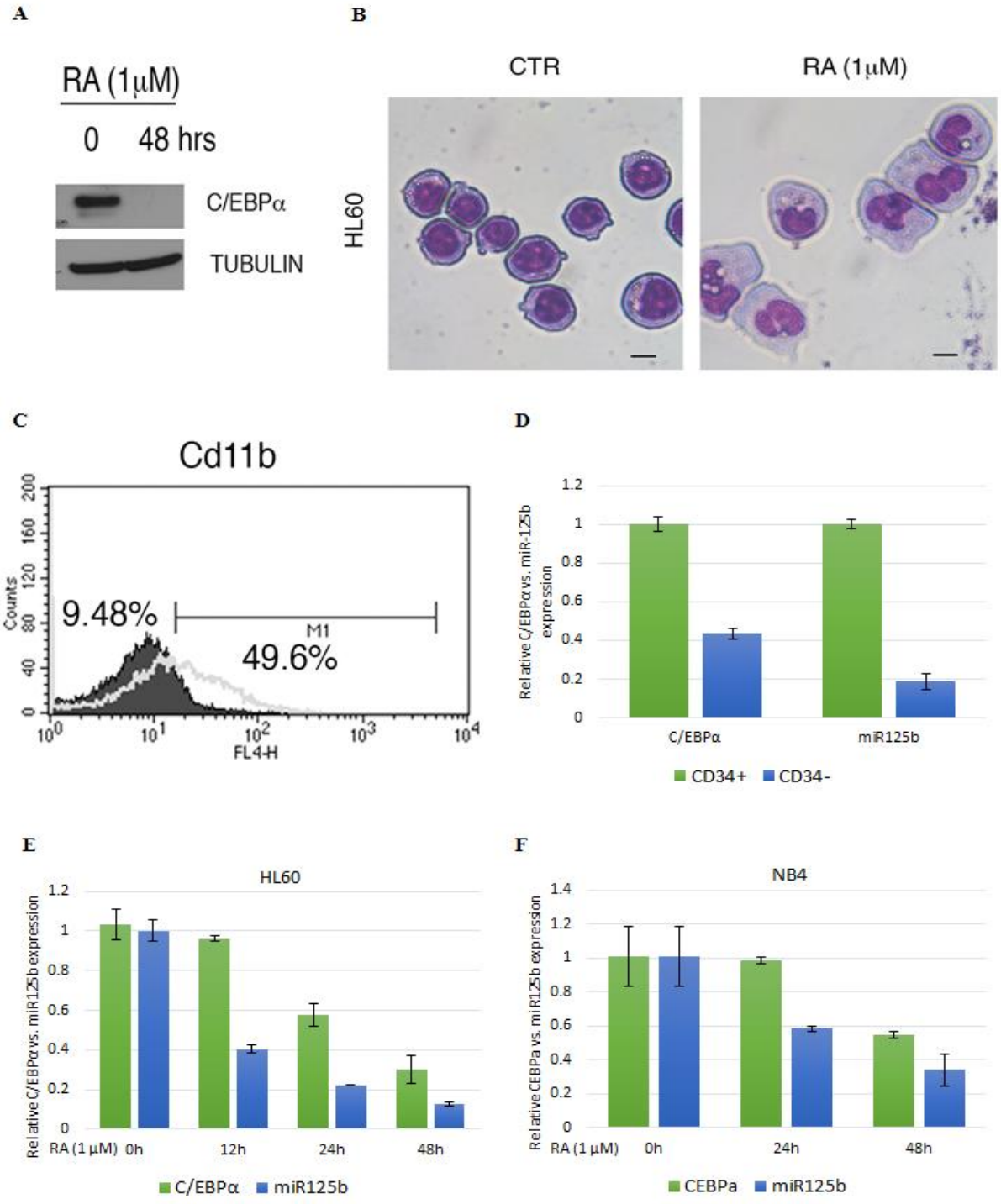

Figure 10. C/EBPα and miR-125b during induced differentiation in AML. (A) C/EBPα expression was analyzed by western blot at 0 and 48 hours after RA treatment of HL60 cells. (B) Changes in morphology by light-field microscopy of Wright-Giemsa stained cells treated as in panel A (Bar 5μM). (C) HL60 RA induce-differentiation was evaluated by CD11b flow cytometry analysis of cells treated as in panel A. (D) MiR-125b and C/EBPα mRNA expression in $CD34^{+}$ vs. $CD34^{-}$ human primary cells by q-PCR. (E) MiR-125b and C/EBPa mRNA expression was analyzed at 0, 12, 24, and 48 hours after RA treatment of HL-60 cells and (F) 0, 24 and 48 hours of RA treatment in NB4, assessed by q-PCR. All q-PCR values were normalized to U6 and GAPDH.

4.6. Characterization of CEBPA gene in AML.

The status of C/EBPα (reference sequence No. NM_004364.3) was evaluated in the primary AML samples (Figure 11A). From six samples characterized, three AML samples showed alterations in CEBPA gene. These mutations have been previously described, and in the cases of AML 6 and AML 9, these alterations were located in the C-terminal region of CEBPA. The first alteration was characterized as a nonsense mutation predicting a truncated protein (Figure 11B AML 6), the second one was described as a synonymous variant (Figure 11B AML 8) and the third one was characterized as an inframe insertion (Figure 11B AML 9). Interestingly, we found that primary samples carrying mutations of this protein had higher levels of miR-125b at the transcriptional level (Figure 5E and 11B).

A

Patient No.	Karyotype	C/EBPα status
1	46, XX	WT
2	47, XX, +8	ND
3	46, XY	WT
4	47, XX, +11	WT
5	46, XY	ND
6	46, XY	c.1000 G>T*
7	46, XY	ND
8	46, XY	c.690 G>T**
9	46, XY	c.928_929 InsCGG***
10	46, XY	ND

Reference sequence for annotation: NM_004364.3

B

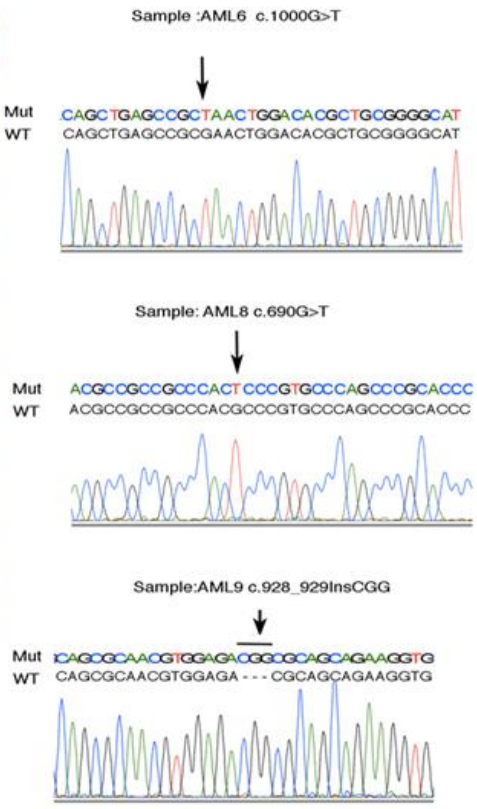

Figure 11. C/EBPα status in primary AML samples. (A) Characteristics of the primary AML samples. Reference sequence for annotation: NM_004364.3. * c.1000G>T. Somatic Mutation Gaa/Taa, consequences: STOP GAINED. Somatic mutations found in AML: COSM18300. **c.690G>T Variation: rs34529039, consequences: Synonymous variant with frequency of 15% in control population. *** Somatic mutation c.928_929InsCGG Codons acg/aCGGcg Amino Acids T/TA, consequences: Inframe Insertion. Somatic mutations found in human cancers: COSM29031. ND: Non Determined. (B) DNA sequence chromatograms for the mutations of C/EBPα in the indicated samples.

