# ZRF1-mediated transcriptional regulation in acute myeloid leukemia

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TESI DOCTORAL UPF / 2013

Aquesta tesi ha estat realitzada sota la direcció del

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Barcelona, desembre de 2013

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# ABSTRACT

Acute myeloid leukemia (AML) is frequently linked to epigenetic abnormalities and deregulation of gene transcription, which lead to aberrant cell proliferation and accumulation of undifferentiated precursors. ZRF1, a recently characterized epigenetic factor involved in transcriptional regulation, is highly overexpressed in human AML, but it is not known whether it plays a role in leukemia progression. In this thesis, we have investigated the function of ZRF1-mediated transcriptional regulation in AML. We demonstrate that ZRF1 depletion decreases cell proliferation, increases apoptosis and induces cell differentiation in human AML cells. Treatment with retinoic acid (RA), a differentiating agent currently used to treat certain AMLs, leads to a functional switch of ZRF1 from a negative regulator to an activator of differentiation. At the molecular level, ZRF1 controls the RA-regulated gene network through its interaction with the RA receptor α (RARα) and its binding to RA target genes. Our genome-wide expression study reveals that ZRF1 regulates the transcription of nearly half of RA target genes. Consistent with our *in vitro* observations that ZRF1 regulates proliferation, apoptosis, and differentiation, ZRF1 depletion strongly inhibits leukemia progression in xenograft mouse models. Finally, ZRF1 knockdown cooperate with RA treatment in leukemia suppression *in vivo*. Taken together, our results show that ZRF1 is a key transcriptional regulator in leukemia progression and suggest that ZRF1 inhibition could be a novel strategy to be explored for AML treatment.

La leucèmia mieloide aguda (LMA) està relacionada freqüentment amb anomalies epigenètiques i desregulació de la transcripció gènica, que provoquen una proliferació cel·lular aberrant i l'acumulació de precursors indiferenciats. ZRF1, un factor epigenètic caracteritzat recentment i implicat en la regulació transcripcional, es troba altament sobreexpressat en la LMA humana, però es desconeix si juga cap paper en la progressió de la malaltia. En aquesta tesi, s'ha investigat la funció de ZRF1 en la regulació transcripcional en la LMA. Es demostra que el silenciament de ZRF1 provoca una disminució de la proliferació, un increment de l'apoptosi i una inducció de la diferenciació en cèl·lules de LMA humana. El tractament amb àcid retinoic (AR), un inductor de la diferenciació que es fa servir actualment per a tractar determinades LMAs, produeix un canvi funcional en ZRF1, que passa de repressor a activador de la diferenciació. A nivell molecular, ZRF1 controla la xarxa de gens regulada per l'AR a través de la seva interacció amb el receptor de l'AR α (RARα) i la seva unió als gens diana de l'AR. El nostre estudi d'expressió a nivell de tot el genoma revela que ZRF1 regula la transcripció de gairebé la meitat dels gens diana de l'AR. En concordança amb les nostres observacions *in vitro* que mostren que ZRF1 regula la proliferació, l'apoptosi i la diferenciació, el silenciament de ZRF1 provoca una forta inhibició en la progressió de la leucèmia en models de xenotrasplantament en ratolí. Finalment, el silenciament de ZRF1 coopera amb el tractament amb AR en la supressió de la leucèmia *in vivo*. Conjuntament, aquests resultats mostren que ZRF1 és un regulador transcripcional clau en la progressió de la leucèmia i suggereixen que la inhibició de ZRF1 podria ser una nova estratègia a explorar en al tractament de la LMA.

# INTRODUCTION

# 1. Acute myeloid leukemia

## 1.1 Leukemia

The types of cancer that affect blood, bone marrow and lymph nodes are known as hematological malignancies. These cancers are commonly divided in leukemias, if they are located mainly in the blood, and lymphomas, if they affect mainly the lymph nodes. Leukemia includes a spectrum of diseases characterized by an abnormal increase of immature blood cells called blasts.[1] Leukemia may derive from either of the two major blood cell lineages: myeloid and lymphoid. Myeloid cells include granulocytes, mast cells, monocytes/macrophages, dendritic cells, megakariocytes and erythrocytes while lymphoid cells include B cells, T cells, and Natural Killer (NK) cells (see Figure I1).[2,3] Based on the kind of blood cell affected, leukemias can be divided into two main groups:

- **Myeloid or myelogenous leukemias**: the cancerous cells derive from cells that were committed to form red blood cells, platelets and white cells other than lymphocytes (mainly granulocytes and monocytes).
- **Lymphoblastic or lymphocytic leukemias**: the cancerous cells derive from cells that were committed to form lymphocytes.

Leukemias can also be divided according to its acute and chronic forms:

- **Acute leukemia**: it is characterized by the fast increase of immature blood cells. Malignant blood cells accumulate into the bloodstream and spread to other organs of the body. These cells do not perform the necessary functions and occupy a space that would allow the development of normal mature cells. Acute forms of leukemia are the most common forms of leukemia in children.
- **Chronic leukemia**: it is characterized by the excessive accumulation of relatively mature, but still abnormal, blood cells. It progresses more slowly and, after months or years, the malignant cells are produced at a much higher rate than normal cells, resulting in many abnormal white cells in the blood. Chronic leukemia mostly occurs in older people, but can affect any age group.

Considering these two classifications, leukemia is divided in four main categories, each of them including specific subtypes:

- Acute myeloid leukemia (AML)
- Chronic myeloid leukemia (CML)
- Acute lymphoblastic leukemia (ALL)
- Chronic lymphoblastic leukemia (CLL)

## 1.2 Acute myeloid leukemia (AML)

AML is a heterogeneous clonal disorder of hematopoietic progenitor cells (blasts), which lose the ability to differentiate normally and to respond to normal regulators of proliferation.[4] AML is characterized by an aberrant proliferation and accumulation of immature myeloid progenitor cells that can affect peripheral blood, the bone marrow, and other tissues such as spleen or liver.[5,6] The most commonly used method of classification for the subtypes of AML is that developed by the French-American-British (FAB) group, which is based on the morphology, the type, the maturation and the cytochemical and immunophenotypic behavior of the leukemic blasts.[7] Table 1 details the AML classification by the FAB system together with the most frequent chromosomal abnormalities corresponding to some of the subtypes.[8,9]

**Table 1: FAB classification of AML and main associated genetic abnormalities**

| Type | Common name | % cases | Cytogenetics | Fusion protein |
|---|---|---|---|---|
| M0 | Acute myeloblastic leukemia with minimal differentiation | 3% | | |
| M1 | Acute myeloblastic leukemia without maturation | 15-20% | | |
| M2 | Acute myeloblastic leukemia with maturation | 25-30% | t(8;21) | AML1-ETO |
| M3 | Acute promyelocytic leukemia (APL) | 5-10% | t(15;17) | PML-RARα |
| M4 | Acute myelomonocytic leukemia | 20% | 11q23 | MLL-fusions |
| M4eo | Acute myelomonocytic leukemia with abnormal eosinophils | 5-10% | inv(16), t(16;16) | CBFβ-MYH11 |
| M5 | Acute monoblastic (M5a) and Acute monocytic leukemia (M5b) | 2-9% | 11q23 | MLL-fusions |
| M6 | Acute erythroid leukemia | 3-5% | | |
| M7 | Acute megakaryocytic leukemia | 3-12% | | |

AML is the most common type of leukemia in adults and has the lowest survival rate.[10] The incidence increases with age, having older patients worse outcomes than younger patients. AML, as an acute leukemia, progresses rapidly and is usually lethal within weeks or months if is not treated. Treatment for patients younger than 60 years consists of cytotoxic chemotherapy and might cure 20-75%, depending primarily on leukemia-cell cytogenetics. However, chemotherapy produces such a result in less than 10% of elderly patients.[4] Increased understanding of the pathogenesis of AML has fostered the development of targeted therapies. However, successful targeting of each of the numerous genotypic variants of AML is a major challenge.

An important genetic characteristic of AML is the high prevalence of chromosomal rearrangements. In fact, a large proportion of AML cases are associated with non-random chromosomal translocations that often result in gene rearrangements and the formation of fusion proteins.[11] The most frequent chromosomal rearrangements and the corresponding fusion proteins are shown in Table 1. Many of these fusion events involve a gene encoding a transcription factor necessary for myeloid differentiation and another gene encoding a transcriptional protein that is capable of interacting with co-repressor complexes. The resulting fusion protein maintains the DNA-binding capability of the wild-type transcriptional regulator and also is able to interact with co-repressors through its fusion moiety, leading to aberrant expression of target genes.[9] Thus, this causes an aberrant silencing of genes necessary for myeloid development. This defect acts in concert with other genetic abnormalities resulting in leukemic transformation.

## 1.3 Differentiation therapy in AML

Although AML is a heterogeneous group of diseases caused by diverse genetic aberrations, these always comprise different abnormalities that confer two main properties to the leukemic cells: impaired differentiation (due to, for example, expression of PML-RARα or AML1-ETO fusion

proteins) and enhanced proliferation/survival (caused for example by activating mutations in FLT3, RAS, or KIT proteins).[12] Therefore, a characteristic abnormality of leukemia cells, and in particular of AML cells, is that they are blocked at an early stage of their development and fail to differentiate into functional mature cells.[13] As a result, there is an aberrant proliferation and accumulation of myeloid precursors in blood and bone marrow, usually granulocyte or monocyte progenitors at different maturation stages.[5]

In the mid 1980s, several studies demonstrated the capability of certain chemicals to induce malignant cells to overcome their block of differentiation and undergo terminal differentiation, as an alternative to killing them by cytotoxic chemotherapy. This strategy could theoretically limit exposure to unwanted side effects of cytotoxic therapies and improve complete remission and cure rates.[13] The first reports addressing the differentiation therapy included studies demonstrating the differentiating capability of dimethyl sulfoxide (DMSO) on erythropoiesis[14] and the effect of different substances in the differentiation of myeloid leukemia.[15] One of the main results of initial *in vitro* experiments was achieved in differentiating HL60 cells (an AML cell line) with all-*trans* retinoic acid (ATRA or RA).

Treatment with RA has been the best proof of principle for differentiation therapy. In fact, this strategy is used in patients with acute promyelocytic leukemia (APL), a subtype of AML characterized in most of the cases by the presence of the fusion protein PML-RARα. APL leukemic cells treated with RA undergo proliferative arrest, terminal granulocytic differentiation, and finally apoptosis.[13] However, the attempt to use other compounds such as PPARγ agonists and vitamin D or different classes of substances such as hematopoietic cytokines or drugs affecting the epigenetic landscape has not been as successful as RA in APL. Nevertheless, triggering differentiation by manipulation of transcription factors is still a promising possibility to be further explored.[13] A schematic representation of differentiation therapy in leukemia in the context of hematopoiesis is shown in Figure I1.

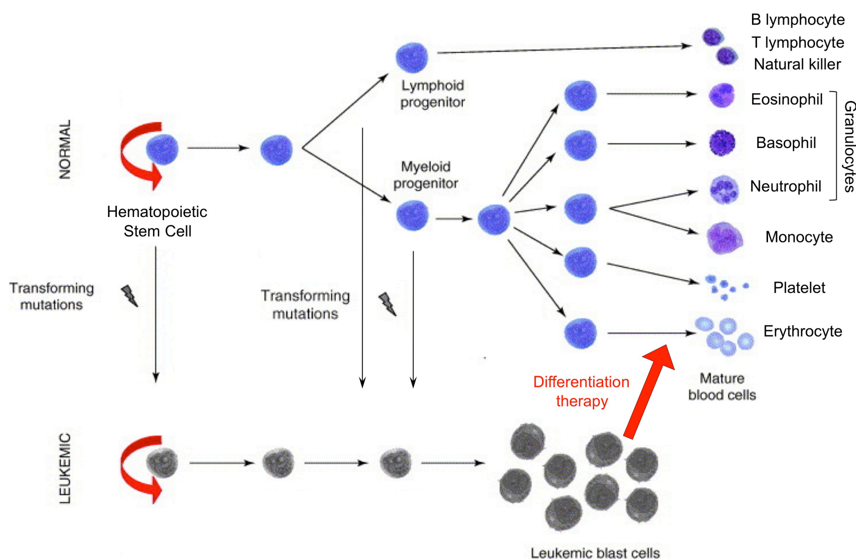


**Figure I1: Hematopoiesis, leukemogenesis and differentiation therapy.** The hematopoietic stem cells originate the lymphoid and myeloid progenitors that then differentiate to form the different types of mature blood cells. Transforming mutations switch normal hematopoietic cells to leukemic cells arrested in different cellular maturation stages. The purpose of differentiation therapy is to convert leukemic cells to normal mature cells. Modified from *Wang et al.*, 2005.[16]

Despite its effectiveness in APL treatment, use of high-dose RA in monotherapy has been related to toxic secondary effects known as Retinoic Acid Syndrome (RAS) and, moreover, to the appearance of resistance. Development of RAS, which occurs in about 15% of patients receiving RA treatment, is recognized as a complication with an associated mortality of about 2%.[17] Therefore, chemotherapy such as cytarabine (AraC), and the differentiating agent arsenic trioxide (ATO) are currently used in APL as co-adjuvant to reduce RA doses and improve cure rates.[18] Treatment with RA/ATO in combination with chemotherapy in APL patients leads to complete remission rates over 90%, with a 5-year overall survival rates close to 100%,[19] showing the high potential of differentiation therapy in leukemia treatment.

Although RA-based differentiation therapy is very effective in PML-RARα-associated APL, this strategy has not been successfully extended to other AML subtypes. At present, AraC is the main therapy in non-APL

AMLs.[20] Althought this could suggest that RA is effective selectively in cells containing PML-RARα several data suggest that this is not the case. First, RA was initially identified to induce differentiation in the HL60 cell line, which lacks PML-RARα. Second, several clinical studies have shown the effectiveness of RA when used in combination with other agents such as conventional chemotherapy or drugs afecting the epigenetic state such as histone deacetylase inhibitors (HDACi) or DNA methyltransferase inhibitors (DNMTi).[21-23]

It has been proposed that for RA-based differentiation therapy to be succesful in non-APL AML, treatments will need to be combined with drugs that afect leukemic cell proliferation/survival and/or with drugs that sensitize AML cells to RA,[24] as exemplified in Figure I2.

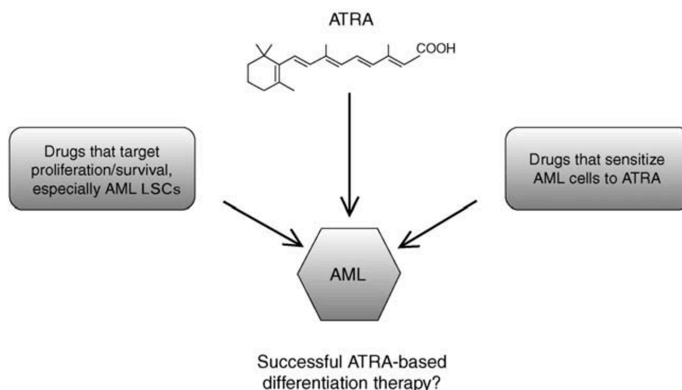


**Figure I2: RA-based differentiation therapy in AML.** Proposed strategy for successful combination therapy in non-APL AML. From *Petrie et al., 2009*.[24]

Current investigations in AML therapy are focused upon developing new and better differentiation-based combination therapies. These treatments could be either targeted against specific abnormalities underlying the pathogenesis of a given AML subtype, or take advantage of characteristics shared by different AMLs. In particular, the future of differentiation therapy may lie in the manipulation of transcription factors that aberrantly lead to impaired differentiation as these have emerged as common abnormalities in AML and other cancers.[13]

# 2. Retinoic acid signaling pathway

## 2.1 RA metabolism and signaling

Vitamin A and its active metabolites, collectively called retinoids, are non-steroid hormones that play a critical role in the development and homeostasis of many vertebrate tissues through their regulatory effects on cell differentiation, proliferation and apoptosis.[25] In contrast with most differentiation regulators, which bind to cell-surface receptors to initiate intracellular signaling pathways, RA enters the nucleus and binds directly to target genes via nuclear receptors.[26] Specifically, RA signaling is carried out predominantly by binding to the retinoic acid receptor (RAR) family of nuclear receptors.

Mammals cannot synthesize vitamin A, which has to be taken from food sources and it is absorbed in the form of retinol.[27] In the blood, retinol binds to a retinol-binding protein (RBP4), and it is taken into cells mainly via the cell membrane receptor Stra6, which recognizes RBP4.[28] Thus, the retinol concentration in cells is regulated primarily by Stra6. In the cytoplasm, retinol is oxidized to all-*trans* retinaldehyde (retinal) by retinol dehydrogenases (RoDH). Then, retinal is further metabolized by retinaldehyde dehydrogenases (RALDH) to produce the main biologically active form of retinoids, the all-*trans* retinoic acid (RA). Importantly, the levels of these enzymes change greatly among different types of cells and at different stages of cell differentiation. This leads to the regulation of the intracellular concentrations of retinoids depending on the cellular and developmental context.[29]

Degradation of RA is carried out by cytochrome P450 (CYP26) enzymes. These enzymes also display unique tissue-specific patterns of expression during development, thus influencing RA signaling.[26] Finally, RA levels are also regulated by binding of retinol to cellular retinol binding proteins (CRBPs) and of RA to retinoic acid binding proteins (CRABPs).[30] It should be noted that important aspects of RA metabolism remain unclear. The metabolic pathway of RA in the context of RA signaling is summarized in Figure I3.

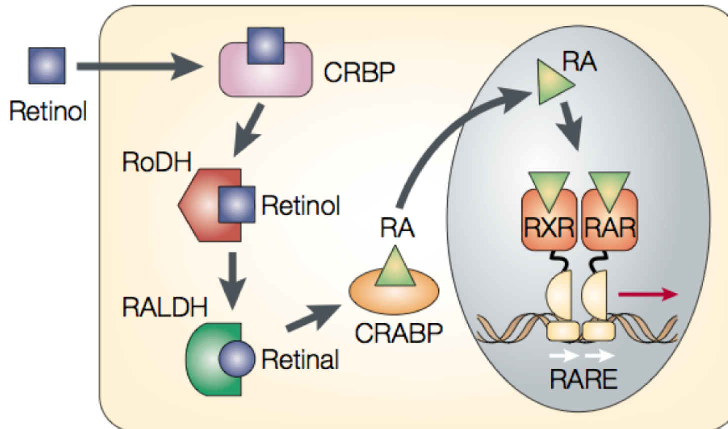


**Figure I3: RA metabolism and signaling pathway.** Retinol (vitamin A) is metabolized to retinal by RoDH and then to RA by RALDH. CRBPs can bind retinol and CRABPs can bind RA. RA enters the nucleus and (as explained below) binds to the RA receptor (RAR), which form heterodimers with retinoid X receptor (RXR) and bind to RA-responsive elements (RARE) of DNA. From *Maden et al. 2002*.[31]

## 2.2 Role of RA in differentiation, apoptosis and proliferation

RA has an essential function during vertebrate development. It is involved in the regulation of numerous processes of organogenesis and differentiation in tissues from all ectoderm, mesoderm and endoderm origins.[26] In early development, RA organizes the trunk by controlling the formation of posterior neuroectoderm, foregut endoderm and by regulating mesoderm differentiation. At later stages, RA contributes to the development of specific organs. Thus, RA regulates many developmental processes including neurogenesis, cardiogenesis, body axis extension, and development of the limb buds, gut and eye.[26]

Additionally, RA is known to play important roles in the development of the hematopoietic system.[32,33] Several reports show that RA stimulates the growth and differentiation of early granulocyte progenitors, and this occurs at the expense of other lineages, particularly erythroid and

macrophage.[34-37] Other studies with knockout mice demonstrate the importance of retinoid signaling during the establishment of fetal liver erythropoiesis.[38] In addition, several studies have used different vitamin A-deficient animal models (commonly known as VAD) and have shown, for instance, that retinoids are important for the regulation of primitive erythropoiesis and for myelopoiesis.[39,40] More recently, it has been demonstrated that RA signaling is essential for embryonic hematopoietic stem cell (HSC) development.[41]

Interestingly, some of these studies and others show essentially opposite functions of RA on distinct cell populations during hematopoiesis, blocking differentiation and stimulating self-renewal in some contexts, while inducing differentiation in others.[32,42,43] Moreover, studies using RARα$^{-/-}$ mice and other approaches have shown that this RA receptor is a bidirectional modulator of granulopoiesis.[44] Thus, RARα, the main isoform of RAR expressed in myeloid leukemic cells,[45] actively blocks granulocytic differentiation in the absence of RA (by repressing specific target genes), while it stimulates differentiation in the presence of RA (by activating these and additional target genes).

Remarkably, the RA signaling pathway not only controls cell differentiation but also directly regulates cell proliferation and apoptosis.[29] On the one hand, RA inhibits proliferation by modulating central components of the cell-cycle machinery, thus leading to a block in the G1 phase of the cell cycle.[46] Via various mechanisms depending on the cellular context, RA controls the expression of cell-cycle inhibitors such as p21 and p27, cyclins such as Cyclin A, B, D and E, and cyclin-dependent kinases such as CDK4 and CDK6, among other factors.[29] On the other hand, the precise mechanism by which RA regulates apoptosis is unclear. Nevertheless, RA is known to induce apoptosis and several important players of the apoptotic cascades are known to be RAR targets. These include Bcl-2 proteins, caspases such as caspase 8 and 9, transcription factors that regulate apoptosis, and genes involved in DNA fragmentation.[47] Moreover, RA has been shown to cause apoptosis in AML cells through the induction of the death ligand TRAIL.[48] It should be noted, however, that antiapoptotic activities of RA have also been reported, although they seem to be RAR-independent.[47]

## 2.3 RA molecular mechanism

As mentioned previously, RA signaling occurs mainly through the binding to the RARs, which are part of the nuclear receptor superfamily. This group of receptors is generally subdivided into three main families: the steroid receptor family, the thyroid/retinoid family (including the RARs), and the orphan receptor family.[49] The RAR subfamily contains three main transcription factors: RARα, RARβ and RARγ. Each of them has different isoforms. RARs can heterodimerize with the Retinoic X Receptor (RXR) subfamily members, formed also by three proteins: RXRα, RXRβ and RXRγ. RAR/RXR heterodimers bind to specific DNA sequences called RA responsive elements (RAREs) localized usually in the promoter regions of target genes.[29] RARE sequences are characterized by direct repeats of two hexamers (AGGTCA) separated usually by five nucleotides (see Figure R19 in the *Results* section).

In the absence of RA, RAR/RXR heterodimers, which are bound to RAREs already in basal conditions, recruit co-repressor complexes, such as N-CoR, SMRT and Sin3A, which contain histone deacetylases (HDACs).[50,51] This process results in histone deacetylation, chromatin compaction and gene silencing. Upon RA binding, RARs undergo a conformational change that promotes the dissociation of the repressive complexes and the recruitment of co-activators. Among them, histone acetyltransferases (HATs) p300 and CBP as well as the chromatin remodeler SWI/SNF are bound,[49,52,53] thus leading to histone acetylation and chromatin decondensation. Finally, the basal transcriptional machinery is recruited and transcription is initiated.[54]

Therefore, RAR/RXR work as transcriptional repressors in the absence of RA and switch to transcriptional activators upon ligand binding, which correlates with their dual function in the regulation of differentiation, as discussed above. A summary of this molecular mechanism is shown in Figure I4.

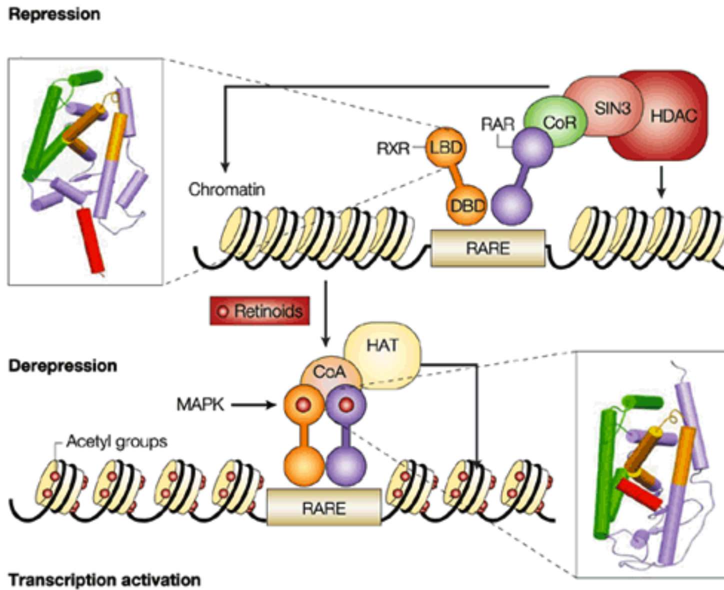


**Figure I4: RA molecular mechanism.** In the absence of RA, RAR/RXR heterodimers bind to co-repressors (CoR), thus repressing transcription. Binding of RA induces a conformational change that leads to recruitment of co-activators (CoA) and transcription activation. LBD: ligand binding domain; DBD: DNA binding domain. Modified from *Altucci et al. 2001.*[54]

## 2.4 Disruption of RA signaling: the example of PML-RARα

The important function of RA signaling in hematopoiesis and, in particular, in the regulation of granulocytic differentiation is the basis of the differentiation therapy in leukemia. As discussed above, the main example of this therapy is RA, which is currently being used to treat APL patients. Specifically, pharmacological doses of RA drives the differentiation of immature APL cells arrested at the promyelocytic stage to mature granulocytes.[29]

The main genetic alteration that causes APL is a chromosomal translocation that involves RARα (in chromosome 17) and (in the vast majority of the cases) the promyelocytic leukemia gene (PML, in

chromosome 15), which leads to the formation of the PML-RARα oncofusion protein.[55,56] PML-RARα contains most of the wild-type RARα sequence, including the DNA binding, ligand binding, RXR heterodimerization and co-activator and co-repressor interaction domains.[57]

PML-RARα retains RARα DNA-binding ability but works as a dominant negative. This fusion protein aberrantly attracts nuclear co-repressors and other transcriptional repressors thus causing abnormal silencing of RARα target genes.[58] Specifically, PML-RARα recruits co-repressor complexes containing HDACs, such as Sin3A and the nucleosome remodeling and deacetylase co-repressor complex (NuRD), and also DNA methyltransferase enzymes (DNMTs) and other repressors such as Polycomb repressive complex 2 (PRC2).[59-61] Moreover, PML-RARα is able to homodimerize through the coiled-coil region of PML, forming a large protein complex surrounded by aberrant amounts of repressors. Altogether, these mechanisms lead to intensive gene silencing. It should be noticed that PML-RARα fusion protein interacts with and disrupts the normal functions of RARα and PML expressed by wild-type alleles from the same cell.[62]

As a result, in APL cells containing PML-RARα, physiological concentrations of RA are not sufficient to activate RARα targets and, therefore, these genes are continuously repressed. This aberrant silencing, which affects differentiation regulators, causes the differentiation arrest characteristic of leukemia.[13] However, pharmacological doses of RA overcome the dominant repression of PML-RARα by triggering the release of co-repressors and the recruitment of co-activators.[63] This leads to transcriptional activation and, therefore, to cell differentiation. The process of aberrant gene repression by PML-RARα and the effect of RA treatment are summarized in Figure I5.

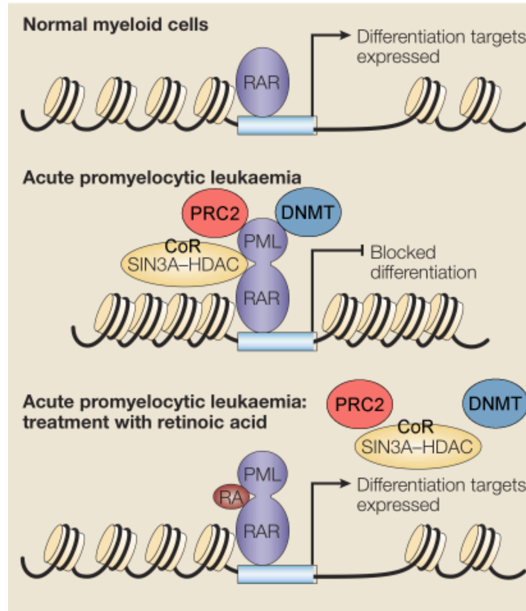


**Figure I5: Molecular pathology associated to PML-RARα and RA differentiation therapy.** PML-RARα binds RARα target genes and aberrantly repress them by recruiting nuclear receptor co-repressors (CoR) containing HDACs, and other transcriptional repressors such as DNMTs and PRC2, leading to a block in differentiation. Treatment with pharmacological doses of RA leads to co-repressor release, transcriptional activation and cell differentiation. Modified from *Roberts et al. 2004*.[64]

# 3. Chromatin and epigenetics in transcription

## 3.1 Chromatin structure

Eukaryotic cells contain approximately 2 meters of genomic DNA that needs to fit into the cell nucleus, which has a diameter of 5–10 µm. To achieve this, DNA is highly organized within the nucleus, with 147 bp of DNA sequence wrapped around histone protein octamers that consist of the histones H2A, H2B, H3 and H4.[65] This structure forms the nucleosome that represents the smallest functional entity of chromatin.[66] Nucleosomes are connected by linker DNA that is bound by the histone H1, which is involved in establishing higher order chromatin structures.[67] A simplified scheme of chromatin structure is shown in Figure I6.

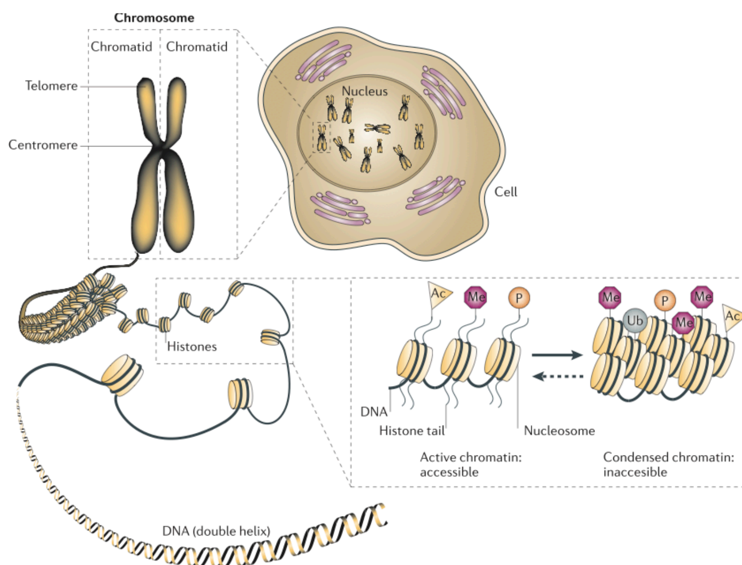


**Figure I6: Chromatin structure.** DNA is wrapped around histone proteins forming the nucleosomes, which are further compacted to form high-order structures. As explained below, histone tails can undergo multiple modifications such as acetylation (Ac), methylation (Me), phosphorylation (P) and ubiquitination (Ub) that regulate chromatin structure and transcriptional activity. From *Sparmann et al. 2004*.[68]

The proper organization of chromatin is crucial not only for DNA compaction within the nucleus but also for the regulation of fundamental cellular functions such as DNA replication, DNA damage repair and transcription.[68] Regarding transcription, chromatin structure has a fundamental role in the control of gene activity. Thus, regions of open chromatin structure (known as euchromatin) permit transcription factors and the basal transcription machinery to access DNA, thereby allowing gene transcription to occur. On the other hand, regions of closed chromatin (known as heterochromatin) are tightly compacted, which prevents access to DNA, thus leading to gene silencing.[69]

## 3.2 Epigenetics

The term epigenetics, in its broad definition, refers to the changes in chromatin that do not alter the DNA sequence. Although this definition includes many different mechanisms, the most characterized epigenetic changes are DNA methylation, histone variants and histone modifications, each of which has key roles in transcriptional regulation. First, DNA methylation is a covalent modification of DNA that occurs on the cytosine within CG dinucleotides and is associated with gene silencing.[69] Second, canonical histones (H2A, H2B, H3 and H4) can be replaced by histone variants with specific functions in transcriptional regulation and other processes. Third, post-translational modifications of the histone proteins are covalent changes that occur mainly in the N-terminal tails of histones, which protrude outward from the nucleosome, as shown in Figure I6.

