Role of the SUMO pathway in Acute Myeloid Leukemias response to treatments

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Preamble

Acute myeloid leukemia (AML) is one of the four types of primary leukemia's, which is characterized by large increase in the number of myeloid precursor cells. These precursors have lost their ability to differentiate and accumulate in the bone marrow. AML is a very heterogeneous disease and most subgroups are treated by chemotherapy composed of a combination of two genotoxics: one anthracycline such as daunorubicin and the nucleoside analogue Ara-C. Unfortunately, a large number of patients relapse. In spite of many efforts in the development of novel chemotherapy, no significant improvement was observed in the survival rates during the past 40 years. Nevertheless, one minor group defined as acute promyelocytic leukemia (APL), is the only one subtype, which is now cured by a differentiation therapy with very high survival rate. It has been shown recently that many cancers have impaired regulation of small-ubiquitin like modifier (SUMO) pathway and this post-translational modification might serve as a new target in the treatment of cancer.

When I arrived in the lab, I first participated to an ongoing study that concerned the role of the SUMO pathway in AML response to chemotherapeutic drugs and its involvement in chemoresistance. We have shown that chemotherapeutic drugs induce a massive desumoylation of cellular proteins through the ROS-dependent inactivation of the SUMO activating and conjugating enzymes. This desumoylation starts rapidly after drug addition and participates in the induction of apoptosis. In particular, we could show that this desumoylation is involved in the translational activation of DDIT3, a gene known to participate in genotoxics-induced apoptosis in AML. In chemoresistant AML, genotoxics do not induce ROS production and desumoylation. However reactivation of the ROS/SUMO axis, by pro-oxydants or pharmacological inhibition of the SUMO pathway restores expression of pro-apoptotoc genes and apoptosis in these cells (patients cells and mouse models). Thus, this work suggested that targeting the ROS/SUMO axis might be a way to overcome chemoresistance in AML and thus improve the treatment of this disease. My participation was to analyze the effects of the treatment with chemotherapeutic drugs on the sumoylation levels in both chemosensitive and chemoresistant AML cell lines. I also compared the sensitivity to Anacardic acid, an inhibitor of sumoylation, of both normal and AML cells. This work was published in 2014 and is detailed in Manuscript 2 (IVOther Projects).

Then, we were interested in the role of the SUMO pathway in AML differentiation therapy. This work constituted the main part of my PhD project. As mentioned previously, prognosis of APL patients is now very good thanks to the use of all-*trans* retinoid acid (ATRA). However its clinical efficiency is very limited in other AML subtypes, in particular because of epigenetic repression of

ATRA-responsive genes. Sumoylation plays a critical role in transcriptional regulation. In this context, using pharmacological inhibitor of sumoylation, 2D08 and Anacardic acid, I showed that inhibition of sumoylation enhances ATRA-induced differentiation in many AML cell lines (HL-60, U937, MOLM 14, THP1) and primary patient samples. To characterize the differentiation, I used flow cytometer to detect differentiating marker of myeloid and monocytic cell lines (CD11b, CD15 and CD14) and the activity of NADPH-oxidase was measured. Julie Kowalczyk, an intern of our lab, also confirmed the pro-differentiating action of sumoylation inhibitors by analysing the morphological changes of cells using May-Grumwald-Giemsa staining. To know if desumoylation could have a benefic effect on ATRA-induced differentiation of chemoresistant cells, I generated U937 resistant cell line to Ara-C by increasing gradually the concentration of Ara-C in the culture media. This also demonstrated that inhibition of sumoylation promotes sensitivity to ATRA even in chemoresistant AML cells. The main results obtained with inhibitors were also confirmed by genetically modulating the SUMO pathway through overexpression of desumoylases, which markedly increased their differentiation by ATRA or SUMO/Ubc9, which limited differentiation. The combination treatment composed of ATRA and inhibitors of sumoylation induce an arrest in AML cells proliferation *in vitro* and *in vivo* in NOD-Scid-IL2rg^{null} mice. Differentiation is a result of transcriptional reprograming process. I could show that inhibition of sumoylation facilitates the ATRA-induced expression of master genes of the myeloid differentiation using qRT-PCR on AML cell lines. Furthermore, I could show that inhibition of sumoylation increase the presence of $H3K4Me_3$, a mark of active transcription, on the promoter of these genes using CHIP-qPCR. Altogether, this work suggests that targeting the SUMO pathway could constitute a promising approach to sensitize AML to differentiation therapies. This project is described in 'Results' part as Manuscript 1, which will be submitted soon.

I Introduction

1 AML

1.1 Normal hematopoiesis and leukemia

1.1.1 Hematopoiesis

Hematopoiesis is a lifelong highly regulated multistages process. Self-renewing hematopoietic stem cells (HSC) commit to specific lineage-committed progenitors. These progenitors have lost the ability to self-renew and commit to both common lymphoid progenitors generating T-and B-lymphocytes or natural killer (NK) cells, and common myeloid progenitors, which give rise to granulocyte-monocyte and megakaryocyte-erythrocyte progenitors (Figure 1) (Rieger and Schroeder 2012; Orkin and Zon 2008).

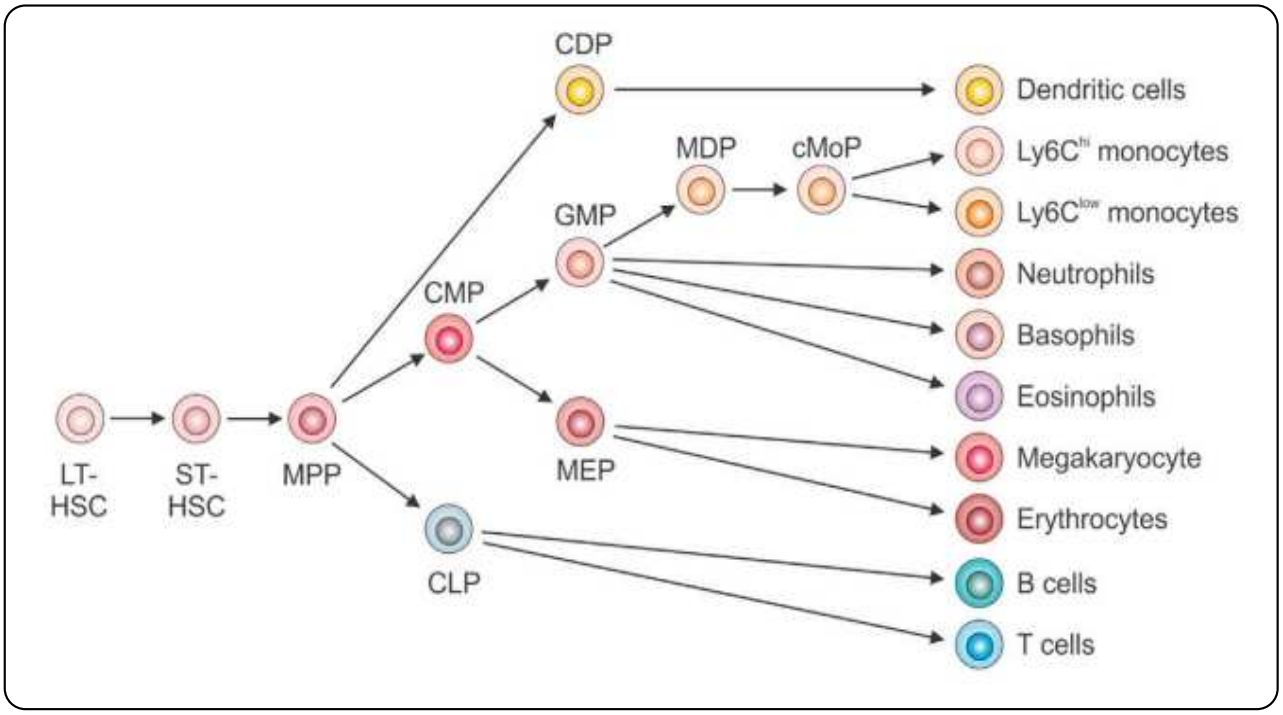


Figure 1: Hematopoiesis. Starting from the stem cell, hematopoiesis process gives two branches, myeloid and lymphoid, which through downstream stages of differentiation are producing normal blood constituents, red blood cells, neutrophils and lymphocytes. LT-HSC: long-term hematopoietic stem cell; ST-HSC: short-term hematopoietic stem cells; MPP: multipotent progenitors; CMP: common myeloid progenitors; CDP: common dendritic cell progenitors; GMP: granulocyte-macrophage progenitors; MEP: megakaryocyte-erythrocyte progenitors and CLP: common lymphocyte progenitors (Schultze and Beyer 2016).

The initial steps of hematopoiesis start in the bone marrow and are tightly regulated by a network of cell extrinsic and intrinsic pathways, which control the HSC/progenitors cell self-renewal capacity and maturation into functional cells. The external signals come from the bone marrow microenvironment and are mediated by soluble cytokines and growth factors mainly provided by stromal cells, cell-cell interactions, and cell-extracellular matrix interactions (Mossadegh-Keller et al., 2013; Rieger et al., 2009; Orkin and Zon 2008). Differentiation also

involves a massive transcriptional reprogramming through the expression of specific transcription factors (Figure 2), epigenetic changes, post-translational modifications of nucleosomal histone proteins, and the expression of small regulatory ncRNA, which all contribute to the irreversibility of cell maturation (Álvarez-Errico et al., 2015; Rosenbauer and Tenen 2007; Fazi and Nervi 2008; Orkin and Zon 2008; Zardo et al., 2008). Terminal differentiation processes that lead to the generation of mature cells take place in the blood or peripheral tissues. It depends on the exposure of precursor cells to cytokines, antigens and other factors (Geissmann et al., 2010).

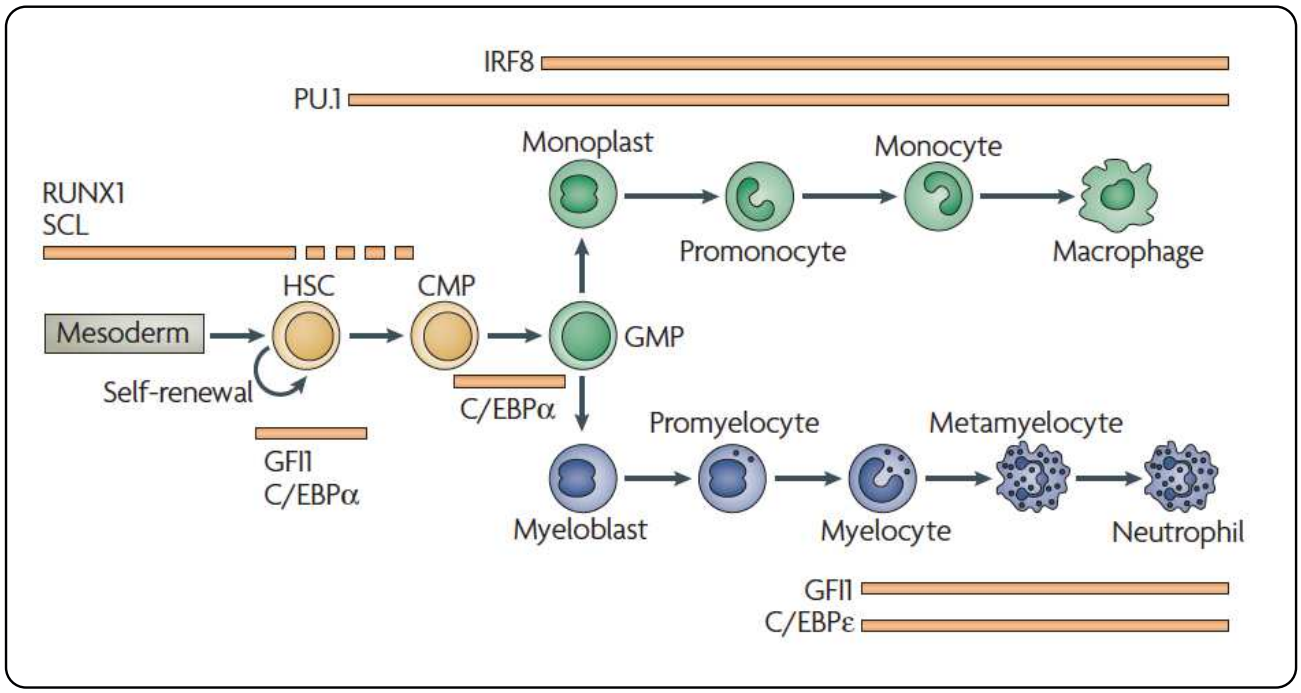


Figure 2: A stepwise requirement for transcription factors during myeloid differentiation. The differentiation of stem cells into the two main myeloid lineages, the monocytic and the neutrophilic lineages, is regulated by a hierarchical network of transcription factors. Runt-related transcription factor 1 (RUNX1) and stem-cell leukaemia factor (SCL) are required for the generation of haematopoietic stem cells (HSCs) whereas growth-factor independent 1 (GFI1) and CCAAT/enhancer binding protein-α (C/EBPα) function in self-renewal of existing HSCs. C/EBPα has another indispensable role in conferring the transition of common myeloid progenitors (CMPs) into granulocyte/monocyte progenitors (GMPs), GFI1, and similarly C/EBPε, are crucial for late-stage neutrophil production. Macrophage production depends on PU.1 and interferon-regulatory factor 8 (IRF8). In this process, PU.1 seems to be essential for all intermediate steps starting from HSCs. (Rosenbauer and Tenen 2007).

Another theory of hematopoiesis has recently emerged stating that lineage commitment during myelopoiesis is not linked to late-stage progenitors with multi-lineage potential. Using single cell transcriptomic of the myeloid progenitor cell compartment Paul et al. (Paul et al., 2015) suggest a much earlier commitment toward distinct lineages, even prior to the common myeloid progenitor state. This hypothesis was also confirmed by Schumacher and colleagues using single-cell fate mapping *in vivo* (Perié et al., 2015) and more recently by Velten et al., who questioned the stepwise progression of hematopoiesis (Velten et al., 2017).

1.1.2 Clusters of differentiations

Many efforts have been done to identify cell-type-restricted surface marker, called clusters of differentiation (CDs) proteins. This characterization enables the discrimination and enrichment of basically all different hematopoietic cell types by flow cytometry or magnetic cell sorting. As mentioned, differentiation and maturation can be monitored by changes in cytomorphology and immunophenotype. Here, I will develop in more detail the characterization by cell surface markers of monocytic, granulocytic and erythroid lineage differentiation stages (Terstappen et al., 1990).

❖ Normal Monocytic differentiation (van Lochem et al., 2004)

Macrophages are differentiated from myelo/monoblast, which becomes pro-monocyte, then monocyte expressing progressively CD11b, CD15 and at late stage, macrophage expressing CD14 (Figure 3).

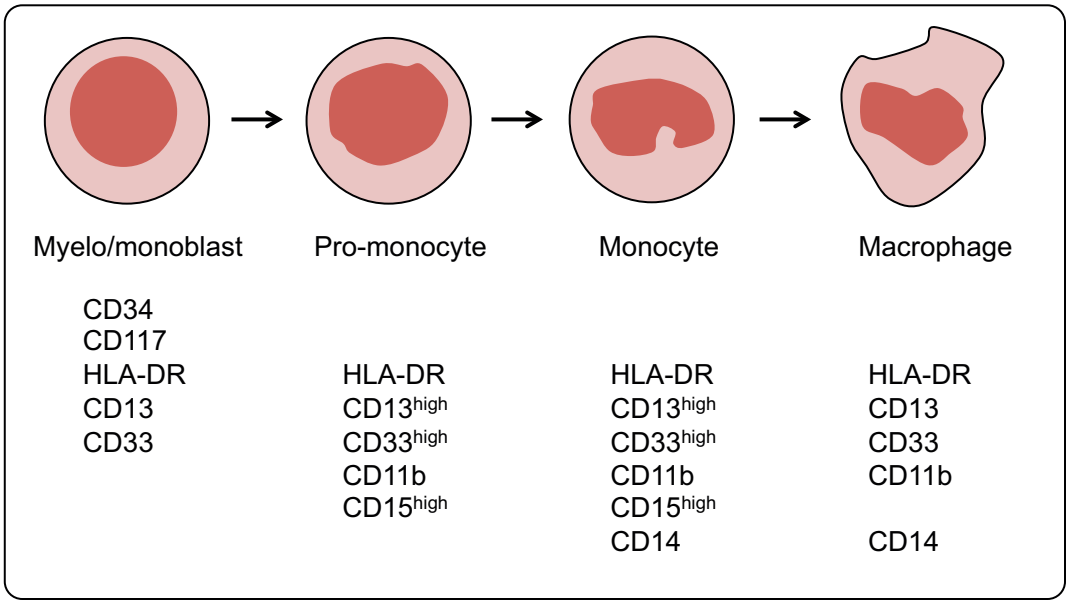


Figure 3: Normal monocytic development in bone marrow. Differentiated macrophage comes from myelo/monoblasts, which becomes pro-monocyte then monocyte. Mature macrophage differs from its precursors by the expression of CD14. Pro-monocyte and monocyte express CD11b and high CD15, which distinguishes them from myelo/monoblast.