DISCUSSION

MiRNAs are small-noncoding RNAs of 21-22 nucleotides that regulate the expression of several genes (Kim, 2005; Fabian and Sonenberg, 2012). Transcribed as primary miRNAs (pri-microRNAs) are processed in the nucleus into 70–80-nt by Drosha-DGCR8 complex, hairpin-shaped precursors, called pre-microRNAs (Kim, 2005; Fabian and Sonenberg, 2012). They are then exported in the cytoplasm by exportin 5 and further processed into mature miRNAs by Dicer, assisted by TRBP, and incorporated in the RNA-induced silencing complex (Krol *et al*., 2010; Fazi and Nervi, 2008). MiR-125b is upregulated in many neoplastic blood disorders, including acute myeloid leukemia (AML) (Bousquet *et al*., 2010; Shaham *et al*., 2012; Sun *et al*., 2013). Enforced constitutive overexpression of miR-125b in mice induces myeloid leukemia (Marcucci *et al*., 2011). It has been indicated that miR-125b in a myeloid context, might act as an oncomiR able to transform cells by targeting multiple genes involved in apoptosis, cell cycle and differentiation (Lobry *et al*., 2014 and references therein). Relevant to myeloid leukemia, C/EBPα is frequently mutated in AML, but surprisingly, none of the observed mutations result in full ablation of the gene (Nerlov, 2004; Ohlsson *et al*., 2013). This indicates that activity of C/EBPα is required for AML, thus in addition to work as a tumor suppressor C/EBPα appears to be required for the development of at least some AML subtypes (Nerlov, 2004; Ohlsson *et al*., 2013; Roe and Vakoc, 2014). Interestingly, C/EBPα in AML is able to induce the expression of miRNAs during myeloid development and leukemia (Eyholzer *et al*., 2010; Eyholzer *et al*., 2010; Pulikkan *et al*., 2010; Paz-Priel and Friedman, 2011; Katzerke *et al*., 2013). Previously has been shown that the manifestation of Hailey-Hailey disease, a rare skin disorder, was in part dependent on Notch1 downmodulation mediated by miR-125b upregulation (Manca *et al*., 2011). Notably, while the involvement of Notch signaling as an oncogene in T-ALL is well characterized, Notch signaling has been described as a possible tumor suppressor in myeloid malignancies (Lobry *et al*., 2013; Lobry *et al*., 2014). Moreover, while T-ALL cells express Notch1 receptor, its activation is silenced in AML (Lobry *et al*., 2014 and references therein). It has been previously shown that miR-125b is overexpressed in AML; thus, we investigated whether miR-125b overexpression might account for the differential Notch1 expression between T-ALL and AML. We compared miR-125b expression pattern in both primary AML and T-ALL leukemia as well as in AML and T-ALL derived cell lines (Figure 5A-C). Both the human primary and AML cell lines samples demonstrated significant upregulation of miR-

125b expression. Conversely, both primary and T-ALL cell lines failed to show significant enrichment of this miRNA.

In order to investigate if the deregulation of miR-125b expression occurs either at the transcriptional or processing level, primary miR-125b expression levels were analyzed in both Jurkat and HL60 cell lines as well as in primary AML samples (Figure 5D and E). Specifically, quantitative reverse transcriptase polymerase chain reaction (q-PCR) was performed to compare the levels of pri-miRNA and mature miRNA. In both evaluated cell lines, the pri-miRNAs levels of the miR-125b were found to follow the same trend of mature miR-125b expression (Figure 5A and D). In most primary AMLs, we found that miR-125b expression was transcriptionally upregulated (Figure 5E). Nevertheless, we observed that in some samples pri-miRNAs basal transcription efficiency was associated with a low abundance of the mature miRNA (Figure 5A ans E, AMLs 8, 9 and 10). Therefore, these observations indicate that in this cellular context there is a general high rate of pri-miR-125b transcription, although an altered processing efficiency might determine the level of mature miRNAs.