Histones are extensively modified by the addition of various molecules. Although many different **histone modifications** have been characterized up to date (see Figure I7), the most studied ones are acetylation, methylation, phosphorylation and ubiquitination. These modifications are deposited in specific residues within the histone tails by specific enzymes and may have two different effects.[70] On the one hand, the presence of different histone modifications affects the interaction between the negatively charged DNA and the positively charged histones, thus altering chromatin structure. On the other hand, several histone modifications

work as an anchoring site for different proteins, which may result in multiple outputs depending on the function of the recruited factor.

Histone modifications have a key role in the regulation of transcription and consequently are very important to establish whether a gene is active or silenced.[69] Specifically, some modifications are associated with active transcription and others with transcriptional repression. A list of the main histone modifications known and their position in the different histone residues is shown in Figure I7.

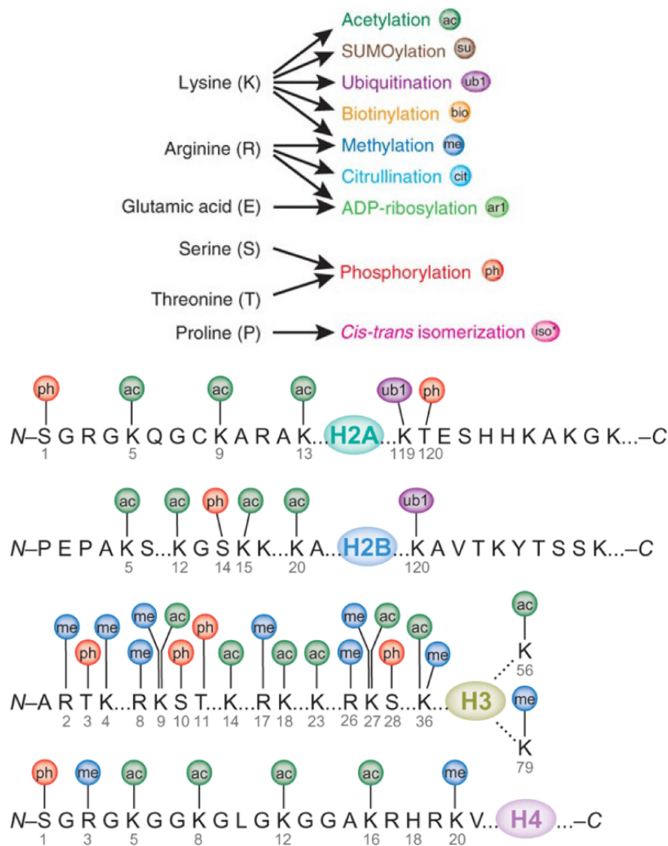


**Figure I7: Histone modifications.** Top panel: main types of histone modifications and the amino acids that they affect. Bottom panel: position of the main modifications within the histone tails (mainly the N-terminal tail), including acetylation (ac), methylation (me), phosphorylation (ph) and ubiquitination (ub1). Globular domains of each core histone are represented as colored ovals. Modified from *Bhaumik et al. 2007*.[71]

Histone modifications influence many fundamental biological processes, including development. In this way, epigenetic mechanisms have crucial roles in cell differentiation and in the determination of lineage-specific gene expression patterns.[71] Moreover, increasing evidences link the alteration in histone modifications with multiple human pathologies and especially with numerous cancers. These alterations may involve the deregulation of enzymes responsible for the deposition or removal of the modifications or factors that are able to bind them and mediate their effect. In fact, deregulation of many genes involved in the regulation of epigenetic mechanism has been shown to be implicated in the carcinogenesis process.[72] Thus, understanding the epigenetic alterations in human cancers could help to discover new therapeutic strategies.

## 3.3 Epigenetic alterations in AML

The correct regulation of the epigenetic marks is fundamental to ensure a correct gene transcription. However, in AML, some of the mechanisms that control these processes are altered. In particular, AML cells are characterized by the aberrant recruitment of epigenetic regulators to the promoters of key differentiation genes, thus disrupting their expression.[6] Specifically, DNA methylation, histone acetylation and histone methylation are often misregulated during tumorigenesis.

As mentioned above, **DNA methylation** occurs at cytosines within CG dinucleotides and is associated with repressed chromatin. This epigenetic modification is catalyzed by enzymes called DNMTs.[73] DNA methylation plays a key role in self-renewal, differentiation and homeostasis and its deregulation is linked with leukemias and other cancers. For instance, as previously mentioned, DNMTs are aberrantly recruited by PML-RARα in APL cells, thus leading to aberrant gene silencing.[61] Similarly, AML1-ETO, another fusion protein that causes a different type of AML, also recruit DNMT enzymes leading to abnormal gene repression.[74] Interestingly, a recent genome-wide DNA methylation analysis has shown the correlation of this major epigenetic modification with unique AML

subgroups, thus predicting clinical outcome.[75] In this regard, DNMT3A, one of the three DNMTs known, is recurrently mutated in AML.[76]

Another essential epigenetic mark altered in AML is **histone acetylation**, a mark that is associated with gene activation. Addition of acetyl groups to lysine residues of histones reduces the interaction between the negatively charged DNA and the positively charged histones thus leading to a more open chromatin state.[65] This modification is deposited by HATs, present in several co-activator complexes, which generally modify more than one lysine residue within the histone tails.[70] Conversely, HDACs are responsible for the removal of the acetyl marks. HDACs can be divided in three main families: the classical class I (HDAC1, 2, 3 and 8) and class II (HDAC4, 5, 6, 7, 9 and 10) and the later discovered class III (that includes the sirtuin family). Class I HDACs are part of several repressive chromatin complexes such as N-CoR, CoREST, NuRD, Sin3A and SMRT and therefore are directly involved in the regulation of transcriptional activity.[77] As mentioned above, HDACs are fundamental components of the RARα-associated corepressor complexes.

Interestingly, deregulation of histone acetylation by aberrant recruitment of HDACs to RA target genes contributes to leukemogenesis in several types of AML.[78] For instance, in AML1-ETO-associated leukemia, this fusion protein recruits HDACs and co-repressors such as N-CoR and Sin3A causing transcriptional repression of differentiation regulators.[79,80] Similarly, CBFβ-MYH11 oncofusion protein also aberrantly recruits co-repressors such as Sin3A and HDACs.[81]

Remarkably, inhibitors of HDAC proteins (HDACi), alone or in combination with other drugs, have been proposed as a possible therapeutic strategy against leukemia since many years.[78] As an example, leukemic cells containing PLZF-RARα (which, as PML-RARα, also aberrantly recruits HDACs) are insensitive to RA treatment but sensitive to a combination treatment of RA and the HDACi trichostatin A (TSA), which is able to overcome transcriptional silencing.[82] Interestingly, HDACi are approved for the treatment of T-cell lymphomas and several clinical trials are ongoing to test the effectiveness of HDACi in AML.[76]

Finally, alterations in **histone methylation** have also been linked to AML. In contrast with HATs, histone methyltransferases (HMTs) are very specific and they usually modify a single lysine or arginine residue. Also unlike acetylation, histone methylation can lead either to transcriptional activation or repression depending on the targeted residue. For instance, some of the best characterized histone modifications associated with gene activation are trimethylation of lysine 4 and lysine 36 at histone H3 (H3K4me3 and H3K36me3). Conversely, other key modifications such as trimethylation of lysine 9 and lysine 27 at histone H3 (H3K9me3 and H3K27me3) are associated with genes silencing.[70] Alterations in these and other methyl marks have been observed in different types of AML. One example of this is, as mentioned above, the aberrant recruitment of PRC2 by the fusion protein PML-RARα in APL, which enhances H3K27me3 thus contributing to the abnormal silencing of RA target genes.[59]

Another example of the link between AML and defects in histone methylation is the important group of leukemias triggered by mixed lineage leukemia (MLL) fusion proteins. MLL protein (the mammalian homolog of *Drosophila* trithorax) is a transcriptional activator that methylates lysine 4 in histone H3 (H3K4) and has a fundamental role in development.[83] MLL gene can fuse with more than 60 different partners thus causing AML and also ALL.[84] MLL fusion proteins usually retain the DNA binding ability of MLL but lose the H3K4 methyltransferase activity and several other domains involved in the interaction with various transcriptional regulators. These lead to the alteration of histone modifications and other transcriptional mechanisms, resulting in gene expression defects.[6]

Figure I8 summarizes the main epigenetic mechanisms deregulated by AML-associated fusion proteins.

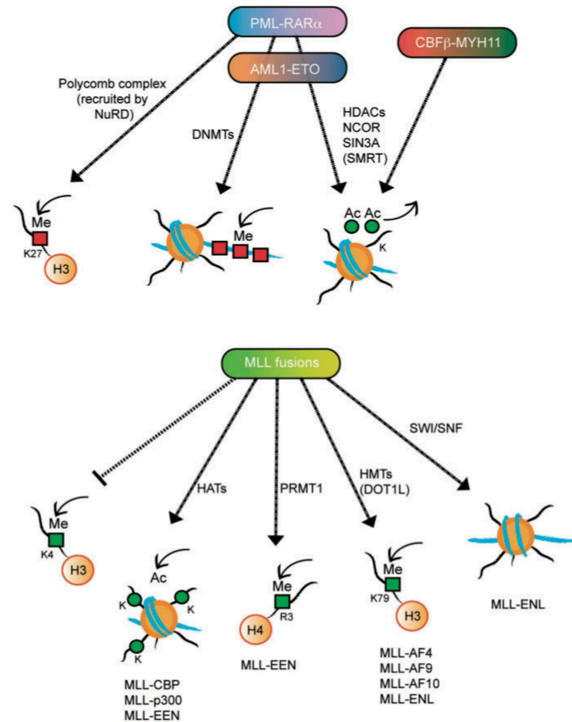


**Figure I8: Epigenetic alterations in AML.** Some of the main fusion proteins that trigger AML disrupt multiple epigenetic mechanisms either by directly affecting the deposition of specific modifications (such as H3K4me3 in MLL fusion proteins) or by aberrantly recruiting epigenetic factors (such as DNMTs, HDACs, HMTs or Polycomb proteins). These lead to different defects in transcriptional regulation thus altering gene expression programs. From *Uribesalgo et al. 2011*.[6]

## 3.4 Polycomb proteins in gene silencing

Polycomb group (PcG) proteins, together with trithorax group (TrxG) proteins, were first identified in *Drosophila melanogaster* as repressors and activators, respectively, of the Hox genes.[65,85] Their role in transcriptional regulation is linked to the control of chromatin structure and epigenetic modifications of histones. PcG and TrxG (including MLL) proteins form multimeric complexes that are crucial regulators of lineage choices during differentiation and development. In mammals, these

complexes also play important roles in cell proliferation, stem cell identity, genomic imprinting and X chromosome inactivation.

PcG proteins form complexes that are involved in transcriptional silencing. Their mechanism of action is believed to rely mainly on the regulation of chromatin structure, in part through post-translational modifications of histones.[86] PcG proteins are found in two major complexes, called Polycomb repressive complex 1 and 2 (PRC1 and PRC2), which are thought to cooperate to silence its target genes. PRC1 is formed by four core subunits, each of them with several homologs: RING1 protein (RING1a and RING1b), CBX protein (CBX2, 4, 6, 7 and 8), HPH protein (HPH1, HPH2 and HPH3) and PCGF protein (PCGF1–PCGF6). RING1 proteins are E3 ligases and catalyze the monoubiquitination of H2AK119 (H2AK119ub).[87,88] PRC2 consists of three core components: EED, SUZ12 and EZH2 or its close homolog EZH1. This complex catalyzes mono-, di- and trimethylation of H3K27 through the methyltransferases EZH2 or EZH1.[89] Given the high number of PcG homologs, especially concerning PRC1 components, PRCs can exist in multiple possible combinations. Moreover, in addition to the core components, PRCs are usually associated with diverse partners in different contexts, such as Jarid2 and PCL proteins in the case of PRC2.[90] The molecular and physiological functions of all this complexity are still poorly understood.

Polycomb-mediated gene silencing, is believed to be regulated by a coordinated action of the two PRCs, although a number of studies have demonstrated that the two complexes also have independent functions.[90] The canonical model for Polycomb-mediated repression involves the successive action of PRC2 and PRC1. The EZH1 or EZH2 subunit of PRC2 trimethylates H3K27. Subsequently, PRC1 is recruited to chromatin through the binding of CBX proteins (one of the PRC1 core components) to the H3K27me3 marks. Afterwards, the RING1 subunit of PRC1 monoubiquitinates H2AK119.[91] As a result, the presence of PRC1 and PRC2 at chromatin leads to chromatin compaction and transcriptional silencing.[90] This sequential model that causes Polycomb-mediated gene silencing is illustrated in Figure I9.

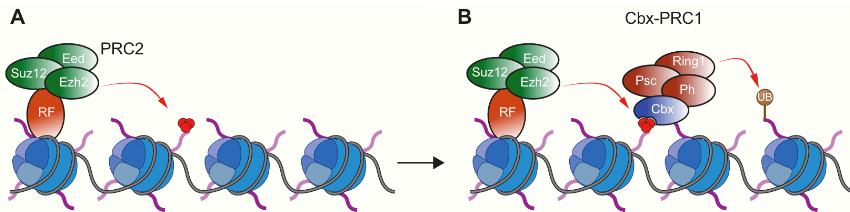


**Figure I9: Canonical model of Polycomb-mediated gene silencing.** Polycomb-target genes are repressed by the sequential action of PRC2, that trimethylates H3K27 (red circles), and Cbx-containing PRC1, that recognizes this mark and ubiquitinates H2AK119. These lead to chromatin compaction and gene silencing. From *Aloia et al. 2013.*[92]

Polycomb-mediated gene silencing is critical for regulating cell identity and differentiation, including hematopoiesis.[93] Thus, in accordance with the concept that cancer is closely linked with differentiation defects, it is not surprising that PcG proteins are frequently found to be mutated and/or deregulated in cancer, including different types of hematological neoplasms.[91] For example, the PRC1 component BMI1 has been extensively linked with leukemogenesis and is commonly upregulated in patients with AML.[93,94] Similarly, the PRC2 component EZH2 has also been related to the development of several hematopoietic neoplasms.[93] It should be noticed, however, that the role of Polycomb in leukemogenesis is controversial, as some PcG proteins (including BMI1 and EZH2) have been reported to behave both as oncogenes and tumor-suppressors, depending on the context.

# 4. ZRF1: Zuotin related factor 1

## 4.1 ZRF1 structure and function

Zuotin (Zuo1) was discovered in *Saccharomyces cerevisiae* as a nucleic acid binding protein, in two different reports. The first one, identified Zuo1 as a protein able to bind Z-DNA,[95] a left-handed configuration of DNA (different from the standard right-handed B-DNA form) with an unclear biological function.[96] A second study showed the Zuo1 also has the capacity to bind to tRNA.[97]

Some years later, the first functional characterization of Zuo1 showed that this protein is a molecular chaperone that associates with the ribosomes and confirmed its RNA-binding properties.[98] Chaperones are proteins that facilitate the folding or unfolding of other proteins and regulate the assembly or disassembly of multimeric complexes.[99] The role of Zuo1 as a ribosome-associated chaperone was confirmed by another report that showed that Zuo1 is part of the ribosome-associated complex (RAC), involved in proper protein folding of newly synthesized polypeptides.[100] Importantly, some years ago this function was shown to be partially conserved in humans; specifically, the Zuo1 homolog Zuotin Related Factor 1 (ZRF1) associates with the ribosomes and forms the human RAC.[101,102] Zuo1 function within the RAC is illustrated in Figure I10.

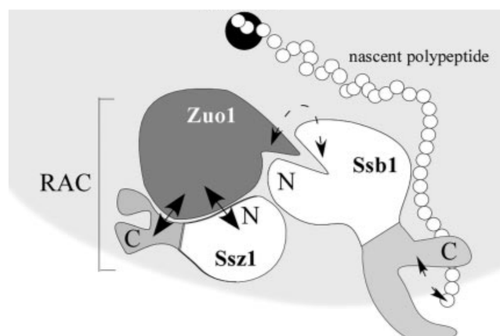


**Figure I10: Role of ZRF1 yeast homolog as a ribosome-associated chaperone.** Zuo1 interacts with the ribosome and (together with Ssz1) forms the RAC, which binds Ssb1, thus facilitating the folding of nascent proteins. This mechanism is partially conserved in mammals. From *Conz et al. 2007.*[103]

ZRF1, also known as MPP11 and DNAJC2, is a poorly characterized protein present both in the cytoplasm and in the nucleus. It has 621 amino acids (aa), although at least one additional isoform of 568 aa has been annotated (data available at http://www.uniprot.org/). ZRF1 is evolutionary conserved, being its N-terminal part homologous to yeast Zuo1.[101] This fragment contains the DnaJ domain, which is conserved from bacteria to higher eukaryotes and mediates the interaction with the heat-shock protein HSP70L1, the other component of the human RAC.[101] In the middle of its sequence, ZRF1 also contains a domain rich in positively charged aa involved in the binding to ribosomes.[104] Consequently, these two domains are directly involved in the function of ZRF1 as a ribosome-associated molecular chaperone.

Compared to Zuo1, ZRF1 contains a C-terminal extension that is shared by most of its homologs from different evolutionary lineages, suggesting that it is not a recent mammalian acquisition.[104] This C-terminal fragment of ZRF1 has two SANT domains. The SANT domain is a highly conserved domain of about 50 aa that consists of three α-helices, arranged in a helix-turn-helix motif.[105] Interestingly, SANT domains have been identified in multiple proteins related to chromatin, such as HATs, HDACs, HMTs and ATP-dependent chromatin remodeling enzymes. Theses include several components of some of the complexes that participate in RARα-mediated gene regulation, such as NuRD and N-CoR. Although the function of the SANT domains is still unclear, it has been proposed to function as a histone-interaction module.[105] Figure I11 shows the domain structure of ZRF1 and its homologs in different species.

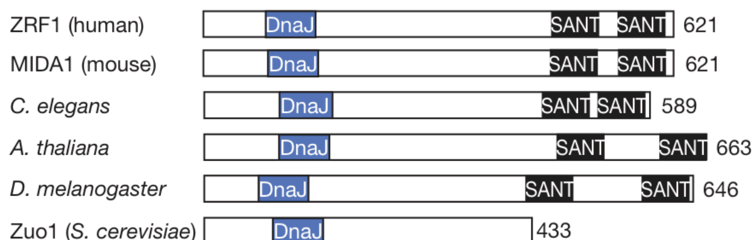


**Figure I11: Domain structure of ZRF1 and its homologs.** ZRF1 contains a DnaJ domain in the N-terminal fragment, evolutionary conserved from yeast to humans, and two SANT domains in the C-terminal fragment, absent in yeast but present in most evolutionary lineages.

The function of ZRF1 in humans, and in general in higher eukaryotes, has been poorly studied. However, some reports have provided several interesting data besides its function within the ribosome. The ZRF1 mouse homolog MIDA1 was shown to interact with Id proteins (in fact MIDA1 stands for Mouse Id Associate 1).[106] Id proteins interact with specific transcription factors and block their capacity to bind DNA. These proteins are important regulators of differentiation and have been implicated in several cancers, including leukemia.[107]

The previous report also suggested that MIDA1 is necessary for normal cell growth in a mouse cancer cell line.[106] Additionally, in a screen designed to identify novel M phase phosphoproteins, ZRF1 was found to be phosphorylated during mitotic M phase.[108] Thus, its alias MPP11 stands for M Phase Phosphoprotein 11. Additionally, GlsA, the homolog of ZRF1 in the green algae *Volvox carteri*, was shown to be required for asymmetric cell division and to associate with the mitotic spindle during cell cleavage.[109] These data suggest that ZRF1 might be involved in cell cycle regulation.

Interestingly, the function of *Volvox* GlsA in asymmetric cell division (which, in contrast to normal symmetric division, produces two daughter cells with different cell fates) is essential for the formation of germ cells.[109-111] Moreover, this function was shown to be conserved in higher plants, where the ZRF1 homolog is essential for male gametic cell formation through this mechanism,[112] and in *C. elegans*, where the homolog of ZRF1 regulates this process during neuroblast development.[113] Therefore, ZRF1 seems to have an evolutionary conserved function and to be important for development. However, we are still very far for having a complete picture of its role in this and other biological processes.

## 4.2 ZRF1 in transcriptional regulation

As mentioned above, ZRF1 was initially identified in yeast (where it is known as Zuo1) as a protein capable of binding to Z-DNA and tRNA (and

presumably also to rRNA) *in vitro*.[95,97,98] Some years later, a report showed that Zuo1 activates pleiotropic drug resistance (PDR) in a ribosome independent manner, and suggested a role of Zuo1 in transcriptional regulation.[114] In addition, it has been proposed that the mouse ZRF1 homolog, MIDA1, has DNA binding activity, not only to Z-DNA but also to standard DNA.[115] Moreover, another study suggested that the SANT domains of MIDA1 bind to DNA in a sequence-specific manner.[116] It should be noted that these studies were performed *in vitro*. In addition, another report showed that overexpression of MIDA1 stimulates the transcription of transfected reporter genes. In contrast with the previous one, this study suggested that the region involved in this process is the DnaJ domain (and not the SANT domains) and that does not involve direct binding of MIDA1 to the DNA.[117] Altogether, these data suggested that ZRF1 might be involved in transcription, although its function and its mechanism remained unknown.

We recently reported that ZRF1 works as a transcriptional regulator in human cells.[118] [(*)] Specifically, ZRF1 was found in affinity purification experiments designed to identify proteins capable of binding ubiquitinated histone H2A (H2Aub), being the first “reader” of this epigenetic modification identified to date. As mentioned above, H2Aub is deposited by PRC1 and, therefore, it is implicated in Polycomb-mediated gene silencing. Using the human embryonal carcinoma cell line NT2 as a model of differentiation upon RA treatment,[119] we found that ZRF1 facilitates transcriptional activation of a subset of Polycomb target genes (such as several Hox genes), that are crucial for the onset of cellular differentiation. Our results suggested that ZRF1 exerts its function by binding to H2Aub and in this way displacing PRC1 from chromatin, thus leading to gene activation,[118] as illustrated in Figure I12.

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(*) The article ‘*Transcriptional activation of Polycomb-repressed genes by ZRF1*’, in which I contributed, is annexed at the end of this thesis.

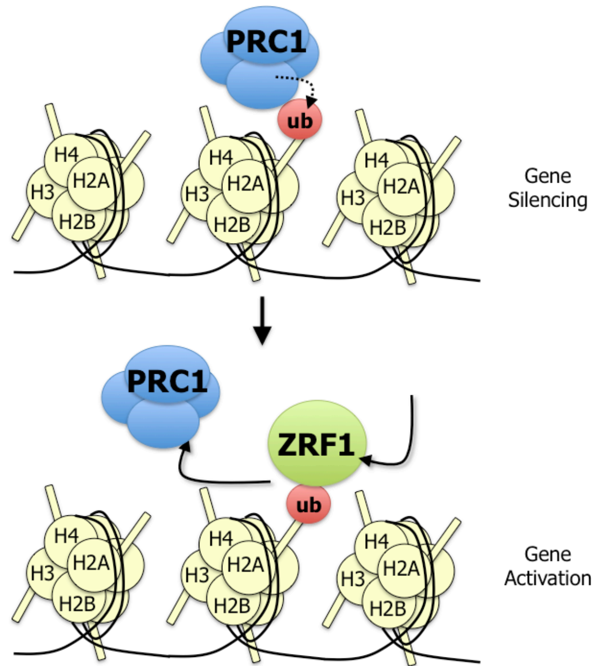



**Figure I12: Model of transcriptional activation of Polycomb-repressed genes by ZRF1.** During gene silencing, PRC1 ubiquitinates histone H2A and remains bound to chromatin. RA treatment (or ZRF1 overexpression) leads to the recruitment of ZRF1 to H2Aub, which displaces PRC1 from chromatin thus leading to gene activation.

Although H2Aub was discovered more than thirty years ago, its function is still poorly understood.[120,121] H2Aub is one of the most abundant epigenetic marks, being present on up to ten percent of total histone H2A. In line with this, our ChIP-on-chip study showed that H2Aub is present in nearly 10,000 target genes.[118] On the other hand, ZRF1 had about 1,000-2,000 target genes and bound only to a subset (about 10-15 %) of H2Aub-containing genes. Moreover, ZRF1 target genes changed depending on the absence or presence of RA and about 50% of them were not PRC1 targets. These data suggested that, first, other factors should be involved in the targeting of ZRF1 to specific sites of the chromatin in addition to H2Aub and, second, that ZRF1 has PRC1/H2Aub-independent transcriptional functions.

## 4.3 ZRF1 in cancer

ZRF1 is a widely expressed gene, with detectable expression in at least 128 different tissues and organs and during at least 135 developmental stages (data from *Bgee Gene Expression Evolution*: http://bgee.unil.ch/). Among them, ZRF1 is highly expressed in testis and in hematological tissues, especially in hematological malignancies (data from the *Gene Atlas*: http://biogps.org/)[122].

The first indication suggesting that ZRF1 could be linked with cancer comes from the fact that the region of chromosome 7 where ZRF1 gene is located (7q22-31) is commonly altered in human cancers, including breast, prostate, pancreatic, ovarian, gastric, colon, germ cell, glioblastoma, head and neck and myeloid malignancies.[123] In fact, the first study of ZRF1 in humans (only thirteen years ago) was about its role in cancer, specifically in head and neck squamous cell carcinoma (HNSCC).[123] This report showed an increase copy number of the ZRF1 gene (termed in this study MPP11) and overexpressed ZRF1 protein levels in HNSCC, which suggest that ZRF1 could have an oncogenic role.

Interestingly, several studies showed that ZRF1 is overexpressed in leukemia, specifically in acute and chronic myeloid leukemias (AML and CML) and in B-cell chronic lymphocytic leukemia (CLL).[124-128] In AML, ZRF1 is highly overexpressed in leukemic blasts from patients (and in AML-derived cell lines) as compared with cells from healthy donors,[124,125] as shown in Figure I13.

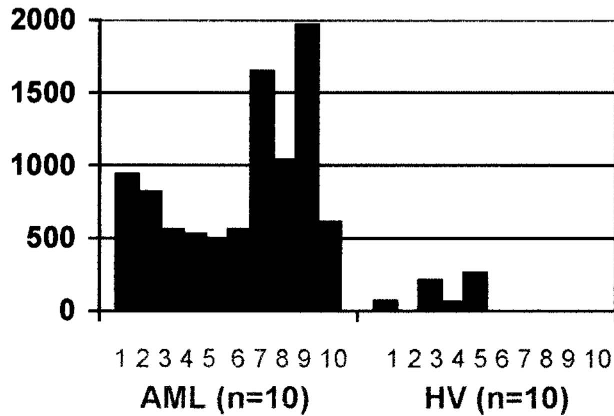



**Figure I13: ZRF1 is overexpressed in AML.** ZRF1 mRNA expression in leukemic blasts from 10 AML patients as compared with peripheral blood mononucleated cells (PBMC) from 10 healthy volunteers (HV). From *Greiner et al. 2004*.[125]

These data show that ZRF1 is overexpressed in several cancers, especially in AML. Although this suggests a putative function of ZRF1 in this disease, it should be noted that none of these investigations provide any functional data. Therefore, it is not known what is the role of ZRF1, if any, in leukemia progression.

# OBJECTIVES

Research in our group is focused on the investigation of transcriptional and epigenetic mechanisms important for differentiation and cancer. ZRF1, a recently characterized epigenetic factor involved in transcriptional regulation, is highly overexpressed in human AML, but it is not known whether it plays a role in leukemia progression. The objective of this PhD thesis was to study the function of ZRF1-mediated transcriptional regulation in AML.

Specifically, the main objectives were:

- To investigate the role of ZRF1 in proliferation, apoptosis and differentiation in AML cells.

- To further characterize the mechanisms by which ZRF1 regulates transcription and their link with the RA pathway.

- To elucidate the effect of ZRF1 inhibition in leukemogenesis potential in AML cells.

# RESULTS

Most of the results presented in this PhD thesis are included in the following original scientific publication:

**Santiago Demajo**, Iris Uribesalgo, Arantxa Gutiérrez, Cecilia Ballaré, Sara Capdevila, Mareike Roth, Johannes Zuber, Juan Martín-Caballero and Luciano Di Croce

**ZRF1 controls the retinoic acid pathway and regulates leukemogenic potential in acute myeloid leukemia**

*Oncogene* 2013 Dec 2 (advance online publication)

I also contributed to the following studies, which are included at the end of this PhD thesis:

Richly H, Rocha-Viegas L, Ribeiro JD, **Demajo S**, Gundem G, Lopez-Bigas N *et al*. **Transcriptional activation of polycomb-repressed genes by ZRF1**. *Nature* 2010; 468: 1124-1128.

Uribesalgo I, Buschbeck M, Gutierrez A, Teichmann S, **Demajo S**, Kuebler B *et al*. **E-box-independent regulation of transcription and differentiation by MYC**. *Nature cell biology* 2011; 13: 1443-1449.

Lange M, **Demajo S**, Jain P, Di Croce L. **Combinatorial assembly and function of chromatin regulatory complexes**. *Epigenomics* 2011; 3: 567-580. Review (not included).

# 1. ZRF1 controls differentiation, proliferation and apoptosis in AML cells

## 1.1 ZRF1 depletion inhibits cell growth

As mentioned in the *Introduction* section, previous studies showed that ZRF1 is overexpressed in several types of cancer, such as acute myeloid leukemia (AML),[124-126] chronic myeloid leukemia,[127] chronic lymphocytic leukemia,[128] and head and neck squamous cell carcinoma.[123] In AML, ZRF1 is highly overexpressed in leukemic blasts from patients and in AML-derived cell lines as compared with cells from healthy donors.