❖ Normal granulocytic differentiation (van Lochem et al., 2004)

Granulocyte (neutrophil) derives from myelo/monoblast, which becomes promyelocyte, then myelocyte and metamyelocyte before being granulocyte. Differentiated granulocytes express CD11b and CD15 compared to the progenitor myelo/monoblasts, which don't express these markers at all (Figure 4).

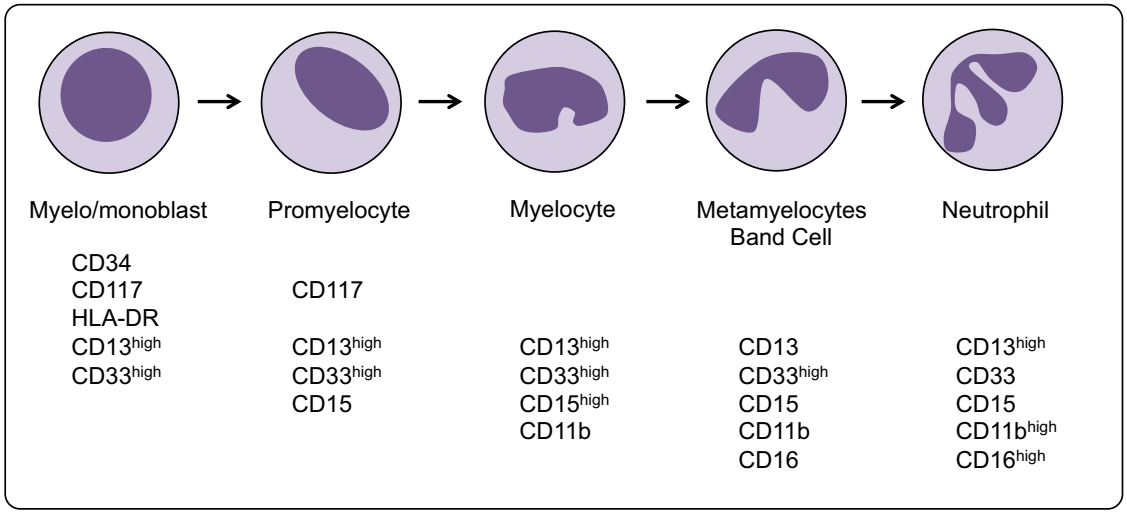


Figure 4: Normal granulocytic development in bone marrow. Myelo/monoblast becomes neutrophil (granulocyte) through a stepwise process. First it begins to express CD15 and becomes promyelocyte. Then appearance of CD11b classifies it as myelocyte. Then it becomes metamyelocytes expressing CD16 and differentiates finally into granulocyte.

❖ Normal erythroid development in bone marrow (van Lochem et al., 2004)

Erythrocyte arises from erythroblast, which is differentiated from pro-erythroblasts (Figure 5).

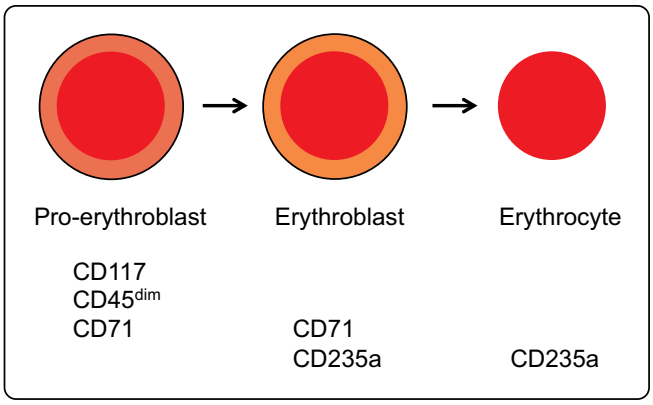


Figure 5: Normal erythroid development in bone marrow. Erythrocyte is derived from pro-erythroblast, which becomes erythroblast before being erythrocyte. Pro-erythroblast express CD117, CD45 and CD71 and erythroblast loose CD117 and express CD235a. Finally erythrocyte expresses only CD235a.

As I mentioned previously, according to the classical hierarchical model of cell differentiation, the differentiation is governed by a major transcriptional reprogramming. Mutations in the genes encoding for many of the critical transcription factors, epigenetic regulators, and miRNA have been found mutated in hematological malignancies or involved in chromosomal rearrangement generating oncogenic fusion proteins responsible for these diseases (Radulovićet al., 2013; Schotte et al., 2012; Zardo et al., 2008). The acquisition of such mutations by leukemic stem cells (LSC) or progenitors impairs the maturation resulting in the generation of a neoplastic clone, expansion of immature progenitor in the bone marrow with abnormal growth properties, uncontrolled cell division, differentiation arrest, and cell death escape. This leads to the accumulation of early blood cell precursors known as blast cells and gives rise to leukemia (Figure 6).

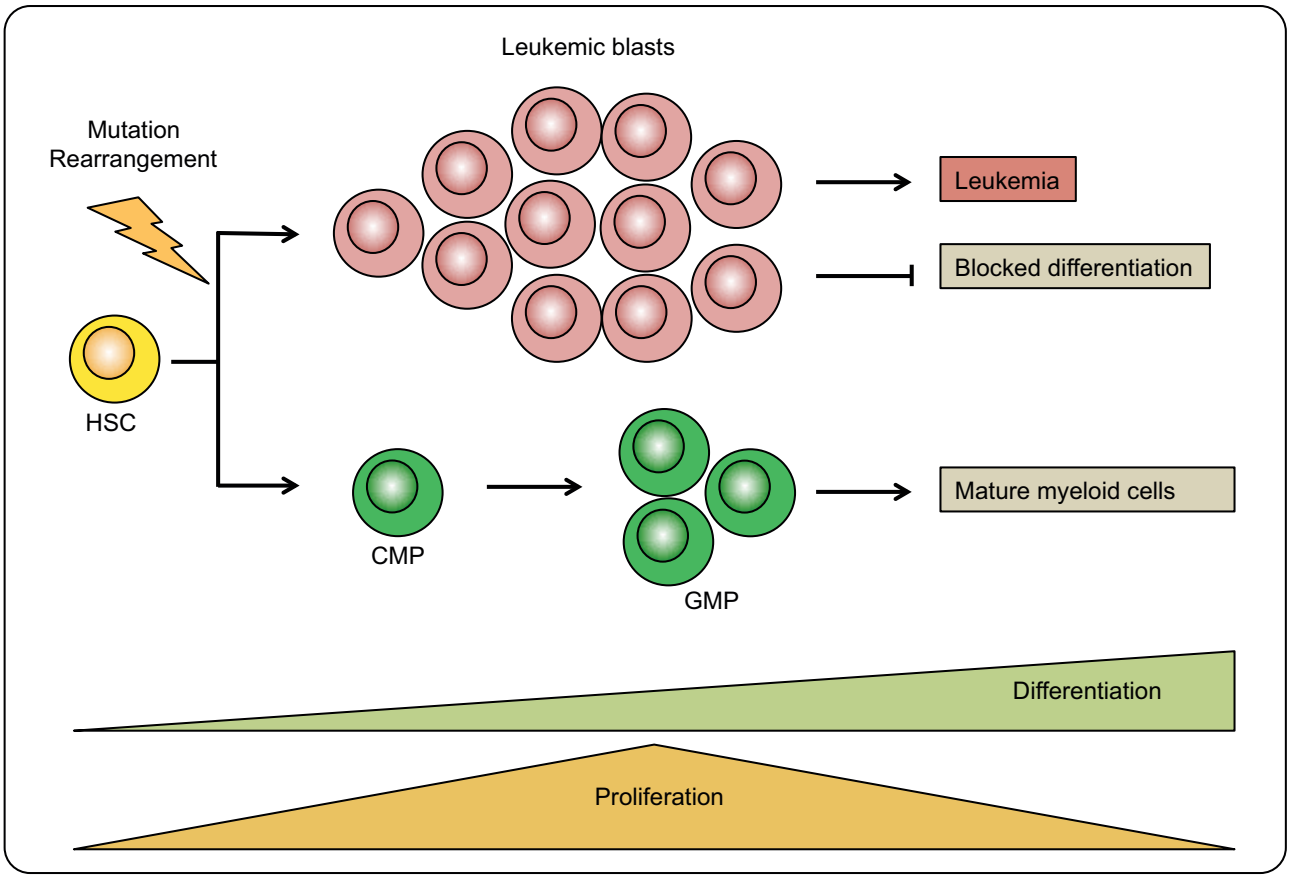


Figure 6: Leukemia development. Normal myeloid progenitors undergo rapid proliferation before they differentiate into functional immune cells. The balance between proliferation and differentiation is well controlled. Oncogenic mutation or chromosome rearrangements of LSC or progenitors cause a block in their differentiation and a prolonged proliferation phase. This gives rise to the leukemia. HSC: hematopoietic stem cell; CMP: common myeloid progenitor; GMP: granulocyte/monocyte progenitor (Adapted from Rosenbauer and Tenen 2007).

1.1.3 Leukemia

Leukemias account for 3.8% of new cancers diagnosed annually and are divided into categorically different types of leukemia depending on the type of white blood cell affected (lymphoid vs. myeloid) and the characteristics of the disease (acute vs. chronic):

Acute leukemias are generally aggressive cancers where cancerous transformation occurs at the early stages of the cell differentiation. Untreated, this disease can be rapidly fatal.

Chronic leukemias are characterized by a slower progression than acute leukemias, and most patients can live with them for many years. However they are generally difficult to cure and the therapy is often conservative and aims at controlling symptoms.

Myeloid leukemias affect the myeloid lineage cells - white blood cells (other than lymphocytes), red blood cells or megakaryocytes. They are also known as myelocytic, myelogenous or non-lymphocytic leukemias.

Lymphocytic leukemias affect immature form of lymphocytes. They are also known as lymphoid or lymphoblastic leukemias when they are developed in bone marrow, and lymphomas when they are found in lymph nodes or other organs.

Thus, we can distinguish 4 different types of leukemias:

- Acute Lymphoblastic Leukemia (ALL)
- Acute Myeloid Leukemia (AML)
- Chronic Lymphoid Leukemia (CLL)
- Chronic Myeloid Leukemia (CML)

Acute Lymphoblastic Leukemia (ALL) is the most common form of leukemia diagnosed in children. It affects B or T precursor cell and cells with B-cell type associated antigen. The incidence of ALL peaks between the ages of 3-7, falls by 10 years of age, and rises again after the age of 40.

Acute Myeloid Leukemia (AML) represents 10-15% of leukemias diagnosed in childhood and is the most common type of acute leukemia diagnosed in adults.

Chronic lymphoid leukemia (CLL) is characterized by the accumulation of fully developed B or T lymphocytes in the blood. These diseases are closely related to lymphomas, in which lymphocytes accumulate in lymph nodes and vessels. CLL mainly affects elderly individuals, with a peak incidence between 60 and 80 years of age. It is the most common form of leukemia in Western countries. CLL follows a variable course, with survival ranging from months to decades. Other types of chronic lymphoid leukemias include Prolymphocytic leukemia, hairy cell leukemia, Plasma cell leukemia, large granular lymphocytic leukemia and T-cell prolymphocytic leukemia.

Chronic Myeloid Leukemia (CML) accounts for approximately 15% of leukemias, and occurs most frequently between the ages of 40 and 60 years. Laboratory tests reveal increased numbers of cells belonging to the myeloid cell line (monocytes, neutrophils, basophils, eosinophils) at various stages of development circulating in the blood stream.

1.2 Acute myeloid leukemia

1.2.1 Incidence and mortality

Acute myeloid leukemia is the most common acute leukemia in adults (Yamamoto and Goodman 2008) and is a primarily a disease of older adults (≥60 years), with a median age at diagnosis of 67 years (National Cancer Institute. SEER stat Fact Sheets: AML, https://seer.cancer.gov/statfacts/html/amyl.html, 2015). The American Cancer Society estimates that

in United States in 2017, AML accounts 34% of all leukemia cases in adults 20 years of age and older (https://old.cancer.org/acs/groups/content/@research/documents/document/acspc-047079.pdf, 2016). The yearly incidence of AML in US is 4,5 cases per 100,000 individuals per year (Patel et al., 2012) and in European adults is 5 to 8 cases per 100,000 individuals with a steep increase in the population aged over 70 years where the incidence reaches 15-25/100,000 per annum and the yearly mortality figures in AML is 4 to 6 per 100,000 (Fey, Buske, and ESMO Guidelines Working Group 2013). The 5-year relative survival rate is 19% for AML (Visser et al., 2012). Of note, 70% of AML patient aged over than 65 years die in the first year after the diagnosis (Meyers et al. 2013).

1.2.2 Acute myeloid leukemia

AML are distinguished by the presence of more than 20% of leukemic blasts in the bone marrow, and AML patients exhibit signs and symptom of the disease. Usually the first step begins with a decrease in the number of normal blood cells, which results in varying degrees of anemia, thrombocytopenia, and neutropenia. Then, the rapid proliferation of immature cells along with their impairment to undergo apoptosis results in their accumulation in the bone marrow, the blood, and, frequently, the spleen and liver. The hyper-proliferation and block in differentiation involves the activation of abnormal genes through chromosomal translocations and/or mutations (Arber et al. 2016). AML diagnosis relies on morphological study of the blasts (FAB criteria (1.2.3.a)), signs of dysplasia as well as cytochemistry, immunophenotyping, cytogenetic study of the bone marrow (caryotype and FISH), and finally, molecular biology study (chromosome rearrangement).

1.2.3 AML classification

1.2.3.a FAB classification

AML is a heterogeneous group of malignancies with varying clinical, morphologic, immunologic, and molecular characteristics. One historical classification called ‘French-American-British’ (FAB) classification was established in 1976 (Bennett et al. 1976). It distinguishes 8 different subgroups of AML (M0 to M7) based on the morphological and cytochemistry features of leukemic cells (Table 1). Subtypes M0 through M5 are blocked at different stages of the myeloid lineage, M6 AML of the erythroid, while M7 AML arises from progenitors of cells that make platelets.

Beside, AML has a pattern of antigen acquisition seen in normal hematopoietic differentiation. Multiparameter flow-cytometry is a useful adjunct to morphology and cytochemistry and is an invaluable tool in the diagnosis of AML (Woźniak and Kopeć-Szlęzak 2008). Flow-cytometry of leukemic cells is largely used to identify AML subtypes and maturation stage as well as the

detection of residual disease: on a CD45/SSC bi-dimensional plot blast, blast cells are located in the so-called “Bermude Area” (Figure 7).

FAB type	*Name*	*% of adult patients*	*Prognosis compared to average for AML*
M0	Undifferentiated acute myeloblastic	5	Worse
M1	Acute myeloblastic leukemia with minimal maturation	15	Average
M2	Acute myeloblastic with maturation	25	Better
M3	Acute promyelocytic leukemia (APL)	10	Best
M4	Acute myelomonocytic leukemia	20	Average
M4 eos	Acute myelomonocytic leukemia with eosinophilia	5	better
M5	Acute monocytic leukemia	10	Average
M6	Acute erytroid leukemia	5	Worse
M7	Acute megakaryoblastic leukemia	5	Worse

Table 1: French-American-British (FAB) classification of AML subtypes.

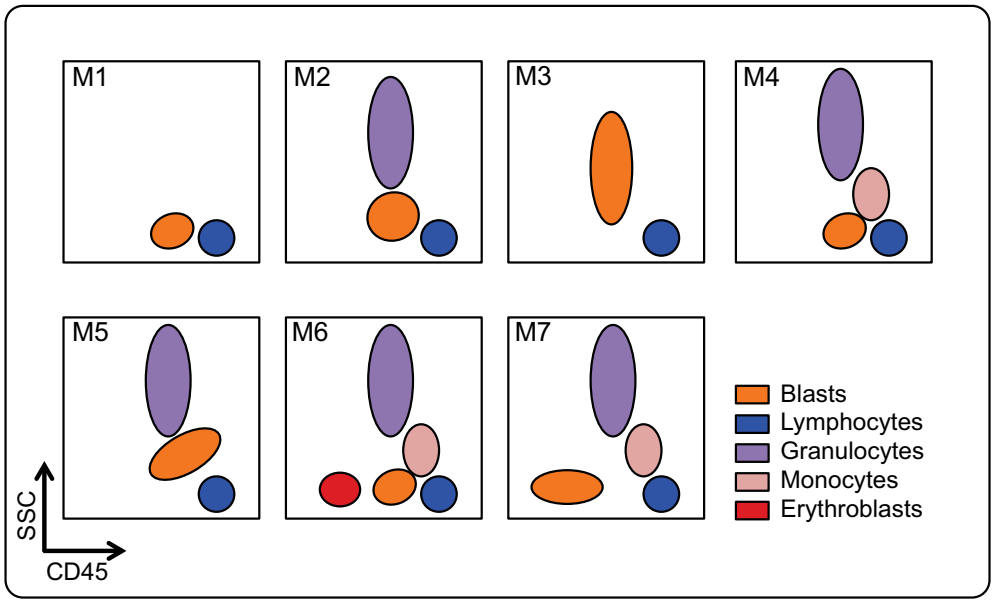


Figure 7: Correlation between different AML FAB subtypes and CD45/SSC dot plot patterns. (Brahimi et al. 2014)

The FAB classification system is useful and is still commonly used to group AML into subtypes. However it doesn’t take into account several prognosis factors. Therefore, the World Health Organization (WHO) has developed a newer classification that includes some of these factors to try to better classify and stratify AML patients.