We found an inverse correlation of miR-125b expression and Notch1 levels in both T-ALL and AML cell lines as well as in primary AML samples (Figure 5A-B and Figure 6A-B). We observed higher level of miR-125b expression in AML when compared with T-ALL samples (Figure 6C). Importantly, the Notch1 target genes Hes-1 and Deltex1 were significantly higher in T-ALL when compared with AML (Figure 6C). Recently, we found that Notch1 is a target of miR-125b (Manca *et al*., 2011). Thus, we analyzed the potential involvement of miR-125b in regulating the differential expression of Notch1 between T-ALL and AML cells. We analyzed Notch1 protein expression after overexpression of either miR-125b or AntagomiR-125b in T-ALL and AML cell lines, respectively. We found that deregulated miR-125b expression impaired Notch1 levels in DND41 (Figure 7A) and although with a lower effect also in Jurkat and HL60 cell lines (Figure 7B). Together, these results suggest that deregulation of miR-125b expression plays a critical role in the differential expression of Notch1 between T-ALL and AML. However, ME-1 cells devoid of miR-125b expression have undetectable level of Notch1 expression (Figure 5A and 6A). Additionally, in the T-ALL derived cell line, Molt3, miR-125b enforced expression did not affect Notch1 expression (data not shown). Therefore, it is likely that other mechanisms, alone or synergistically with the miR-125b, are involved in Notch1 downmodulation in AML (Katzerke *et al*., 2013) or

alternatively an unknown mechanism antagonizes the repressive activity of miR-125b on the 3'-UTR of Notch1 in a cell context specific manner.

To explore the mechanism regulating miR-125b expression, we first characterized the miR-125b promoter region using the Genomatix MatInspector software package, focusing on those transcription factors that have been shown to play a role in either T-ALL or AML. In Figure 8, a scan of 2 kb of genomic sequence located upstream of the predicted pre-miR-125b start site identified putative Hes-1, GATA3 and one C/EBPα consensus binding sites suggesting a possible involvement of those factors in the regulation of miR-125b expression. Due to the relevance of C/EBPα in normal hematopoiesis and in AML and its ability to induce the expression of miRNAs during myeloid development and leukemia (Eyholzer *et al*., 2010; Eyholzer *et al*., 2010; Pulikkan *et al*., 2010; Paz-Priel and Friedman, 2011; Katzerke *et al*., 2013), in protein extracts from AML and T-ALL derived cell lines were first analyzed the expression of this factor. Interestingly, C/EBPα expression was correlated with miR-125b expression in the cell lines examined (Figure 6A and Figure 9A). We next examined the role of this transcription factor in the regulation of miR-125b expression by a generated a miR-125b promoter construct (Manca *et al*., 2011) and tested it in a luciferase reporter assay. As shown in the Figure 9C, we found the induction of miR-125b promoter activity by C/EBPα transfection in a dose-dependent fashion, indicating that C/EBPα might be a transcriptional regulator of miR-125b expression. C/EBPα is a key myeloid transcription factor, frequently mutated in AML, but none of the described mutations result in the full loss of its function (Nerlov, 2004; Ohlsson *et al*., 2013). Recently, it has been shown that C/EBPα dependent activity plays an important role in AML etiology (Roe and Vakor, 2014). Next, we investigated whether C/EBPα directly regulates miR-125b promoter. For this purpose, we performed chromatin immunoprecipitation experiments in both HL60 cells and primary AML samples. In HL60, we were able to observe an enrichment of DNA from the predicted C/EBPα binding sites when compared with the immunoglobulinG (IgG) control (Figure 9D). Additionally, we observed an increased recruitment of C/EBPα onto the miR-125b promoter in AML primary samples highly expressing miR-125b primary transcript (Figure 9E). Finally, we controlled the status of C/EBPα in the primary AML samples (Figure 11), and we found that primary samples carrying mutations of this protein had higher levels of miR-125b at the transcriptional level (Figure 5E). Transduction of miR-125b-1 in bone marrow cells accelerated myeloid tumors induced by a C-terminal mutant of C/EBPα in mice (Enamoto *et al*., 2012), thus, mutations in C/EBPα might be inducing more efficiently the transcription of