To explore the functional role of ZRF1 in AML, we stably knocked down ZRF1 in the human AML cell line HL60. We used two independent shRNA constructs that efficiently downregulated ZRF1 mRNA and protein levels (**Figure R1A-R1B**). Interestingly, ZRF1 depletion led to a strong decrease in growth rates in HL60 leukemic cells (**Figure R1C**).

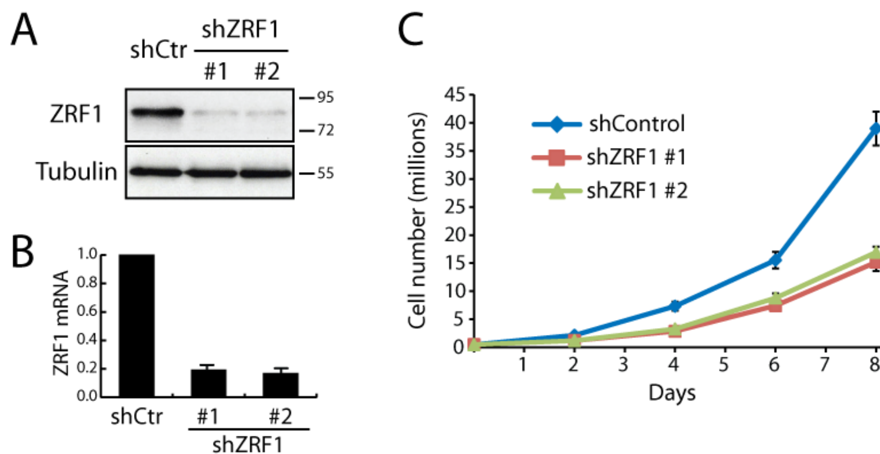



**Figure R1: ZRF1 depletion inhibits cell growth in HL60 cells.** (**A**) Western blot analysis of ZRF1 in control (shCtr) and ZRF1-depleted (shZRF1 #1 and #2) HL60 cells. Tubulin was used as a loading control. (**B**) qRT-PCR analysis of control and ZRF1-depleted HL60 cells. Results are shown relative to shCtr. Data are the means ± s.e.m. of four independent experiments. (**C**) Growth curves of control and ZRF1-depleted cells. Data are the means ± s.e.m. of four independent experiments.

In order to investigate whether the effect of ZRF1 depletion in cell growth inhibition was general for AML cells, we next stably knocked down ZRF1, using the same shRNA constructs, in three additional AML cell lines: NB4, U937 and THP1. Confirming the results in HL60, ZRF1 depletion led to a remarkable decrease in cell growth in these cells (**Figure R2**).

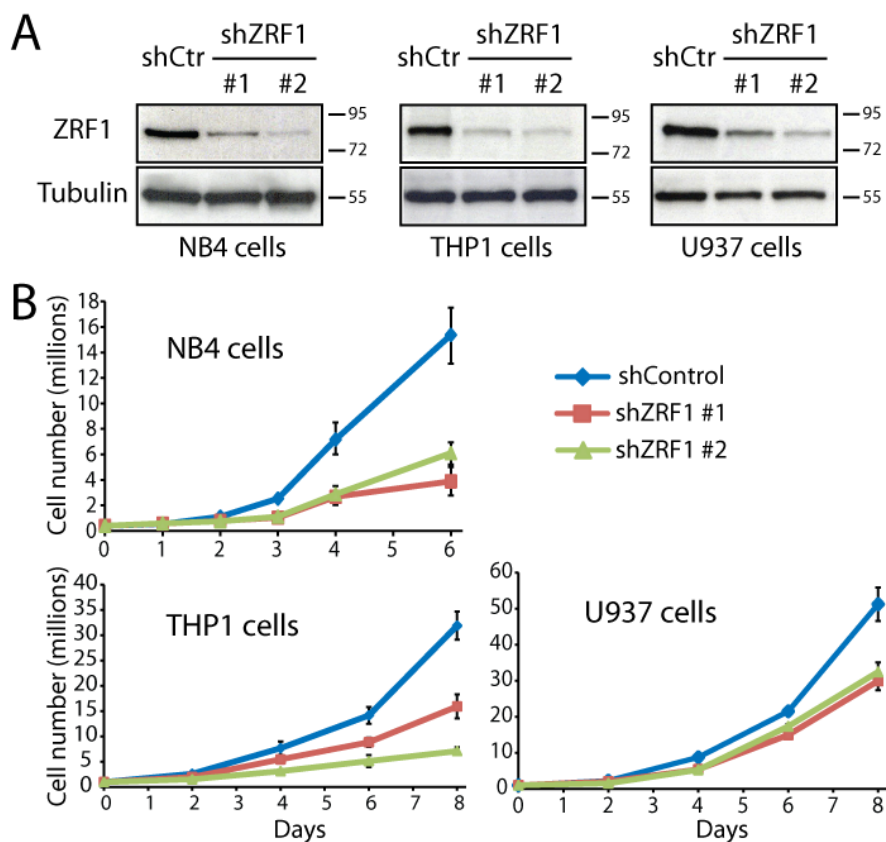



**Figure R2: ZRF1 depletion inhibits cell growth in AML cells.** (**A**) Western blot analysis of ZRF1 in control (shCtr) and ZRF1-depleted (shZRF1 #1 and #2) cells, in three AML cell lines. Tubulin was used as a loading control. (**B**) Growth curves of control and ZRF1-depleted cells. Data are the means ± s.e.m. of three independent experiments.

The four AML cell lines used so far are known to be sensitive to retinoic acid (RA), which causes cell differentiation and proliferation arrest.[29] We then asked whether ZRF1 also regulated cell growth in AML cells that

have a poorer response to RA. Therefore, we used a variant of the NB4 cell line generated under the selective pressure of RA, namely, NB4.007/6.[129] Despite expressing RARα,[130] these cells are about 70 times less sensitive to RA than the original cell line.[129] FACS analysis of the differentiation marker CD11c confirmed that NB4.007/6 cells did not respond to RA concentrations that are sufficient to induce differentiation in the original NB4 cells (**Figure R3A**), although they are sensitive to higher concentrations.[129] We then stably knocked down ZRF1 in these cells and we observed a growth inhibition comparable to the four AML cell lines analyzed previously (**Figure R3B-R3C**).

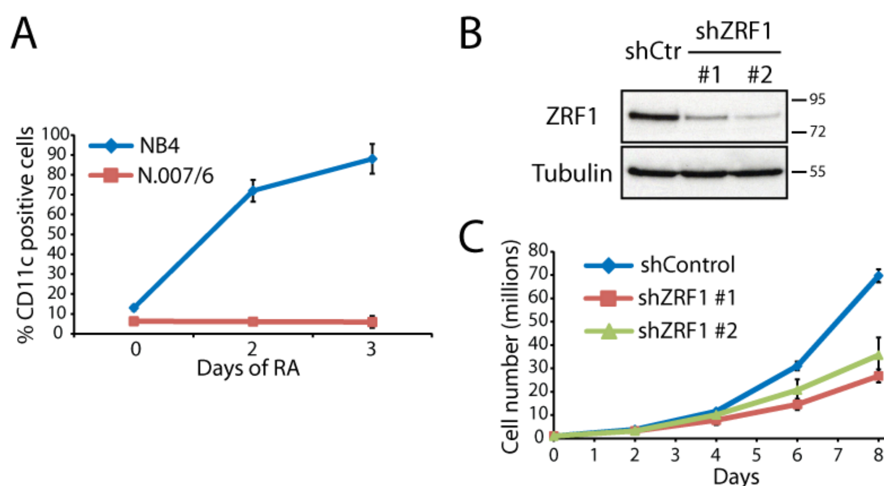



**Figure R3: ZRF1 depletion inhibits cell growth in NB4.007/6 cells.** (**A**) Differentiation assay by CD11c-positive surface marker measured by FACS, in NB4 and NB4.007/6 (N.007/6) cells, untreated (0) or treated with RA (0.1 μM) for 2 and 3 days. Data are the means ± s.e.m. of two independent experiments. (**B**) Western blot analysis of ZRF1 in control (shCtr) and ZRF1-depleted (shZRF1 #1 and #2) NB4.007/6 cells. Tubulin was used as a loading control. (**C**) Growth curves of control and ZRF1-depleted NB4.007/6 cells. Data are the means ± s.e.m. of three independent experiments.

We also analyzed the effect of ZRF1 depletion in a non-leukemic cancer cell line. Specifically, we knocked down ZRF1 in the malignant embryonal carcinoma cell line NT2 and also observed a strong cell growth inhibition (**Figure R4**).

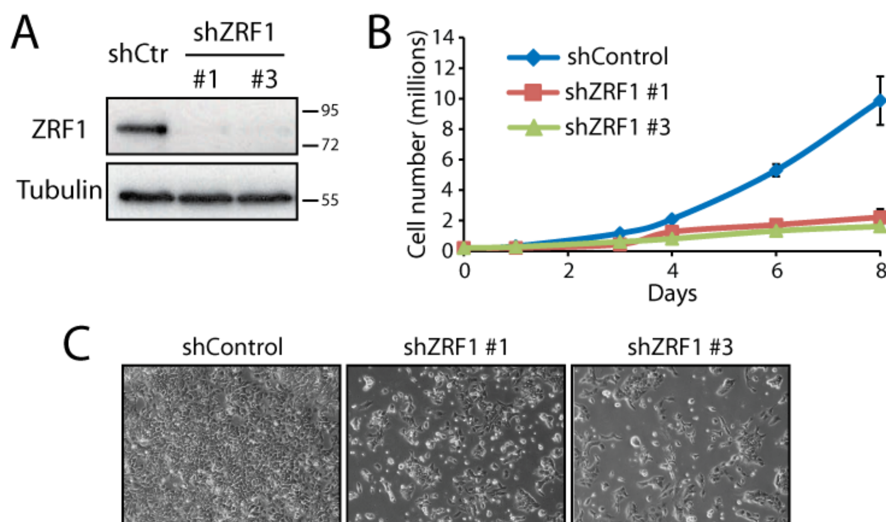



**Figure R4: ZRF1 depletion inhibits cell growth in NT2 cells.** (**A**) Western blot analysis of ZRF1 in control (shCtr) and ZRF1-depleted (shZRF1 #1 and #3) NT2 cells. Tubulin was used as a loading control. (**B**) Growth curves of control and ZRF1-depleted NT2 cells. Data are the means ± s.e.m. of three independent experiments. (**C**) Representative pictures of control and ZRF1-depleted NT2 cells.

We next aimed to study the effect of ZRF1 depletion in combination with RA treatment. As discussed in the *Introduction* section, RA induces cell differentiation and also reduces cell proliferation and increases apoptosis.[29,54] We focused on HL60 cells as a model cell line and performed growth curves in the presence and absence of RA. This assay showed that the effect of ZRF1 depletion in growth inhibition was comparable to the effect observed after RA treatment alone in control cells. Moreover, we found a cooperative effect of RA and ZRF1 knockdown in growth inhibition of leukemic cells (**Figure R5**).

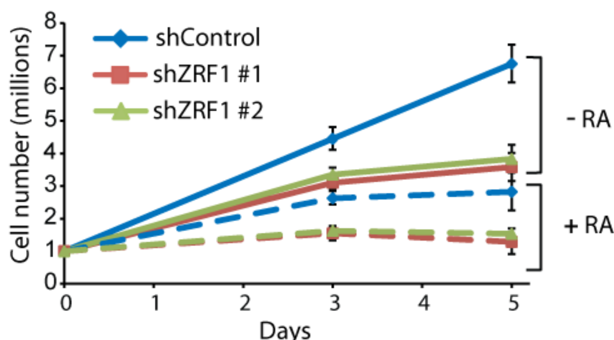



**Figure R5: ZRF1 depletion cooperates with RA in cell growth inhibition.** Growth curves of control (shControl) and ZRF1-depleted (shZRF1) HL60 cells, untreated (solid lines), or treated with RA 1 µM (dashed lines).

## 1.2 ZRF1 depletion inhibits cell proliferation

We next investigated the cause of the observed decrease in cell growth upon ZRF1 depletion. We first studied the proliferation of control and ZRF1-knockdown HL60 cells. Analysis of BrdU incorporation revealed that cell proliferation was significantly decreased in ZRF1-depleted cells compared to control cells. Specifically, we observed a reduction of about 30% in BrdU positive cells (**Figure R6**).

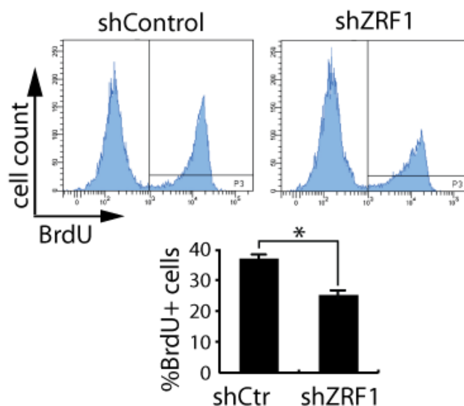



**Figure R6: ZRF1 depletion inhibits cell proliferation in HL60 cells.** BrdU cell proliferation assay in control (shCtr) and ZRF1-depleted (shZRF1) cells. Graphs correspond to a representative experiment. Data are the means ± s.e.m. of six independent experiments. Statistical significance was assessed by a two-tailed Student's t-test, $p = 0.0016$.

We then analyzed cell proliferation in the other four AML cell lines and found a consistent reduction of BrdU incorporation in ZRF1-depleted cells as compared to control cells, ranging from 20% to 40% (**Figure R7**).

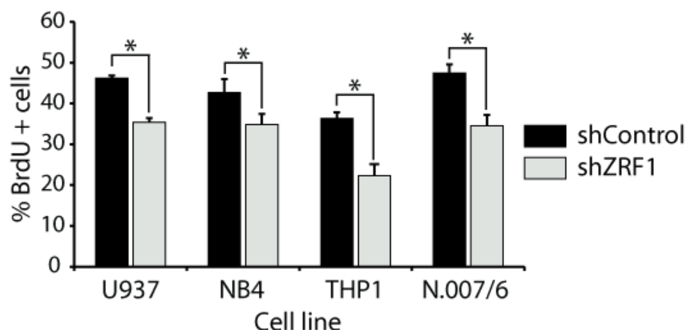



**Figure R7: ZRF1 depletion inhibits cell proliferation in AML cells.** BrdU cell proliferation assay in control (shCtr) and ZRF1-depleted (shZRF1) cells, in four AML cell lines. Data are the means ± s.e.m. of three independent experiments. Statistical significance was assessed by a two-tailed Student's t-test, * $p < 0.05$.

## 1.3 ZRF1 depletion induces cell death

To further understand the basis of the observed decrease in cell growth caused by ZRF1 depletion, we studied cell death in control and ZRF1-knockdown AML cells. We performed trypan blue staining, which is incorporated specifically in dead cells, and found an increased rate of cell death in ZRF1-depleted cells as compared to control cells. We next used Annexin V / Sytox Green double staining followed by FACS analysis to study whether this increase in basal cell death was caused by apoptosis. This assay revealed that indeed ZRF1 depletion induces apoptosis in AML cells (**Figure R8**).

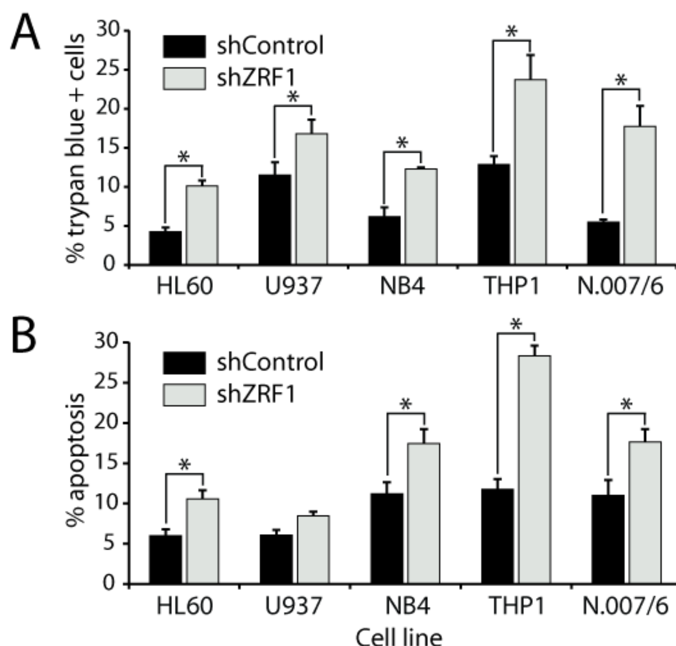



**Figure R8: ZRF1 depletion induces apoptosis in AML cells.** Cell death in control (shControl) and ZRF1-depleted (shZRF1) cells. (**A**) Cell death assay by trypan blue-positive cell count. (**B**) Apoptosis assay determined by FACS after Annexin V / Sytox Green double staining. Apoptotic cells were defined as Annexin V positive and Sytox Green negative cells. Data are the means ± s.e.m. of at least three independent experiments. Statistical significance was assessed by a two-tailed Student's t-test; * $p < 0.05$.

Subsequently, we studied the effect of ZRF1 depletion in combination with RA treatment. We treated control and ZRF1-knockdown HL60 cells with RA and performed trypan blue staining and Annexin V / Sytox Green double staining. We found that, in untreated conditions, cell death was around 4-10% (depending on the method) in control cells and around 10-15% in shZRF1 cells. After treatment with RA for 5 days, cell death increased to 15-25% in control cells and to 40-50% in shZRF1 cells. Therefore, ZRF1 depletion strongly enhanced RA-induced apoptosis, thus decreasing leukemic cell viability up to 50% (**Figure R9**).

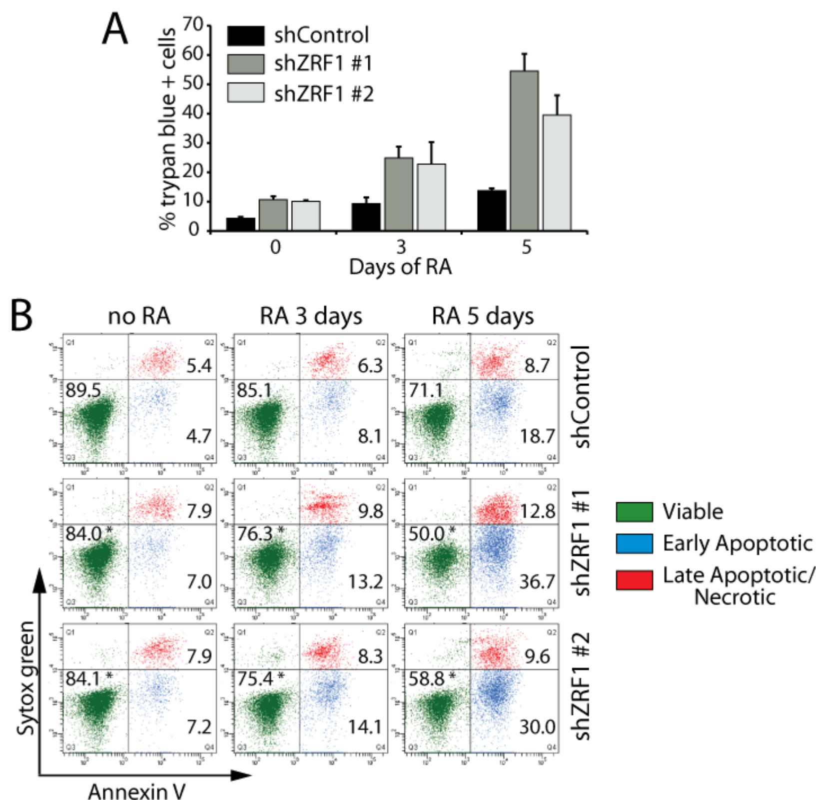



**Figure R9: ZRF1 depletion cooperates with RA in apoptosis induction.** Cell death in control (shControl) and ZRF1-depleted (shZRF1) cells, untreated or treated with RA (1 μM) for 3 or 5 days. (**A**) Cell death assay by trypan blue-positive cell count. Data are the means ± s.e.m. three independent experiments. (**B**) Apoptosis assay determined by FACS after Annexin V / Sytox Green double staining. Viable cells in green (Q3), apoptotic cells in blue (Q4), late apoptotic or necrotic cells in red (Q2). Graphs are representative of three independent experiments; numbers represent percentage of cells. Statistical analysis was performed between the two shZRF1 and the shControl cells at each of the three conditions, by a two-tailed Student's t-test; * $p < 0.05$.

Taken together, these results show that ZRF1 depletion leads to a cell growth inhibition in AML cells due to both a decrease in cell proliferation and an increase in apoptosis. Moreover, these data reveal a cooperative effect of RA treatment and ZRF1 downregulation in growth inhibition and apoptosis induction in leukemic cells.

## 1.4 ZRF1 controls cell differentiation

We next studied the role of ZRF1 in leukemic cell differentiation. We first analyzed the expression of ZRF1 during RA-induced differentiation in HL60 cells. We observed a decrease in ZRF1 mRNA levels upon RA induction, which were reduced by about 50% after three days of treatment. However, this decrease did not result into a reduction in ZRF1 protein levels, which were stable during RA-induced differentiation (**Figure R10**).

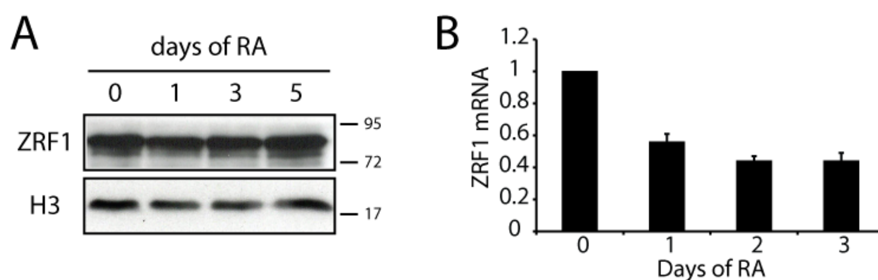



**Figure R10: ZRF1 expression during RA-induced differentiation.** (**A**) Western blot analysis of ZRF1 during HL60 cell differentiation: untreated (0) or treated with RA (1 µM) for 1, 3, or 5 days. Histone H3 was used as a loading control. (**B**) qRT-PCR analysis of ZRF1 in HL60 cells untreated (0) or treated with RA (1 µM) for 1, 2 or 3 days. Results are shown relative to the untreated condition. Data are the means ± s.e.m. of three independent experiments.

We next investigated the effect of ZRF1 depletion in cell differentiation. We stably knocked down ZRF1 in HL60 cells by using four independent shRNA constructs that efficiently downregulated ZRF1 expression (shZRF1 #3 and #4 had a similar efficiency than shZRF1 #1 and #2, see Figure R1). We then analyzed the differentiation status of control and ZRF1-knockdown cells by performing FACS analysis of CD11b (cluster of differentiation 11b), a surface marker belonging to the integrin family that is expressed specifically in differentiated granulocytes. This analysis showed a consistent increase in the rate of basal differentiation after ZRF1 depletion. Specifically, the percentage of CD11b positive cells increased from about 10% in control cells to 16-27% in ZRF1-knockdown cells (**Figure R11A**). We subsequently extended our study to the other four

AML cell lines. We observed a higher proportion of cells expressing CD11b (or CD11c, the typical differentiation marker in NB4 cells) in ZRF1-depleted cells as compared to control cells, which revealed an increased differentiation status (**Figure R11B**).

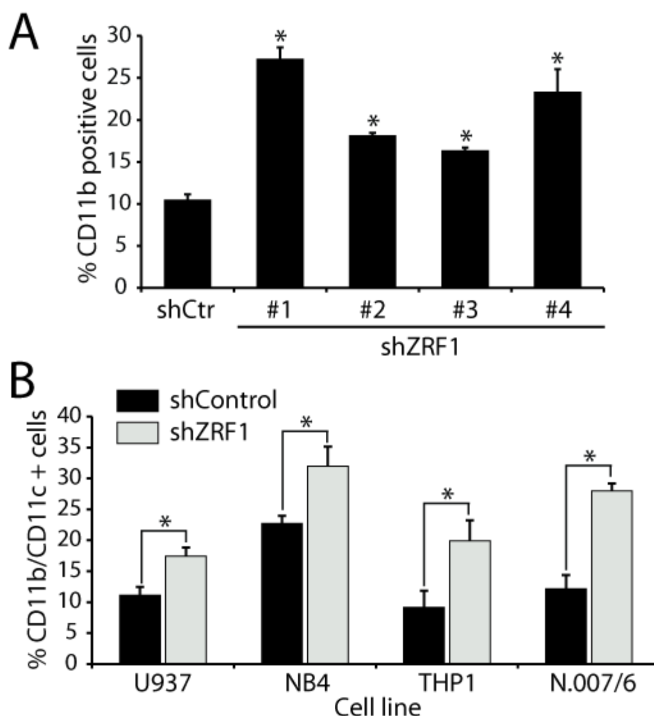



**Figure R11: ZRF1 depletion induces basal cell differentiation.** (**A**) Differentiation assay by CD11b-positive surface marker measured by FACS, in control (shCtr) and ZRF1-depleted (shZRF1) HL60 cells. (**B**) Differentiation assay in control and ZRF1-depleted AML cells; results are shown as percentage of CD11b (or CD11c, in the case of NB4 cells) positive cells. Data are the means ± s.e.m. of at least three independent experiments. Only viable cells were considered for the analysis. Statistical significance was assessed by a two-tailed Student's t-test; * $p < 0.05$.

We then focused on HL60 cells as a model cell line to study the effect of ZRF1 depletion during RA-induced differentiation. Unexpectedly, although ZRF1-knockdown cells had an increased basal differentiation status as compared to control cells, ZRF1 depletion led to a reduction in differentiation potential upon RA treatment. This effect was observed

after one day of RA treatment and was significant from day two (**Figure R12A**). The decrease in differentiation levels in ZRF1-knockdown cells became even clearer when we evaluated the increase in CD11b-positive cells with respect to the untreated situation (**Figure R12B**). In addition, in basal conditions, ZRF1 knockdown not only increased the percentage of CD11b positive cells, but also the level of CD11b expression within them. Conversely, upon RA treatment, ZRF1 knockdown not only decreased the number of CD11b positive cells but also CD11b expression levels within them (**Figure R12C**).

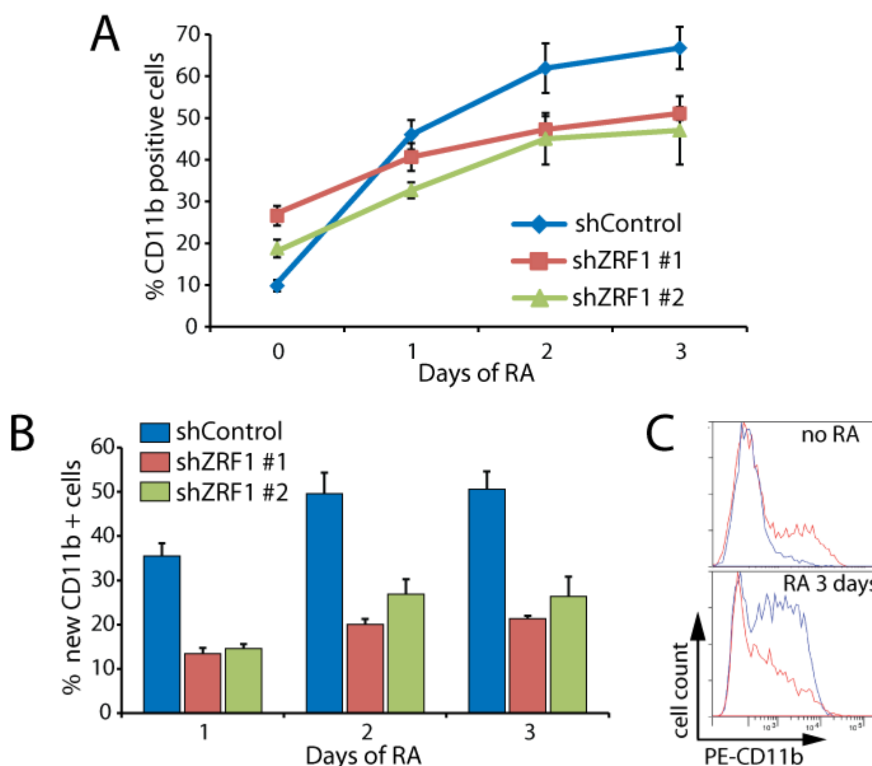



**Figure R12: ZRF1 depletion induces basal differentiation and inhibits differentiation upon RA treatment.** (**A**) Differentiation assay by CD11b-positive surface marker measured by FACS, in control (shCtr) and ZRF1-depleted (shZRF1) HL60 cells, untreated (0) or treated with RA (1 µM) for up to 3 days. Only viable cells were considered for the analysis. Data are the means ± s.e.m. of four independent experiments. (**B**) Differentiation increase with respect to the untreated condition. Data were calculated subtracting in each case the basal differentiation status (i.e. the percentage of CD11b positive cells in untreated condition, corresponding to each cell line). (**C**) FACS profiles of a representative experiment, showing shControl (blue) and shZRF1 #1 (red) cells.

It should be noticed that in all these differentiation experiments, only viable cells were considered for the analysis (which are easily distinguished by FACS), thus excluding the effect of ZRF1 depletion in apoptosis induction.

To support these results we evaluated cell differentiation using nitroblue tetrazolium (NBT) assay, a technique based in the ability of mature granulocytes to reduce the NBT compound. In basal conditions, we observed a slight increase in NBT-positive cells in ZRF1-depleted cell lines as compared with control cells. The fact that the difference between CD11b-positive shZRF1 and shControl cells is higher than for the case of the NBT assay fits with previous reports that showed that CD11b expression is detected faster than NBT-positive staining upon RA induction, and that other differentiation inducers increase CD11b expression to a higher extent than NBT staining.[131,132] In contrast, after treatment with RA for two days, we observed a decrease in NBT-positive staining in ZRF1-depleted cells as compared to control cells, which was consistent with our CD11b expression data (**Figure R13**).

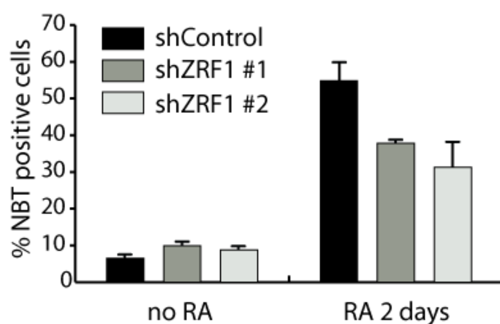



**Figure R13: NBT differentiation assay in ZRF1-depleted cells.** Differentiation assay by nitroblue tetrazolium (NBT) reduction test in control and ZRF1-depleted HL60 cells, untreated (no RA) or treated with RA (1 μM) for 2 days. Data are the means ± s.e.m. of three independent experiments.