1.2.3.b Cytogenetic classification

Recently, considerable progress has been performed to decipher AML molecular genetic and epigenetic to find novel diagnosis and prognosis markers. In 2001, WHO classification has been proposed. It distinguishes different subgroups of AML depending on the cytogenetic and genetic abnormalities. In 2016, this classification has been revised again and now includes the morphology, immunophenotyping as well as clinical presentation in order to divide 6 major AML subtypes (Arber et al. 2016).

1. **AML with certain genetic abnormalities**
   - AML with t(8;21)(q22;q22) ; RUNX1-RUNX1T1
   - AML with inv(16)(p13.1q22) or t(16;16)(p13.1q22) ; CBFB-MYH11
   - APL with PML-RARA
   - AML with t(9;11)(p21.3;q23.3) ; MLLT3-KMT2A
   - AML with t(6;9)(p23;q34.1) ; DEK-NUP214
   - AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2) ; GATA2,MECOM
   - AML (megakaryoblastic) with t(1;22)(p13.3;q13.3) ; RBM15-MKL1
   - AML with BCR-ABL1(provisional entity)
   - AML with mutated NPM1
   - AML with biallelic mutations of CEBPA
   - AML with mutated RUNX1 (provisional entity)
2. **AML with myelodysplasia-related changes**
3. **Therapy-related myeloid neoplasms**
4. **AML not otherwise specified** (This includes cases of AML that do not fall into one of the above groups, and is similar to the FAB classification.)
   - AML with minimal differentiation (M0)
   - AML without maturation (M1)
   - AML with maturation (M2)
   - Acute myelomonocytic leukemia (M4)
   - Acute monocytic leukemia (M5)
   - Acute erythroid leukemia (M6)
   - Acute megakaryoblastic leukemia (M7)
   - Acute basophilic leukemia
   - Acute panmyelosis with fibrosis
5. **Myeloid sarcoma (also known as granulocytic sarcoma or chloroma)**
6. **Myeloid proliferations related to Down syndrome**

Sometimes ALL with myeloid markers may be included in the AML group and are called AML with lymphoid markers, or mixed lineage leukemias or undifferentiated or biphenotypic acute leukemias (with both lymphocytic and myeloid features).

Acute promyelcytic leukemia (APL) constitutes a specific group of AML classified as M3 or AML3. APL is a rare condition, through extremely malignant because of its very rapid spontaneous evolution and occurrence of sudden hemorrhages mainly caused by coagulation disorders. APL is associated with specific chromosomal translocation that always involve the retinoic acid (RA) receptor α (RARα) gene on chromosome 17 to create a variety of X-RARα fusion, the most common one being t(15,17) translocation encoding the PML/RARα fusion (de Thé et al. 1990, 1991) which is associated with >98% of APL cases where PML/RARα is most often the only driving genetic alteration (Welch et al. 2011).

The WHO classification stratifies AML patients according to their prognosis factor: low risk group; intermediate risk group and poor risk group (Figure 8). Importantly the treatment depends on this stratification (1.2.4), age and the comorbidities of the patients. On note, most of the APLs are defined as a particular subtype having a very good prognosis factor (1.2.4.b). The patients in the low-risk cytogenetic group constitute 10-15% of all AML patients, intermediate group 50-60% and poor risk group accounts for 15-20%.

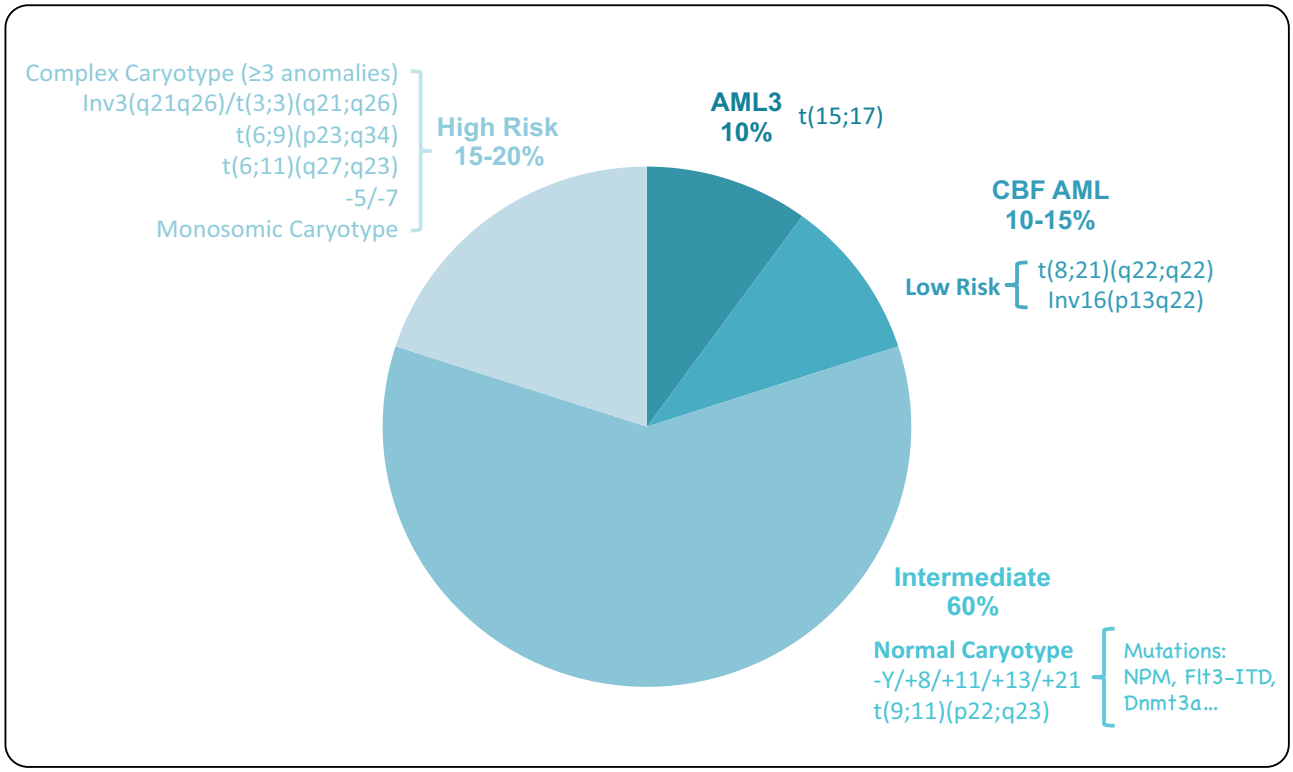


Figure 8: Cytogenetic classification of acute myeloid leukemia. CBF: Core Binding Factor; AML3: Acute Myeloid Leukemia 3; NPM: Nucleophosmin; Flt3-ITD: Internal tandem duplication (ITD) of the fms-like tyrosine kinase 3 (Flt3). Dnmt3a: DNA methyl-transferase 3a.

1.2.4 AML treatment

At diagnosis, the number of AML blasts in patients can reach up to 95% of total blood cells, which implies an urgent treatment. Despite important progresses in the molecular characterization and prognosis refinement of this disease (Network, The Cancer Genome Atlas Research. 2013), AML treatments have not significantly improved during the past 40 years except for the M3 AML subtype (APL).

1.2.4.a Standard chemotherapy

Front line induction therapy (<60 years)

The standard therapy of AML is an induction chemotherapy with cytarabine (Ara-C) and an anthracycline such as daunorubicin (DNR) or idarubicin, sometimes in association with other drugs. The standard combination is the '7+3', with a 7 days continuous infusion of cytarabine at the dosage of 100 or 200mg/m^2/day on days 1 to 7 and daunorubicin at 60-90 mg/m^2 or idarubicin at 12 mg/m^2/day on days 1 to 3.

After induction chemotherapy, most of the patients enter in a complete remission (CR). In AML, remission is defined as a normal peripheral blood cell count (absolute neutrophil count >1,000/mm^3 and platelet count >100,000/mm^3) (Cheson et al. 1990) and normocellular bone marrow with less than 5% blasts and no signs or symptoms of the disease. Recently a new definition of complete remission has been suggested, including the cytogenetic remission, in which a previously abnormal karyotype reverts to normal, and the molecular remission, in which interphase fluorescent *in situ* hybridization (FISH) or multiparameter flow cytometry are used to detect minimal residual disease (Cheson et al. 2003).

Low-risk cytogenetic group has a CR rate of 90-95% with an overall survival in 5 years of 50-60% and a longer median duration of remission. Intermediate-risk patients have CR rate of 70-85% and a 5-year overall survival between 24-45%. CR rate of high-risk patients is only between 25-50% and a 5 year overall survival around 20%. Relapses are largely due to the persistence of leukemic stem cells (LSCs) or leukemic progenitors, which are refractory to chemotherapeutic drug-induced cell death (Vergez et al. 2011).

To limit the occurrence of relapses, AML patients receive a post-remission therapy also called consolidation therapy, which differs depending on the AML subgroup.

Post-remission therapy

Standard consolidation after CR:

Low-risk patients, who reached CR, generally receive several dose of Aracytin during 5 days at day 1, 3, and 5. Unlike favorable group patients, high-risk patients receive 1 to 2 dose of consolidation therapy before undergoing Hematopoietic Stem Cell Transplantation (HSCT). In alternative protocols, patients undergo HiDAC (high Dose Ara-C) consolidation courses using cytarabine twice daily at a $3g/m^2$ dose on days 1,3 and 5 (Mayer et al. 1994). However the optimal cytarabine dose, schedule of administration, and number of cycles are still to be defined (Richard F. Schlenk 2014).

Allogeneic HSCT:

For patients with favorable-risk AML, the relapse risk may be low enough and the salvage rate high enough to postpone HSCT (Koreth et al. 2009). Most young patients with intermediate- and unfavorable-risk AML are generally considered candidates for allogenic HSCT from sibling or fully-matched unrelated donors after the first CR.

Treatment for older AML patients (>60)

For older patients, outcome remains dismal with lower CR rates and very few long-term survivors compared with younger patients. General health status and the presence of organ dysfunctions or comorbidities affect intensive chemotherapy tolerance. Similar to younger patients, their response depends on the cytogenetics characteristics of the AML. When it is possible, they receive intensive chemotherapy but in many cases they are not fit enough to tolerate it. Low-dose cytarabine (LDAC) has also been introduced as a possible standard treatment for elderly. However a clinical trial in adverse cytogenetics has not shown a significant benefit for this treatment (Burnett et al. 2007). More recently, new drugs are also used, in particular hypomethylating agents including decitabine and azacitidine, which result in a longer median and higher 1-year survival than those observed in LDAC arms, even if this did not result in a higher proportion of long-term survivors (Dombret et al., 2015). Many other new molecules are being investigated to treat older patients (1.2.5).

Refractory and Relapse therapy

Primary refractory AML and early relapse remain among the most important challenge in the management of AML. Primary refractory AML is defined by an absence of CR with 5% or more remaining blast count after several induction therapy (Cheson et al., 2003). Currently, treatment of

relapsed AML patients is not well defined. In most AML subsets (other than APL), the principal objective is to prepare the patients to receive HSCT through either targeted therapies such as FLT3 inhibitors or with standard intensive chemotherapy. If patients don't enter in CR, HSCT is performed after 5 days of conditioning regimens including chemotherapy, monoclonal antibody therapy, and radiation to the entire body to prevent the patient's body from rejecting the transplanted cells, and to kill any remaining cancer cells.

1.2.4.b Differentiation therapy (APL, PML-RARα)

Among all AML subtypes, only APL receives a specific therapy called differentiation therapy. APL accounts for around 10% adult AML cases and APL is associated with chromosomal translocation that disrupts RARα gene located on the short arm of chromosome 17(q21) and results in an arrest of the early stage of granulocytic differentiation (promyelocytes) (H. de Thé et al. 1991). The genetic hallmark of 98% of APL is characterized by t(15;17)(q22;q11-12) that results in the generation of the PML-RARα onco-fusion protein that initiates the disease by promoting a block in myeloid differentiation and proliferation of the promyelocytic blasts (de Thé and Chen 2010). In 2% of morphologically defined APL, patients carry other variants of 17q chromosome translocation (Redner 2002) (Table 2).

Translocation	Translocation partner	Epidemiology
t(11;17) (q23;q21)	PLZF (promyelocytic leukemia zinc-finger protein)	1% of APL patients
t(5;17) (q35;q21)	NPM1 (nucleophosmin 1)	Exceptional (only two well-documented cases)
t(11;17) (q13;q21)	NUMA (nuclear mitotic apparatus)	Exceptional (only one case fully described)
der(17)	Stat5b (signal transducer and activator of transcription)	Exceptional (only one case fully described)

Table 2: Variant translocations in APL. (Marchwicka et al., 2014 and http://atlasgeneticsoncology. org/index.html)

In the last two decades, the treatment of APL with all-*trans*-retinoic acid (ATRA) (2.1.1) in combination with arsenic trioxide (ATO) has transformed this aggressive form of leukemia into a highly eradicable disease, (Lallemand-Breitenbach and de Thé 2013) (2.1.2 and 3.5.2.a). The current treatment results in long-term survival rates up to 90%, at least for low-risk APL patients. The high-risk patients are defined as those exhibiting >10 x 10^9/L white blood cells (WBC) at diagnosis. Their immunophenotypic features have been associated expression of CD34, CD56, T

cell antigen CD2. At the molecular level, the short PML/RARα isoform and FLT3-internal tandem duplication (ITD) mutations have also been associated with increased relapse risk. The same was found it the case of additional chromosomal abnormality such as trisomy 8 and abn(7q), abnormally expressed gene such as LEF1, ERG and mutation of the epigenetic modifier gene including DNMT3A, MLL, IDH1, IDH2, and TET2 (Testa and Lo-Coco 2016). For high-risk disease, current clinical trials leave open the issue of the most appropriate regimen (Cicconi and Lo-Coco 2016).

Even though APL is considered as a curable disease, there are still some complications and limitations in their treatment. Differentiation syndrome (DS) is a relatively common and potentially life-threatening complication that can occur during the first days or weeks after the beginning of ATRA and ATO. Several clinical signs and symptoms exist, most common are dyspnea, interstitial pulmonary infiltrates, unexplained fever etc. Therefore, ATRA-ATO regimens now include a steroid prophylaxis (Sanz and Montesinos 2014). Other complications are *Pseudotumor cerebri* (PTC), which is a peculiar complication of ATRA therapy. The common well-documented side-effect of ATO is cardiac death and hepatic toxicity (Cicconi and Lo-Coco 2016).

1.2.5 Novel therapeutical approaches

Current AML treatment still rely largely on intensive chemotherapy and allogeneic hematopoietic stem cell transplantation (HSCT), at least for younger patients who can tolerate such intensive treatment (Döhner et al. 2010). Unfortunately the outcome of most AML patients and especially those older than 60 years or with secondary AML after cancer therapy remain poor (Döhner et al. 2010). Therefore it is essential to develop new, effective and less-toxic agents that, either alone or in combination to increase the response rate and survival.

Many therapeutic strategies aim at changing the doses of cytotoxic chemotherapy or at incorporating new agents in combination with standard chemotherapy. Here are listed some examples:

- Liposomal formulation of cytarabine and daunorubicin in a 5:1 molar ratio, called CPX-351.
- Vasaroxin. Vasaroxin is a quinolone derivative that inhibits topoisomerase II without the production of oxygen free radicals, which causes cardiac toxicity particularly in patients with preexisting hear failure.
- Guasdecitabine. Hypomethylating agents are used for patients with AML who are ineligible for conventional cytotoxic induction chemotherapy. Study on the guadecitabine showed that it could be a supplant hypomethylating agents.
- Antibody-drug conjugates (anti-CD33).

- Addition of third agents such as Purine analogs, Sorafenib (multikinase inhibitor), Gemtuzumab ozogamicin.

As AML is a heterogeneous disease presenting several mutations and translocations (Table 3), various molecularly targeted agents targeting the mutated proteins were investigated. Among others:

- Flt3 inhibitors. Flt3 internal tandem duplication (Flt3-ITDs) accounts almost 30% of patients with de novo AML and patient carrying these mutations have a very poor prognosis.
- IDH1 and IDH2 inhibitors. IDH2 and IDH1 mutations are seen in approximately 10-15% and 5-10% of AML patients respectively.
- DOT1L, BCL-2, BET bromodomain inhibitors, and histone deacetylase inhibitors.