miR-125b in AML suggesting that miR-125b overexpression and alterations in C/EBPα collaborates in the pathogenesis of myeloid malignancies. Due to the capacity of C/EBPα interacting with other transcriptional factors implicated in leukemia (Tenen *et al*., 1997; Friedman, 2007; Paz-Priel and Friedman, 2011), it is possible that these interactions could render more efficiently the transcriptional activation of miR-125b.

The myeloid cell lines provide an important in vitro model system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells of the granulocyte/monocyte/macrophage lineage. To explore further the role of C/EBPα in the induction of miR-125b expression, we compared C/EBPα and miR-125b expression after RA treatment (Figure 10E and F). Treatment with RA (1μM) strongly decreased both C/EBPα protein and mRNA expression (Figure 10A and E), simultaneaously with induction of granulocytic differentiation (Figure 10B and C). Notably, in both cell lines HL60 and NB4, the downregulation of C/EBPα expression by RA parallels that one of miR-125b (Figure 10E and F). Interestingly a similar trend was observed in $CD34^+$ and $CD34^-$ primary cells (Figure 10D). Finally, siRNA against C/EBPα in HL60 decreased the basal level of miR-125b expression (Figure 9B) further supporting our finding that miR-125b is a direct target of C/EBPα.

In summary, several studies have made important advances in elucidating the contribution of both C/EBPα and miR-125b into the molecular mechanisms of acute myeloid leukemia development. Our study implicates the transcription factor C/EBPα as a critical determinant of miR-125b expression in AML, supporting a model whereby C/EBPα functions to enhance miR-125b expression to regulate a group of genes whose deregulation leads to acute myeloid transformation. Additionally, our results suggest that miR-125b dysregulation plays a critical role in the differential expression and activity of Notch1 between T-ALL and AML.

CONCLUSIONS

As previously reported, we found miR-125 upregulation in AML, both derived cell lines and primary patient samples. Its overexpression was at the transcriptional level (pri-miR-125b1 and pri-miR-125b-2). Conversely, both T-ALL primary samples and T-ALL derived cell lines failed to show significant enrichment of miR-125b. We suggested a possible mechanism of miR-125b dysregulated expression mediated by C/EBPα, throught direct binding of C/EBPα to its promoter region. Also, mutations of C/EBPα could render more efficiently the transcription of miR-125b by interaction with other transcriptional factors that have putative binding sites to its promoter. Additionally, we observed that in some samples pri-miRNAs basal transcription efficiency was associated with a low abundance of the mature miRNA (AMLs 8, 9 and 10). Therefore, these observations indicate that in this cellular context there is a general high rate of pri-miR-125b transcription, even though an altered processing efficiency might determine the level of mature miRNAs.

Based on the evidence that miR-125b might target Notch1 and the inverse correlation of expression between miR-125b and Nocth1 observed in T-ALL and AML in our results, we performed transient transfections or lentiviral transduction to upregulated or downregulated miR-125b expression. In these cellular contexts, we found that Notch1 is targeted by miR-125b, thus, miR-125b overexpression might account for the differential Notch1 expression between T-ALL and AML. Although, it is likely that other mechanisms, alone or synergistically with miR-125b, are involved in Notch1 downmodulation or alternatively an unknown mechanism antagonizes the repressive activity of miR-125b on the 3'-UTR of Notch1 in a cell context specific manner.

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