We further studied the role of ZRF1 in RA-induced differentiation by analyzing the effect of ZRF1 depletion in later differentiation time points. We extended our CD11b FACS analysis up to five days of RA treatment, when HL60 differentiation becomes maximal, and we observed a

significant decrease of CD11b expression in ZRF1-depleted cells as compared to control cells (**Figure R14A**). We additionally analyzed the expression of CD11c, a late differentiation marker in HL60, by Western blot. In consistency with our previous data, we observed a decreased expression of CD11c in ZRF1-depleted cells, which was significant at day five of RA treatment (**Figure R14B**).

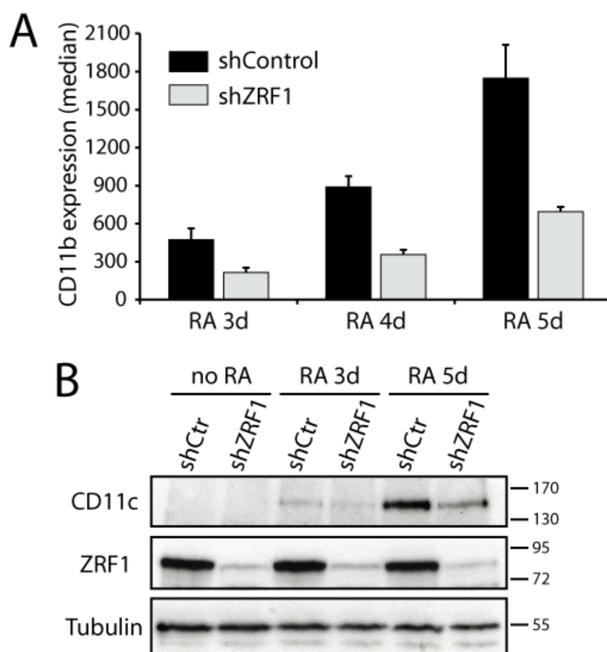



**Figure R14: ZRF1 depletion inhibits RA-induced differentiation at late stages.** (**A**) Differentiation assay by CD11b-positive surface marker measured by FACS, in control (shControl) and ZRF1-depleted (shZRF1 #2) HL60 cells, treated with RA (1 µM) for 3, 4, and 5 days. Results are shown as median fluorescence intensity. Data are the means ± s.e.m. of two independent experiments. (**B**) Western blot analysis of the differentiation marker CD11c and ZRF1 in control and ZRF1-depleted HL60 cells, untreated (no RA) or treated with RA (1 µM) for 3 or 5 days. Tubulin was used as a loading control.

In summary, our data show that ZRF1 depletion increases the basal differentiation state of leukemic cells and inhibits proper differentiation upon RA treatment.

We next investigated the effect of increasing ZRF1 expression in RA-induced cell differentiation. We thus generated an HL60 cell line that stably overexpressed ZRF1 and the corresponding control cell line (infected with an empty vector). As observed in **Figure R15A**, we obtained a mild expression of exogenous HA-tagged ZRF1, with about 50% overexpression as compared to endogenous ZRF1 levels. We then performed differentiation assays by treating control and ZRF1-overexpressing cells with RA. Consistent with our results upon ZRF1-depletion, FACS analysis of CD11b expression showed that ZRF1 overexpression increased the cell differentiation potential following RA administration (**Figure R15B**).

Since our HA-ZRF1 plasmid contained GFP as a reporter, we were able to measure CD11b expression in the cells expressing the highest levels of ZRF1. We first checked that ZRF1 expression correlated with the expression of GFP by sorting out the 'GFP high' population. By analyzing ZRF1 mRNA and protein levels of this 'GFP high' population, we found that indeed these cells expressed higher levels of ZRF1 as compared to the whole ZRF1-overexpressing cell line (**Figure R15C**). CD11b expression analysis showed that 'GFP high / ZRF1 high' cells had the highest rate of differentiation, which confirmed that ZRF1 overexpression increased RA-induced differentiation and indicated that this effect was dose dependent (**Figure R15B**).

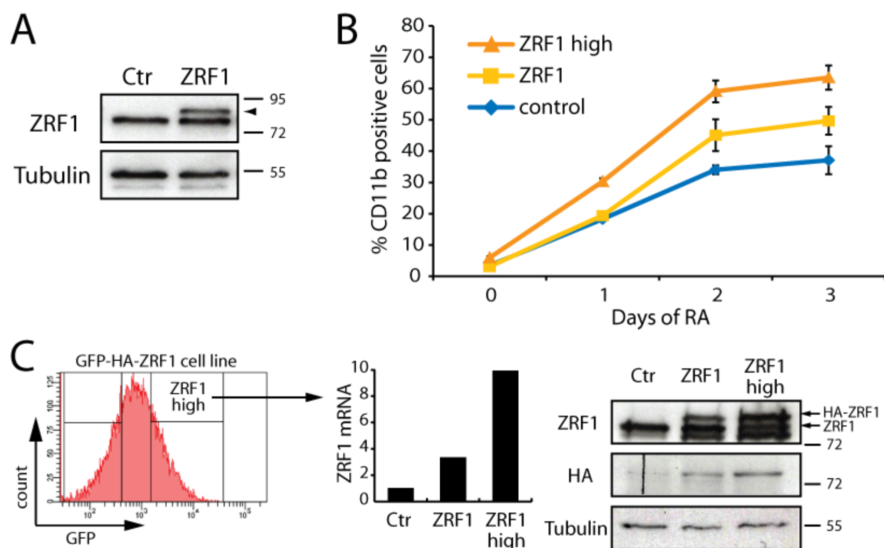



**Figure R15: ZRF1 overexpression enhances RA-induced differentiation.** (**A**) Western blot analysis of ZRF1 in HL60 cells infected with GFP-HA-empty (control, Ctr) or GFP-HA-ZRF1 (ZRF1) expression vector. Arrowhead indicates HA-ZRF1 fusion protein. Tubulin was used as a loading control. (**B**) Differentiation assay (CD11b-positive surface marker) in control and ZRF1-overexpressing HL60 cells, untreated (0) or treated with RA (100 nM) for up to 3 days. ZRF1 high corresponds to the cells overexpressing the highest levels of ZRF1. Only viable cells were considered for the analysis. Data are the means ± s.e.m. of four independent experiments. (**C**) Left: FACS plot showing GFP expression of ZRF1-overexpressing cells (GFP-HA-ZRF1 cell line); 'GFP high'/'ZRF1 high' cells were defined as the 25% of cells with the highest GFP expression. Right: qRT-PCR analysis of ZRF1, and Western blot analysis of ZRF1 and HA, after sorting 'ZRF1 high' cells, as compared with control (Ctr) and total ZRF1-overexpressing cells (ZRF1). Tubulin was used as a loading control. qRT-PCR data were calculated relative to control.

On the other hand, ZRF1 overexpression did not change the differentiation status in basal conditions. It should be noticed that, in the absence of RA, the amount of differentiated cells in normal situations (i.e. in wild-type cells or cells infected with empty plasmid) is already low (about 5–10%). This can be considered to be background rather than real differentiated cells and thus a differentiation status lower than this would not be expected.

Taken together, these results show that ZRF1 regulates cell differentiation in AML cells. ZRF1 seems to have a dual role, as a differentiation repressor in basal conditions but then switching to an activator following RA induction. Interestingly, as mentioned in the *Introduction* section, previous studies have reported a similar dual function of RARα in differentiation.[32]

# 2. ZRF1 regulates RA target gene expression

## 2.1 Functional overlap between ZRF1 and RA

In order to study the molecular mechanisms underlying the effect of ZRF1 in cell proliferation, apoptosis and cell differentiation, we performed a genome-wide expression analysis in ZRF1-depleted and control HL60 cells. We did the study in three different conditions: untreated (RA0) and treated with RA for 4 h (RA4h) or 48 h (RA48h). These early and late RA treatment time points were selected to be able to study both the direct effect of RA in transcription (RA4h) and the transcriptome at the onset of RA-induced differentiation (RA48h).

Comparing ZRF1-depleted and control cells, we found that the expression of more than 5000 genes was altered in each of the three RA conditions, with approximately half downregulated and half upregulated. Using our gene expression array data, we also extracted the RA-activated genes by comparing untreated control cells to RA4h-treated control cells and found 1075 genes to be direct RA targets (**Figure R16**).

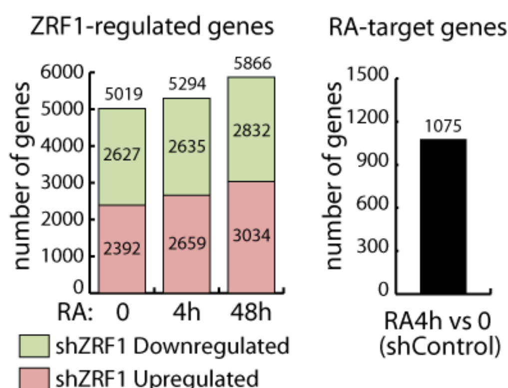



**Figure R16: Gene expression microarray in HL60 cells.** Left: number of genes downregulated and upregulated in ZRF1-depleted cells as compared with control cells, at the three time points of the experiment: untreated or treated with RA (1 μM) for 4 h or 48 h. Right: number of RA direct target genes defined as the genes upregulated at RA 4 h (RA4h) as compared with untreated (RA0), in control (shControl) cells. The experiment was performed in quadruplicates.

To determine the pathways and networks that were significantly regulated by RA and ZRF1, we next used the Ingenuity Pathway Analysis (IPA) software. Interestingly, we found that the cohort of genes regulated by ZRF1 both in basal and RA conditions were in the same functional categories as the genes directly regulated by RA. In particular, among the five most significantly overrepresented categories both for RA- and ZRF1-regulated genes, we found 'cell development', 'cell growth and proliferation' and 'cell death and survival' (**Figure R17**). This analysis supported our previous findings of the cellular functions regulated by ZRF1 (see chapter 1 of the *Results* section). In addition, these data suggested a functional link between ZRF1 and the RA pathway.

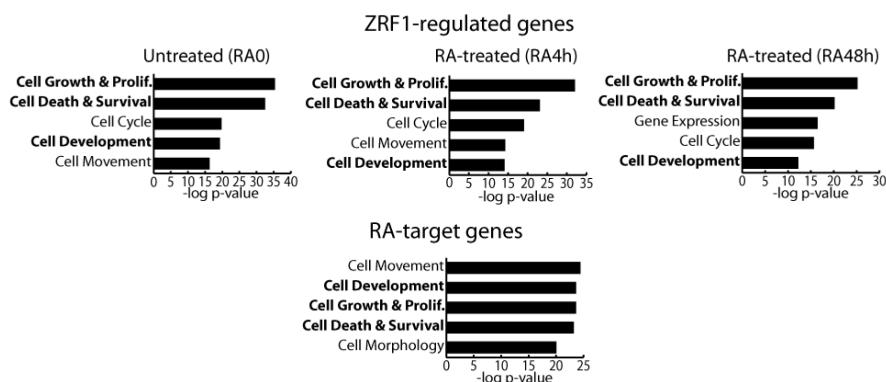



**Figure R17: ZRF1 and RA regulate very similar gene functional categories.** Ingenuity Pathway Analysis (IPA) of the gene expression microarray showing the top five most significantly overrepresented categories of the cohort of RA direct targets (as defined in Figure R16) and ZRF1-regulated genes (both up- and downregulated) corresponding to RA 0, RA 4 h and RA 48 h. Categories correspond to IPA 'Molecular and Cellular Functions' classification. In bold, the categories shared in the four analyses.

To further understand the link between ZRF1 and the RA pathway, we next studied the overlap between the 1075 direct RA target genes and the genes regulated by ZRF1, both in basal and RA conditions. Remarkably, we found a very significant overlapping, with almost half of the RA targets in HL60 cells co-regulated by ZRF1 (**Figure R18**).

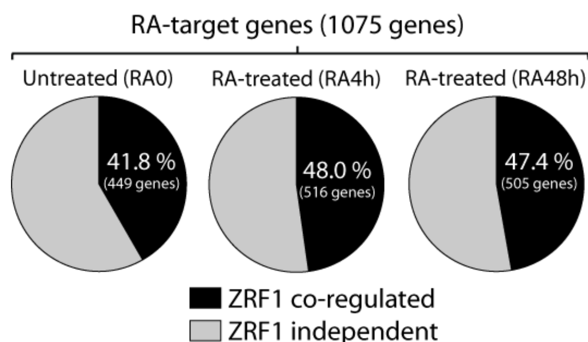
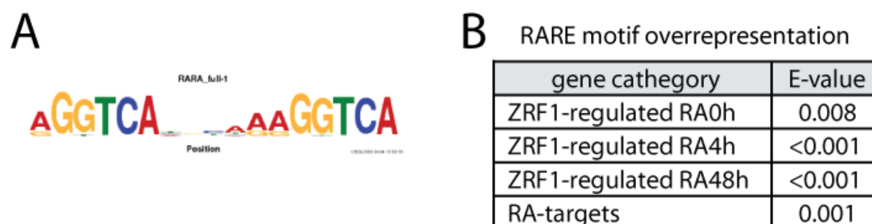



**Figure R18: ZRF1 regulates almost half of the RA target genes.** Proportion of RA direct target genes co-regulated by ZRF1 (comprising both upregulated and downregulated genes in shZRF1 cells as compared to shControl) in the three experimental conditions: RA 0, RA 4 h and RA 48 h. Statistical analysis by a Fisher's test showed that the overlap was very significant, with $p < 2{,}2 \times 10^{-16}$.

We then asked whether the genes regulated by ZRF1 had an enrichment of the retinoic acid responsive element (RARE), the DNA sequences recognized by RA receptors (RARs).[29] We used the Clover software[133] to perform a motif analysis of the promoters of ZRF1-regulated genes and found a significant overrepresentation of the RARE sequence (**Figure R19**). This result corroborated the role of ZRF1 in the regulation of RA target genes.




| gene cathegory | E-value |
|---|---|
| ZRF1-regulated RA0h | 0.008 |
| ZRF1-regulated RA4h | <0.001 |
| ZRF1-regulated RA48h | <0.001 |
| RA-targets | 0.001 |

**Figure R19: The RARE motif is overrepresented in the promoters of ZRF1-regulated genes.** (**A**) For the analysis, consensus sequences corresponding to the different RARs and RXRs motifs were used, as described before.[134] The image shows the consensus sequence corresponding to RARα (**B**) Clover bioinformatic analysis of RARE motif overrepresentation in the promoters of ZRF1-regulated genes when compared with a random group, at the three RA time points (0, 4, and 48 h). Promoters were defined as the region 5 kilobases upstream of the transcription start site. RA direct targets were used as a positive control. The four E-values indicate significant overrepresentation.

Taken together, these results show that ZRF1 regulate a very significant proportion of RA target genes and reveal an important functional link between ZRF1 and the RA pathway.

## 2.2 Dual role of ZRF1 in transcriptional regulation of RA target genes

We further analyzed the importance of ZRF1 in the regulation of the RA pathway by overlapping the RA target genes with the genes upregulated or downregulated in ZRF1-knockdown cells as compared to control cells, in the three experimental conditions. These analyses reveled that, overall, ZRF1 carried out opposite transcriptional roles in untreated and RA-treated cells, specially comparing the basal (RA0) and the 48 h RA treatment (RA48h) conditions (**Figure R20** and **Figure R21**).

As shown in Figure R20, in the absence of RA, 29.5% of the RA target genes were upregulated in the ZRF1 knockdown cells while 12.3% were downregulated. In contrast, with 48 h RA treatment, 29.7% of RA target genes were downregulated in the ZRF1-depleted cells while 17.7% were upregulated. With 4 h RA treatment, we observed an intermediate pattern, with nearly the same percentage of upregulated genes than in basal conditions (30.6%) and an increase in downregulated genes (17.4%).

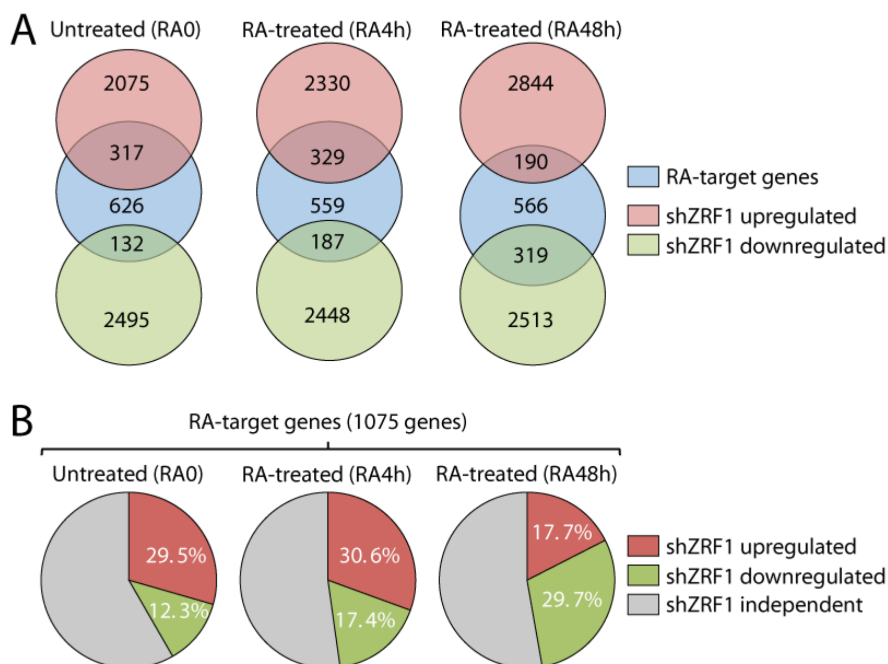



**Figure R20: Overlap of RA targets and ZRF1-regulated genes.** (**A**) Venn diagrams of RA-target genes and genes upregulated and downregulated in shZRF1 as compared with shControl cells, either untreated or treated with RA for 4 h or 48 h. Total number of genes are indicated. (**B**) Proportion of RA-target genes upregulated and downregulated in shZRF1 cells as compared to shControl, corresponding to RA 0, RA 4 h and RA 48 h.

We then focused on the subsets of genes co-regulated by RA and ZRF1 in untreated (RA0) and 48 h RA-treatment (RA48h) conditions. As observed in the heat-map (Figure R21), in the absence of RA (RA0), 70.6% of the 449 co-regulated genes were upregulated in the ZRF1 knockdown cells. In contrast, with RA treatment (RA48h), 62.7% of the 505 co-regulated genes were downregulated in the ZRF1 depleted cells. These data suggest that ZRF1 works predominantly as a repressor in basal conditions and mainly as an activator in RA-treated cells.

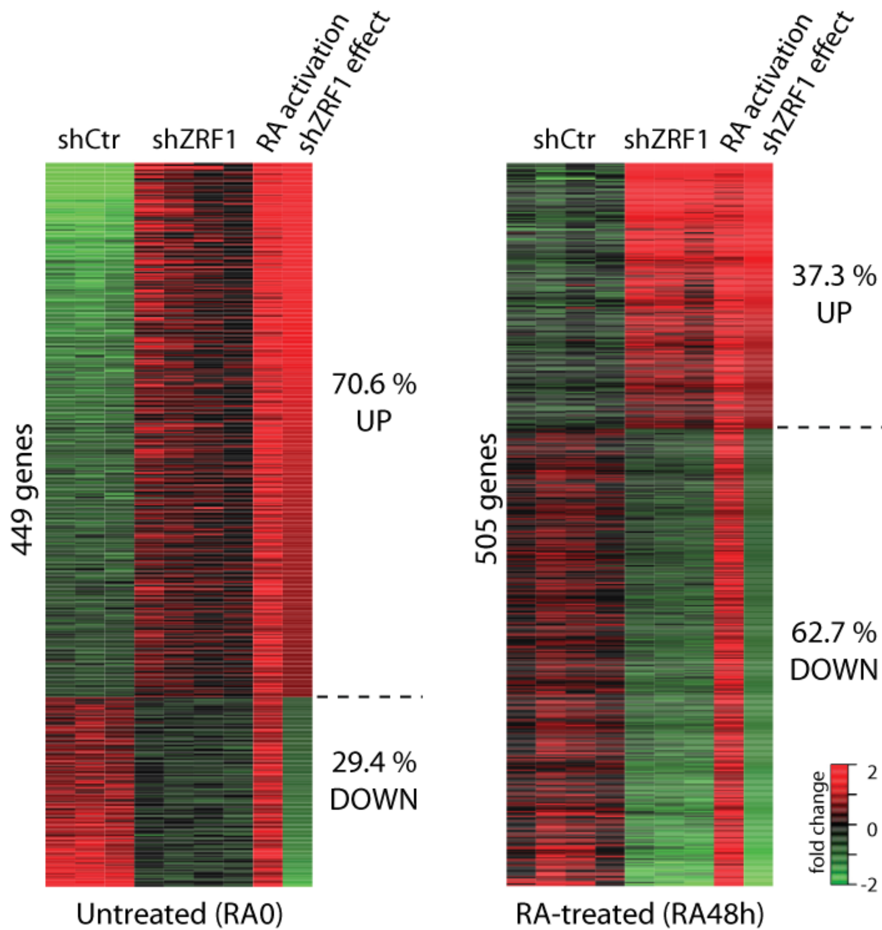



**Figure R21: Dual role of ZRF1 in transcriptional regulation of RA target genes in basal and RA conditions.** Microarray heat-map of RA and ZRF1 co-regulated genes corresponding to untreated cells (RA0, left) and RA-treated cells (RA48h, right). In each case, the first columns corresponds to the expression levels of control (shCtr) and ZRF1-depleted (shZRF1) replicates; the last two columns correspond to the direct effect of RA (RA activation: comparing the expression at RA4h with RA0, in control cells) and the effect of ZRF1 (shZRF1 effect: comparing shControl with shZRF1 cells). Genes were sorted by how they were affected by shZRF1, from the most upregulated to the most downregulated. The percentage of genes upregulated (UP) and downregulated (DOWN) in each case are shown. For gene expression validation, see Figures R25 and R26.

We next analyzed the overlap between the RA target genes upregulated and downregulated by ZRF1 knockdown in untreated (RA0) versus RA-treated (RA48h) conditions. These data revealed that ZRF1 appears to work either as a repressor or as an activator in different subsets of genes. In fact, the two main groups observed in Figure R21 (i.e. 'RA0 upregulated' and 'RA48h downregulated') showed a low overlap, with only 20 genes switching from being upregulated at RA 0 to downregulated at RA 48 h (**Figure R22A**).

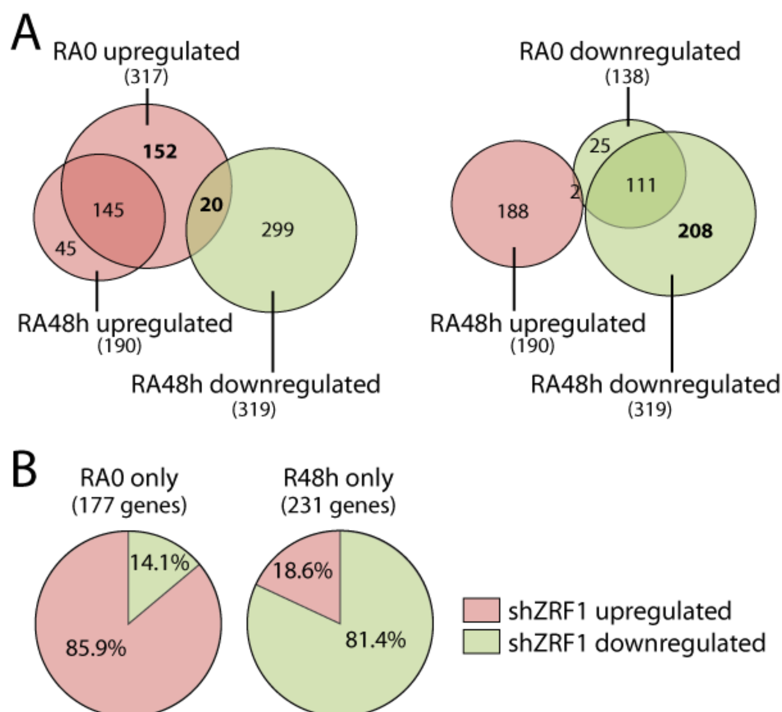



**Figure R22: ZRF1 works either as a repressor (predominantly in untreated conditions) or as an activator (predominantly in RA-treated conditions) in different subsets of genes.** (**A**) Overlap of shZRF1-regulated genes (upregulated and downregulated) between untreated (RA0) and RA-treated (RA48h) conditions. About 54% [(152+20)/317] of the genes upregulated in shZRF1 at RA 0 were no longer upregulated at RA 48 h; among these, 12% [20/(152+20)] were downregulated at RA 48 h. About 65% [208/319] of the genes downregulated in shZRF1 at RA 48 h were not downregulated at RA 0. Gene numbers are shown in brackets. (**B**) Genes whose expression was regulated exclusively at RA 0 (RA0 only) or RA 48 h (RA48h only), and the proportion of them upregulated and downregulated in shZRF1 as compared with shControl cells.

Further analysis of these data reinforced the finding that ZRF1 worked predominantly as a repressor at RA 0 and predominantly as an activator at RA 48 h. Specifically, about 54% of the genes upregulated in ZRF1-depleted cells at RA 0 were no longer upregulated at RA 48 h; among these, 12% even became downregulated (the 20 genes mentioned above). In contrast, at RA 48 h, a new group of genes that were downregulated by shZRF1 appeared. In particular, about 65% of the genes downregulated in shZRF1 at RA 48 h were not downregulated at RA 0 (**Figure R22A**).

We then focused on the RA target genes regulated by ZRF1 exclusively at RA 0 or exclusively at RA 48 h (i.e. the genes whose expression was changed by shZRF1 only in one of the conditions). These data showed that 85.9% of the genes regulated exclusively at RA 0 were upregulated in ZRF1-depleted cells. In contrast, 81.4% of the genes regulated exclusively at RA 48 h were downregulated in ZRF1-knockdown cells (**Figure R22B**).

We then further analyzed the function of ZRF1 in gene induction during the process of RA-induced differentiation. To do that, we first obtained the ratios between gene expression at RA 48 h and RA 0 in ZRF1-depleted and control cells. Second, we compared the ratios corresponding to both cell lines. This allowed us to define two subset of genes in which ZRF1 worked as an activator (when the ratio was higher in shControl than in shZRF1 cells) or as a repressor (when the ratio was lower in shControl than in shZRF1 cells). Finally, we overlapped these two subsets of genes with the RA target genes that we had defined before (see Figure R16). This analysis confirmed the role of ZRF1 as an activator during RA-induced differentiation. Specifically, we found that 40.2% of RA targets depended on ZRF1 for proper gene induction after RA administration (**Figure R23**).

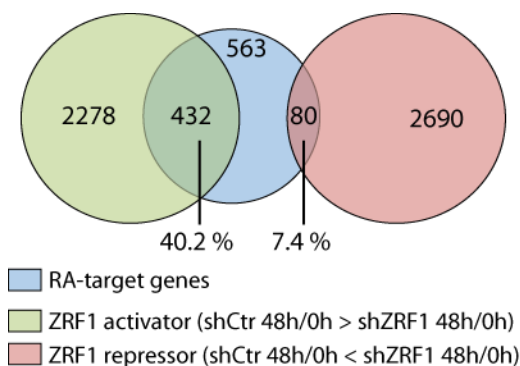



**Figure R23: ZRF1 acts as an activator during RA-induced differentiation.** Venn diagram of RA targets with the genes in which ZRF1 acts as an activator (defined as the genes in which the ratio RA48h/RA0 is higher in shControl that in shZRF1 cells) or as a repressor (defined as the genes in which the ratio RA48h/RA0 is higher in shZRF1 that in shControl cells). Percentages refer to RA-target genes. For gene validation, see Figure R27.

Taken together, these results show that ZRF1 works both as a transcriptional repressor and as a transcriptional activator of RA target genes, being the repressive function predominant in basal conditions and the activating function predominant in RA-treated cells.

## 2.3 Differential transcriptional regulation of proliferation, apoptosis and differentiation by ZRF1

As shown in chapter 1 of the *Results* section, ZRF1 regulates proliferation, apoptosis and differentiation in AML cells. Moreover, our study on the gene functional categories regulated by ZRF1 (see Figure R17) confirmed these data at the transcriptional level. However, our results in AML cell lines showed that ZRF1 had a dual role in cell differentiation while it had a single function in proliferation and apoptosis. Specifically, ZRF1 depletion induced basal differentiation and inhibited differentiation potential upon RA treatment and, in contrast, led to proliferation inhibition and apoptosis induction in both conditions.

We asked whether these observations could be supported by our genome-wide expression data. Thus, we used the DAVID software to further analyze the ZRF1/RA-coregulated genes in the absence (RA0) or presence (RA48h) of RA. One useful characteristic of this software is that it gives information about positive and negative regulation on the gene ontology (GO) categories. Focusing on the categories related to apoptosis, proliferation and differentiation, this analysis showed that ZRF1 depletion up-regulated 'positive regulators of apoptosis' and 'negative regulators of proliferation', both in untreated (RA0) and RA-treated (RA48h) conditions. In contrast, ZRF1 depletion upregulated differentiation regulators in basal conditions and downregulated this group of genes in RA-treated cells (**Figure R23**). These data correlated nicely with the fact that ZRF1 depletion induced apoptosis and inhibited proliferation both in untreated and RA-treated conditions, while it had a dual effect in differentiation.

DIFFERENTIATION

| | RA-targets shZRF1 UP-regulated | RA-targets shZRF1 DOWN-regulated |
|---|---|---|
| RA0 | positive reg. of developmental process<br>hemopoietic or lymphoid development<br>myeloid cell differentiation | - |
| RA48h | - | hemopoietic or lymphoid development<br>myeloid cell differentiation |

APOPTOSIS

| | RA-targets shZRF1 UP-regulated | RA-targets shZRF1 DOWN-regulated |
|---|---|---|
| RA0 | positive regulation of apoptosis | - |
| RA48h | positive regulation of apoptosis | - |

PROLIFERATION

| | RA-targets shZRF1 UP-regulated | RA-targets shZRF1 DOWN-regulated |
|---|---|---|
| RA0 | negative regulation of cell proliferation | - |
| RA48h | negative regulation of cell proliferation | - |

**Figure R24: GO analysis of ZRF1/RA co-regulated genes.** Gene ontology (GO) analysis of RA-targets upregulated and downregulated in shZRF1 cells as compared with shControl, both in untreated (RA0) and in RA-treated (RA48h) conditions. The most overrepresented categories corresponding to the groups of differentiation, apoptosis, and proliferation are shown, with only the categories that include 'positive regulation' or 'negative regulation' for apoptosis and proliferation. Only the categories that were also overrepresented in the analysis of the whole group of RA-targets are shown. In the cases of empty boxes, no categories fulfilling these criteria were identified.

We next further analyzed the two main cohorts of genes identified before (see Figure R20 and R21): on the one hand, the RA targets that were upregulated by the ZRF1 knockdown in basal conditions and, on the other hand, those downregulated by ZRF1 depletion upon 48 h of RA treatment. As predicted previously, these lists of genes included important regulators of development and differentiation in the myeloid linage, such as ICAM1, HOXA5, RGS2, THBD, CSF1R, ICAM4, ICAM3, CSF3R, CD11c, DHRS3, HOXA4 and GATA6. Their expression was measured in independent experiments by quantitative PCR with reverse transcription (qRT-PCR), and validated in both ZRF1-depleted cell lines (**Figure R25**). As mentioned above, the RA target genes upregulated by shZRF1 at RA 0 and those downregulated at RA 48 h correspond to different subsets of genes, although some of them are common.