Gene	Epigenetic function	Type of abnormalities described (percentage of AML)
DNMT3A	De novo DNA methylation	Mostly frameshifts; rare missense- and non-sense mutations (6-36%)
TET2	Conversion of 5-methylcytosine to 5-hydroxymethylcytosine	Frameshift, nonsense and missense mutations (8-27%)
IDH1 and *IDH2*	Enzymes that convert isocitrate to α-ketoglutarate (α-KG), a cofactor for TET2	Missense mutations (5-16% for *IDH1*; 6-19% for *IDH2*)
CREBBP (CBP)	Histone lysine acetyltransferase	Rearrangements: fusion genes
KAT6A (MYST3/MOZ)	Histone lysine acetyltransferase	Rearrangements: fusion genes
EP300 (p300)	Histone lysine acetyltransferase	Rearrangements: fusion genes
HDAC2 and *HDAC3*	Histone deacetylase	Missense mutations
KMT2A (MLL/MLL1)	H3K4 methyltransferase	Rearrangements: fusion genes (1-10%); partial tandem duplications (4-7%)
EZH2	H3K27 methyltransferase, enzymatic component of PRC2	Mutations (2%)
NSD1	H3K36 methyltransferase	Rearrangement involving *NUP98* (2-5%)
ASXL1	Recruitment of PRC2 to target loci	Mostly frameshifts or nonsense mutations (3-25%)
ASXL2	Homolog of *ASXL1*; function unknown	Mutations (23% of AML with RUNX1-RUNX1T1)
JARID2	Recruitment of PRC2 to target loci	Deletion in transformation of MDS or MPN to AML
SUZ12	Member of PRC2	Missense mutations, insertions and deletions
KDM5A (JARID1)	Histone lysine demethylase	Rearrangement involving *NUP98*
KDM6A (UTX)	Histone lysine demethylase	Missense mutations

Table 3: Recurrently mutated or translocated genes with epigenetic function in AML. (Wouters and Delwel 2016)

Apart from introducing new chemotherapy agents or targeting oncogenic drivers, various other therapeutic strategies are under investigation. Among others:

- Stem cell targeting (Horton and Huntly 2012)
- Immunotherapy to target AML LSC (Snauwaert, Vandekerckhove, and Kerre 2013)
- Targeting aberrant glutathione metabolism and oxidative phosphorylation to eradicate human AML cells (Pei et al. 2013; Lagadinou et al. 2013).

1.2.6 AML cell lines

Immortalized cell lines are used in research in place of primary cells to study biological processes. These cell lines offer several advantages. They are cost effective, easy to use, provide an unlimited supply of material, bypass ethical and provide also a pure population of cells, which gives reproducible results. Numerous human cell lines were established according to several publications as well as American Type Culture Collection (ATCC) Cell biology Collection. ATCC consists of over 3,600 cell lines from over 150 different species.

Unfortunately, cell lines do not always accurately replicate the primary cells. Indeed, cell lines are genetically manipulated which can alter their phenotype, functions and their responsiveness to stimulis.

Several human AML cell lines were also established having different cytogenetic characteristics and mutations (Table 4:). These cell lines provide model systems to study for instance the differentiation as well as the normal myeloid development. During my thesis, I decided to use 4 different AML cell lines: HL60, Molm14, U937 and THP1. They are blocked at different steps of maturation and each of them has different and frequent cytogenetics and mutations. Moreover, they are one of most used in the literature to study the AML differentiation.

	FAB	Cytogenetics	Molecular
HL-60	M2	MYC amplification	N-ras, CCND2 C238T, p53 homozygous deletion
MOLM 14	M4 post MDS	MLL-AF9	FLT3/ITD
U937	M4	t(10;11)(p14;q23)	p53
THP1	M5 de novo	MLL-AF9	N-ras, p53

Table 4: AML cell lines used in the project

2 ATRA induced-reactivation of differentiation in AML

In this part, I will develop why differentiation therapy is promising to treat non-APL AML using ATRA. For that, first, I will describe the mechanism of differentiation via ATRA and its own receptors, retinoic acid receptors (RAR) and its use in APL will be addressed. Then I will present current promising work using ATRA to treat non-APL AML. Finally I will describe the deregulations of RAR transcriptional activity in AML cells and the pertinence of their targeting.

2.1 Differentiation therapy

2.1.1 Retinoids and Retinoid acid receptors

Retinoids are a class of chemical compounds derived from vitamin A called also all-*trans* retinol (Figure 9) or are chemically related to it. All-*trans* retinol and retinyl esters are the most abundant retinoids in the diet and can be converted to all-*trans* retinaldehyde. Then retinaldehyde dehydrogenases can catalyze retinaldehyde oxidation to all-*trans*-retinoic acid (ATRA).

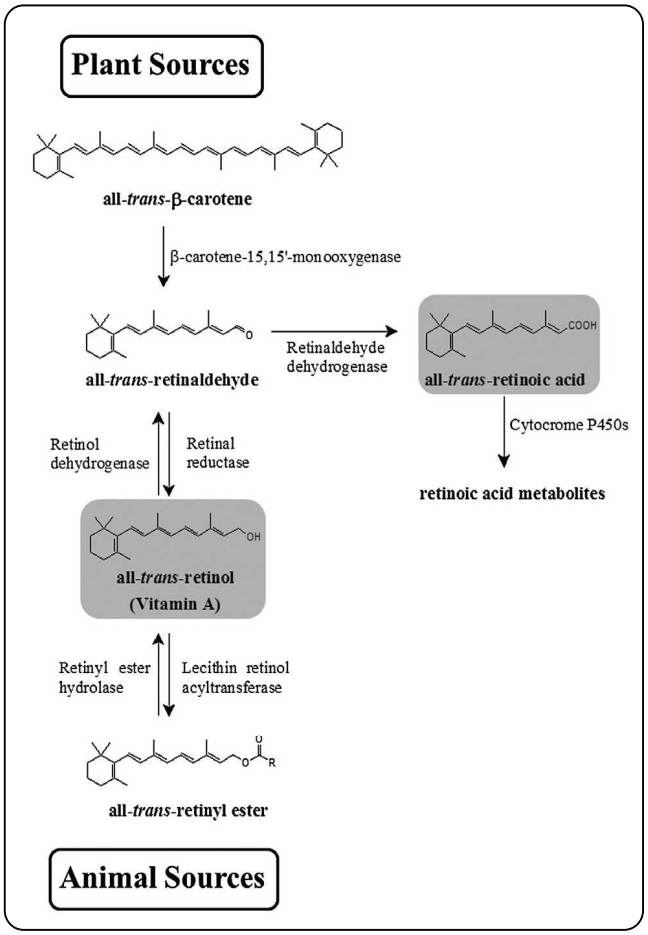


Figure 9: Retinoid metabolism. Retinoids are either from plant source (all-*trans*-β-carotene) or from animal source all-*trans*-retinyl ester. All-trans-retinoic acid (ATRA) is converted from all-trans-retinaldehyde, which is from all-trans-ß-carotene as well as from all-trans-retinol (vitamin A) provided from all-trans-retinyl ester of animal source. Finally, all-*trans*-retinoic acid is oxidized by cytochrome P450s to retinoic acid metabolites (di Masi et al. 2015).

ATRA is the differentiation agent used in APL as mentioned in the section 1.2.4.b. Here I will describe the mechanisms of ATRA-induced differentiation. ATRA is recognized by its own receptor called retinoic acid receptor (RAR). RAR has three different forms, RARα, RARβ, RARγ, which are critical regulators during myeloid differentiation (Collins 2008). Many RAR target genes are involved in myelopoiesis (Balmer and Blomhoff 2002), including CCAAT/enhancer-binding proteins (C/EBPs), PU.1 and HOX proteins. Many other are implicated in regulation of the cell cycle, and intrinsic and extrinsic apoptotic pathways such as p21, c-myc, several cyclin proteins, and FAS and FASL. All cytogenetic aberrations found in APL concern RARα gene, which plays a central role in APL pathogenesis (Redner 2002). RARα is a principal mediator of ATRA activity and regulates various transcription factors PU.1 (Iwasaki et al. 2005) and (C/EBPs): C/EBPα (Friedman et al. 2003), C/EBPβ (Duprez et al. 2003) and C/EBPε (Morosetti et al. 1997). In the absence of ligands, RAR dimerizes with RXR and tethers to target promoters a complex with co-repressor proteins that contain histone deacetylase activity. This complex modulates target chromatin structure and actively represses gene expression (Nagy et al., 1997). Upon ATRA binding, RAR undergoes a major change in conformation (Nagy et al., 1999). Co-repressor complex is thus released, and a domain is exposed that allows RAR to interact with co-activator complex. This results in the recruitment of RNA polymerase to initiate gene transcription implicated in the differentiation **(**Figure 10**)**.

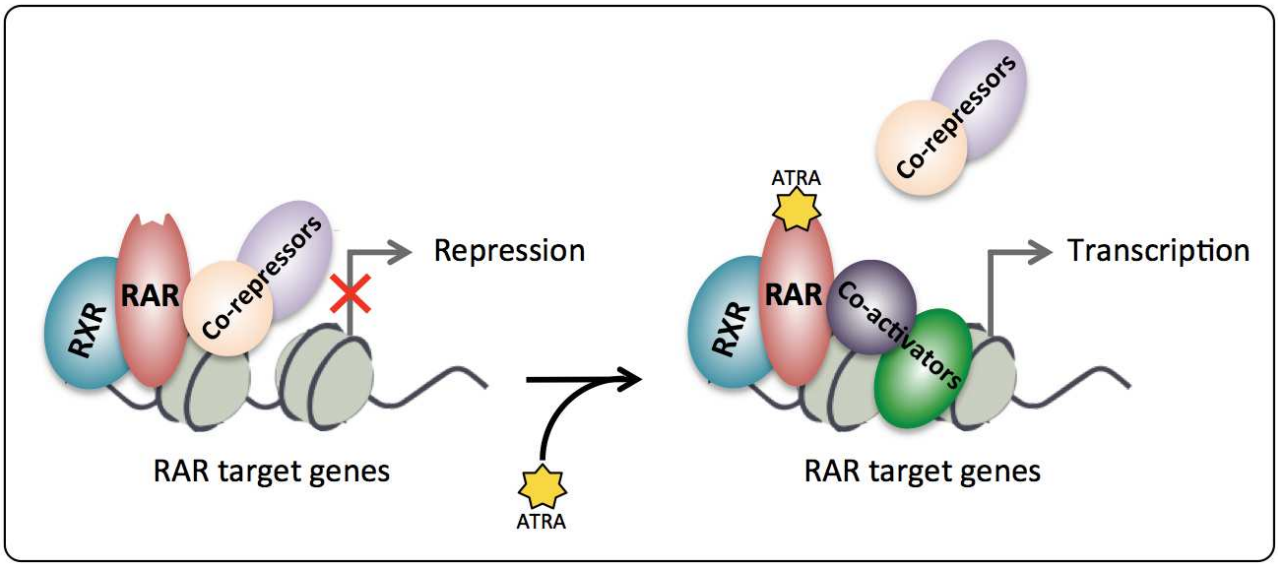


Figure 10: Mechanism of transcription regulation by RARs. Interaction of RARs with corepressors and coactivators upon ATRA binding.

2.1.2 ATRA in the treatment of APL

In APL, PML-RARα antagonizes the transactivational function of wild-type RARα on retinoic acid (RA)-inducible promoters by homodimerizing through PML coiled–coil domains and acts as a dominant-negative for RARα signaling. This blocks the conformational change, which impairs the release of co-repressor complex (SMRT and N-CoR), methyltransferase, and histone desacetylases (HDACs) (de Thé and Chen 2010) and lead to histone H3 modifications (Saeed et al. 2011) which repress RARα target genes involved in differentiation. Thus, RARα loses its potential to respond to physiological concentrations of ATRA and acts as a constitutive repressor resulting in the inhibition of the differentiation of APL (de Thé and Chen 2010). It is only upon ATRA treatment that APL cells differentiate into matures granulocytes-like cells and enter into programmed cell death (Petrie et al., 2009). A breakthrough in APL treatment was the combination of ATRA with arsenic trioxide (ATO) (Lallemand-Breitenbach and de Thé 2013). The most important effect of ATO is the degradation of PML-RARα (de Thé et al., 2012). ATO binds to Cys residues of Zn-fingers located within the RBCC motif in PML-RARα and in PML (Jeanne et al. 2010) and induces PML and PML-RARα sumoylation (Tatham et al. 2008) which is followed by its ubiquitination and degradation by the proteasome (de Thé and Chen 2010) (more details about sumoylation of PML in APL, 3.5.2.a).

2.1.3 Differentiation therapy: a promising approach in AML treatment

AML chemotherapies are mostly aiming at inducing the death of highly proliferating cancer cells. However, as mentioned previously, in spite of efforts in the development of such chemotherapy, no significant improvement has been made during past 40 years except for APL and the relapse rate is still considerably high. Most malignant cells are blocked in their differentiation, and the success of differentiation therapies in APL has led to consider the potential effect of such therapies for other AMLs. The induction of tumor cell differentiation has been demonstrated to be effective in the *in vitro* and *in vivo* treatments of several types of cancer cells (Leszczyniecka et al., 2001). Many molecules have also been proposed to induce differentiation via several mechanisms of action. Here, I will mention rapidly the most common differentiating agents (other than ATRA):

- **Vitamin D3**

Vitamin D3 (VD) is the ligand of nuclear receptors called vitamin D receptors (VDR). VD has with a high differentiating efficacy but its use is limited to life-threatening cardiotoxicity. Many studies are carried out to develop this agents as well as derivatives that would limit its hypercalcemic side effect (Hughes et al. 2010) (for more details, 2.2.1).

- **PPARgamma ligands**

Peroxisome proliferator-activated receptor (PPAR) gamma ligands bind to specific receptors of the nuclear hormone receptor family, and PPAR heterodimerizes with RXR. It shows efficiency *in vivo* on myeloid leukemic cells (Konopleva et al. 2004). However clinical trials have not shown any significant effect so far (Veliceasa et al. 2008).

- **G-CSF**

Granulocyte colony-stimulating factor (G-CSF) can differentiate leukemia cells *in vivo* (Souza et al. 1986). However it is more often used to enhance immune defenses in leukemia rather than as a differentiating agent. Several cytokines were also shown in various cell lines (Table 5) to enhance differentiation. However, they were usually not efficient *in vivo*.

Cytokine	Leukemia lines/Primary leukemia cells	Differentiation lineage
EPO	K562	Erythrocytic
G-CSF	U937	Monocytic
GM-CSF	U937, ML-1	Monocytic
IL-4	U937	Monocytic
IL-6	K562	Megakaryocytic
SCF	AML blasts	Monocytic
SCF or IL-3	AML blasts	Granulocytic
TGF-β	K562	Erythrocytic
TGF-β	HL-60, ML-1, THP-1, U937	Monocytic
TNF-α	HL-60, ML3, U937, AML blasts	Monocytic
IFN-α + GM-CSF	CML mononuclear cells	Dendritic
TGF-β + TNF-α	U937	Monocytic
IL-3 + SCF + TPO	AML blasts	Megakaryocytic
GM-CSF + TNF-α + IL-4	CS-1, KG-1, MUTZ-3, THP-1, AML blasts, CML blasts	Monocytic

Abbreviations: AML, acute myeloid leukemia; EPO, erythropoietin; CML, chronic myeloid leukemia; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; SCF, stem cell factor; TGF, transforming growth factor; TNF, tissue necrosis factor; TPO, thrombopoietin.

Table 5: Cytokines can induce differentiation of human myeloid leukemia cells. (Koeffler, 2010)

- **Tyrosine kinase inhibitors**

Gefitinib, an epidermal growth factor receptor (EGFR) inhibitor targets the tyrosine kinase. Gefitinib or its analog R406 promote the differentiation of HL60 and U937 (Stegmaier et al. 2005). The spleen tyrosine kinase (SYK) inhibitor R406 can also induce differentiation in acute lymphoblastic leukemia (ALL) B lymphocytes (Wossning et al. 2006).

- **Epigenetic modulating agents**

DMSO, a polar planar compound, was discovered as inducer of differentiation and other polar compounds were introduced including hexamethylamine bisacetamide (HMBA) (Reuben et al. 1976) and also suberoylanilide hydroxamic acid (SAHA), a second-generation polar compound and HDAC inhibitor, which can induce differentiation (Richon et al. 1996). Chromatin modifying enzymes such as HDAC and DNA methyl-transferase play an important role in the regulation of gene transcription and hence differentiation (2.3.2.a). Drugs targeting these enzymes can have some anti-leukemic and anti-myelodysplasia syndrome (anti-MDS) effects, and both hypomethylating agents, azacitidine and decitabine, have been approved by the Food and Drug Administration (FDA) for treatment of advanced MDS.

In addition to these well-studied differentiating agents, many natural compound and pharmaceuticals have been shown to activate leukemia cell differentiation (Morceau et al. 2015) (Figure 11).