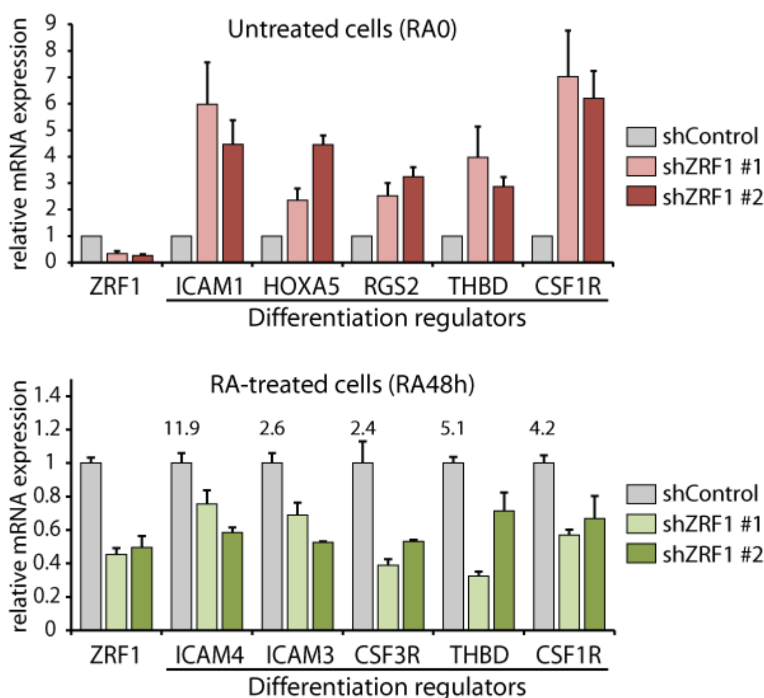



**Figure R25: Dual function of ZRF1 in transcriptional regulation of differentiation genes.** qRT-PCR analysis of representative RA-target genes previously reported to be involved in differentiation, in shZRF1 relative to shControl cells. Top panel: genes upregulated in shZRF1 at RA0. Bottom panel: genes downregulated in shZRF1 at RA48h; the numbers above the shControl bars correspond to the fold induction as compared with untreated cells. Expression was normalized to the PUM1 housekeeping gene. Data are as the means ± s.e.m. of four independent experiments.

We also analyzed the RA target genes upregulated by ZRF1 knockdown in both untreated and RA-treated cells. As predicted previously, and supporting the phenotypical effects observed upon ZRF1 depletion (see chapter 1 of the *Results* section) this list of genes included important positive regulators of apoptosis and negative regulators of proliferation. Among the positive regulators of apoptosis, we found genes such as several caspases (CASP8 and CASP10), HIPK2, CD38 and NLRC4. The negative regulators of proliferation included genes such as CDK inhibitors (CDKN1C), RUNX3, BTG2 and SMAD3. Their expression was measured in independent experiments by qRT-PCR (**Figure R26**).

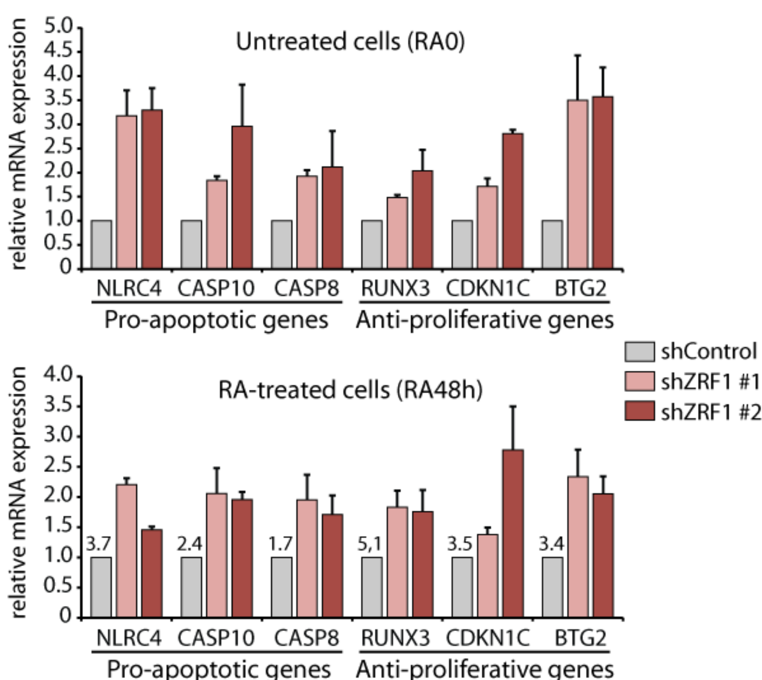



**Figure R26: ZRF1 represses positive regulators of apoptosis and negative regulators of proliferation.** qRT-PCR analysis of representative RA-target genes previously reported to be involved in apoptosis induction or proliferation inhibition, upregulated in shZRF1 relative to shControl, in both untreated (RA0) and RA-treated (RA48h) conditions. The numbers above the shControl bars in RA 48 h correspond to the fold induction as compared to RA 0. Expression was normalized to the PUM1 housekeeping gene. Data are the means ± s.e.m. of three independent experiments.

Furthermore, among the genes downregulated upon ZRF1 depletion in both untreated and RA-treated cells we found important regulators of proliferation, but this time positive regulators of this process, such as E2F2, cyclin E2 and CDC25C. This observation also correlated with the effect of ZRF1 knockdown on cell proliferation inhibition that we previously characterized.

Additionally, we also validated the effect of ZRF1 depletion on RA-mediated gene induction. As shown in Figure R23, about 40% of RA target genes require ZRF1 for proper gene activation. This gene subset included some important differentiation regulators such as ICAM1, THBD, RGS2, CSF1R, ICAM4, CD11c, DHRS3 and GATA6. By measuring their expression in independent experiments, we found that all these genes had a decreased gene induction in the absence of ZRF1, as shown in **Figure R27**.

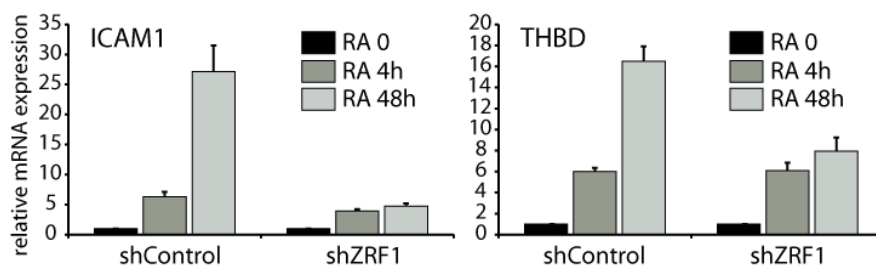



**Figure R27: ZRF1 is an activator of differentiation regulators during RA induction.** qRT-PCR analysis of two representative RA-target genes previously reported to be involved in differentiation, during RA-mediated gene activation, in shZRF1 and shControl cells. Results are shown relative to the untreated condition (RA 0) for each cell line. Data are the means ± s.e.m. of four independent experiments.

Finally, we extended our study to NB4 cells, in which we validated some of the data obtained in our microarray by analyzing the expression of several RA target genes involved in differentiation regulation. Similarly to HL60 cells, we observed that, in basal conditions, several of these genes were upregulated by ZRF1 knockdown while, in RA-treated cells, they were downregulated by ZRF1 depletion (**Figure R28**).

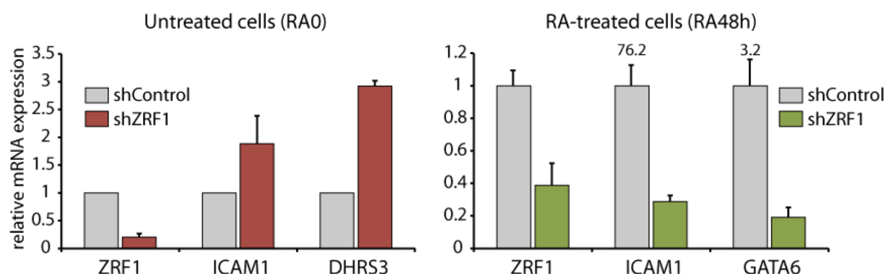



**Figure R28: Dual function of ZRF1 in transcriptional regulation of differentiation genes, in NB4 cells.** qRT-PCR analysis of representative RA-target genes previously reported to be involved in differentiation, in shZRF1 relative to shControl cells. Left panel: genes upregulated in shZRF1 at RA0. Right panel: genes downregulated in shZRF1 at RA48h; the numbers above the shControl bars correspond to the fold induction as compared with untreated cells. Data are as the means ± s.e.m. of four independent experiments.

Taken together, these data confirm, at the gene expression level, our previous finding of the cellular functions regulated by ZRF1. Thus, our results indicate that ZRF1 (*i*) works as a positive regulator of proliferation mainly by repressing anti-proliferative genes; (*ii*) works as a negative regulator of apoptosis mainly by repressing pro-apoptotic genes; and (*iii*) has a dual function in the regulation of differentiation by repressing differentiation regulators in basal conditions and activating this group of genes in RA-treated cells.

# 3. Transcriptional regulation by ZRF1: interaction with RARα and other molecular mechanisms

## 3.1 ZRF1 interacts with RARα

Our genome-wide expression analysis showed that ZRF1 is an important regulator of the RA transcriptome, controlling almost half of the RA target genes. As mentioned in the *Introduction* section, the RAR family of nuclear receptors mediates the physiological effects of RA.[54] As previously reported, RARα is the main isoform of RAR expressed in myeloid leukemic cells.[45] We confirmed these observations by analyzing the expression of the three RARs in our main cellular model, the HL60 cells (**Figure R29**).

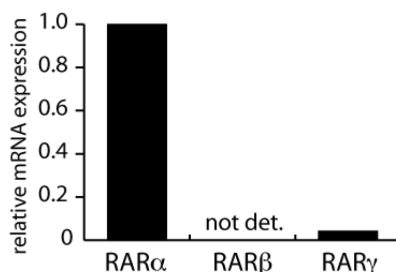



**Figure R29: RARα in the main RAR in HL60 cells.** qRT-PCR analysis of the three RAR isoforms in HL60 cells. Data are represented relative to RARα. RARβ expression was not detectable (not det.).

Given the important effect that ZRF1 depletion had on RA-regulated gene network, we hypothesized that ZRF1 controls RA target gene expression through its interaction with RARα. We thus performed pulldown experiments using His-ZRF1 recombinant protein and nuclear extracts obtained from HEK293T cells. This analysis suggested that ZRF1 and RARα indeed interacted.

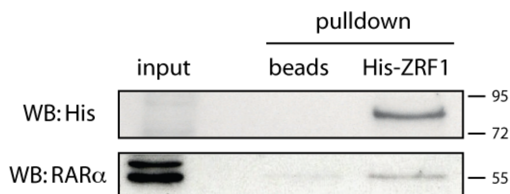



**Figure R30: ZRF1 interacts with RARα.** His-pulldown assay with recombinant His–ZRF1 and 293T nuclear extracts, followed by Western blot analysis of His and RARα. Empty beads were used as a control.

We next studied the interaction of ZRF1 and RARα in HL60 cells by performing endogenous co-immunoprecipitation (co-IP) assays. Importantly, these experiments confirmed the ZRF1-RARα interaction. Specifically, we observed this interaction by immunoprecipitating RARα and co-eluting ZRF1 as well as by immunoprecipitating ZRF1 and co-eluting RARα (**Figure R31**).

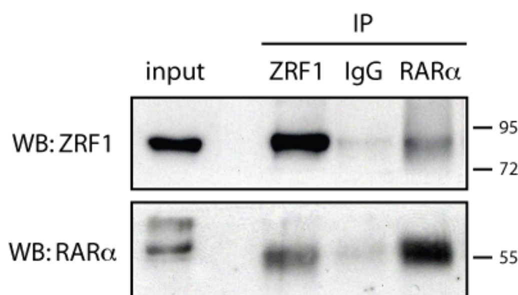



**Figure R30: ZRF1 interacts with RARα in HL60 cells.** Co-immunoprecipitation (IP) assay of endogenous RARα and ZRF1 in HL60 cells, followed by western blot. IgG was used as a negative control.

We then asked whether the interaction between ZRF1 and RARα was direct or indirect. To address this question, we produced recombinant proteins, His-ZRF1 and GST-RARα, and performed *in vitro* pulldown assays. These experiments showed that indeed ZRF1 interacted directly with RARα. Furthermore, we investigated whether RA modulated this interaction by performing *in vitro* pulldown assays in the presence of different concentrations of RA. These experiments showed that ZRF1 and

RARα interacted irrespective of the presence or absence of RA (**Figure R31**).

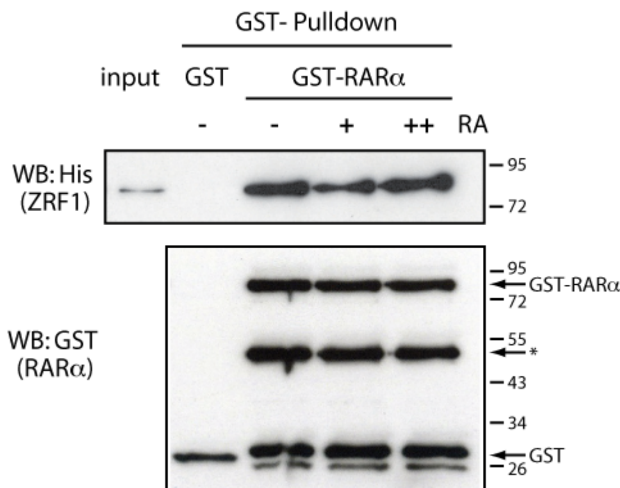



**Figure R31: ZRF1 and RARα interact directly and independently of RA.** *In vitro* GST pulldown assay with recombinant GST-RARα and recombinant His-ZRF1, in the absence (–) or presence (+: 100 nM; ++: 1 μM) of RA, as detected by anti-His and anti-GST immunoblottings. GST protein was used as a control. As observed in the GST blot, part of GST-RARα spontaneously degrades, forming mainly GST and RARα alone (*).

We then mapped the interaction between ZRF1 and RARα. To do that, we produced truncated forms of recombinant His-ZRF1. Specifically, we divided the protein into two parts: the N-terminal part, containing the DnaJ domain together with the UBD region (that binds H2Aub),[118] and the C-terminal part, containing the two SANT domains. We next performed *in vitro* pulldown assays with these ZRF1 fragments and GST-RARα. These experiments showed that the binding to RARα was mediated by the N-terminal part of ZRF1 (**Figure R32**). Further experiments using smaller fragments corresponding to the N-terminal part of ZRF1 are needed to know the exact fraction of the protein that interacts with RARα. Unfortunately, due to technical limitations, we were no able to produce GST-RARα truncated proteins and therefore the domain of RARα involved in the interaction with ZRF1 remains unknown.

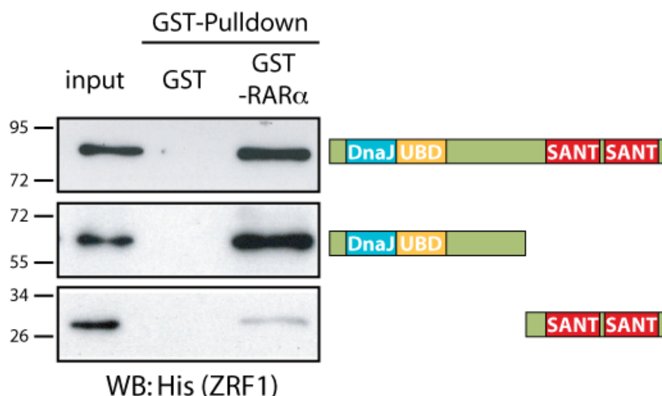



**Figure R32: ZRF1 interacts with RARα through its N-terminal part.** *In vitro* GST-pulldown assay with recombinant GST-RARα and recombinant His-ZRF1, full-length or truncated forms, as indicated in the corresponding diagram; detected by anti-His immunoblotting. GST protein was used as a control. Anti-GST immunoblotting was also performed as a control (data not shown). The different domains of ZRF1 are indicated.

Taken together, these results show that ZRF1 interacts directly with RARα, strongly suggesting that ZRF1 regulates the transcription of RA target genes through its binding to this transcription factor.

## 3.2 ZRF1 binds to RA target genes

Our biochemical results showed that ZRF1 and RARα interacted in HL60 cells and that their interaction was direct. We therefore hypothesized that ZRF1 controls RA target gene transcription through its binding to RARα. To evaluate the presence of ZRF1 at RA target genes, we performed chromatin immunoprecipitation (ChIP) experiments. Specifically, we studied whether ZRF1 was bound to the RAREs, the DNA sequences where RARα binds,[54] of several of the RA targets that we had seen to be upregulated upon ZRF1-depletion (see Figure R25). ChIP experiments in HL60 cells showed that ZRF1 indeed occupied RAREs on the promoters of RA target genes. Since our ChIP signals were low (probably due to limitations of our ZRF1 antibody) we checked the specificity of the ZRF1 enrichment by performing ChIP assays in ZRF1-depleted and control

cells. These experiments confirmed that ZRF1 occupied RARE sequences located on the promoters of RA target genes (**Figure R33**).

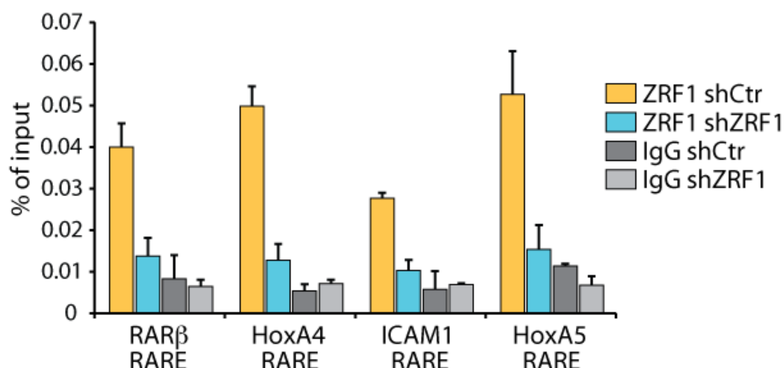



**Figure R34: ZRF1 binds to RARE sequences on RA target genes.** Chromatin immunoprecipitation (ChIP) of ZRF1 in HL60, control (shControl), and ZRF1-depleted (shZRF1) cells, followed by qRT-PCR analysis of the indicated RA target genes at the RARE regions. IgG was used as a control. Results are shown as percentage relative to input. Data are the means ± s.e.m. of three independent experiments.

We next extended our ChIP analysis to HL60 cells treated with RA. These experiments showed that ZRF1 was bound to RA target genes to a similar extent in untreated and RA-treated conditions (**Figure R35**).

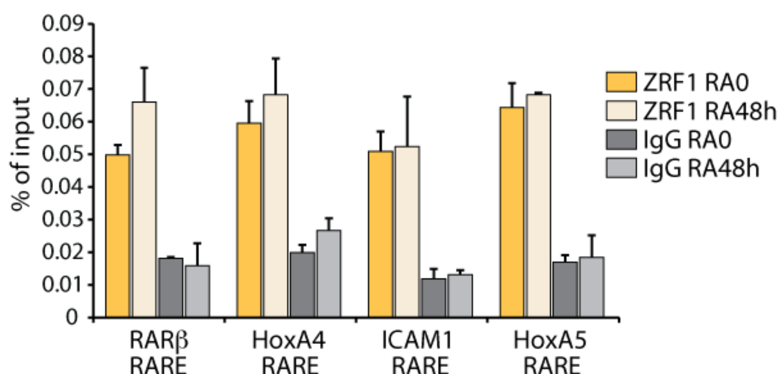



**Figure R35: ZRF1 binds RA target genes independently of RA.** ChIP of ZRF1 in untreated (RA0) and RA-treated (RA48h) HL60 cells, followed by qRT-PCR analysis of the indicated RA target genes at the RARE regions. IgG was used as a control. Results are shown as percentage relative to input. Data are the means ± s.e.m. of three independent experiments.

These results indicated that, as predicted by our *in vitro* pulldown experiments (see Figure R31), the ZRF1 binding to RA targets is independent of the presence or absence of RA.

Taken together, these data show that ZRF1 occupies RARE sequences on the promoters of RA target genes to regulate transcription.

## 3.3 ZRF1 as a transcriptional repressor: interaction with HDACs and regulation of histone acetylation

Our data show that, in proliferating AML cells, ZRF1 depletion inhibits cell proliferation and induces apoptosis and differentiation (see chapter 1 of the *Results* section). Moreover, our gene expression results show that, in these conditions, ZRF1 represses differentiation, proapoptotic and antiproliferative genes (see chapter 2 of the *Results* section). Given the promising anti-leukemic effects of ZRF1-knockdown, we focused on studying ZRF1 regulation of RA target genes in proliferating untreated leukemic cells.

Therefore, we aimed to further understand the molecular basis of ZRF1 function as a transcriptional repressor. With this objective, we tried to identify novel protein interactors that could shed new light on the repression mechanism. Therefore, we attempted to purify ZRF1 protein complex by performing Flag-purification experiments coupled to mass spectrometry. Specifically, we transiently transfected 293T cells with Flag-ZRF1 and then we carried out a Flag-purification in Flag-ZRF1 and Flag-control cells. We then analyzed the samples by SDS-PAGE and silver staining followed by mass spectrometry analysis of the specific bands.

Among the proteins identified in our mass spectrometry experiment, we found several components of the ribosome and the heat shock protein Hsp70, which confirmed previously publish data concerning ZRF1 function as part of the ribosome-associated complex.[101] Moreover, we

identified all the core histones, H2A, H2B, H3 and H4, which confirmed that ZRF1 associated to chromatin, as we recently characterized[118] and as we had seen in our ChIP experiments. Our mass spectrometry analysis did not identify, though, any other interactor with high score (i.e. high confidence) that could explain the role of ZRF1 as a transcriptional repressor.

Nevertheless, among the low score interactors identified in our mass spectrometry analysis, we found the histone deacetylase HDAC2. As mentioned in the *Introduction* section, HDACs are fundamental components of some of the main corepressor complexes, including those necessary for RARα-mediated transcriptional repression.[54] HDACs deacetylate histone lysine residues therefore leading to chromatin compaction and gene silencing.[69,135] We then wanted to validate the interaction between ZRF1 and HDAC2. We thus obtained 293T cells transiently expressing Flag-tagged HDAC2 or ZRF1 and we performed co-immunoprecipitation experiments. These assays showed that ZRF1 indeed interacted with HDAC2. Specifically, we demonstrated the ZRF1-HDAC2 interaction by immunoprecipitating Flag-HDAC2 and co-eluting ZRF1 as well as by immunoprecipitating Flag-ZRF1 and co-eluting HDAC2 (**Figure R36**). Moreover, by performing similar experiments we found that ZRF1 interacted not only with HDAC2 but also with HDAC1 and HDAC3, all of them members of the class I HDACs (data not shown).

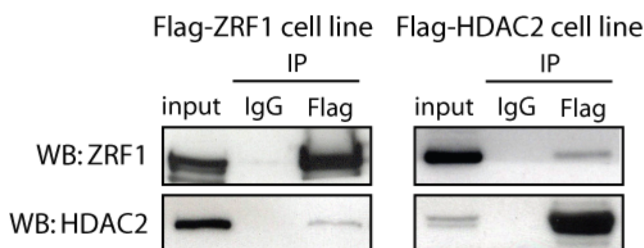



**Figure R36: ZRF1 interacts with HDAC2.** Co-immunoprecipitation (IP) assay in 293T cells transfected either with Flag-ZRF1 or Flag-HDAC2, followed by western blot analysis of ZRF1 and HDAC2. IgG was used as a control. Similar results were obtained in additional experiments using as a control cells transfected with a Flag-empty plasmid.

Since ZRF1 interacted with HDAC proteins, we speculated that ZRF1 might repress transcriptional activity in proliferating AML cells through the regulation of histone acetylation levels. To test this hypothesis, we studied the effect of ZRF1 depletion in histone acetylation by performing ChIP experiments in control and ZRF1-knockdown HL60 cells. Specifically, we analyzed global histone H3 acetylation (H3ac) and histone H3 acetylation at lysine 27 (H3K27ac), both of which are marks associated with active chromatin,[69] in the four RA target genes analyzed previously (see figure R34). These experiments revealed that ZRF1 depletion increased the level of histone acetylation in RA targets (**Figure R37**).

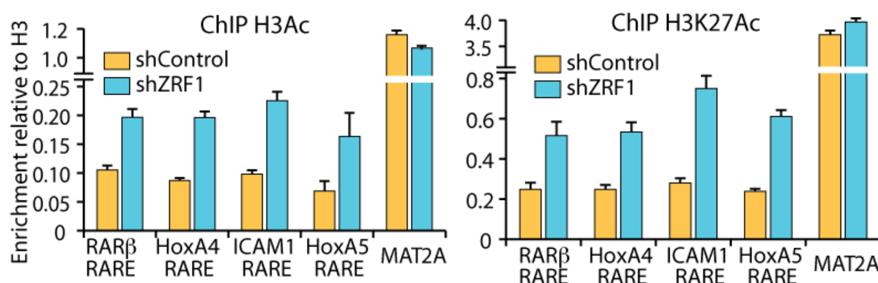



**Figure R37: ZRF1 depletion increases histone acetylation of RA target genes.** ChIP of global histone H3 acetylation (H3ac) and histone H3 lysine 27 acetylation (H3K27ac) in shControl and shZRF1 HL60 cells, followed by qRT-PCR analyses of the indicated RA target genes at the RARE regions. Results are shown as enrichment relative to total histone H3. MAT2A gene was used as a positive control for acetylation.[136] Data are the means ± s.e.m. of three independent experiments.

The increased acetylation level upon ZRF1 depletion correlated with our genome-wide expression results, which showed that a large proportion of RA target genes were upregulated following ZRF1 knockdown in basal conditions (see chapter 2 of the *Results* section).

Taken together, these data show that ZRF1 interacts with HDACs and regulates histone acetylation levels of RA target genes. Specifically, this ZRF1-HDACs interaction would result in a reduction of histone acetylation levels, which could explain, at least partially, the role of ZRF1 as a transcriptional repressor.

## 3.4 Other transcriptional mechanisms regulated by ZRF1

Our genome-wide expression study shows that ZRF1 works predominantly as a transcriptional repressor in basal conditions, in proliferating AML cells (see chapter 2 of the *Results* section). Indeed, we found that ZRF1 interacts with HDACs, which act as transcriptional co-repressors, and regulate histone acetylation levels. However, in RA-treated cells, our expression data indicate that ZRF1 is predominantly an activator. Therefore, we aimed to further understand the molecular basis of ZRF1 function as a transcriptional activator.

As mentioned in the *Introduction* section, we initially identified ZRF1 as an activator of Polycomb-repressed genes through the binding to H2Aub.[118] Although the exact role of H2Aub in transcriptional regulation is not clear, recent reports have proposed some molecular mechanisms linking this histone mark with gene repression. First, H2Aub was reported to inhibit the recruitment of the elongation factor FACT.[137] Second, H2Aub was shown to inhibit H3K4me3, a mark associated with transcriptional initiation deposited by the MLL complex.[83,138] We therefore hypothesized that ZRF1 could counteract this inhibition and attract the FACT complex and/or the MLL complex as a possible mechanism to induce gene activation.

To analyze whether ZRF1 interacted with FACT and/or MLL complexes, we performed Flag-purification experiments coupled to western blot analysis in 293T cells that we previously transfected with Flag-ZRF1. These experiments suggested that indeed ZRF1 interacted both with FACT and MLL complexes. First, we found that ZRF1 interacted with SPT16, one of the two components of the FACT complex. Second, we found that ZRF1 interacted with ASH2L and MLL1, both part of the multimeric MLL complex[83] (**Figure R37**).

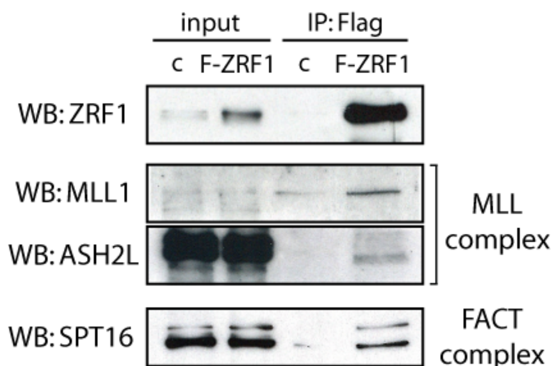



**Figure R38: ZRF1 interacts with components of the MLL and FACT complexes.** Flag co-immunoprecipitation (IP) experiment in 293T cells transiently transfected either with Flag-ZRF1 (F-ZRF1) or with Flag-empty (c) vector, followed by western blot analysis for ZRF1, MLL1, ASH2L and SPT16.

We also analyzed whether ZRF1 interacted with other proteins that had been previously linked with H2Aub and, additionally, had been reported to be involved in transcriptional activation. Specifically, we checked the interaction between ZRF1 and the H2A deubiquitinases USP21 and 2A-DUB,[121] and the H3K27 demethylases UTX and JMJD3.[139] Similarly to the previous experiment, we performed Flag-purifications followed by western blot analysis and found that (at least in our experimental conditions) ZRF1 did not interact with any of these proteins.

Altogether, these data suggest that the interaction with FACT and/or MLL complexes could represent two molecular mechanisms implicated in ZRF1-mediated transcriptional activation. However, further research is needed to confirm this hypothesis. Moreover, the molecular basis for the dual function of ZRF1 as a transcriptional repressor and as a transcriptional activator in different gene subsets and in different cellular conditions remains unclear. These aspects are further addressed in the *Discussion* section.

# 4. ZRF1 regulates leukemogenic potential *in vivo*

## 4.1 ZRF1 depletion inhibits leukemia progression *in vivo*

Our data show that ZRF1 depletion leads to cell growth inhibition due to a decrease of proliferation and an induction of apoptosis and basal differentiation in AML cells (see chapter 1 of the *Results* section). These results, together with the fact that ZRF1 is highly overexpressed in human AML,[124-126] suggested that targeting ZRF1 could be a potential novel strategy to be explored for leukemia treatment.

Therefore, we decided to study the anti-leukemic potential of ZRF1 inhibition *in vivo*. For that purpose, we used xenotransplantation of human AML leukemic cells into severe combined immunodeficient (SCID) mice. This experimental model recapitulates most of the features of the human malignancy, such as the presence of leukemic cells in peripheral blood and their homing to the bone marrow and spleen.[140] We first generated an HL60 cell line that stably expressed luciferase to be able to monitor leukemia progression by *in vivo* imaging. Afterwards, we stably knocked down ZRF1 in this cell line. We then intraperitoneally injected control and ZRF1-depleted cells in SCID mice. Specifically, we performed two independent experiments, with eight mice in total for each group.

After injecting ZRF1-knockdown and control cells, we followed leukemia progression through bioluminescent imaging every three or four days. Remarkably, we observed a strong inhibition of leukemia progression in the mice injected with ZRF1-depleted cells as compared with the control mice. Regarding the ‘shControl’ group, all the mice showed high levels of leukemic cell growth and dissemination. Specifically, HL60 cells typically progressed from the inoculation point to the area of the spleen and liver and afterwards spread throughout the body. On the other hand, only two out of eight mice injected with ZRF1-depleted cells showed a comparable level of leukemic cells propagation. In fact, four of the mice from this group had a complete or almost complete suppression of leukemic cells

after three week, while in the other two leukemia progression was clearly delayed as compared to control mice (**Figure R39** and **R40**).

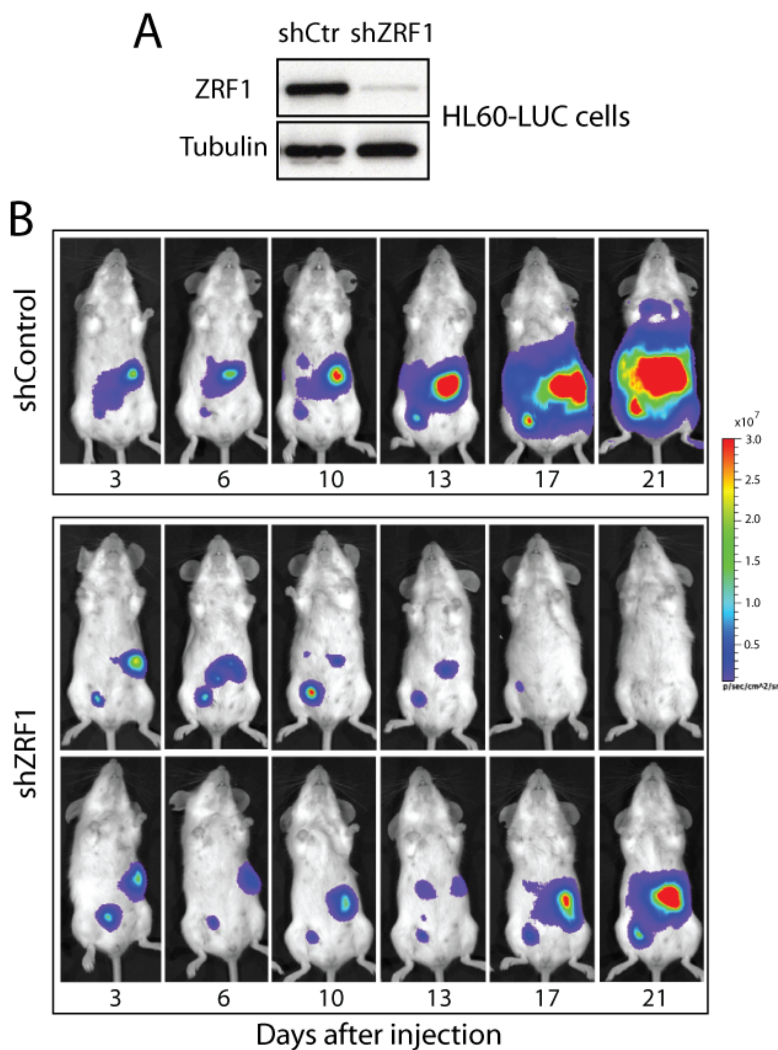



**Figure R39: ZRF1 depletion inhibits leukemia progression *in vivo*.** (**A**) Western blot analysis of ZRF1 in control (shCtr) and ZRF1-depleted (shZRF1) HL60 cells previously infected with a luciferase (LUC) expression vector. shZRF1 #2 was used. Tubulin was used as a loading control. (**B**) Bioluminescent imaging of xenografted SCID mice injected with control (shControl) or ZRF1-depleted (shZRF1) HL60 cells during leukemia progression. One representative mouse from the shControl group (which was quite homogeneous) and two representative mice from the shZRF1 group are shown.

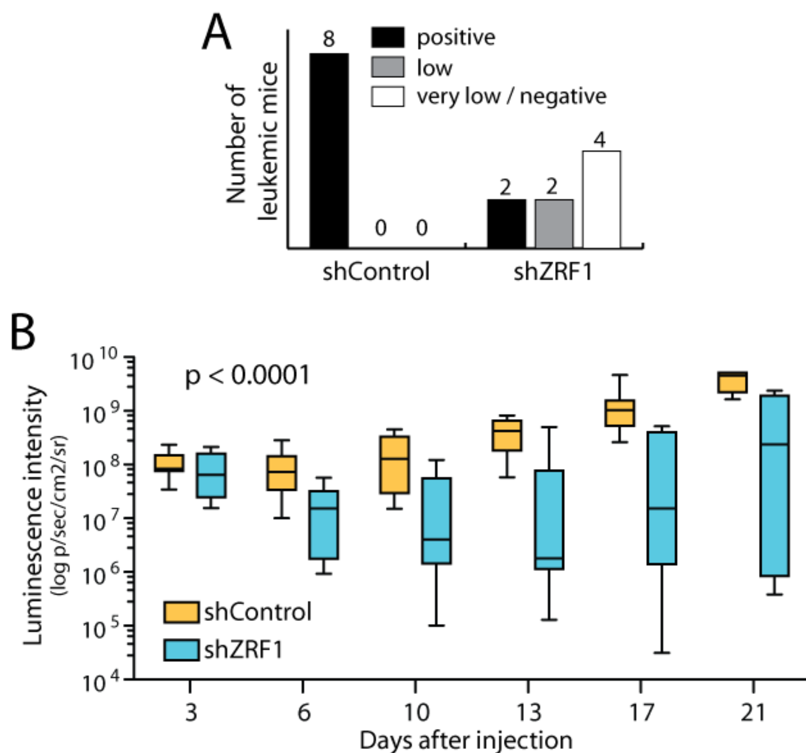



**Figure R40: ZRF1 depletion inhibits leukemia progression *in vivo*.** (**A**) Summary of the *in vivo* xenograft experiment, showing the number of mice with high levels of leukemic cell propagation ('positive') and those with a reduced ('low') o very reduced ('very low / negative') amount. (**B**) Bioluminescent quantification (in photons/sec/cm2/steradian) of xenografted SCID mice. Data are represented on a logarithmic scale as box-and-whisker plots of the eight mice in each group, at the corresponding days after injection; boxes represent the quartiles and whiskers mark the minimum and maximum values. Statistical significance was determined with a two-way ANOVA.

These data showed that leukemia progression was strongly inhibited in the mice injected with ZRF1-depleted cells as compared with the mice injected with control cells. Specifically, the effect of ZRF1 knockdown was observed already at day six after injection of the cells and was very significant by day ten. At the last day of the experiment, the mean bioluminescent signal of 'shZRF1' mice was about 7-fold lower as compared with 'shControl' mice.

Three weeks after cell injection, mice were sacrificed and post-mortem necropsies were performed. These studies showed that all the mice injected with control cells presented several intraperitoneal solid tumors, while only two out of eight mice injected with ZRF1-depleted cells showed tumor masses, which were also smaller than those in the control mice. Moreover, some mice injected with control cells had an enlargement of the spleen, a characteristic feature of leukemia, whereas those with ZRF1-depleted cells did not (**Figure R41**).

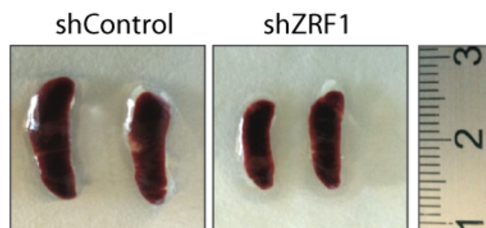



**Figure R41: Spleen enlargement in control but not in ZRF1-depleted mice.** Representative pictures showing an enlargement in the spleen of some of the mice injected with shControl cells as compared with the normal size of mice injected with shZRF1 cells.

Additionally, we further analyzed the leukemic phenotype. For that purpose, we collected samples from peripheral blood and spleen from each mouse to check the presence of leukemic cells. After processing the samples, we performed FACS analysis of human CD33, a myeloid cell surface marker, which allowed us to differentiate the human leukemic cells from the endogenous mouse cells. In agreement with the bioluminescence results, we detected leukemic cells in both peripheral blood and spleen in all control mice. In contrast, we observed a strong reduction in the amount of leukemic cells in the mice injected with ZRF1-depleted cells. In particular, most of the mice from the 'shZRF1' group showed an undetectable or almost undetectable level of HL60 leukemic cells in the blood and had a decreased amount of cells in the spleen (**Figure R42**).

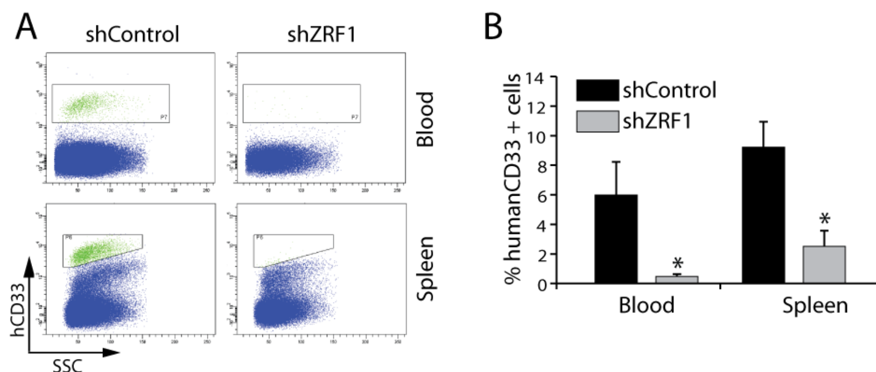



**Figure R42: ZRF1 depletion decreases leukemic cell propagation in blood and spleen.** FACS analysis of human CD33 positive cells in peripheral blood and spleen of mice injected with shControl or shZRF1 cells. (**A**) FACS plot examples. For 'shControl' group, one representative mouse is shown, while for 'shZRF1' group, one of the mice with undetectable levels of HL60 is shown. Leukemic cells are shown in green. SSC: Side Scatter. (**B**) Quantification of the abundance of leukemic cells. Results are shown as percentage of human CD33 positive cells within the gated population; data are the means ± s.e.m of the eight mice in each group. Statistical significance was assessed by a two-tailed Student's t-test; * $p < 0.05$.

Taken together, these results show that depletion of ZRF1 leads to a strong inhibition of leukemogenic potential in a mouse xenograft model, confirming *in vivo* the effect of ZRF1 knockdown that we had seen *in vitro*.

## 4.2 ZRF1 depletion cooperates with RA treatment in the inhibition of leukemia progression

Our data show that ZRF1 depletion leads to a decrease in cell proliferation and an increase in apoptosis and cell differentiation in AML cells, which results in inhibition of leukemia progression *in vivo*. Moreover, as previously mentioned, RA also inhibits leukemic cell growth and it is currently being used in the treatment of certain types of AML.

Therefore, we next aimed to study the effect of ZRF1 depletion in combination with RA treatment *in vivo*. We thus repeated the mouse xenograft experiments including RA administration. After inoculating control and ZRF1-depleted cells in SCID mice, we treated half of the mice with RA and the other half with vehicle. Both substances were injected intraperitoneally twice a week starting at day seven. We then had four groups: ‘shControl’, ‘shZRF1’, ‘shControl + RA’ and ‘shZRF1 + RA’ (**Figure R43** and **R44**).

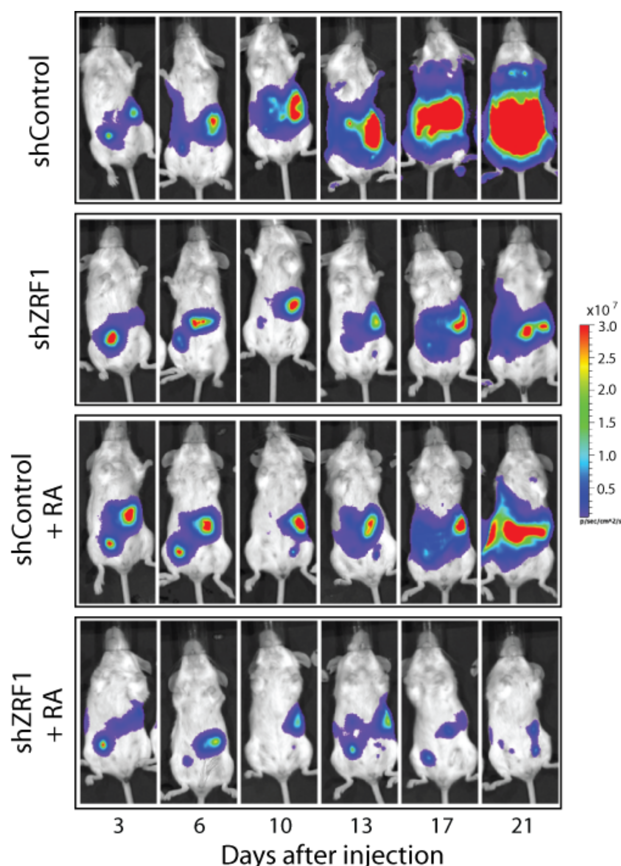



**Figure R43: ZRF1 depletion cooperates with RA treatment in the inhibition of leukemia progression *in vivo*.** Bioluminescent imaging of xenografted SCID mice injected with control (shControl) or ZRF1-depleted (shZRF1) HL60 cells, treated with RA or vehicle, during leukemia progression. RA treatment started at day seven. One representative mouse from each group is shown.

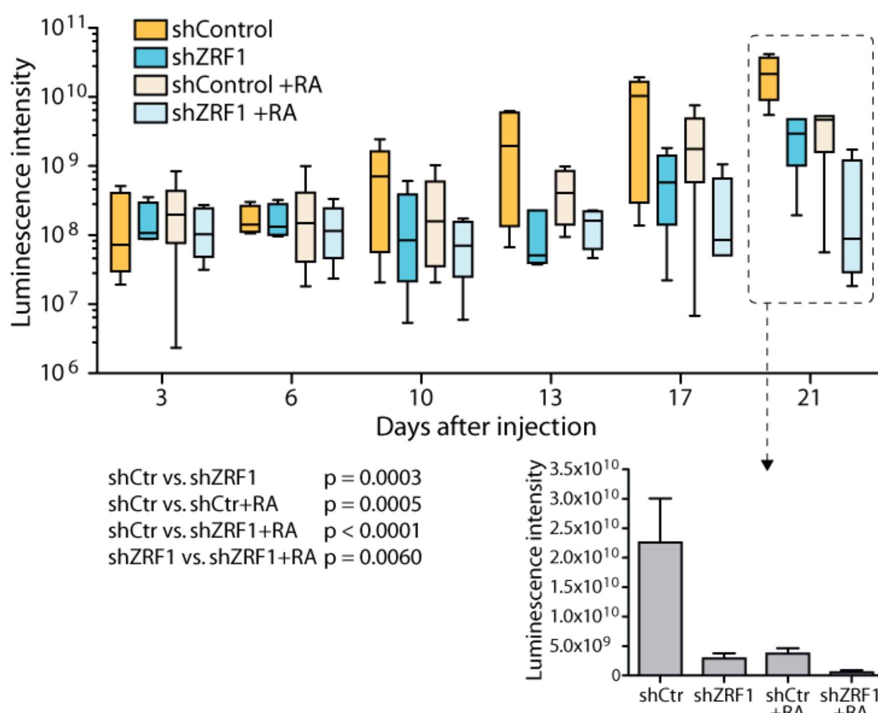



**Figure R44: ZRF1 depletion cooperates with RA treatment in the inhibition of leukemia progression *in vivo*.** Bioluminescent quantification (in photons/sec/cm2/steradian) of xenografted SCID mice. Data are represented on a logarithmic scale as box-and-whisker plots at the corresponding days after injection; boxes represent the quartiles and whiskers mark the minimum and maximum values. Statistical significance was determined with a two-way ANOVA. Lower panel: detail of the bioluminescent data on a linear scale corresponding to day 21; data are the means ± s.e.m of the five mice in each group.

This experiment showed that ZRF1 depletion inhibited leukemia progression to a similar extent as RA treatment, and, remarkably, that a combination of both had a cooperative effect in leukemia suppression. Specifically, the inhibitory effects of ZRF1 knockdown as well as of RA treatment were evident starting from day thirteen. At the day before sacrificing the animals (day 21), RA treatment and ZRF1-depletion alone caused about 6-fold and 8-fold decrease, respectively, in luminescence intensity as compared with the control. Remarkably, the combination of both inhibited leukemic cell progression up to 36-fold (Figure R44).

Statistical analysis indicated that ZRF1 depletion as well as RA treatment had a very significant effect in leukemia inhibition, which was even higher when the two factors were combined.

We next examined the presence of leukemic cells in peripheral blood and spleen. This analysis confirmed our *in vivo* imaging results. First, we observed that ZRF1 depletion inhibited leukemia progression to a similar extent as RA treatment. Second, we found a cooperative effect of ZRF1 knockdown and RA treatment in leukemia inhibition. In fact, mice injected with ZRF1-depleted cells and treated with RA had almost undetectable levels of leukemic cells in both organs (**Figure R45**).

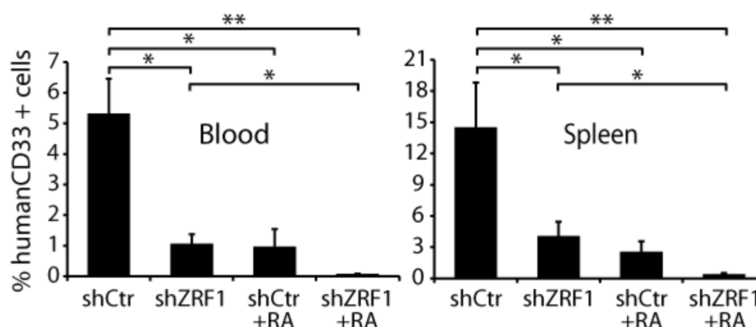



**Figure R45: ZRF1 depletion cooperates with RA treatment in leukemic cell suppression in blood and spleen.** FACS analysis of HL60 leukemic cells abundance in peripheral blood and spleen corresponding to the four experimental groups. Results are shown as the percentage of human CD33 positive cells within the gated population. Data are the means ± s.e.m. Statistical significance was assessed by a two-tailed Student's t-test; * $p < 0.05$, ** $p < 0.01$.

Since depletion of ZRF1 in AML cells in culture had a triple effect on cell proliferation, apoptosis and differentiation, we then aimed to further investigate the cause of its inhibitory effect on leukemia progression *in vivo*. For that purpose, we incorporated in our FACS experiments the analysis of the differentiation marker CD11b. In agreement with our *in vitro* results, this analysis showed that ZRF1 knockdown cells had a higher level of differentiation as compared with control cells, both in peripheral blood and spleen. In fact, ZRF1 depletion increased the differentiation state of HL60 cells to a similar extent as RA treatment (**Figure R46**). These data suggested that the inhibitory effect of ZRF1

depletion on leukemia progression was caused, at least partially, by an increase in cell differentiation. We were not able to perform this analysis in ZRF1-depleted cells after RA treatment because, as observed in Figure R45, the levels of leukemic cells in the 'shZRF1 + RA' group were undetectable or near background.

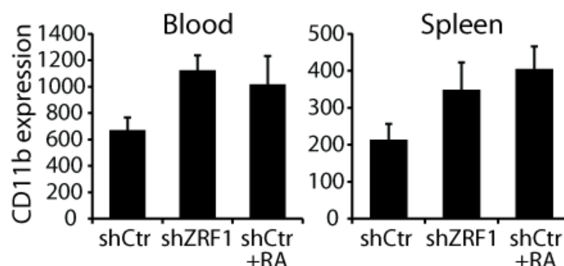



**Figure R46: ZRF1 depletion increases cell differentiation *in vivo*.** FACS analysis of the differentiation state of HL60 cells in peripheral blood and spleen. Results are shown as expression of human CD11b (mean fluorescent intensity) corresponding to the CD33 positive population. Data are the means ± s.e.m. In mice from 'shZRF1+RA' group, CD33 positive cells were not detectable.

Given the strong effect that ZRF1 depletion had in leukemia inhibition, we wanted to extend our study by performing a mouse xenograft experiment using a different cell line. Similarly as we did for HL60 cells, we first obtained an NB4 cell line stably expressing luciferase. We then stably knocked down ZRF1, and intraperitoneally injected control and ZRF1-depleted NB4 cells in SCID mice. We administrated RA to half of the mice to be able to validate also the effect of ZRF1 depletion in combination with RA treatment. In total, as in the previous experiment, we had four groups: 'shControl', 'shZRF1', 'shControl + RA' and 'shZRF1 + RA'.

Our xenograft experiment with NB4 cells confirmed our previous result obtained with HL60 cells. Thus, we observed a strong inhibitory effect of ZRF1 depletion in leukemia progression. Moreover, we found a cooperative effect of ZRF1 knockdown and RA treatment in leukemia suppression. Specifically, the inhibitory effects of ZRF1 knockdown as well as of RA treatment were evident starting from day nine. At the day before sacrificing the animals (day 22), ZRF1-depletion and RA treatment

alone caused about 5-fold and 3-fold decrease, respectively, in luminescence intensity as compared with the control. Remarkably, the combination of both inhibited leukemia progression up to 37-fold. In fact, two out of five mice from the 'shZRF1 + RA' group were completely negative (**Figure R47**).

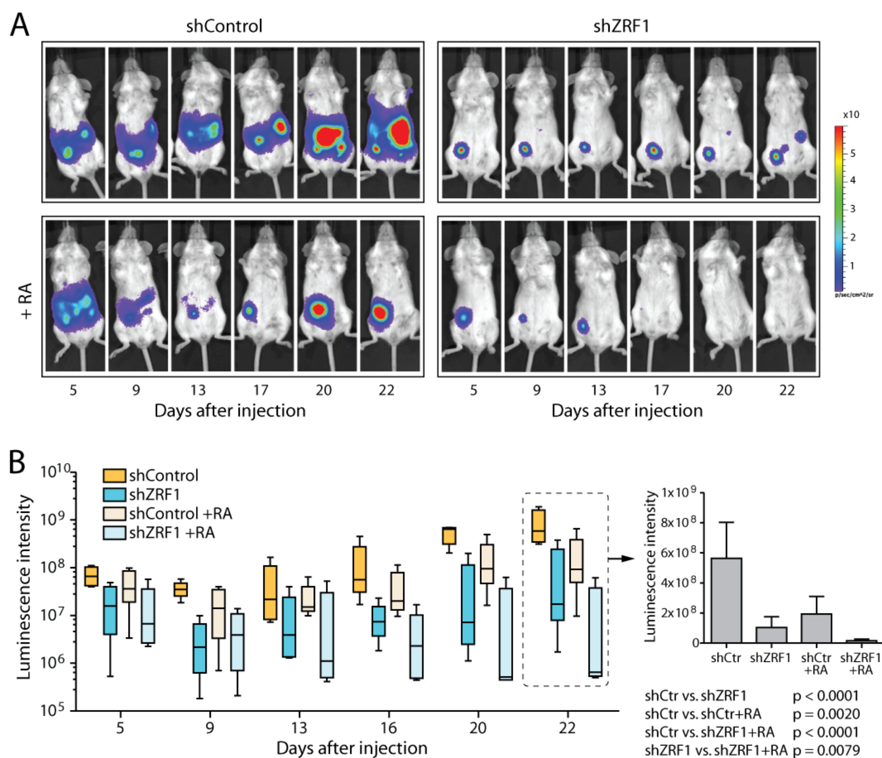



**Figure R47: ZRF1 depletion inhibits leukemia progression in an NB4 xenograft model, and cooperates with RA treatment.** (**A**) Bioluminescent imaging of xenografted SCID mice injected with control (shControl) or ZRF1-depleted (shZRF1) NB4 cells, treated with RA or vehicle, during leukemia progression. One representative mouse from each group is shown. (**B**) Bioluminescent quantification (in photons/sec/cm2/steradian) of xenografted SCID mice injected with control (shControl) or ZRF1-depleted (shZRF1) NB4 cells, treated with RA or vehicle. Statistical significance was determined with a two-way ANOVA. Right panel: bioluminescent data on a linear scale corresponding to day 22. .

We then further studied the leukemic phenotype by examining the presence of leukemic cells in peripheral blood and spleen. In this case, no

CD33-positive cells were detected in blood samples of mice from any of the groups. Concerning the spleen samples, we only detected considerable levels of leukemic cells in the 'shControl' group, while mice from the other three groups were almost negative (**Figure R48**). Indeed, luminescence intensity was approximately 25-fold lower in the NB4 xenografted mice as compared with the HL60 xenografted mice (compare Y-axis of the graphs shown in Figures R44 and R47). This suggests that the amount of cells that were able to progress in the *in vivo* environment was considerably lower in the case of the NB4 xenograft than in the case of HL60, which would explain why NB4 cells could be detected by FACS only in the spleens of control mice. In fact, the percentage of CD33-positive cells in spleen was about 40-fold lower in 'shControl' mice from the NB4 experiment than in 'shControl' mice from the HL60 experiment (compare Figures R45 and R48). In any case, the luminescence results of our NB4 xenograft nicely confirmed our HL60 xenograft data.

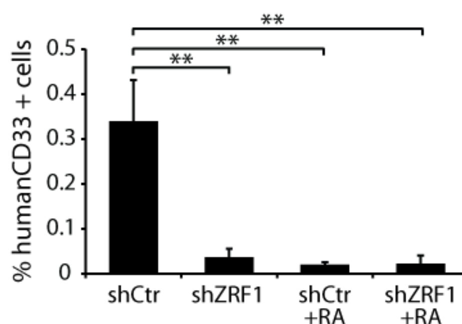



**Figure R48: ZRF1 depletion inhibits leukemic cell propagation in spleen.** FACS analysis of NB4 leukemic cell abundance in spleen corresponding to the four experimental groups. Results are shown as the percentage of human CD33 positive cells within the gated population. Data are the means ± s.e.m. Statistical significance was assessed by a two-tailed Student's t-test; ** $p < 0.01$. Blood samples were also analyzed, but no CD33-positive cells were detected in any of the groups.

Taken together, these results show that depletion of ZRF1 strongly suppresses leukemia progression *in vivo* and that a combination of ZRF1 knockdown with RA treatment has a cooperative effect in leukemia inhibition.

# DISCUSSION

Intensive research on the process of leukemic cell differentiation in recent years has provided important clues both for understanding the mechanisms underlying cell fate transitions and for discovering novel therapeutic drugs. In this thesis, we have studied the function of ZRF1, a recently characterized epigenetic factor that is overexpressed in AML but with an unknown role in leukemia development. We have shown that ZRF1 is an important regulator of proliferation, apoptosis, and differentiation in AML cells, all of which are fundamental processes altered in leukemia. Our data demonstrate that ZRF1 depletion strongly inhibits leukemia progression in mouse xenograft models. At the molecular level, we have shown that ZRF1 has an important interplay with the RA pathway through its binding to RARα, thus regulating RA target gene transcription.

## ZRF1 regulates essential biological functions in AML cells

Our data provide evidence that ZRF1 is a pleiotropic regulator in AML cells since it is involved in the control of proliferation, apoptosis and differentiation processes. Specifically, our results suggest that ZRF1 regulates the expression of the gene networks that control these fundamental biological functions. As a result, ZRF1 depletion leads to an inhibition of cell proliferation and an induction of apoptosis and basal differentiation, which results in a reduction of cell growth. Remarkably, we have observed this effect in five different AML cell lines and therefore the function of ZRF1 in AML seems to be general.

ZRF1 is also involved in the regulation of RA-induced differentiation in AML cells. Interestingly, the effect of ZRF1 depletion in differentiation varies upon treatment with RA: ZRF1 knockdown leads to an increased differentiation in basal conditions and to a reduced potential to differentiate when cells are treated with RA. Therefore, ZRF1 functions as a repressor of differentiation in proliferating cells but turns into an activator upon RA-induced differentiation. In contrast with this dual role of ZRF1 in differentiation, our data show a single function for ZRF1 in

proliferation and apoptosis, since ZRF1 depletion inhibits proliferation and induces apoptosis both in the absence and presence of RA. As a result, upon RA treatment, ZRF1 knockdown cells do not differentiate properly, but they mainly become apoptotic. The multiple effects of ZRF1 depletion in AML cells are summarized in Figure D1.

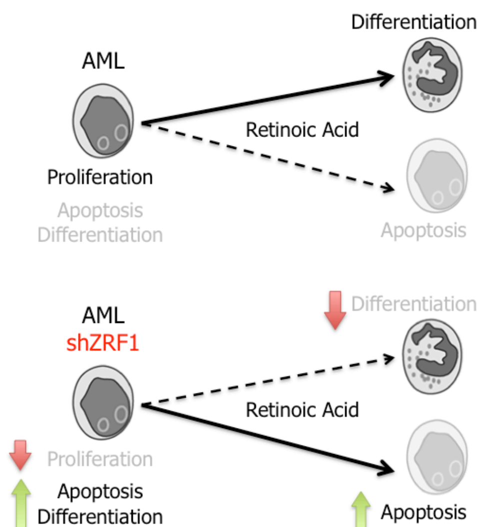



**Figure D1: Effect of ZRF1 depletion in AML cells.** Top panel: normal AML cells proliferate rapidly and have low levels of apoptosis and basal differentiation. RA treatment induces cell differentiation and, to a lesser extent, a certain level of apoptosis. Bottom panel: ZRF1 depletion (shZRF1) causes an inhibition of cell proliferation and an induction of apoptosis and basal differentiation that results in a decreased cell growth. Upon RA treatment, ZRF1-knockdown cells have a reduced potential to differentiate and an increased apoptosis.

It should be noted that the phenotypic effects caused by ZRF1 depletion, both in untreated and RA-treated cells, correlate with the functional categories of the ZRF1-regulated genes. In line with this, ZRF1 seems to be a novel regulator of the RA signalling pathway, which, as previously explained, not only controls cell differentiation but also directly regulates cell proliferation and apoptosis.[29] Accordingly, ZRF1 depletion alters the expression of nearly half of the RA-target genes. Altogether, this highlights the importance of ZRF1 as a transcriptional regulator of essential biological functions in AML cells and, in particular, in the control of RA-regulated gene network.

## ZRF1 in cell growth regulation

As mentioned in the introduction, one of the first functional studies about ZRF1 showed that its depletion led to cell growth inhibition in a mouse cancer cell line.[106] Moreover, a recent publication reported the same effect in the human cancer cell line HeLa.[104] Additionally, we have also shown that ZRF1 knockdown causes cell growth inhibition not only in AML cells, but also in another human cancer cell line: the embryonal carcinoma cells NT2.[119] Furthermore, unpublished data from our laboratory show that ZRF1 depletion also affects normal growth in mouse neural progenitor cells (NPC) and mouse embryonic fibroblasts (MEFs). In contrast, a recent study from our laboratory revealed that ZRF1 knockdown does not lead to cell growth inhibition in human keratinocytes,[141] and other unpublished data show that mouse embryonic stem cells (ESC) are not affected either. Taken together, these data demonstrate that growth inhibition by ZRF1 depletion is cell type-dependent and suggest that it might predominantly (although not exclusively) affect cancer cells.