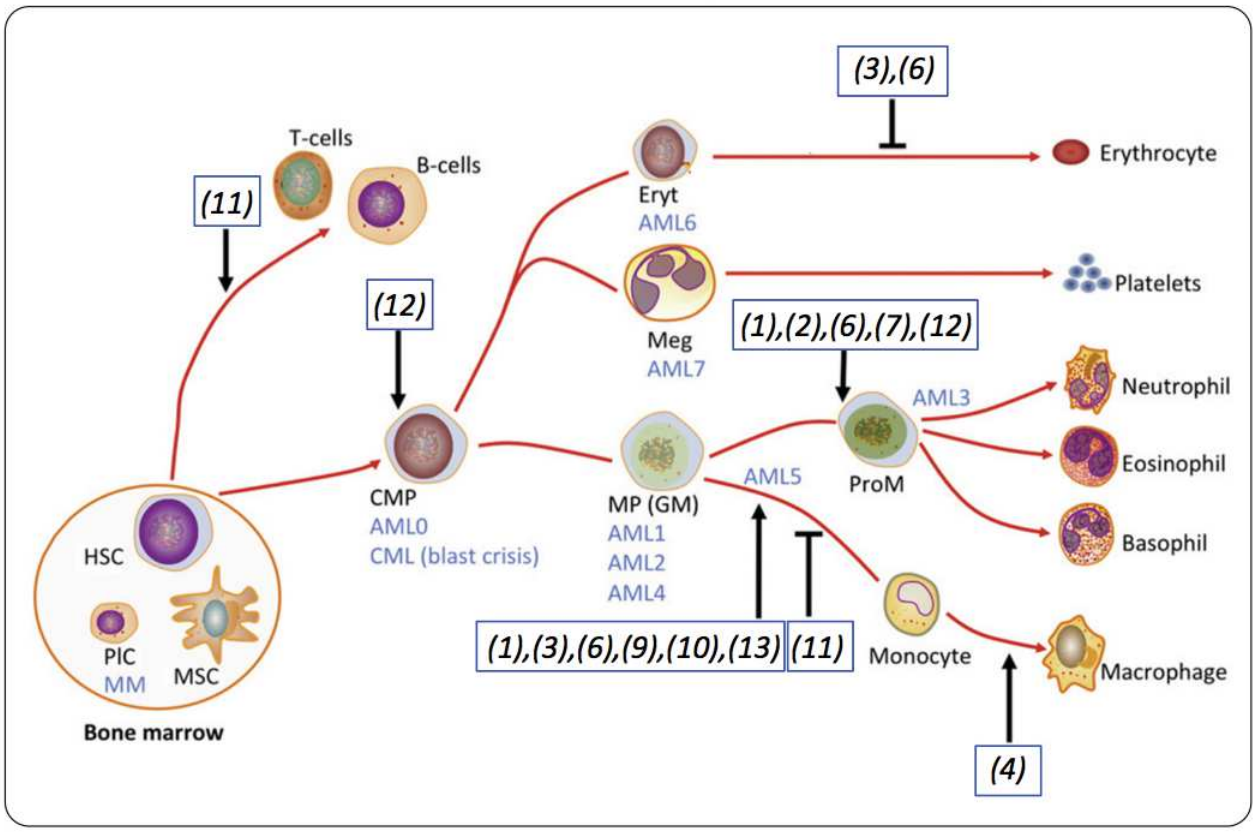


Figure 11: Induction of AML cell differentiation by natural compounds and pharmaceuticals. Several compounds have been reported to be able of inducing AML cell differentiation, which leads to cell growth arest and/or apoptosis. Association arrows and sticks indicate the inducing or the inhibiting effect on differentiation, respectively. HSC: hematopoietic stem cell, MSC : mesenchymal stem cell, AML : acute myeloid leukemia; CML : chronic myeloid leukemia; MM: multiple myeloma; PIC : plasma cells; CMP : common myeloid progenitor; MP (GM): myeloid precursor (granulocyte-monocyte); Meg : megakaryoblast; Eryt : erythroblast, and ProM : promyelocyte. *(1)*1,alpha,25-Dihydroxyvitamin D3; *(2)*all-trans retinoic acid (ATRA); *(3)*Valproic acid; *(4)*Securinine; *(5)*5-aza-2'-deoxycytidine; *(6)*Cyclopamine; *(7)*Tomatidine; *(8)*Verticinone; *(9)*Tryptanthrin; *(10)*Cotylenin A; *(11)*Berberine; *(12)*Wogonine; *(13)*Wogonoside. Figure adapted from (Morceau et al., 2015).

In conclusion, although many efforts were carried out to identify new differentiating therapies for the treatment of non-APL AML none of them are used in clinical practice yet.

2.2 Differentiation therapies using ATRA in non-APL AML

2.2.1 Effect of retinoids in non-APL AML

Because of the success of ATRA on APL patients with CR >90%, many studies intended to determine its efficacy in non-APL AML. Indeed ATRA has been already well known to effectively mediate the differentiation of non APL-AML cell lines (Brown and Hughes 2012) including HL60, U937, THP-1, MOLM14 and HF6 (Collins 2002). It was also shown to differentiate many other type of solid tumor cell, including osteosarcoma, glioma (Campos et al. 2010) and breast cancer (Ginestier et al. 2009) cells. In spite of broad differentiating activity of ATRA *in vitro*, results have been disappointing when used *in vivo*. Combining ATRA with other molecules has emerged as a more effective strategy.

For example, ATRA has been combined with ligand of peroxisome proliferator activator gamma (PPARγ). PPARγ is a nuclear receptor and functions as a ligand-dependent transcription factor responsible for lipid metabolism (Konopleva et al. 2004). Interestingly, its ligands can force cells to differentiate toward macrophages (Tontonoz et al. 1998). In particular ligand of PPARγ, troglitazone, can inhibit clonal proliferation of myeloid monocytic leukemic cells U937 in combination with ATRA and/or RXR ligands (Asou et al. 1999).

Retinoid X receptor is another important target in AML. RXRs are receptors for vitamin A metabolites like 9-*cis*-RA and interact with other members of the steroid/thyroid hormone receptor superfamily, including RARs, VDRs, and PPARs (Rowe 1997) to play a role in transcriptional activation. RXR agonist, bexarotene, was studied as an inhibitor of growth and inducer of differentiation toward neutrophils in HL60 and patient's cells. Furthermore, Phase I clinical trials with bexarotene in non-APL patients demonstrated that co-stimulation of both RAR and RXR receptors could be involved in differentiation of non-APL AML (Tsai et al. 2008).

Vitamin D3 (VD), 1a, 25-dihydroxyvitamin D3 (carcitriol), and vitamin D derivatives (VDDs) are important differentiating agent (Marchwicka et al. 2014). Vitamin D receptor (VDR) heterodimerizes with RXR and turns on a variety of genes. VD can differentiate HL60 cells into macrophage-like cells (Hughes et al. 2010). However VD causes hypercalcemia in patients at clinical doses, which can lead to fatal heart failure (Krishnan et al. 2010). Therefore VD analogs were developed but the toxicity of VDDs still remains high. The idea of using VD or VDDs in

combination with RA has been investigated to limit the doses to use and hence their toxicity. The study was performed in HL60 and NB4 using VDD, 20-epi-22oxa-24a,26a,27a-tri-homo-1,25$(OH)_2D_3$ (KH1060) and 9-*cis*-RA. This combination promoted the differentiation and inhibited the growth of the cell lines, reduced anti-apoptotic bcl-2 and increased pro-apoptotic bax expression (Elstner et al. 1996). Other VDDs were also tested in combination with RA in vitro on HL60 cells revealing a pro-differentiating effect (Doré et al. 1994).

Securinine was also shown to enhance the differentiating activities of ATRA, as well as that of cytidine analog 5-aza-2'-deoxycytidine (decitabine or Dacogen) and VD on HL60 cells suggesting the benefic effect of natural alkaloid in a combination therapy (Gupta et al. 2011). Securinine triggers growth arrest in cell lines, patient samples and AML tumors in nude mice, confirming its clinical potential.

In same way, plant-derived steroidal jerveratrum alkaloid cyclopamine from the corn lily Veratrum californicum Durand improves HL60 cells differentiation in combination with ATRA by up-regulating T cell marker CD44. This effect was also observed in primary cells from patients with induction of the myeloid markers CD11b, CD14 and CD15 (Takahashi et al. 2011). The isosteroidal alkaloid verticinon from the bulbs of Fritillaria usuriensis Maxim was also shown to differentiate HL60 into granulocytic lineage and to increase the differentiating activity of ATRA (Pae et al. 2002).

All these observation, as well as many others not listed here, suggest that differentiation observed in APL through the RAR pathway might also occur even in non-APL AML by reactivating ATRA pathway. However the clinical efficiency of ATRA has been mostly limited to cell lines and/or was observed primarily under *in vitro* condition.

2.2.2 ATRA in combination with existing chemotherapy

ATRA as a part of the induction chemotherapy was tested in several clinical trials. However the results are controversial and disparate. Patients with relapsed or refractory AML were treated in a Phase II trial with idarubicin 10mg/d x 3d and cytarabine 1000 mg/m^2 12h for 6 days with or without ATRA 45 mg/m^2/d from day 1 until remission (Belhabri et al. 2002). No significant effect of ATRA was observed. Other 405 patients with high-risk AML were treated either with 2 courses of ADE (cytarabine 100 mg/m^2 q12h d 1-10; daunorubicin 50 mg/m^2 d1,3,5; etoposide 100 mg/m^2 qd d1-5) vs 2 courses of FLA (fludarabine 30 mg/m^2 d1-5; cytarabine 1 or 2 mg/m^{2}qd d1-5), +/- ATRA 45 mg/m^2 for a maximum of 90 days, +/- G-CSF. Again, no advantage for ATRA or G-CSF was observed (Milligan et al. 2006). Furthermore, low dose cytarabine (20 mg sq bid x 10d every 4-

6 weeks) or hydrea +/- ATRA 45 mg/m^2 qd for 60 days had no significant benefit in survival or remission rate (Burnett et al. 2007). 1075 patients were induced with daunorubicin 50 mg/m^2 d1,3,5; cytarabine 100 or 200 mg/m^2 d1-10 q12h; and thioguanine 100 mg/m^2 d1-10. Then followed second induction cycle of 8 days duration +/- ATRA at a dose of 45 mg/m^2 day 1-6 (Burnett et al. 2010). Again no benefic effect from ATRA addition was observed. Finally, randomized high risk patients received fludarabine 30 mg/m^2 qd x 4 plus cytarabine 2 mg/m^2/d d 1-4, and idarubicin 12 mg/m^2 days 2-4 +/- G-CSF +/- ATRA 45 mg/m^2/d day-2 though d7 with 53-55 patients each arm revealed here again no favorable effect (Estey et al. 1999).

However, in contradiction with the above results, some benefic effects of ATRA were observed in combination with existing chemotherapy. In a Phase III trial, 242 elderly AML patients were randomized to receive either conventional chemotherapy for induction and consolidation or the same regimen with ATRA. The ATRA receiving arm had a statistically significant improvement in the remission rate (38.0% vs. 27.5%) and overall survival (estimated median survival 11.3 versus 7 months). Interestingly among these patients, ATRA was more benefic for NPM1 mutated patients without FLT-ITD mutation (Schlenk et al. 2004). Moreover in younger AML patients with NPM1 mutation, response rate, event-survival, and overall-survival increased in the ATRA-treated cohort. Different trials were performed with similar association, but they could not reproduce the effect of ATRA treatment on NPM1 mutated patients (Burnett et al. 2010). Finally a trial with 63 patients including low and high-risk cytogenetics showed that the treatment with timed-sequential therapy comprising cytarabine, idarubicin and etoposide and ATRA 45 mg/m^2 on day 1-6 had 60% of CR (Bolaños-Meade et al., 2003) which is very high compared to prior studies (Ma et al., 2017).

It is thus still difficult to conclude on the efficiency of ATRA as a part of induction chemotherapy because of conflicting results and clinical trials that differs in age, entry criteria, chemotherapy regimens, dose and duration of administration of ATRA. In spite of this contradictory results, certain patients may benefit from retinoid treatment.

2.2.3 Effect of ATRA in specific AML subtypes

The use of next generation sequencing identified new mutations in AML. As mentioned above, ATRA treatment might benefit to patients carrying specific mutations.

Isocitrate dehydrogenase (IDH) 1/2 mutations are found in 15% of AML patients (Mardis et al. 2009). IDH is a metabolic enzyme that converts isocitrate to α-ketoglutarate. The mutations found in AML result in the aberrant production of the oncometabolite (R)-2-hydroxyglutarate (2-HG) and leads to DNA hypermethylation (Figueroa et al. 2010; Turcan et al. 2012). This hypermethylation is

in particular observed in genes of the RA pathway (Chou et al., 2012). Interestingly, cells expressing IDH1 oncogenic mutation express a transcriptional program of ATRA-responsiveness (Boutzen et al., 2016). Accordingly, their treatment with ATRA enhances terminal granulocytic differentiation and death of AML cell lines, primary patient samples, and a xenograft mouse model-carrying mutant IDH1 (R132H) (Boutzen et al., 2016).

Combination of ATRA with ATO has strongly improved APL treatment (Lo-Coco et al. 2013). The question was addressed whether the ATRA/ATO strategy might also be used for non-APL leukemia and this idea was tested on AML with NPM1 mutation. NPM1 is a gene encoding a nucleolar shuttling protein and is frequently mutated in AML (30%). Even though this mutation has a favorable prognosis, relapses often occur. The oncogenic mutations cause the delocalization of NPM1 mutant from the nucleolus with a disorganization of PML nuclear bodies. ATRA/ATO treatment on NPM1 mutant cells has been showed to induce selective proteosomal degradation of the mutant NPM1 protein accompanied by a nucleolar redistribution of wile-type NPM1, apoptosis and/or differentiation. Importantly, this treatment induces also oxidative stress and p53 activation (Hajj et al. 2015; Martelli et al. 2015).

Flt3 mutation is another common mutation in AML with Flt3/ITD (20-25%) (Network, The Cancer Genome Atlas Research. 2013). Flt3 is a receptor tyrosine kinase and it is expressed during normal hematopoietic development. Its mutation leads to a constitutive activation and leads to a block in differentiation and increases proliferation. Several studies confirm the synergy in combining ATRA and Flt3 inhibitor (TKIs) in AML treatment. For instance, Ma et al., determined that TKIs with ATRA efficiently eliminate Flt3/ITD LSCs *in vitro* and also in patient samples. This was extended in mouse model, which revealed a prolonged survival of leukemic mice and engraftment of mutated patients cells in mice was reduced upon this combination therapy. They stated that the synergic effect is through the regulation of the antiapoptotic BCL2 (Ma et al. 2016). Some clinical trials are ongoing using sorafenib or midostaurin in combination with ATRA in Flt3/ITD mutated subgroup (Ramsingh et al., 2014; Guenounou et al., 2014).

2.3 Deregulations of ATRA-mediated transcriptional activity in AML cells and pertinence of its targeting

2.3.1 Inhibition of RAR activity by leukemic oncoproteins

First argument why it is pertinent to focus on ATRA-mediated differentiation in AML is that many of the genes mutated or aberrantly expressed in AML impact RAR either directly or

indirectly. For instance, SKI is a nuclear oncogene frequently overexpressed in AML and is described to bind to RAR directly to silence RAR transcriptional activity (Ritter et al. 2006). Recently it has been revealed that PRAME, a member of the RAR-associated co-repressor complex is overexpressed in AML (Bullinger et al. 2013). RAR also serves as a component of the macromolecular complex formed by the t(8,21) fusion protein RUNX1-CBFA2T1. This oncogene suppresses RAR activity by both sequestrating RAR and potentially recruiting co-repressor activities to RAR target gens (Fazi et al. 2007). Along this line, a clinical trial showed that high level of PRAME is associated with ATRA responsiveness.

2.3.2 Epigenetic modifications synergize with ATRA in non-APL AML by controlling RAR signaling

In the nucleus, the DNA is packaged together with histones to form chromatin. Chromatin can be in a condensed, transcriptionally repressed form (heterochromatin) or in a decondensed, and transcriptionally active form (euchromatin). The regulation of chromatin state affects accessibility to DNA, allowing, control transcription, replication, recombination, and DNA repair. And different epigenetic mechanisms affect the chromatin state. This consists of histone post-translational modifications (Rothbart and Strahl 2014), DNA modifications (Koh and Rao 2013), replacement of canonical histones with histone variants (Biterge and Schneider 2014), ATP-dependent nucleosome remodeling (Hargreaves and Crabtree 2011), non-coding RNA (ncRNAs) (Wilusz et al. 2009), and others (Avvakumov et al. 2011). The repeating unit of chromatin called nucleosome is composed of a histone octamer core, which consists of two copies of each histone H2A, H2B, H3, and H4 proteins, and a short segment of DNA, between 145 and 147 base pairs, which is wrapped around it. Post-translational modifications (PTMs) occurs at histone N-terminal tails such as acetylation, methylation, (Morera, Lübbert, and Jung 2016), phosphorylation, ubiquitination, sumoylation, and others (Zentner and Henikoff 2013). But they also occur in the core of the histones and in the C-terminal regions (Huang et al. 2014). The enzymes, which add chemical groups onto either histones tails or DNA itself, are commonly termed ‘writers’. Proteins that recognize these specific epigenetic changes are called ‘readers’ and finally the ‘erasers’ can remove them. In histone tails, lysine and arginine residues are the main sites of modifications. And several histone lysines can be substrates of methylation as well as of acetylation processes (Figure 12**)**. DNA methylation and histone methylation were among the first epigenetic targets to be addressed for drug development and inhibitors of DNA methyltransferases and histone deacetylases are approved by FDA for clinical use in cancers (Arrowsmith et al. 2012).