In line with the effect of ZRF1 knockdown in cell growth inhibition, our genome-wide expression study shows that ZRF1-regulated genes have a significant overrepresentation of proliferation and apoptosis regulators. Among those, ZRF1 controls the expression of a significant group of RA-targets involved in this processes. Thus, ZRF1 appears to repress anti-proliferative genes, including important regulators of the cell cycle such as the cyclin-dependent kinase inhibitor CDKN1C (also known as p57),[142] among many others. On the other hand, ZRF1 seems to repress pro-apoptotic genes, including central players of the apoptotic pathway such as caspases 8 and 10.[143] This suggests that ZRF1 overexpression in AML might promote proliferation and inhibit apoptosis through the downregulation of anti-proliferative and pro-apoptotic genes respectively. Therefore, ZRF1 could directly contribute to the alteration of these processes, which is characteristic of leukemia.

Interestingly, in human cells, ZRF1 was initially identified as a protein that is phosphorylated during mitosis, thus it was termed M-phase phosphoprotein 11 (MPP11).[108] Moreover, this study proposes, based on

immunofluorescence data, that ZRF1 might associate with the mitotic spindle. In fact, the homolog of ZRF1 in the green algae *Volvox*, GlsA, associates with the mitotic spindle during asymmetric cell division.[109,110,144] These studies also demonstrate that this is essential for the proper regulation of this process and suggest that the function of GlsA is through the binding to chromatin. The homolog of ZRF1 in *C. elegans*, dnj-11, has also been reported to regulate asymmetric cell division and, at the same time, to control the apoptotic pathway.[113] Additionally, a recent study in our laboratory provides evidence that ZRF1 directly regulates the INK4-ARF locus, which encodes three key cell cycle regulators: p15, p16 and ARF.[141]

Altogether, these data suggest that first, ZRF1 is indeed an important regulator of cell growth; second, ZRF1 might play an evolutionary conserved function in cell growth regulation; and third, ZRF1-mediated cell growth regulation possibly relies not only in its role as a transcriptional regulator, but also in other molecular mechanisms. Considering that, in *Volvox*, ZRF1 binds to the mitotic spindle together with the heat shock protein Hsp70,[110] one possibility is that ZRF1 worked as a chromatin-associated chaperone in this process, mimicking its role in the ribosome. The hypothetical function of ZRF1 as a chromatin chaperon is further discussed below. Therefore, ZRF1 might combine transcriptional-dependent and transcriptional-independent functions to regulate cell growth.

## Emerging role of ZRF1 as a developmental regulator

Our data reveal that ZRF1 regulates RA-induced granulocytic differentiation in AML cells. We have shown that ZRF1 functions as a repressor of differentiation in proliferating cells but turns into an activator when cells are treated with RA. It is worth noting that, as previously explained, this dual role is also found in RARα itself.[32] Several lines of evidence have shown that RARα actively blocks differentiation in the absence of RA (by repressing specific target genes), while it stimulates granulocytic differentiation in the presence of RA (by activating these and additional target genes).[145] Additionally, we have found that ZRF1

interacts directly with RARα and regulates nearly half of the RA target genes. Altogether, these suggest a functional cooperation between ZRF1 and RARα in RA-induced differentiation in AML cells.

An interesting observation comes from a genome-wide study of expression patterns during normal murine myeloid development.[146] This report reveals that ZRF1 (termed DNAJC2 in that study) is highly expressed during early phases of differentiation, and particularly at the promyelocyte stage, and then becomes downregulated upon the formation of mature myeloid cells. The fact that the expression of ZRF1 is highly regulated during myeloid differentiation, together with our functional data in AML cell differentiation, points to ZRF1 as an important regulator of normal myelopoiesis. In addition, since AML cells are arrested at early phases of differentiation, the high expression of ZRF1 in this developmental stage fits with the finding that ZRF1 is highly overexpressed in AML. Therefore, this suggests that ZRF1 overexpression might contribute significantly to the differentiation defects characteristic of leukemia.

Taking into account that RA has an essential function during vertebrate development,[26] it would be interesting to elucidate the role of ZRF1 in other RA-regulated differentiation processes. In fact, in our previous study we showed the important transcriptional function of ZRF1 in NT2 cells,[118] which can be differentiated into neuronal cells upon RA treatment.[119] Remarkably, RA is an essential regulator of the neuroectoderm linage.[26] Unpublished data from our laboratory indicate that ZRF1 is indeed an important regulator of neural development. Thus, ZRF1 is specifically required for the differentiation of mouse ESC into neural progenitors. Moreover, the function of ZRF1 in neural differentiation seems to be conserved, as it has also been recently reported in *C. elegans*.[113]

Additionally, ZRF1 has been shown to be a key regulator in other developmental processes. As mentioned above, the homolog of ZRF1 in *Volvox*, GlsA, is an essential regulator of asymmetric cell division during germ cell specification.[109] This function is conserved in higher plants, where the homolog of ZRF1 is fundamental for male gametic cell formation.[112] Interestingly, as mentioned in the introduction, ZRF1 is

highly expressed in human testis, which suggests that ZRF1 could also regulate the formation of gametes in the human system. Furthermore, RA has a key role during mammal gametogenesis.[147] Consequently, it can be hypothesized that ZRF1 might regulate spermatogenesis in cooperation with RARα.

Taken together, these data strengthen the role of ZRF1 as a regulator of RA-mediated differentiation processes, as we have observed in AML cells. In addition, they place ZRF1 as an emerging regulator of multiple developmental processes, a function that seem to be conserved throughout evolution.

## Nuclear and cytoplasmic functions of ZRF1

One important element to consider is whether the effects that we observe upon ZRF1 depletion are exclusively related to its function as a transcriptional regulator. Besides its recently characterized role in transcription, ZRF1 is known to have a cytoplasmic function as a molecular chaperone. As exposed in the introduction, ZRF1 associates with the ribosomes and facilitates proper folding of newly synthesized proteins,[101,102] a function that is conserved in yeast.[98] Thus, lack of ZRF1 leads to alterations in translational fidelity in yeast.[148] A recent report suggests that ribosome number and function might also be affected in ZRF1-depleted human cells, although the data are less conclusive than in yeast.[104] On the other hand, as previously discussed, ZRF1 has been reported to have additional functions. Particularly, at least in some contexts, ZRF1 seems to directly regulate cell division as a mitotic spindle-associated factor, although this has not been shown in mammals.

To shed new light on this subject, it would be interesting to perform rescue experiments in ZRF1-depleted AML cells with ZRF1 mutants that either cannot enter the nucleus or that are retained there, and evaluate their effect both at the phenotypic and at the molecular level. These studies would allow discriminating the relative importance of ZRF1 function in the nucleus and in the cytoplasm. As the protein does not have any described nuclear localization sequence (NLS), the simplest approach

could be using a mutant form of ZRF1 fused with a strong NLS that would restore the nuclear function but not the cytoplasmic one.

It should be noted that while ZRF1 is abundant both in the cytoplasm and in the nucleus in human cells, in yeast it is exclusively (or very predominantly) expressed in the cytoplasm.[98] Moreover, the yeast homolog of ZRF1 lacks the SANT domains that are present not only in humans but also in the rest of multicellular organisms (including vertebrates, invertebrates, plants and multicellular algae, see figure I11). Additionally, as discussed above, in multicellular organisms the function of ZRF1 regarding cell growth and differentiation seems to be cell type-specific and depend on the developmental stage. Altogether, these data argue in favour of a more specialised function of ZRF1 in complex organisms. For this reason, we hypothesize that, in this context, ZRF1 might predominantly function in the regulation of specific transcriptional programs rather than being just a general regulator of translation.

Altogether, these data suggest that ZRF1 is a multifunctional protein. ZRF1 could combine, among others, a nuclear role as a transcriptional regulator and a cytoplasmic role as a translational regulator. It could de speculated that ZRF1 might therefore control the whole gene expression process, from gene transcription in the nucleus to protein synthesis in the ribosomes. Further research is required to understand these complex regulatory mechanisms.

## Link between ZRF1 and RARα in transcriptional regulation

We previously reported that ZRF1 is an epigenetic regulator that facilitates transcriptional activation of Polycomb-repressed genes in the context of RA-induced differentiation in NT2 cells. Mechanistically, we found that ZRF1 binds to ubiquitinated histone H2A (H2Aub) and displaces the Polycomb repressive complex 1 (PRC1) from chromatin, hence leading to gene activation.[118] The function of ZRF1 as an activator of Polycomb-repressed genes was recently confirmed by another report in

our laboratory, this time in the context of oncogene-induced senscence.[141] However, two main questions still remained unsolved. First, the mechanism by which ZRF1 is targeted to specific genes. Second, the mechanism by which ZRF1 regulates transcription once it is bound to chromatin. These two issues are discussed below.

As just mentioned, we previously reported that ZRF1 is recruited to chromatin through the binding to H2Aub.[118] However, H2Aub is one of the most abundant epigenetic marks, being present on up to ten percent of total histone H2A. Given that ubiquitination is thought to occur usually at only one of the two H2A proteins within the nucleosome, this means that on average every fifth nucleosome is marked by this epigenetic mark.[149] ZRF1 does not seem to bind to all these genomic locations. In fact, the ChIP-on-chip analysis from our previous study showed that ZRF1 is present in about 10-15% of the H2Aub sites.[118] Moreover, this report revealed that, at least in NT2 cells, ZRF1 target genes changed depending on the absence or presence of RA. This strongly suggested that there should be additional mechanisms to target ZRF1 to specific genomic sites and in response to different stimuli.

Here we have shown that binding to RARα is a possible mechanism by which ZRF1 is recruited to chromatin. Our data reveal that ZRF1 interacts directly with RARα through its N-terminal part, indicating that the interaction is not dependent on its SANT domains. In HL60 cells, ZRF1 binds to RARE sequences located in the promoters of RA-target genes, the regions where RARα proteins are placed. The importance of ZRF1 in the regulation of RA-controlled transcriptome has been further demonstrated by our genome-wide expression data, which show that ZRF1 regulates nearly half of the RA target genes.

These results point to ZRF1 as a novel RARα-associated factor. However, ChIP-sequencing experiments of ZRF1 and RARα would be needed to corroborate these findings. In addition, ZRF1 ChIP-sequencing would reveal to what extent the effects that we see in RA-regulated transcriptome upon ZRF1 depletion are direct. Although we have tried to address this question, the low quality of both ZRF1 and RARα antibodies (as observed by the low percentage of input in our ChIP experiments,

specially in HL60 cells) has made it very challenging. Furthermore, it would be interesting to perform ChIP experiments of ZRF1 in RARα-depleted cells to definitively prove that ZRF1 binding to RARE-containing regions is mediated by RARα. It is important to consider, though, the possibility of compensatory effects by RARβ and RARγ, which would complicate the analysis in the case that ZRF1 was also able to bind them. In fact, RARγ is expressed at low levels in HL60 cells and, more importantly, RARβ gene is directly repressed by RARα thus its expression would be activated upon RARα knockdown.[29] In this sense, it would be interesting to study whether ZRF1 can also bind RARβ and RARγ, which could be relevant in other cellular contexts in which these isoforms predominate over RARα. In summary, although our data clearly suggest that ZRF1 is a novel regulator of RARα-controlled gene expression, further research is required to understand the exact mechanism.

Additionally, other mechanisms apart from binding to RARα and H2Aub could be involved in ZRF1 recruitment to chromatin. First, one obvious possibility is that ZRF1 might bind directly to DNA, an option that has been suggested by previous *in vitro* studies with the mouse and yeast homologs of ZRF1.[95,115,116] One of these studies even identified a sequence (GTCAAGC) as a putative binding motif for the SANT domains of ZRF1.[116] Although the experiments were performed *in vitro* and using only the SANT domains (which are conserved domains between different proteins), it may be worth studying whether this motif is overrepresented in the genes that we have found to be regulated by ZRF1 in HL60 cells. A second additional mechanism of recruiting ZRF1 to chromatin could be through the binding to any kind of non-coding RNA, a possible mechanism of protein recruitment to chromatin that is being intensively studied in recent years. In this sense, ZRF1 was found to have the ability to bind RNA, at least in yeast, which is thought to be important for its function in the ribosome[97,98] but might also have a role in the chromatin context. Even a third mechanism of regulating ZRF1 binding to chromatin could involve Id proteins, which were shown to bind ZRF1 in mouse cells,[115] a finding that we confirmed in human cells.[118] Since Id proteins bind to various transcription factors thereby preventing them from binding to chromatin,[107] this mechanism might also apply to ZRF1. In summary,

this suggests that, apart from the binding to RARα that we have characterized, multiple mechanisms could be playing a role in ZRF1 targeting to chromatin, probably depending on the cellular and developmental context.

## ZRF1-mediated transcriptional mechanisms

Another mechanistic issue that remained unsolved in our previous report was how ZRF1 regulates transcription once bound to chromatin. We previously showed that, in a subset of Polycomb-repressed genes, ZRF1 displaces PRC1 and facilitates transcriptional activation.[118] In this sense, here we have provided initial evidence that ZRF1 may interact with two different complexes that are known to participate in transcriptional activation. First, the FACT complex, which works as a histone chaperone that facilitates nucleosome remodelling during transcriptional elongation.[150] Second, the MLL complex, which trimethylates H3K4, a mark associated with transcriptional initiation.[83] Further biochemical and ChIP experiments are required to confirm these interactions and to understand whether any of these complexes are indeed involved in ZRF1-mediated transcriptional activation.

One element to contemplate is the link between, on the one hand, the mechanism of ZRF1 in association with RARα and, on the other hand, the role of ZRF1 in relation to H2Aub/PRC1. In a hypothetical combined mechanism, ZRF1 could be targeted specifically to chromatin through its binding to RARα and then, after a certain signal such as RA treatment, bind to H2Aub and displace PRC1. Nevertheless, our ChIP experiments in HL60 cells reveal that neither PRC1 nor H2Aub are present in the RA-targets in which we find ZRF1 (figure R34 and data not shown). Besides, in HL60 cells, ZRF1 binds RA-target genes both in the absence and in the presence of RA (figure R35). Therefore, the association with RARα and H2Aub/PRC1 are probably two independent mechanisms by which ZRF1 regulates transcription.

In this sense, it should be noted that, although the expression of nearly half of the RA-target genes are affected by ZRF1 depletion, this

relationship is significantly different in the other way. Thus, more than 80% of ZRF1-regulated genes are not RA targets (i.e. they are not activated by RA administration, see figure R20), although we cannot exclude that some of them are bound by RARα anyway. This strongly suggests that ZRF1 has as well RARα-independent transcriptional functions and one of them would be the link with H2Aub and PRC1. Another possibility is that the two mechanisms are operating in different cell types, which it is likely since both RA pathway and Polycomb (and probably also ZRF1) are involved in particular lineage specification processes.

Interestingly enough, our results in AML cells suggest that ZRF1 may have a dual role in transcriptional regulation and, specifically, in the control of RA target genes. Thus, our gene expression analysis shows that, in proliferating cells, ZRF1 depletion leads to the upregulation of about two-thirds of the ZRF1/RA-coregulated genes. Conversely, in RA-treated cells, ZRF1 knockdown leads to the downregulation of about two-thirds of the ZRF1/RA-coregulated genes (see figures R20 and R21). Our data thus suggest that ZRF1 works both as a transcriptional repressor and as a transcriptional activator of different subsets of RA target genes, being the repressive function predominant in basal conditions and the activating function predominant in RA-treated cells. As mentioned above, RARα has also a dual role in transcriptional regulation, as a repressor in the absence of RA and as an activator when RA is present,[54] hence reinforcing the finding of a functional cooperation between the two proteins.

Although our data suggest that ZRF1 works mainly as a transcriptional repressor in untreated cells and mainly as a transcriptional activator in RA-treated cells, the molecular basis for this switch remains unknown. One possible explanation is that the protein undergoes a post-translational modification during differentiation. Interestingly, a genome-wide phosphoproteomic study suggested that ZRF1 is phosphorylated during RA-induced differentiation in mouse P19 cells.[151] Specifically, four serines present in the N-terminal end of the protein seem to be phosphorylated at a higher level in differentiated cells than in basal conditions. We performed multiple sequence alignment comparing different species and found that these residues are conserved in vertebrates

but not in other phylogenetic groups, thus suggesting that this mechanism could be involved in specialized developmental functions. In this regard, we recently reported that the oncogene Myc cooperates with RARα to repress RA target genes in undifferentiated AML cells and, upon RA treatment, becomes phosphorylated and activates gene expression.[152] (*) Understanding whether a similar mechanism controls the ZRF1 switch from a repressor to an activator will be a focus of future research. In fact, preliminary immunoprecipitation experiments coupled to mass spectrometry in untreated and RA-treated HL60 cells suggest that ZRF1 may be indeed phosphorylated upon RA administration in this cellular system, although this has yet to be confirmed.

Finally, based on several studies about ZRF1 one could speculate on some additional mechanisms that could explain the function of ZRF1 as a transcriptional regulator. First, since ZRF1 may have the ability to bind RNA,[97,98] it could be involved in the recruitment of non-coding RNA and, in particular, long non-coding RNA, which are emerging in the last years as important players in transcriptional regulation.[153] Second, one intriguing characteristic of ZRF1 is that it may bind to Z-DNA,[95,115] a left-handed configuration of DNA, different from the standard right-handed B-DNA form.[96] Z-DNA configuration is thought to occur transiently during the process of transcription and to have a function in its regulations (although it is unclear); therefore ZRF1 could be involved in this process. Third, an attractive hypothesis is that ZRF1 could function as a histone chaperone, mimicking its function within the ribosomes. Histone chaperones regulate chromatin structure and are important regulators of transcription.[69] In this line, some reports in *Volvox* and in yeast, suggest that ZRF1 could have chromatin-related functions in association with Hsp70.[114,144] Since the two proteins work together as molecular chaperons in the ribosome, it could be speculated that they might have the same role in transcriptional regulation.

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(*) The article 'E-box-independent regulation of transcription and differentiation by MYC', in which I contributed, is annexed at the end of this thesis.

## A novel function of ZRF1 as a repressor: potential implications in leukemogenesis

Here we have revealed a potential novel role of ZRF1 as a transcriptional repressor. More specifically, in proliferating AML cells, ZRF1 seems to repress a large proportion of RA target genes. Our ChIP data show that ZRF1 depletion leads to its displacement from RA targets, which results in increased acetylation levels. In addition, we have shown that ZRF1 interacts with HDAC proteins and, particularly, with HDAC2. Altogether, this suggests that ZRF1 may repress gene transcription through the recruitment of HDACs, thus decreasing histone acetylation levels. As previously mentioned, RARα-mediated repression also involves the regulation of histone acetylation, being HDACs fundamental components of the RARα-associated corepressor complexes.[54]

Interestingly, deregulation of histone acetylation by aberrant recruitment of HDACs to RA target genes contributes to leukemogenesis in several types of AML.[78] This is the case, for instance, of leukemia-associated fusion proteins such as PML-RARα and AML1-ETO, which causes aberrant gene silencing through the recruitment of several complexes containing HDACs. Accordingly, we hypothesize that overexpression of ZRF1 in AML cells could contribute to leukemia through a similar mechanism. In this sense, high levels of ZRF1 would result in anomalous recruitment of the protein to RA target genes involved in the control of proliferation, apoptosis and differentiation processes. This would lead to aberrant recruitment of HDACs to these genes, followed by histone deacetylation and chromatin compaction, which would result in gene silencing. Specifically, since our data show that ZRF1 repressed genes are involved in differentiation and also in proliferation inhibition and apoptosis induction, this would lead to differentiation arrest, increased proliferation and reduced apoptosis. In this way, overexpression of ZRF1 could contribute significantly to these basic features of leukemia.

In contrast, ZRF1 inhibition would results in induction of differentiation and apoptosis and inhibition of proliferation, as we see in our ZRF1 depleted AML cells. Mechanistically, ZRF1 knockdown would lead to

HDAC displacement, increased histone acetylation, chromatin de-compaction and gene activation. In this sense, preliminary ChIP experiments suggest that indeed HDACs (and specifically HDAC2) are displaced from RA target genes upon ZRF1 depletion, at least at some targets. A model for the molecular mechanism that could link ZRF1 overexpression with leukemia induction and ZRF1 depletion with leukemogenesis inhibition is illustrated in Figure D2.

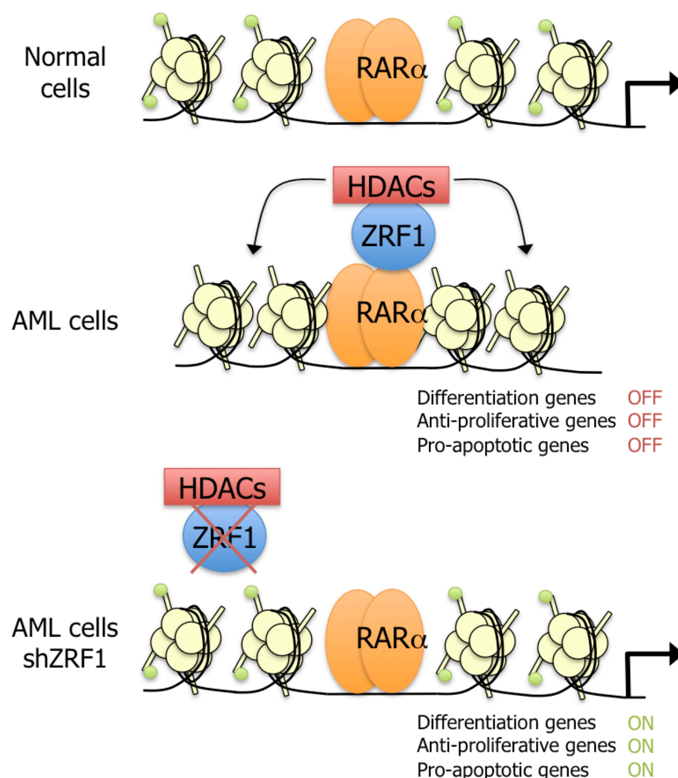


**Figure D2: Model of leukemogenesis induction by ZRF1 overexpression and leukemogenesis inhibition by ZRF1 depletion in AML cells.** The negative or low expression of ZRF1 in normal myeloid cells would not interfere with RA target gene regulation. In AML cells, ZRF1 overexpression might contribute to leukemia development by silencing RA target genes through the recruitment of HDACs, which would lead to histone deacetylation and chromatin compaction. Specifically, ZRF1 overexpression would result in repression of differentiation, anti-proliferative and pro-apoptotic genes, thus contributing to differentiation arrest, increased proliferation and inhibited apoptosis. In contrast, ZRF1 depletion in AML cells would cause displacement of HDACs, histone reacetylation and chromatin decompaction, resulting in activation of these genes and thus causing inhibition of leukemogenesis. Green circles indicate acetyl groups.

## ZRF1: a novel target for AML treatment?

As mentioned above, previous studies showed that ZRF1 is overexpressed in several types of cancer, including leukemia and, specifically, in AML.[124-127] Additionally, one of these reports reveals that more than 80 % of the AML patients have detectable levels of antibodies against ZRF1 in the blood, whereas this is not observed among healthy donors. In fact, these studies suggest that ZRF1 is a highly immunogenic leukemia-associated antigen (LAA). LAAs, and in general tumor-associated antigens, are factors produced specifically by cancer cells that can trigger antitumoral immune responses. Consequently, LAAs are considered to be promising potential targets for immunotherapy in AML.[154]

Here, we have shown that ZRF1 inhibition in AML cells leads to a decrease in cell proliferation and an increase in apoptosis and basal differentiation, which results in a reduced leukemogenic potential. Remarkably, we have observed this effect in five cell lines corresponding to different subtypes of AML. Specifically, HL60 cells correspond to acute myeloblastic leukemia (M2), NB4 correspond to acute promyelocytic leukemia (M3), and THP1 and U937 correspond to acute monocytic leukemia (M5) (see Table 1). In addition, NB4.007/6 are NB4-derived cells that, despite expressing RARα,[130] require much higher concentrations of RA (as compared with the original NB4) to become differentiated.[129] Altogether, this shows that ZRF1 depletion affects various types of AML cells, suggesting that its function is of broad relevance in AML.

By performing mouse xenograft experiments, we have shown that ZRF1 depletion strongly reduces leukemia progression *in vivo*. Moreover, we have validated *in vivo* that ZRF1 knockdown induces differentiation, as we had previously seen in cell culture experiments. With our data, though, it is not possible to distinguish between the relative contribution of proliferation inhibition, apoptosis induction and differentiation promotion by ZRF1 knockdown in leukemia suppression. Nevertheless, we believe that leukemia inhibition by ZRF1 depletion is most probably due to the triple function of ZRF1 in these processes. Remarkably, the effect observed in our xenograft experiments seems to be stronger than the one

obtained in our cell culture experiments, which suggests that the lack of ZRF1 also hinders cell engraftment and/or dissemination in an *in vivo* environment.

Furthermore, we have shown that ZRF1 depletion cooperates with RA treatment in leukemia suppression *in vivo*. One element to consider concerning our cell culture experiments is that, on the one hand, they show a cooperative effect of ZRF1 knockdown and RA treatment in cell growth (inhibition) and apoptosis (induction) but, on the other hand, ZRF1 knockdown inhibits RA-induced differentiation, thus showing an opposite role. Nevertheless, it should be noted that, in our *in vivo* experiments, during the period that lasts from the day in which we infect the cells with shZRF1 (five days before the inoculation into the mice) until the day in which we start the RA treatment (seven days after inoculation) the effect of the ZRF1 knockdown is already occurring. Therefore, we believe that this initial induction of differentiation caused by the ZRF1 knockdown may be added to the enhancement of apoptosis and inhibition of proliferation to produce the cooperative effect of ZRF1 depletion and RA treatment in leukemia suppression. Remarkably, ZRF1 knockdown and RA treatment might have a synergistic effect in leukemia inhibition, since the two factors together seem to produce a greater effect than the sum of both individually. In conclusion, the cooperative effect between ZRF1 inhibition and RA treatment in leukemia suppression that we have observed opens the possibility to investigate combination therapies.

An additional observation with potential therapeutic consequences is the link between ZRF1 and HDACs. Specifically, ZRF1 inhibition could potentially result in the displacement of HDACs and the reactivation of aberrantly silenced genes (see figure D2), mimicking, at least partially, the effect of HDAC inhibitors (HDACi). Remarkably, in accordance with the notion that aberrant recruitment of HDACs to certain genes is a leukemogenic mechanism in some AMLs, HDACi have been proposed as possible therapeutic drugs against leukemia.[78] Indeed, as previously mentioned, some HDACi are even under clinical trials for AML treatment.[76]

Overall, our data suggest that ZRF1 inhibition, alone or in combination with RA treatment, is a potential novel strategy to be explored for AML treatment. Unfortunately, there are no ZRF1 inhibitors known so far. Since ZRF1 does not have any characterized enzymatic activity, which makes it difficult to design one, further characterization of its molecular functions is required. Nevertheless, considering our results and the fact that ZRF1 is highly overexpressed in AML blasts, we hypothesize that a potential inhibitor of ZRF1 may specifically affect leukemic cells, which would be interesting to test in the future with the appropriate experimental models.

# CONCLUSIONS

The following conclusions can be drawn from the results presented in this PhD thesis:

1. ZRF1 depletion inhibits cell growth in AML cell lines corresponding to different AML subtypes and in other cancer cell lines. ZRF1 depletion cooperates with RA in cell growth inhibition.

2. ZRF1 depletion inhibits cell proliferation in AML cells.

3. ZRF1 depletion induces apoptosis in AML cells. ZRF1 depletion cooperates with RA in apoptosis induction.

4. ZRF1 regulates differentiation through a dual role. ZRF1 depletion induces basal cell differentiation in AML cells. In contrast, ZRF1 depletion inhibits differentiation upon RA treatment and ZRF1 overexpression enhances RA-induced differentiation.

5. ZRF1 controls the RA-regulated transcriptome. ZRF1 depletion deregulates the expression of almost half of the RA target genes.

6. ZRF1 works both as a transcriptional repressor and as a transcriptional activator of RA target genes in different subsets of genes, being the repressive function predominant in basal conditions and the activating function predominant in RA-treated cells.

7. ZRF1 mainly represses differentiation regulators in basal conditions and activate this class of genes in RA-treated cells. ZRF1 represses positive regulators of apoptosis and negative regulators of proliferation.

8. ZRF1 directly interacts with RARα. The interaction is independent of the presence or absence of RA and is mediated by the N-terminal part of ZRF1.

9. ZRF1 binds to RARE sequences on the promoters of RA target genes both in the absence and presence of RA.

10. ZRF1 interacts with HDACs and regulates histone acetylation levels of RA target genes. ZRF1 depletion leads to increased histone acetylation. This could mediate the function of ZRF1 as a transcriptional repressor.

11. ZRF1 may interact with FACT and MLL complexes. This could potentially mediate the function of ZRF1 as a transcriptional activator.

12. ZRF1 depletion inhibits leukemia progression in mouse xenograft models of AML, both with HL60 and NB4 cells. ZRF1 depletion leads to an increased cell differentiation *in vivo*.

13. ZRF1 depletion cooperates with RA treatment in the inhibition of leukemia progression *in vivo*.

# MATERIALS AND METHODS

# Cell culture, proliferation, differentiation and apoptosis methods

## Cell culture

HL60, NB4, U937, THP1, and NB4.007/6 cells were cultured at 37°C and 5% $CO_2$ in RPMI medium supplemented with 10% fetal bovine serum. HEK293T, GP2-293 and NTERA-2cl.D1 (NT2) cells were cultured at 37°C and 5% $CO_2$ in DMEM supplemented with 10% fetal bovine serum. To monitor cell growth, AML cells were seeded at approximately 2.5 x $10^5$ cells / mL and counted and diluted every two days. NT2 cells were harvested by trypsinization, seeded at approximately 1 x $10^6$ cells in a p10 plate and counted and spitted every two to three days.
For RA treatment, all-*trans* retinoic acid (ATRA, Sigma) was dissolved in dimethyl sulphoxide (DMSO). Before use, it was dissolved in cell culture medium to obtain the desired concentration.

## Lentiviral and retroviral infection

To produce lentivirus, HEK293T cells were transfected with 5 μg of pCMV-VSV-G, 6 μg of pCMVDR-8.91 and 7 μg of the plasmid of interest, either pLKO-shRNA (Sigma) for knockdown or pEV833 for overexpression. To produce retrovirus, GP2-293 cells were transfected with 9 μg of pLPNIG plasmid together with 4.5 μg of pCMV-VSV-G plasmid. In both cases, 48 hours after transfection, virus were collected, filtered using a 0.44 μm filter, and then used to transduce AML cells by spinoculation (1000g, 90 min, 32ºC) in presence of protamine sulfate followed by an additional 3 hours incubation at 37ºC in 5% $CO_2$; the protocol was repeated for 2 consecutive days. In the case of NT2, cells were infected by spinoculation (1200rpm, 90 min, 32ºC) in presence of polybrene (Sigma). 24–48 hours after infection, cells were either selected with 2 μg/ml of puromycin (Sigma) or FACS-sorted for GFP positive cells. FACS sorting was performed at the CRG/UPF FACS Unit.

## Cell transfection

HEK293T or GP2-293 cells were plated at a density of 2 x $10^6$ cells in a p10 plate. The day after, the calcium phosphate-DNA precipitates were prepared with different amounts of DNA depending on the vector used in each assay and 0.25M $CaCl_2$. While overtaxing, 1 volume of the prepared calcium-DNA solution was mixed with equal volume of 2-fold HeBS solution (HEPES-buffered saline solution, pH 7.05: 0.28M NaCl, 0.05M HEPES, 1.5mM $Na_2HPO_4$) at room temperature. After 10 min, the calcium phosphate-DNA-HeBS suspension was added to the cells. After 16 hours the medium was replaced by fresh one. For transient overexpression experiments, the cells were collected after 32–38 additional hours. For virus production, the medium was collected 48 hours after transfection, replaced by fresh one and collected again after 24 additional hours.

## Cell proliferation

AML cells were treated with 10μM of BrdU solution for 30 min and then analyzed for BrdU incorporation using the APC BrdU Flow Kit (BD Pharmingen) according to the manufacture's protocol. The percentage of BrdU-positive cells was analyzed by flow cytometry on Becton Dickinson FACSCanto.

## Cell differentiation

AML cells, after treatment with RA where indicated, were rinsed twice with PBS and incubated 20 min with CD11b-PE or CD11c-APC antibodies (see antibody table), at room temperature and protected from light. After washing twice with PBS, cells were analyzed by flow cytometry on Becton Dickinson FACSCanto. Analysis was performed using FACSDiva Software (BD Bioscience).
The nitroblue tetrazolium (NBT) assay was performed using a commercially available NBT (Sigma). 200 μl of cell suspension at a density of 2 x $10^5$ cells were mixed with 200 μl of filtered 0.2% NBT solution and 3 μl of TPA (1 μM) and further incubated for 30 min at 37ºC.

Subsequently, cytocentrifuge slides were prepared (200 rpm, 4 min). NBT positive cells were determined by counting cells under a light microscope.

### Cell viability and apoptosis

AML cells, after treatment with RA where indicated, were stained for cell viability by diluting them 1:2 with Trypan blue. Cell viability was determined by counting cells under a light microscope. Apoptosis analysis was performed using Violet Annexin V / Dead Cell Apoptosis Kit (Invitrogen) according to the manufacture's protocol. After staining, cells were analyzed by flow cytometry on Becton Dickinson LSRII.

## Protein analysis methods

### Western blot

Cell extracts for western blot analysis were prepared in lysis buffer (25mM Tris-HCl, pH 7.6, 1% SDS, 1mM EGTA, 1mM EDTA), incubated 10 min at 100ºC, sonicated 30 sec in a Bioruptor (Diagenode), and centrifugated 30 min at maximum speed at 4ºC. Protein extracts were quantified by Bradford assay (Bio-Rad), diluted with 5X Laemmli Buffer, heated for 5 min and analyzed by SDS-PAGE using acrylamide gels in Running Buffer (25mM Tris-base, 200mM glycine, 0.1% w/v SDS). Proteins were then transferred onto nitrocellulose membranes at 300mA for 1 hour on ice in Transfer Buffer (25mM Tris-HCl pH 8.3, 200mM glycine, 20% v/v methanol). Protein transfer was checked by staining with Ponceau S (Sigma). Transferred membranes were blocked 30 min at room temperature in 5% w/v skimmed milk in TBS-T (10mM Tris-HCl, pH 7.5, 100mM NaCl and 0.1% Tween-20). Membranes were incubated overnight at 4ºC with the corresponding primary antibody (see antibody table) diluted in 5% skimmed milk in TBS-T. After three washes of 1 min with TBS-T, membranes were incubated for 1 hour at room temperature with the corresponding secondary antibody conjugated to horseradish peroxidase (1:5000, Dako) diluted in TBS-T. After four washes of 5 min

with TBS-T, protein detection was performed by enhanced chemiluminiscence with Pierce ECL Western Blotting Substrate (Thermo Scientific).

## Immunoprecipitation

For co-immunoprecipitation assays, cells were washed in PBS, diluted in lysis buffer (25mM Tris-HCl, pH7.4, 150mM NaCl, 1mM EDTA, 1% NP40, 5% glycerol, P.I.) and sonicated for 1 min (6 cycles of 10 sec) in a Bioruptor (Diagenode). After centrifugation for 30 min at 13.000 rpm, soluble material was quantified by Bradford. Antibodies were crosslinked to protein A sepharose beads (GE Healthcare) using Bis(sulfosuccinimidyl) suberate (BS3, Thermo Scientific), following the supplier's guidelines, and saturated with BSA. Lysates were incubated overnight with the antibodies conjugated to the beads in rotation at 4ºC. Immunoprecipitated material was washed four times with lysis buffer and eluted in parallel with Laemmli buffer and 0.1M glycine-HCl pH 2.8 (to avoid co-elution of antibody heavy chain with the target antigen). Eluates were loaded into SDS-PAGE gels.

## Flag-affinity purification

After transfection with Flag-containing plamids, HEK293T cells were washed in PBS and diluted in lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, P.I.). Lysates were incubated 30 min in rotation at 4ºC. After centrifugation for 30 min at 13.000 rpm, soluble material was quantified by Bradford. Flag immunoprecipitation was performed with anti-FLAG M2-agarose affinity gel (Sigma) or with anti-FLAG antibody coupled to G-agarose beads (Amersham Biosciences) in rotation overnight at 4ºC. After immunoprecipitation, beads were washed twice with lysis buffer and twice with TBS (10mM Tris-HCl, pH 7.5, 100mM NaCl). Two sequential elutions were performed with, in total, three resin volumes of 500 ng/μL FLAG peptide (Sigma). Eluates were diluted with 5X Laemmli Buffer and analyzed by SDS-PAGE. For mass spectrometry experiments, gels were silver stained, and the specific bands were analyzed at the Proteomics Unit of the CRG.

## Pulldown assays

For *in vitro* GST-pulldown assays, 15 μg of recombinant GST or GST-RARα and His-ZRF1 (or His-ZRF1 fragments) were mixed with 30 μL of glutathione-Sepharose beads and incubated in PBS containing 0.1% Triton X-100 for 3 hours at 4ºC. Where indicated, RA was also added at the corresponding concentration, and tubes were protected from light. After binding, beads were washed four times with PBS - 0.1% Triton X, and the bound proteins were next eluted by boiling the samples for 5 minutes in Laemmli Buffer at 95ºC. Samples were analyzed by SDS-PAGE, and proteins were detected by immunoblotting.
For His-ZRF1 pulldown assays, 20 μg of His-ZRF1 were mixed with 30 μL of Ni-NTA beads and incubated in IP Buffer (1% Triton X-100, 150mM NaCl, 2mM EDTA pH 8, 20mM Tris-HCl pH 8, P.I.) for 3 hours at 4ºC. In parallel, HEK293T cells were harvested, washed twice with PBS and resuspended in Lysis Buffer I (5mM PIPES pH.8, 85mM KCl, 0,5% NP-40, P.I.) to break the cell membranes. Nuclei were then resuspended in IP Buffer and sonicated with Branson sonicator three times at 10% output for 10 seconds. Lysates were centrifuged 30 minutes at 13.000 rpm at 4ºC and quantified using Bradford. Cell extracts were incubated with the Ni-NTA beads previously bound to His-ZRF1 (or empty control) overnight at 4ºC in rotation. After binding, beads were washed four times with IP Buffer and the bound proteins were eluted by boiling the samples for 5 minutes in Laemmli Buffer at 95ºC. Samples were analyzed by SDS-PAGE, and proteins were detected by immunoblotting.

## Chromatin immunoprecipitation

For chromatin immunoprecipitation (ChIP), HL60 cells were crosslinked with 1% of formaldehyde at 37ºC for 10 min, and the reaction was stopped by the addition of glycine (0.125 M). Cells were rinsed twice with cold PBS, resuspended in lysis buffer (1% SDS, 10mM EDTA pH 8, 50mM Tris-HCl pH 8, P.I.) and sonicated 19 cycles in a Bioruptor (Diagenode), in high intensity and 30 sec on/off. After checking DNA size and concentration, lists were centrifuged 30 minutes at 13.000 rpm at 4ºC and supernatants were diluted 1:10 with IP buffer (1% Triton X-100,

150mM NaCl, 2mM EDTA pH 8, 20mM Tris-HCl pH 8, P.I.) and then incubated overnight with specific antibodies (see antibody table). 30 μL of protein A agarose bead slurry (Amersham Biosciences), previously blocked with BSA, were added to the lysates for 2 hours. Then, beads were washed three times with Washing buffer 1 (1% Triton X-100, 0.1% SDS, 150mM NaCl, 2mM EDTA pH 8, 20mM Tris-HCl pH 8, P.I.) and one time with Washing buffer 2 (1% Triton X-100, 500mM NaCl, 2mM EDTA pH 8, 20mM Tris-HCl pH 8, P.I.). The immunoprecipitated material was then eluted with 400μL of Elution Buffer (1% SDS, 100mM $NaHCO_3$) for 30 min in rotation at room temperature. The eluted material was incubated overnight at 65ºC with a final concentration of 200mM NaCl; here the input material was included. The samples were then incubated with 8μL of 500mM EDTA, 16 μL of Tris-buffer 1M pH 6.5 and 2μL of proteinase K (10mg/mL) for 2 hours at 45ºC before phenol/chloroform-extraction and ethanol-precipitation with 3 volumes of Ethanol 100%, 200mM of NaCl and 10 μg of glycogen for 30 min at -80ºC followed by 30 minutes of centrifugation at 13.000 rpm at 4ºC. DNA was washed with 70% EtOH and centrifuged for 5 min more at 13.000rpm 4ºC. Precipitated DNA was dried at 37ºC and resuspended in 30−50μL of water. qPCRs were performed with 2 μL of immunoprecipitated DNA to a final volume of 10 μL in Lightcycler SYBR Green (Roche) and the corresponding primers (see primer table) using a Lightcycler Detector 480 (Roche).

## Gene expression methods

### qRT–PCR analysis

RNA was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacture's protocol. cDNA was generated from 1 μg of RNA with the First Strand cDNA Synthesis Kit (Fermentas) using oligo-dT primers according to the manufacture's protocol. cDNA was diluted to 100 μl with water, and 2 μl of sample were used for each qRT-PCR reaction, using SYBR green (Roche) and the corresponding primers (see primer table).

## Agilent gene expression microarray

RNA from four independent experiments was isolated with the miRNeasy Kit (Qiagen) according to the manufacture's protocol. The microarray experiment was performed at the CRG Genomics Core Facility. 100 ng of total RNA was labeled using LowInputQuick Amp Labeling kit (Agilent) following manufacturer's instructions. The labeled cRNA was hybridized to the Agilent SurePrint G3 gene expression 8x60K microarray according to the manufacturer's protocol. The arrays were washed, and scanned on an Agilent G2565CA microarray scanner at 100% PMT and 3 μm resolution. Intensity data were extracted using the Feature Extraction software (Agilent) and processed. In short, raw data were corrected for background noise, normalized, and corrected for multiple testing by using bioconductor project (http://www.bioconductor.org/).
Two samples (one corresponding to shControl RA0 and the other to shZRF1 RA48h) were excluded from the analysis due to its low quality as compared to the rest of the samples. ZRF1-regulated and RA-target genes were selected by considering all probe sets with an adjusted p-value lower than 0.05 and a fold-change cutoff of at least 1.2. Results were validated using RNA obtained from independent experiments. The overlap analyses between the different gene subsets were performed using the Genomatix Software (http://www.genomatix.de). The obtained gene lists were analyzed with Ingenuity Pathways Analysis (IPA; Ingenuity Systems; http://www.ingenuity.com) and DAVID Gene Functional Classification (http://david.abcc.ncifcrf.gov/).[155]
Analysis of RARE motif overrepresentation was performed at the CRG Bioinformatics Unit. In summary, for each group of genes analyzed, the sequences located within 5 kilobases upstream of the transcription start site were extracted. Sequences were scanned with the Clover software[133] using a matrix describing the RARE motif.[134] E-values were estimated for each group by comparing the obtained scores with a 1000 random sample obtained from the non-regulated genes.

## *In vivo* studies

### Xenotransplantation models

Animal studies were carried out in the AAALAC international accredited Animal Facility of the Biomedical Research Park of Barcelona (PRBB) in accordance with approved protocols from the Institutional Animal Care and Use Committee. HL60 and NB4 stable cell lines expressing luciferase were obtained by infecting these cells with the LPING plasmid. These cell lines were subsequently infected with pLKO-shControl or pLKO-shZRF1 (shZRF1 #2 was used for HL60 and shZRF1 #1 for NB4). Five days after starting the selection with puromycin, $4 \times 10^6$ cells were intraperitoneally inoculated into 8-week-old female CB17 SCID/beige mice. In the experiment without RA, eight mice per group were used divided in two independent experiments (four mice per group in each one). In the two experiments with RA (one with HL60 and another with NB4), five mice per group were used.
Both the vehicle and RA were injected intraperitoneally twice a week at 40 mg/kg, starting at day 7. RA (Sigma) was initially dissolved in Cremophor EL (Sigma) and then diluted 1:15 in PBS just before use. For whole-body bioluminescent imaging, mice were injected intraperitoneally with 50 mg/kg of D-luciferin (Gold Bio Technology), and analyzed after 6 min using an IVIS Imaging System (Caliper LifeSciences). Images were quantified with Living Image software (Caliper LifeSciences). The whole study was performed under specific pathogen-free (SPF) conditions.
Mice were euthanized on day 22 or 23, and necropsies were performed. The presence of tumours and other abnormalities were macroscopically evaluated. Spleens and peripheral blood were collected (the latter in the presence of EDTA). The spleens were mechanically sliced and samples were separately prepared using 1×RBC lysis buffer (eBioscience), following the supplier's guidelines, and were incubated 30 min with PE-Cy5-CD33 and, if indicated, with PE-CD11b. The presence of CD33-positive cells and the cell differentiation status were analyzed by flow cytometry with a Beckton Dickinson FACScanto.

# Other general methods

## Production of recombinant proteins

GST fusion expression constructs were transformed into *E. coli* BL21. Protein expression was induced with 0.3mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 17°C for GST and GST-RARα. Induced recombinant proteins were bound to glutathione beads and eluted with 15mM reduced glutathione in 50mM Tris-HCl pH.8 containing 10mM DTT and dialyzed against PBS / 5% glycerol overnight. His-ZRF1, His-ZRF1 N-terminus and His-ZRF1 C-terminus expression constructs were transformed into *E. coli* BL21. Protein expression was induced with 0.2mM IPTG 4 hours at 37°C. Induced recombinant proteins were bound to Ni-NTA beads and eluted with 250mM imidazole in 50mM $NaH_2PO_4$ pH.8 containing 300mM NaCl, 0.1% Glycerol and 0.05% Tween 20 and buffer exchanged to PBS / 5% glycerol in Vivaspin columns (GE Healthcare). Protein concentration was determined by Coomassie blue staining of SDS-PAGE gels in comparison with a standard.

## Bacterial transformation and preparation of plasmid DNA

The *E. coli* DH5α or BL21 competent cells (50μL) were transformed with 5μL of ligation mix or the desired plasmid by the heat shock protocol, which consists in incubation of the DNA/bacteria mixture on ice for 30 min, a heat shock step at 42ºC for 45 seconds and 5 min incubation on ice. Subsequently, 1 mL of LB medium was added and the transformed cells were shacked at 37ºC for 45 min. Cells were plated on LB plates with ampicilin or kanamicin and incubated at 37ºC overnight. Single colonies were picked the next day and incubated overnight with agitation in LB medium with the adequate selection.
For both mini- and maxi-scale DNA preparation, the bacterial culture was spinned down at 4ºC (4500rpm for 4 min in mini-preps, 6000g for 15 min in maxi-preps). Plasmid DNA was isolated using Exprep Plasmid SV Kit (GeneAll) for mini-preps or HiPure Plasmid Filter MaxiPrep Kit (Invitrogen).

# Antibodies, primers and plasmids

## Antibodies

| Protein | Application | Company | Cathalog num. |
|---|---|---|---|
| **ZRF1** | WB, IP, ChIP | Di Croce Lab. | - |
| **Tubulin** | WB | Abcam ab7291 | ab7291 |
| **Histone H3** | WB | Abcam ab1791 | ab1791 |
| **HA** | WB | Covance | HA.11 PRB101P |
| **CD11c** | WB | Abcam | ab52632 |
| **RARα** | WB | Santa Cruz | sc-551 |
| **RARα** | IP | Diagenode | CS-155-100 |
| **His** | WB | Qiagen | 34660 |
| **GST** | WB | Di Croce Lab. | - |
| **H3Ac** | ChIP | Millipore | 06-599 |
| **H3K27Ac** | ChIP | Millipore | 07-360 |
| **HDAC2** | WB | abcam | ab7029 |
| **SPT16** | WB | Millipore | 07-255 |
| **ASH2L** | WB | Bethyl | A300-489A |
| **MLL1** | WB | Shilatifard Lab. | - |
| **control IgG** | IP, ChIP | Abcam | ab46540 |
| **Flag** | IP | Sigma | Flag M2 |
| **CD11b-PE** | FACS | BD Pharmingen | 555388 |
| **CD11c-APC** | FACS | BD Pharmingen | 559877 |
| **CD33-PECy5** | FACS | BD Pharmingen | 551377 |

WB: Western blot; IP: Immunoprecipitation; ChIP: Chromatin immunoprecipitatio

## Primer sequences: ChIP-qPCR

| Gene | **Sequences** (F: forward; R: reverse) |
|---|---|
| **RARβ RARE** | **F:** AATCCTGGGAGTTGGTGATG<br>**R:** AGACCCTCCTGCCTCTGAA |
| **HOXA4 RARE** | **F:** TTTAGAAGCGCAAAGTCCAAG<br>**R:** GCCTTCACTAGCCGACATTT |
| **HOXA5 RARE** | **F:** AGAAGCAGGGCATCCTGAGA<br>**R:** CAACCACTAGGGTTCACCTG |
| **ICAM1 RARE** | **F:** GGTGTAGACCGTGATTCAAGC<br>**R:** CCGGAATTTCCAAGCTAAAG |

## Primer sequences: qRT-PCR

| Gene | Sequences (F: forward; R: reverse) |
|---|---|
| ZRF1 | **F:** CGGTCGTCCTGAGGATAAAA<br>**R:** CGTACGTGAGGCGTGAGTAA |
| ICAM1 | **F:** CTTGAGGGCACCTACCTCTG<br>**R:** TGCAGTGCCCATTATGACTG |
| HOXA5 | **F:** CCCAGATCTACCCCTGGATG<br>**R:** GGGTCAGGTAACGGTTGAAG |
| RGS2 | **F:** GATTGGAAGACCCGTTTGAG<br>**R:** CCTCAGGAGAAGGCTTGATG |
| THBD | **F:** CACAGGTGCCAGATGTTTTG<br>**R:** AACCGTCGTCCAGGATGTAG |
| CSF1R | **F:** TGGCTGTGAAGATGCTGAAG<br>**R:** CCTTCCTTCGCAGAAAGTTG |
| ICAM4 | **F:** CTCCAGGATCACCGCCTAC<br>**R:** GGCAGCGCAAAGTGTATTTC |
| ICAM3 | **F:** GCTCACGAGGCAAATACACC<br>**R:** AGTAACACCGCCACGAAGAC |
| CSF3R | **F:** CAGGCCCTTTCAGCTCTATG<br>**R:** TGCTTTAGATGCAGCTCTGG |
| NLRC4 | **F:** AGTTTGGTCCTCAGCACCTG<br>**R:** CCCAAGCTGTCAGTCAGACC |
| CASP10 | **F:** TCTTGGAAGCCTTACCGCAG<br>**R:** TCATCCTGTACACAGCTGCC |
| CASP8 | **F:** GAGAGAAGCAGCAGCCTTGA<br>**R:** TCCCCGAGGTTTGCTTTTCA |
| RUNX3 | **F:** ATGGCAGGCAATGACGAGAA<br>**R:** GGGGTTGGTGAACACAGTGA |
| CDK1C | **F:** CGATCAAGAAGCTGTCCGGG<br>**R:** GCTCTTTGGGCTCTAAATTGGC |
| BTG2 | **F:** GCGAGCAGAGGCTTAAGGT<br>**R:** TTGTGGTTGATGCGAATGCAG |
| DHRS3 | **F:** CCATCGACTACTGCACATCC<br>**R:** GGGAAACCTGACTCTCATGC |

## Plasmids and cloning

- pLKO-shZRF1: for ZRF1 knockdown, plasmids were purchased from Sigma, MISSION pLKO.1-puro. The shRNA sequences are the following (only the specific target sequence is shown):
  shZRF1 #1: ACAGATCAAAGCAGCTCATAA (TRCN0000254055)
  shZRF1 #2: CTGGAAGAACCAAGATCATTA (TRCN0000254058)
  shZRF1 #3: TACTTCACTTGCATAACTAAA (TRCN0000254057)
  shZRF1 #4: AGCAGCTGGTGAACCAATAAA (TRCN0000254054)

- pEV833-HA-ZRF1: for ZRF1 overexpression, ZRF1 was subcloned from a pet28A[118] to a lentiviral pEV833 plasmid.

- Plasmids for recombinant proteins production GST-RARα and His-ZRF1 (full-length and deletion mutants) were described before.[118,152]

- Plasmids for the ectopic expression of Flag-ZRF1, Flag-RARα, Flag-HDAC1, Flag-HDAC2 and Flag-HDAC3 were described before.[60,118,152]

- pLPNIG (MSCV-Luc2-PGK-Neo-IRES-GFP) was generated at the Dr. Johannes Zuber laboratory (IMP, Viena) by replacing the miR30 cassette in pLMN [156] with a mammalian codon-optimized luc2 transgene (taken from pGL4.13, Promega), which compared to conventional firefly luciferase results in a >100-fold increase in bioluminescent signal.

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# ABBREVIATIONS

**aa**: amino acid
**AML**: acute myeloid leukemia
**AML1**: acute myeloid leukemia 1
**ALL**: acute lymphoblastic leukemia
**APL**: acute promyelocytic leucemia
**AraC**: cytarabine: cytosine β-D-arabinofuranoside
**ASH2L**: ash2 (absent, small, or homeotic)-like (*Drosophila*)
**ATRA**: *all-trans* retinoic acid
**BMI**: B lymphoma Mo-MLV insertion region 1 homolog
**BrdU**: bromodeoxyuridine (5-bromo-2'-deoxyuridine)
**CASP8**: caspase 8, apoptosis-related cysteine peptidase
**CASP10**: caspase 10, apoptosis-related cysteine peptidase
**CBX**: chromobox
**CD11b**: cluster of differentiation molecule 11b
**CD11c**: cluster of differentiation molecule 11c
**CDK**: cyclin-dependent kinase
**ChIP**: chromatin immunoprecipitation
**CLL**: chronic lymphoblastic leukemia
**CML**: chronic myeloid leukemia
**CSF1R**: colony stimulating factor 1 receptor
**CSF3R**: colony stimulating factor 3 receptor
**CYP26**: cytochrome p450
**DBD**: DNA binding domain.
**DNAJC2**: DnaJ (Hsp40) homolog, subfamily C, member 2
**DNMT**: DNA methyltransferase
**EED**: embryonic ectoderm development
**EZH1**: enhancer of zeste 1
**EZH2**: enhancer of zeste 2
**ETO**: Eight twenty-one
**FACS**: fluorescence-activated cell sorting
**FACT**: facilitates Chromatin Transcription Complex
**GFP**: green fluorescent protein
**H2Aub**: histone H2A monoubiquitinated at lysine 119
**H3ac**: acetylated histone H3
**H3K4me3**: histone H3 trimethylated at lysine 4
**H3K27ac**: histone H3 acetylated at lysine 27
**H3K27me3**: histone H3 trimethylated at lysine 27
**HAT**: histone acetyltransferase
**HDAC**: histone deacetylase
**HDACi**: HDAC inhibitors
**HMT**: histone methyl transferase
**HOX**: homeobox
**HOXA4**: homeobox protein A4
**HOXA5**: homeobox protein A5

**HSC**: hematopoietic stem cell
**ICAM1**: intercellular adhesion molecule 1
**ICAM3**: intercellular adhesion molecule 3
**ICAM4**: intercellular adhesion molecule 4
**IgG**: immunoglobulin G
**IP**: immunoprecipitation
**LBD**: ligand bindig domain
**MLL**: mixed lineage leukemia
**MPP11**: M-phase phosphoprotein 11
**N.007/6**: NB4.007/6 cells
**N-CoR**: nuclear receptor co-repressor
**NLRC4**: NLR family, CARD domain containing 4
**NuRD**: nucleosome remodeling and deacetylase
**PcG**: Polycomb group proteins
**P.I.**: protease inhibitors
**PLZF**: promyelocytic leukemia zinc finger
**PML**: promyelocytic leukemia protein
**PPAR**: peroxisome proliferator activated receptor
**PRC1**: Polycomb repressive complex 1
**PRC2**: Polycomb repressive complex 2
**RA**: retinoic acid; *all-trans* retinoic acid
**RAC**: ribosome-associated complex
**RoDH**: retinol dehydrogenase
**RALDH**: retinaldehyde dehydrogenase
**RAS**: retinoic acid syndrome
**RARα**: retinoic acid receptor alpha
**RARβ**: retinoic acid receptor beta
**RARγ**: retinoic acid receptor gamma
**RARE**: retinoic acid responsive element
**RING**: really interesting new gene
**RGS2**: regulator of G-protein signaling 2
**RXR**: Retinoid X receptor
**SANT**: Swi3, Ada2, N-Cor, and TFIIIB
**shRNA**: short hairpin RNA
**SIN3A**: SIN3 transcription regulator family member A
**SMRT**: silencing mediator for retinoid and thyroid hormone receptor
**SPT16**: suppressor of Ty 16 (SUPT16H)
**SUZ12**: suppressor of zeste 12
**THBD**: thrombomodulin
**TrxG**: Trithorax group
**ZRF1**: zuotin related factor 1

# ACKNOWLEDGMENTS

Si he llegado hasta aquí es porque este viaje, que ha sido largo y muchas veces duro, lo he compartido con mucha gente. Gracias Luciano por la oportunidad de hacer el doctorado en tu grupo, por mostrarme siempre tu confianza, por tu apoyo y porque, a pesar de contestar la mayoría de emails con un 'ok', siempre has tenido la puerta de tu despacho abierta para cualquier cosa. Gracias a toda la gente del laboratorio, a la que está ahora y la que ya se ha ido. A Holger por haberme pasado la herencia de ZRF1. A Arantxa por estar ahí desde el primer día hasta el último, siempre con una historia para contar, por tu positivismo y tu pragmatismo, y por haber trabajado también en "la enfermedad humana". Al resto de gente que ha pasado por el lab: Luciana, Paola, Martin, Celia, Lilli, Sophia, Joana. Gràcies Iris, per haver estat la meva postdoc (sense ser-ho), per haver compartit plegats tantes reflexions, tant científiques com vitals, per haver-me passat els teus coneixements d'AML i per haver-me ajudat a tirar endavant el projecte... Ha estat un plaer treballar amb tu. Y a los que estáis ahora, Luigi, compañero de ZRF1 y de tertúlia política, Lluís, por tu buen humor, Àlex, companya de PhD (junior) i d'MH, Pedro, porque has traído buen ambiente al lab y por las correcciones, Livia, por ser completely different, Malte, compañero de bench, Cecilia, por haber compartido penas (¡y alegrías!) con ZRF1 y estar siempre dispuesta a ayudar, Payal, Enrique... gracias a todos y a todas por vuestra ayuda en el día a día. Al resto de gente que ha colaborado en el proyecto, Sara Capdevila y Juan Martín Caballero. A la gente del FACS, Microarray, Bioinformática, Proteómica... y a la gente de administración, cocina,... por el día a día. A Chiara por compartir cafés y reflexiones. Y a tanta gente del departamento por su ayuda.

Y cuando llegas hasta aquí piensas también en el camino (científico) que has recorrido antes. Gràcies Montse Forns per haver-me passat la teva passió per la biologia des del batxillerat. I tantes i tants professors de la Universitat que et fan emocionar amb la replicació del DNA, les vies de senyalització, la regulació del metabolisme o la biologia molecular del càncer. I a la gent amb la que he tingut l'oportunitat de treballar abans del doctorat, el Ricard Guerrero (i també el Jordi Urmeneta, la Laura, el Javi,...) a la UB, la Lynn Margulis a Massachussetts, el Jaume Reventós a la Vall d'Hebron, el Jos Jonkers i el Bastiaan Evers al NKI d'Amsterdam. I a la Coral i l'Albert per ser també companys de professió.

Gracias mamá, gracias papá, por estar siempre, siempre ahí. Por estar en los momentos más complicados y por dármelo todo. Por el apoyo incondicional y por haberme enseñado tanto. Lu, gracias por ser la mejor hermana, por haber crecido juntos, por poder compartir tantas cosas contigo. Muchas gracias a los tres.

Gràcies a l'Alba, la Maria, el Roger, la Marta i la Laura per tantes coses viscudes des de fa tants anys. Per seguir estant junts des de l'institut (o des de l'escola). Gracias Elsa por haber estado ahí en momentos duros de este camino, pero sobretodo por haber compartido commigo tantos años, en los que hemos crecido juntos.

I gràcies a tota la gent de la uni, han estat 10 anys genials... i els que queden. Al Marcel i la Magda, des del primer dia. A l'Èlia per haver compartit tantes coses junts. A la Júlia, el Fernando, la Rosa, la Laia, l'Àlex, la Bea, la Lara... i a molta altra gent. A la Marta, companya de pis i de viatges. A l'Alba, per tantes coses viscudes junts, per totes les reflexions compartides, sobre la ciència i la vida, i per haver compartit el camí de la tesi (ho hem aconseguit!). Y gracias Ale por haberme acompañado en el tramo final de este largo viaje.

# SUPPLEMENTARY ARTICLES

Richly H1, Rocha-Viegas L, Ribeiro JD, Demajo S, Gundem G, Lopez-Bigas N, Nakagawa T, Rospert S, Ito T, Di Croce L. Transcriptional activation of polycomb-repressed genes by ZRF1. Nature. 2010 Dec 23;468(7327):1124-8.

Uribesalgo I1, Buschbeck M, Gutiérrez A, Teichmann S, Demajo S, Kuebler B, Nomdedéu JF, Martín-Caballero J, Roma G, Benitah SA, Di Croce L. E-box-independent regulation of transcription and differentiation by MYC. Nat Cell Biol. 2011 Oct 23;13(12):1443-9.