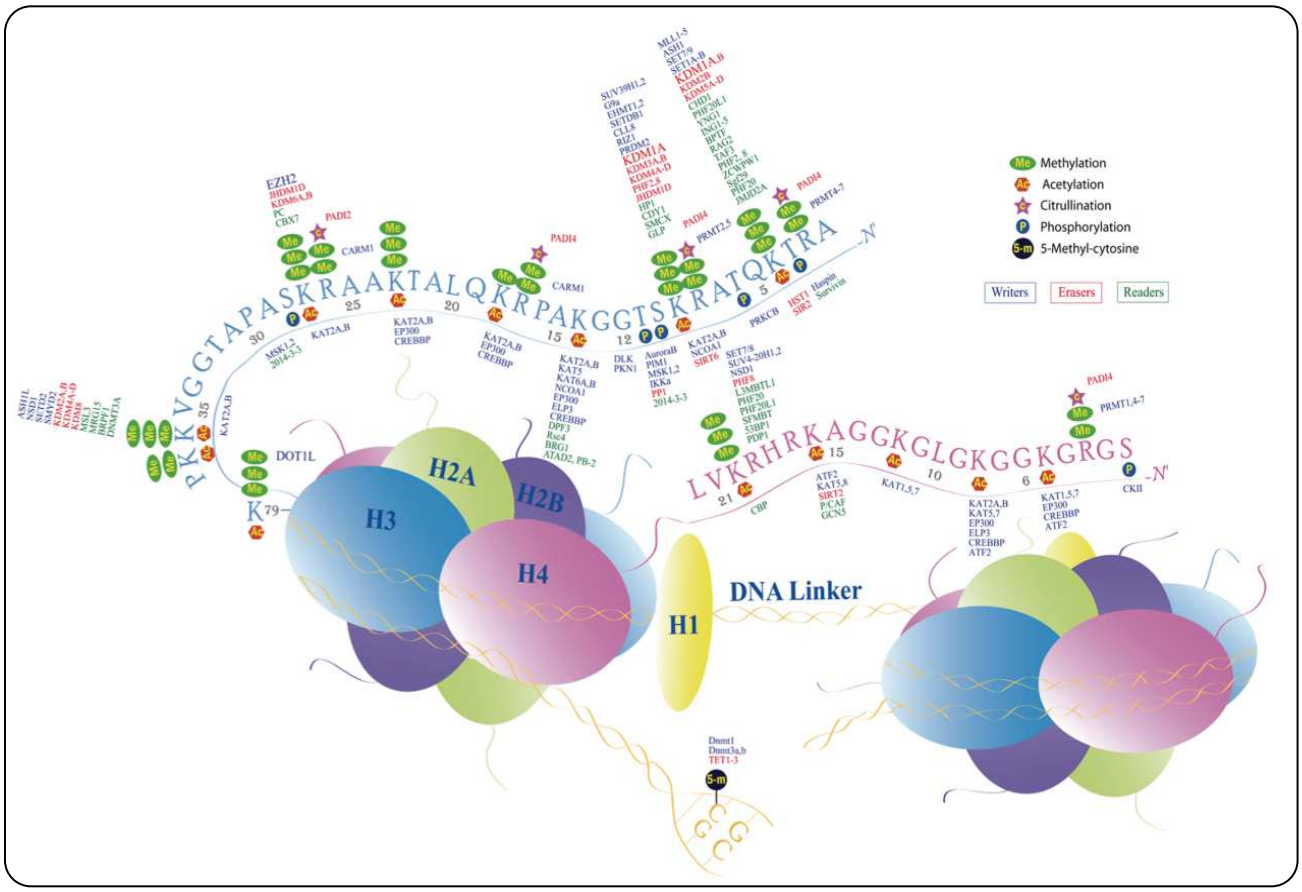


Figure 12: Nucleosome structure and principal modification sites on H3, H4 and DNA. (Morera et al., 2016)

Many proteins that are aberrantly expressed in AML affect the epigenome (Fazi et al. 2007). As I mentioned previously, recent use of next generation sequencing technologies defined new mutations in AML such as DNMT3A, EZH2, TET2, IDH1, and IDH2 (Network, The Cancer Genome Atlas Research. 2013). DNMT1, 3A, and 3B alter DNA methylation; MLL, TET1 and 2 and IDH1 and 2 modulate histone or DNA methylation; fusion oncoprotein such as AML1-ETO recruits histone deacetylases to name a few. AML blasts show global changes in the epigenome (Figueroa et al. 2010). Theses elements suggest that epigenetic modifications could silence the promoters of RAR target genes and prevent the transactivating effects of liganded-RAR. Unlike genetic abnormalities, epigenetic changes of DNA or chromatin status can be reversed with the use of small molecules like HDACs inhibitors (valproic acid (VPA), romidepsin, and vorionostat), and DNA-demethylating drugs (azacytidine and decitabine) approved for clinical use and used as single agents or in combination. Unfortunately, almost half of the AML patients treated with epigenetic agents as single agents have not shown a clinical response. This suggests combinatorial studies with different epigenetic modulators, chemotherapy, and/or biological agents such as retinoids would be a way to release the block of differentiation (Petrie et al. 2009). In next parts, I will develop the efficiency of ATRA in combination with epigenetic targeting agents in blasts differentiation.

2.3.2.a Histone Acetylation

Histone acetylation is a transfer of acetyl groups to lysine residues in histone proteins. The processes of acetylation and deacetylation are regulated by histone lysine acetyltransferases (HATs) and histone desacetylases (HDACs), respectively. Acetylation of lysine residues results in open chromatin conformations whereas deacetylation results in condensed and closed chromatin.

Interestingly, a pan-histone desacetylase inhibitor Trichostatin A combined with ATRA showed an increase expression of a series of RA-responsive genes and enhanced differentiation of all primary blasts from 23 AML patients (Ferrara et al. 2001). Another study was done with clinically available histone desacetylase inhibitor, Valproic acid (VPA), in combination with ATRA on AML cell line OCI/AML-2 as well as in 6 AML primary samples and there was an increased expression of retinoic response genes. In another study combination with VPA and ATRA increased p21 expression in *ex vivo* treated AML samples leading cell cycle arrest and apoptosis (Trus et al., 2005). These results lead several clinical trials. However the remission rates remains low (Cimino et al., 2006; Kuendgen et al., 2005).

Concerning MLL rearrangement (MLL-r), t(4;11) (MLL-AF4) and t(9;11) (MLL-AF9) are the most common in AML and acute lymphoblastic leukemia (ALL) and its presence is related to poor response to conventional chemotherapy. One study revealed that Trichostatin A combined with ATRA induces the death of MLL-AF9 expressing Molm14 (Iijima et al., 2004). HDAC inhibitor was also shown to increase ATRA sensitivity on AML1/ETO cells, in line with the fact that AML1/ETO represses RA signaling through HDAC-dependent mechanism (Ferrara et al. 2001).

Finally, the combination of ATRA and HDAC inhibitors has also been successfully used in ATRA-resistant APL containing the PZLF-RARα fusion where these inhibitors sensitize cells to ATRA treatment (Grignani et al. 1998).

2.3.2.b Methylation

The enzymatic methylation of histones is performed by lysine methyltransferases (KMTs) and arginine methyltransferases (PRMTs), with *S*-adnosyl-*L*-methionine (SAM) as the methyl donor. Histone methylation can involve the transfer of up to three methyl groups, thus resulting in mono-, di-, or trimethylated lysine, respectively, and in mono- or di- methylated arginine. Importantly, the same modifications could lead to opposite activities for instance $H3K4me_2$ and $H3K4me_3$ (Greer and Shi 2012).

Targeting DNA methylation with the DNA methyltransferase inhibitor, azacitidine, has been used as a strategy to reactivate RAR transactivation. For instance, in RUNX1-CBFA2T1 blasts, alteration of methylation by azacitidine restored retinoic acid-mediated differentiation (Fazi et al. 2007). A synergic effect of ATRA with decitabine (a derivative of azacitidine) was also observed in K562 resulting an increased expression of the p16 tumor suppressor (Xiang et al. 2014). With these promising results, clinical trials were performed. For instance, a Phase II trial using azacitidine with valproic acid and ATRA in 53 patients (Soriano et al. 2007) had a benefic effect on the overall response rate of 42% although the degree of hypomethylation, histone acetylation, or p21 expression were not correlated in term of clinical outcomes. Some other studies confirmed the benefic effect of combining ATRA and inhibitors of methyltransferases (Ma et al., 2017).

MLL-rearrangement (MLL-r) results in hypermethylation of promoter regions of genes involved in leukemogenesis (Niitsu et al., 2001). Niitsu et al. demonstrated that azacitidine treatment promoted sensitivity to ATRA of MLL-r AML cell lines (Niitsu et al., 2001). Another study done by Fujiki et al. confirmed this benefic effect of combination treatment of ATRA and azacitidine on MLL-AF9 expressing cells through an increase in C/EBPα expression. They concluded that ATRA as mono-therapy is ineffective and need RA pathway to be reactivated (Fujiki et al. 2012). Finally, MLL-AF4 cells were also shown to be sensitized to ATRA upon co-treatment with lysine-specific demethylase 1 inhibitor, tranylcypromine (Sakamoto et al. 2014).

Promoters of RAR target genes are epigenetically silenced in AML and they are characterized by decreased methylation of H3K4me$_2$. Demethylation of H3K4 is regulated by the LSD1 demethylase, which is overexpressed in AML. In fact, inhibition of LSD1 enhanced ATRA-driven RAR target gene expression. LSD1 inhibitor tranylcypromine promoted differentiation of ATRA-sensitive cell lines as well as primary AML cells and decreased AML engraftment in xenotransplantation models (Schenk et al. 2012). This strongly suggests that LSD1 may contribute to the ATRA resistance of non-APL AMLs. Various clinical trials combining ATRA to LSD1 inhibitors are ongoing in AML as well as in other hematomalignancies. Finally RARα can itself be methylated on Lys residues. This modification affects ATRA sensitivity, co-regulator interaction, and heterodimerization with RXR (Huq et al. 2008).

Epigenetic modifying agents might thus appear as a promising approach in combination with ATRA. Several clinical studies are ongoing to confirm this hypothesis.

3 Sumoylation

3.1 Ubiquitin

Ubiquitin, a 7 kDa protein of 76 aminoacids, is a highly conserved protein, present in all eukaryotic cells. Its most recognized role concerns the regulation of protein turnover by the proteasome. Ubiquitination consists in the covalent ligation of ubiquitin carboxyl-group of C-terminal glycine to ε-amino-group of a lysine side-chain on the targeted protein. This process starts by an ATP-dependent activation of ubiquitin C-terminal by a specific, usually single activating enzyme E1. An ubiquitin-adenylate is bound to the cysteine residue of the active site of the E1 via the formation of a thioester bond. Activated ubiquitin is then transferred to one of 40 ubiquitin conjugating enzymes E2, and finally, with the help of specific E3 ligases, ubiquitin is bound to lysine side-chains on the substrate proteins via the formation of an isopeptide bond (Hershko and Ciechanover 1998). Around 600 different E3 ligases ensure the specificity of this process (Figure 13). Ubiquitination is a highly dynamic process due to the deubiquitination carried out by around 100 isopeptidases, which are cysteine or metalloprotein proteases. Ubiquitin can be conjugated to its target proteins either as a monomer or a polymer. Monomeric ubiquitination is generally not involved in proteasomal degradation. For example, monoubiquitination of the histone variant γH2aX is involved in DNA damage repair via homologous recombination pathway (Kocyłowski et al. 2015). In the case of polyubiquitination, ubiquitin is polymerized through multiple ubiquitination reactions involving one of its seven lysines residues (K6, K11, K27, K29, K33, K48 and K63). Depending on which lysine residues of ubiquitin are used, the polyubiquitin chains can have different functions (Komander and Rape 2012).

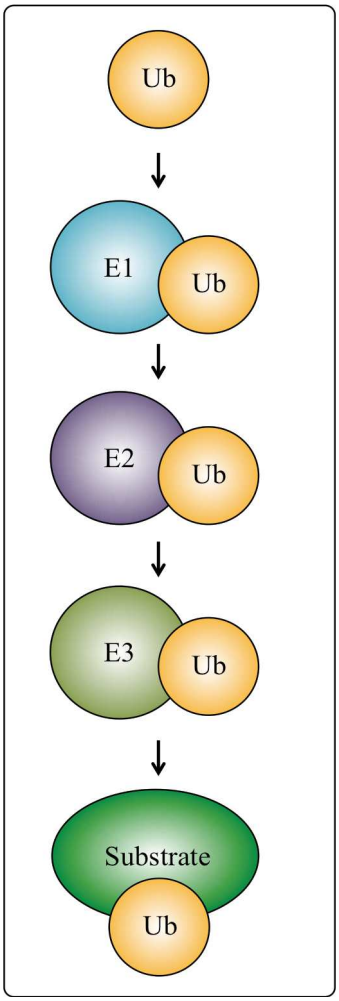


Figure 13: The ubiquitin pathway. Free ubiquitin (Ub) is activated by a ubiquitin-activating enzyme (E1). E1 is then transferred to one of the many ubiquitin-conjugating enzyme (E2s). E2 joins to E3, the ubiquitin protein ligase, which allows the polymerization of one or more ubiquiti molecules on target proteins.

The first recognized and best-characterized types of chains are K48 chains, which are responsible for protein degradation via the 26S proteasome complex. Chains must contain at least four ubiquitins for the target protein to be degraded. This chain is recognized by a specific sequence found on Rpn10 proteasome subunit, which is called ubiquitin interacting motif (Glickman and Ciechanover 2002).

3.2 SUMO: a post-translational modifier of the ubiquitin family

3.2.1 SUMO protein

Small ubiquitin-like modifier (SUMO) proteins are conjugated as a post-translational modification (PTM) to more than 3000 proteins in mammalian cells (Hendriks and Vertegaal 2016). Despite a limited sequence similarity of 18%, SUMO is structurally related to ubiquitin with a similar protein fold including ββαββαβ (Bayer et al. 1998). All eukaryotes express at least one member of the SUMO protein family and SUMO is conserved from yeast to plants and vertebrates. Humans express five SUMO proteins. SUMO-2 and SUMO-3 are about 50% similar to SUMO-1 and are 97% similar to each other, which renders them indistinguishable by immunoblotting techniques. Moreover they are not clearly functionally differentiated (Saitoh and Hinchey 2000). However it is important to note that despite their similarity, SUMO-3 knockout mice are viable while a SUMO-2 knockout is embryonic lethal (L. Wang et al. 2014) showing that SUMO-2 and SUMO-3 do have non-redundant functions, at least during development. SUMO-4 belong to another family member and very few things are known about this family (Guo et al. 2004). SUMO-4 is 87% similar to SUMO-2/3. Unlike SUMO-1 and SUMO-2/3, SUMO-4 has only been detected at mRNA levels in specific organs such as spleen, lymph nodes, and kidney (Bohren et al., 2004). Very recently, a SUMO-5 protein was identified as a tissue-specific member of the SUMO family that's highly conserved among primate species (Liang et al. 2016) (Figure 14).

```
SUMO1 MSDQEAKPSTEDLGDKKEGEYIKLKVIGQDSSEIHFKVKMTTHLKKLKESYCQRQGVPMN 60
SUMO2 MSEE--KPK--EGVKTE-NDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMR 55
SUMO3 MADE--KPK--EGVKTENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMR 56
SUMO4 MANE--KPT--EEVKTENNNHINLKVAGQDGSVVQFKIKRQTPLSKLMKAYCEPRGLSMK 56
SUMO5 MSDQEAKLSTEHLGDKIKDEDIKLRVIGQDSSEIHFKVKMTTPLKKLKKSYCQRQGVPVN 60

SUMO1 SLRFLFEGQRIADNHTPKELGMEEEDVIEVYQEQTGGHSTV------- 101
SUMO2 QIRFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGGVPESSLAGHSF 103
SUMO3 QIRFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGGVY--------- 95
SUMO4 QIRFRFGGQPISGTDKPAQLEMEDEDTIDVFQQPTGGVY--------- 95
SUMO5 SLRFLFEGQRIADNHTPEELGMEEEDVIEVYQEQIGGHSTV------- 101
```

Figure 14: Alignment of amino acid sequences of SUMO-1, SUMO-2, SUMO-3, SUMO-4 and SUMO-5

All SUMO proteins are conjugated by the same enzymatic machinery. SUMO-2, -3 and -5 are able to multimerize, forming polymeric chains as ubiquitin (Matic et al. 2008). Interestingly, SUMO-1 seems to be preferentially conjugated under normal conditions, whereas SUMO-2/3 is mobilized in response to cellular stress (Saitoh and Hinchey 2000). However, although having unique specificities, SUMO-2/3 can compensate for the loss of SUMO-1 upon knockdown of SUMO-1 in mice (Zhang et al. 2008).

3.2.2 Sumoylation enzyme

Similar to ubiquitination, sumoylation occurs through a series of biochemical steps catalyzed by a set of enzymes (Figure 15) (For list of enzymes c.f. Table 6). SUMO is expressed as an inactive precursor protein. To be activated, SUMO is processed by cysteine-specific SUMO proteases (ULPs in yeast, SENPs in mammals, 3.2.3) that remove a small peptide from the C-terminus. This exposes a di-glycine motif, which is subsequently linked to the SUMO-activating E1 complex, a dimer consisting of Sae1 and Sae2 in human (Aos1 and Uba2 in yeast). This step involves the covalent attachment of SUMO to a reactive cysteine residue in Sae2 through ATP-dependent thioesterification (Gareau and Lima 2010).

Protein function	*S. cerevisiae*	*H. sapiens*
Sumo	Smt3	SUMO-1,-2,-3
E1 activating enzyme	Aos1•Uba2	Sae1•Sae2
E2 conjugating enzyme	Ubc9	UBC9
E3 ligase	Siz1, Siz2, Cst9, Mms21	PIAS1,-2,-3,-4; MZIZ1; NSE2; RanBP2; Pc2; MUL1; TOPORS; HDAC4,-7; TRAF7; FUS; RSUME
Sumo protease	Ulp1, Ulp2	SENP1,-2,-3,-4,-5,-6,-7; DESI1,-2; USPL1

Table 6: The sumoylation machinery in S. cerevisiae and mammals. (Enserink, 2015)

Through thioester linkage, SUMO is transferred to a cysteine residue of the E2 conjugating enzyme Ubc9 (also known as UBE2I) (Desterro et al. 1997). Contrarily to ubiquitin E2, Ubc9 can directly bind the substrates to which it transfers SUMO. Then E3 ligases can serve as a scaffold that brings together SUMO-charged Ubc9 and the substrate. It promotes efficiency and specificity of the sumoylation process.

The largest group of E3 ligases are protein inhibitor of activated signal transducer and activator of transcription (PIAS) family, which contain RING-finger-like structure essential for their function as E3 ligases. They bind directly to Ubc9 and to selected SUMO targets, and stimulate their modification. Mammals have six PIAS family members: PIAS1, PIAS3, PIAS4 PIASxa, PIASxb

and PIASy. Beyond their SP-RING domains, PIAS family members share additional conserved motifs, including an N-terminal scaffold attachment factor (SAF)-A/B, acinus, PIAS (SAP) motif, a PINIT motif, a SIM, and a C-terminal domain that is rich in serine and acidic amino acids (S/DE domain). The SAP domain directs the localization of PIAS proteins to chromatin within the nucleus (Palvimo 2007). The SP-RING domain is required for the activation of the Ubc9-SUMO thioester, whereas the PINIT domain directs sumoylation to the correct target lysine (Wang and Dasso 2009). Another SUMO E3 ligase is the vertebrate specific 358-kDa protein, RanBP2/Nup358 (Pichler et al. 2002). RanBP2 is a component of nuclear pore complexes. A 30-kDa domain of RanBP2 is sufficient for catalytic activity but this domain does not contain a RING-finger motif and shows no obvious homology to ubiquitin E3 ligases. It binds stably to Ubc9 and RanGAP1*SUMO-1 forming a complex, but it doesn't bind to targets (Pichler et al. 2004). Another E3 SUMO ligases is the human Polycomb member Pc2 (Kagey et al. 2003) which is not related to PIAS or RanBP2 proteins. Polycomb group (PcG) proteins form large multimeric complexes (PcG bodies) that are involved in gene silencing. There are only few substrates of Pc2 protein, such are transcriptional co-repressor CtBP, transcriptional regulator SIP1, HIPK2 and Dnmt3a (Gareau and Lima 2010). Tripartite motif-contatining (TRIM) proteins are members of nearly 70 member protein family (Hatakeyama 2011), and among them, some members have ubiquitin E3 ligase activity, while others are SUMO E3 ligases requiring intact RING and specific B-box domain in order to interact both with Ubc9 and substrates. Some of the known SUMO E3 ligases in TRIM family are TRIM19 (PML), which stimulates sumoylation of p53 (Chu and Yang 2011), TRIM27 which sumoylates Mdm2 (Hatakeyama 2011) and TRIM28 which sumoylates NPM1 with the help of p14ARF (Neo et al. 2015). The Mms21/Nse2 subunit of the Smc5/6 complex possesses a SUMO ligase activity and uniquely prevents inappropriate recombination intermediates in meiosis (Xaver et al., 2013). This protein is needed for nucleus-to-cytoplasm transport and myogenic differentiation (Berkholz, Michalick, and Munz 2014). Several other SUMO E3 ligases such as topoisomerase I binding, arginine/serine-rich (Topors) (Braun et al. 2012), TNF receptor-associated factor 7 (TRAF7) (Morita et al. 2005), SLX4 (Guervilly et al. 2015) and ZNF451 (Eisenhardt et al. 2015) have been reported to have SUMO E3 activity (Schulz et al. 2012).

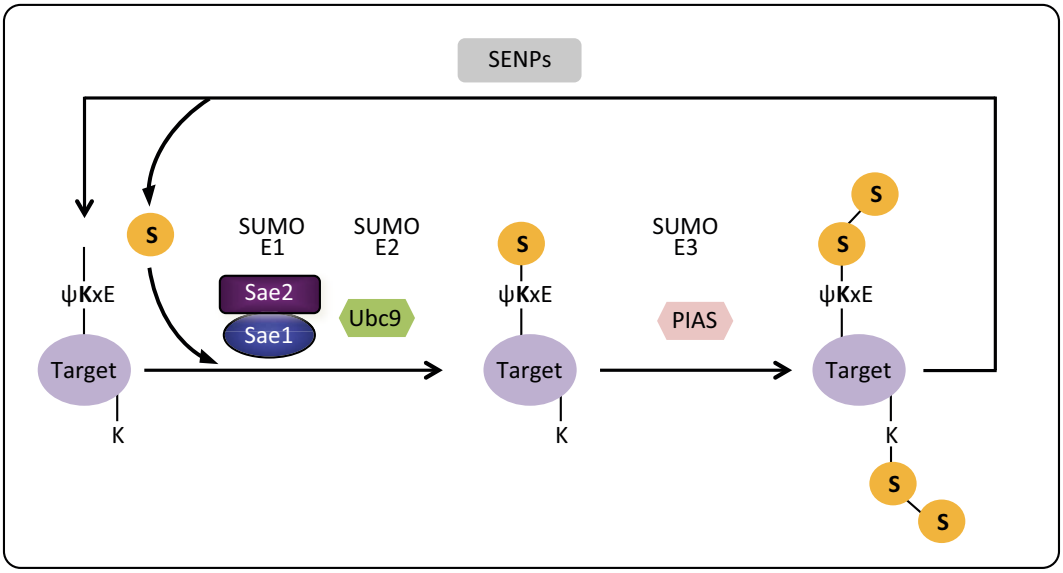


Figure 15: The SUMO pathway. Small ubiquitin-like modifiers (SUMOs) are covalently attached to Lys residues in target proteins through an enzymatic cascade involving the dimeric E1 activating enzymes Uba2 and Aos1 and the E2 conjugating enzyme Ubc9. E3 ligases enhance the efficiency of the SUMO conjugation. Sumoylation process is reversible: SUMO-specific proteases SENPs are capable of deconjugating SUMO from target proteins. Furthermore, SENPs are essential for the maturation of SUMO.

Similarly to ubiquitin, SUMO proteins can form chains (Vertegaal 2010) through Lys11 of SUMO-2 and SUMO-3 and SUMO chain formation can also occur through other internal Lys residues. Furthermore, SUMOs can form mixed chains such as SUMO-1 as the distal SUMO in the chain (Matic et al. 2008) (Figure 16)

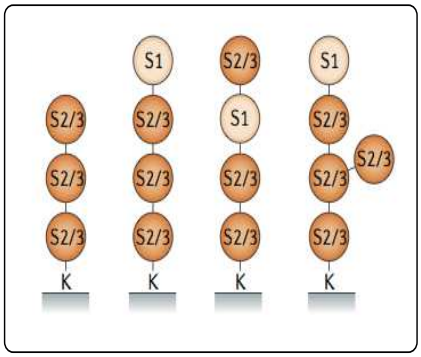


Figure 16: Formation of SUMO chain. (Hendricks and Vertegaal, 2016)

3.2.3 Desumoylase

An important aspect of protein sumoylation is that this modification is a dynamic and reversible process. Sumoylated proteins can be desumoylated by the same proteases that cleave the inactive SUMO precursor to its reactive form, called sentrin specific isopetidase (SENPs). There are seven homologues in humans, SENP1-SENP7. Two of these enzymes are found in yeast (Ulp1 and Ulp2) (Li and Hochstrasser 2000). SENPs isoforms differ from each other in their localization (Mukhopadhyay and Dasso 2007), catalytic activity on precursor and conjugated SUMO and also in specificity to SUMO paralogs. For example SENP1 deconjugates equally SUMO-1, SUMO-2/3

whereas other SENPs deconjugate more efficiently SUMO-2/3 than SUMO-1. In addition, SENP6 and SENP7 are more active on SUMO-2/3 chains than on monosumoylated substrates (Di Bacco et al. 2006) (Table 7). Recently, three new SUMO proteases in humans were identified, desumoylating isopeptidase 1 (DeSI1), DeSI2 (Shin et al. 2012), and ubiquitin-specific protease-like 1 (USPL1). USPL1 is a SUMO isopeptidase with essential, non-catalytic functions, which share little sequence similarity with the SENP protease class (Schulz et al. 2012).

Name	*Processing*	*Substrate*	*Localization*
SENP1	SUMO-1/2/3	Equal	Nucleus
SENP2	SUMO-1/2/3	high SUMO-2/3 low SUMO-1	Nuclear envelope
SENP3	SUMO-2/3	SUMO-2/3	Nucleolus
SENP5	SUMO-2/3	SUMO-2/3	Nucleolus
SENP6	-	SUMO-2/3 chains	Nucleoplasm
SENP7	-	SUMO-2/3 chains	Nucleoplasm

Table 7: Classification of SENP isopeptidases

SUMO proteases have important functions in spatial regulation of SUMO turnover (Mukhopadhyay and Dasso 2007). In S. cerevisiae, the activity of Ulp1 and Ulp2 towards sumoylated proteins is dependent upon their localization; Ulp1 activity is highly localized at nuclear pore complexes, whereas Ulp2 is more active towards proteins located in the nucleoplasm (Li and Hochstrasser 2000). This reversibility of sumoylation is crucial for many cellular processes including chromosome cohesion, mitosis and transcription (Pelisch et al. 2017, Texari and Stutz 2015). However, how SUMO proteases are regulated is currently not well understood.

3.2.4 SUMO consensus motif

Sumoylation of substrates preferentially occurs on a lysine residue in the canonical SUMO consensus motif ΨKx(D/E), in which Ψ is a large hydrophobic residue and x is any amino acid followed by an acidic residue (Vertegaal et al. 2004). The C-terminal domain of Ubc9 can directly interact with this SUMO-motif to transfer SUMO to the target substrates (Bernier-Villamor et al. 2002). There are also variant of this consensus site, such as phosphorylation dependent SUMO site (PDSMs) found in PML, HSF1, HSF4b, EXO9 and in the PIAS proteins, as well as negatively charged amino-acid dependent SUMO site (NDSMs) (Yang et al. 2006) and inverted site (ISCM) (Matic et al. 2010) and hydrophobic dependent site (HCSM) (Matic et al. 2010). PDSMs are

extended versions of the canonical SUMO motif (ψKx(D/E)xxSP), and phosphorylation of this motif by proline-directed kinases generally increases sumoylation efficiency (Yang et al. 2006). Phosphorylated PDSMs and NDSMs likely promote sumoylation efficiency by increasing the stability of the interaction between Ubc9 and the substrate, because the negatively charged phosphate (in PDSM) or negatively charged amino acid (in NDSM) interact with basic residues on the surface of Ubc9 (Yang et al. 2006). Sumoylation can also occur on lysines that do not conform to know SUMO consensus motif (Blomster et al. 2010) and data from high throughput studies indicate that non-consensus sumoylation may be relatively common event (Tammsalu et al. 2014). Still, how these sites are recognized remains unknown. In some proteins, mutating a known sumoylated lysine into arginine results in increased sumoylation on some other lysines not necessarily in consensus site, without any apparent effect on the functional outcome of substrate sumoylation. For instance, sumoylation of the DNA helicase mutated in patients with Bloom syndrome (BLM) was found to be very promiscuous (Eladad et al. 2005). This protein is primarily sumoylated at K317. Surprisingly, mutation of K317 resulted in enhanced modification at secondary sites such as K331, K344, and K347. However these sites share little or no resemblance with a consensus SUMO-motif. Another example is based on Rap1, transcription factor in yeast, which has nine potential SUMO sites but only one lysine (K651) included in a canonical SUMO-motif. Mutating this lysine into arginine had no effect on the Rap1 sumoylation status. All nine lysines have to be mutated to abolish totally Rap1 sumoylation (Chymkowitch, Nguéa P, and Enserink 2015). Recently, Hendriks and Vertegaal unified SUMO sites from several studies that reported at least 100 sites, and analyzed using the latest MaxQuant software. They found 5,032 SUMO sites in more than 3000 proteins and 32,7% of these sites being consensus sites (Hendriks and Vertegaal 2016). In an even newer study, Hendricks et al., identified 40,765 SUMO acceptor sites corresponding to 6,747 human proteins (Hendriks et al. 2017).

3.2.5 SUMO Interacting Motif (SIM)

SUMO-interacting motifs (SIMs) consist of several bulky hydrophobic residues (Keusekotten et al. 2014) and thus protein-protein interactions between SIM-containing proteins and covalently sumoylated proteins (Figure 17-**A**). For instance, sumoylated proteins may be targeted by SIM-containing SUMO-targeted ubiquitin ligases (STUbLs), which are a subset of ubiquitin E3 ligases that specifically recognize and ubiquitylate sumoylated proteins (Nagai et al. 2011). The main example in human is the E3 ubiquitin-protein ligase ring-finger 4 (RNF4), which has a pivotal role in arsenic-induced degradation of PML proteins (Lallemand-Breitenbach et al. 2008; Tatham et al. 2008). It was also suggested that functional clusters of proteins might be modified in concert by sumoylation owing to the recruitment of SIM-containing proteins to sumoylated proteins or the

capacity of SIM-containing proteins to recruit SUMOs and in turn become sumoylated (González-Prieto et al. 2015) (Figure 17-**B**). Sumoylation machinery proteins themselves contain SIMs and may be immobilized in SUMO rich cellular structures such as PML bodies to sumoylate more proteins that come into proximity. Protein complexes, nuclear bodies, chromatin or other nuclear structures could be solidified through multiple SUMO-SIM interactions. This could serve to either regulate or degrade entire protein complexes (Hendriks and Vertegaal 2016). Interestingly, modification of SUMO, such as acetylation, can also determine selectivity and dynamics of SUMO-SIM interactions and prevent specific SUMO-SIM interactions (Ullmann et al., 2012).

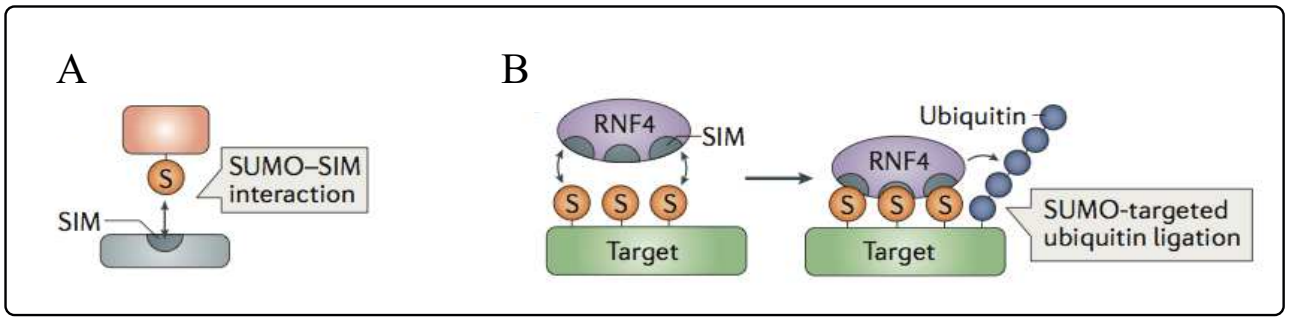


Figure 17: SUMO interacting motifs (SIMs). A/ SIMs are composed of multiple bulky hydrophobic residues and an acidic residue and recognize SUMO. **B**/ RNF4, the SUMO-targeted ubiquitin E3 ligase ring-finger 4 recognizes multi-sumoylated proteins and ubiquitinates them in order to degrade them by the proteasome. (Hendricks and Vertegaal, 2016)

3.3 Targeting the SUMO pathway

SUMO modification regulates numerous cellular activities and its deregulations are thought to be involved in various pathologies including cancers (see 3.5). Therefore, drugs, which selectively and efficiently disrupt SUMO modification could have important implications for their treatment. The first sumoylation inhibitor was identified in 2004 (Boggio et al. 2004) and their development has continued to progress (da Silva et al. 2013). However, only few sumoylation inhibitors have been identified today (Table 8) and the most efficient ones are only efficient in the micromolar range. The most recent inhibitor of sumoylation is 2D08, an inhibitor of the SUMO E2 enzyme (Kim et al. 2014) and the most used in the literature is Anacardic acid, an inhibitor of the SUMO E1 (Fukuda et al. 2009). These two inhibitors are routinely used in our laboratory.

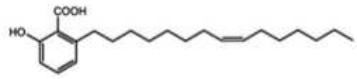
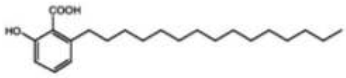
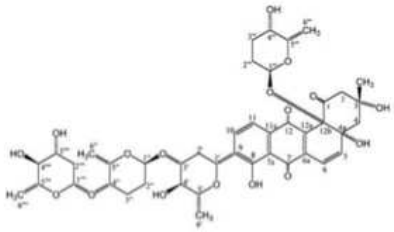
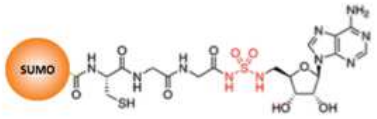
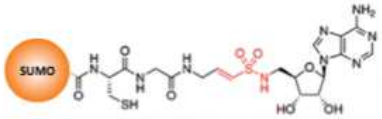
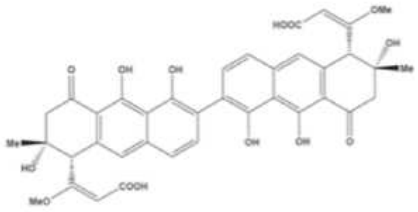
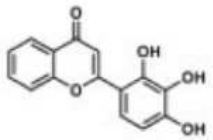
Name	Structure	IC50 (µM)	Studied model	Reference
Gam1	Adenoviral protein		in vitro	Boggio et al. 2004
Gingkolic acid		3	in vitro	Fukuda et al. 2009
Anacardic acid		2.2		
Kerriamycin B		11.7	in vitro	Fukuda et al. 2009
SUMO-AMSN			in vitro	Lu et al. 2010
SUMO-AVSN				
Spectomycin B1		4.4	in vitro in vivo (mice)	Hirohama et al. 2013
2D08		6	in vitro	Kim et al. 2013

Table 8: Sumoylation inhibitors.

3.4 Regulation of transcription by SUMO

Whereas ubiquitination, acetylation and phosphorylation modify proteins throughout the cell, sumoylation occurs predominantly in the nucleus and has been involved in pre-mRNA splicing, transcription, viral transcription, chromatin remodelling, ubiquitin-ligase activity, the DNA Damage

Response, DNA replication and nuclear body organization, as well as protein synthesis and, to a less extent, the cell cycle (Hendriks and Vertegaal 2016; Flotho and Melchior 2013). Recently, it was revealed that the major clusters of sumoylated proteins correspond to proteins involved in pre-mRNA splicing, the ribosome and ribosome biogenesis. Then following clusters are implicated in a wide range of nuclear functions, including chromatin remodeling, the DNA damage response, cell cycle regulation, transcriptional and pathway in cancer (Hendriks and Vertegaal 2016). Although sumoylation controls many cellular functions, one of its best-characterized role is the regulation of transcription via the modification of histones, transcription factors and co-factors, chromatin-modifying enzymes and basal transcription machinery (Raman et al. 2013).

In most of cases, sumoylation was shown to inhibit gene expression. For example, SUMO is known to modify histones (Hendriks et al. 2014) and SUMO can regulate HDAC1 (David et al. 2002), HDAC2 (Yang and Sharrocks 2004) and HDAC4 (Kirsh et al. 2002) generally serving to repress transcription. SUMO can modify Lys-specific demethylase 5B (KDM5B) and KDM5C and coordinates transcription repression in response to DNA damage (Hendriks et al. 2015).

Importantly, several mechanisms have been described by which SUMO can inhibit transcription (Figure 18). For instance, SUMO can inhibit nuclear entry of transcription factors such as Atf7 (Hamard et al. 2007). The SETDB1 histone H3K9 methyltransferase has a SIM motif on its N-terminal important for its chromatin targeting and thereby participates in local heterochromatin and PML nuclear bodies formation (Cho et al. 2013) (Figure 18-**A**). SUMO can also prevent the recruitment of general transcription factors to promoters (Figure 18-**B**). Another example is the histone demethylase LSD1 partner CoREST1, which binds SUMO-2 chains via its SIM. This helps the recruitment of LSD1 and HDAC proteins to target promoters and prevent the expression of target genes (Ouyang et al. 2009). Sumoylation can block binding of transcription factors to specific sequences in the promoter (Figure 18-**C**); sumoylation can compete with other modification for given lysine such as acetylation, methylation, or ubiquitination implicated in transcriptional activity (Figure 18-**D**). For instance, sumoylation and ubiquitination compete for steroid hormone nuclear receptors and the sumoylation prevents the ubiquitin-mediated proteosomal turnover (Faus and Haendler 2006). Because ubiquitination dependent turnover is required to achieve full transactivation activity, sumoylation of nuclear receptors results in reduced transcriptional output (Chymkowitch et al. 2011).

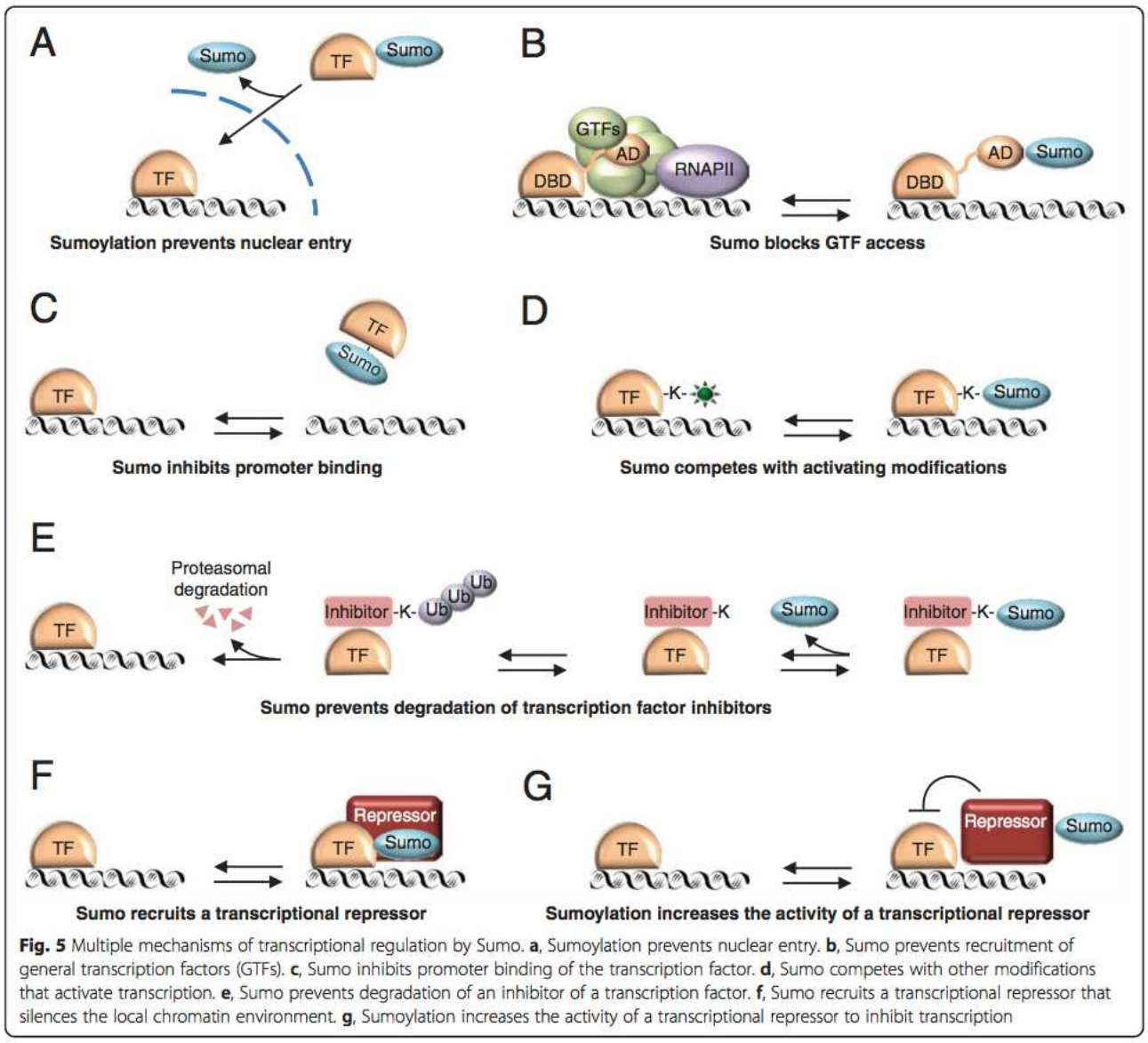


Fig. 5 Multiple mechanisms of transcriptional regulation by Sumo. **a**, Sumoylation prevents nuclear entry. **b**, Sumo prevents recruitment of general transcription factors (GTFs). **c**, Sumo inhibits promoter binding of the transcription factor. **d**, Sumo competes with other modifications that activate transcription. **e**, Sumo prevents degradation of an inhibitor of a transcription factor. **f**, Sumo recruits a transcriptional repressor that silences the local chromatin environment. **g**, Sumoylation increases the activity of a transcriptional repressor to inhibit transcription

Figure 18: SUMO regulates transcription through multiple mechanisms. A/ Sumoylation prevents nuclear translocation. **B**/ SUMO prevents recruitments of general transcription factors. **C**/ SUMO inhibits promoter binding of the transcription factor. **D**/ Competition between SUMO and other post-translational modification that activate transcription. **E**/ SUMO prevents degradation of an inhibitor of transcription. **F**/ SUMO recruits a transcriptional repressor. **G**/ Sumoylation increases the activity of a transcriptioinal repressor to inhibit transcription. (Enserink, 2015)

Histone sumoylation was also shown to inhibit transcription (Nathan et al. 2006). Indeed, sumoylation of H4 and H2B prevents acetylation of H4 and ubiquitination of H2B thus repressing transcription, and tethering SUMO to histone tails was sufficient to inhibit transcriptional activation. SUMO can also prevent ubiquitin-mediated degradation of transcriptional inhibitors (Figure 18-**E**), as described for IκBα (Desterro, Rodriguez, and Hay 1998), which is an inhibitor of NFκB. Sumoylation of transcription factors can also result in the recruitment of transcriptional repressors (Figure 18-**F**). This is the case for Elk-1, which sumoylation induce the recruitment of HDAC-2 (Yang and Sharrocks 2004). Along the same line, sumoylation of the HAT p300 promotes the interaction of p300 with HDAC6 to counteract the positive effect of p300 in transcription (Girdwood et al. 2003). Sumoylation can activate directly transcription repressors to create a

repressive chromatin environment (Figure 18-**G**) including sumoylation of HDAC1, which promotes transcriptional repression in vivo (David et al. 2002). Sumoylation of the transcriptional co-repressor Tup1 promotes its binding to the ARG1 promoter (Ng et al. 2015).

However, sumoylation has also been shown to activate transcription in some cases. For instance, sumoylation of GATA-1 promotes transcription through the co-regulator Friend of GATA-A (FOG-A) (H.-Y. Lee et al. 2009). Pax6 is a transcription factor for eye and brain development and sumoylation of Pax6 favors its DNA binding and its transcriptional activity (Yan et al. 2010). Gli is a transcription factor activated by sumoylation which regulates the Hedgehog pathway (Cox et al. 2010). In addition, SUMO has been associated with actively transcribed genes, although in most cases involved in the limitation of their transcription. Our lab has shown that sumoylated c-Fos binds to its targets promoter at the onset of transcriptional activation, and not during transcriptional termination, using an antibody specific for the sumoylated form of c-Fos (Tempé et al. 2014). Sumoylation of AP1 occurs on actively transcribed genes and limits both reporter gene induction and appearance of histone marks of activation on the promoter. AP-1 sumoylation would thus serve to buffer target gene activation to maintain their transcription within physiological windows (D. Tempé et al. 2014). Various high throughput studies have then addressed the distribution of SUMO on the chromatin. In particular, using ChIP-Seq, Neyret-Kahn et al. found that SUMO, although distributed over the whole genome, is highly enriched on gene promoter regions (Neyret-Kahn et al. 2013). More surprisingly, they found that SUMO has strong association with active promoters, mainly of histones and protein biogenesis genes, as well as Pol I rRNAs and Pol III tRNAs. Another large-scale approach confirmed that SUMO is enriched in regions containing genes, notably in promoters, and SUMO paralogs are commonly centered and symmetrically distributed within 500 bp around transcription start sites (Chang et al. 2013). Similar results for promoter occupancy were obtained in yeast, where both SUMO and Ubc9 were found enriched on active and induced promoters. Inhibition of Ubc9 produces an increase in transcription suggesting that, as shown in mammalian cells, SUMO can facilitate transcriptional silencing (Rosonina et al. 2010)**.** Niskanen et al. (Niskanen et al. 2015) reported that heat shock induces a gain in PIAS1 binding and sumoylation on promoters and enhancers of various transcription factors, and a loss of sumoylation at the intergenic chromatin associated with CTCF-cohesin complex and SetdB1 methyltransferase complex. Finally, Seifert et al. (Seifert et al. 2015) reported that upon heat-shock, SUMO-2 accumulated at nucleosome-depleted and active DNA regulatory elements, which are binding sites for large protein complexes. They propose that conjugation of SUMO-2 to chromatin-associated proteins would be part of the proteotoxic stress response by contributing to the maintenance of protein complex homeostasis. Interestingly, in these examples, it seems that

SUMO does not need to be conjugated to specific proteins within large protein complexes to exert its biological effects. This led to the concept of "grouped sumoylation", which was first discovered in the context of DNA repair. It states that sumoylation of a complex, rather than that of individual proteins within the complex, is important, in particular to serve as a platform for the recruitment of SIM-containing proteins (Psakhye and Jentsch 2012).

Together, these studies illustrate the complexity of SUMO's function in regulating transcription. Because of the high number of SUMO targets, the promiscuity, and high versatility of sumoylation, more studies need to be done to understand the many functions of SUMO in the regulation of transcription. In particular, the upstream signals and pathways that control sumoylation, as well as many critical SUMO targets and the effector proteins that bind SUMO remain largely unknown.

3.5 SUMO in cancer

3.5.1 SUMO in carcinogenesis

With many enzymes being involved in SUMO conjugation and considering the high number of SUMO targets and regulators, deregulations of this system are expected to impact cellular behavior and facilitate the onset and progression of various human diseases, in particular cancer (Figure 19) (Seeler and Dejean 2017; Flotho and Melchior 2013).

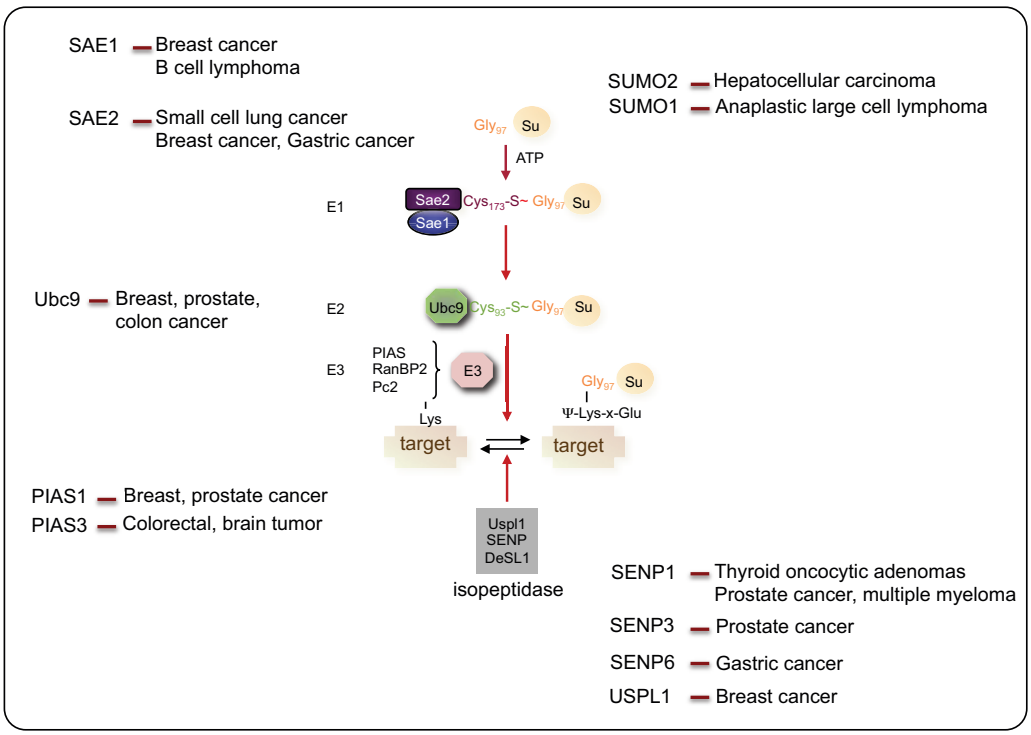


Figure 19: Deregulations of the SUMO pathway in various types of cancer. Both sumoylating and desumoylating enzymes as well as SUMO itself were found to be deregulated in cancers.

- **Sumoylation enzymes in cancer**

First, the SUMO E1 enzyme, SAE1 and SAE2 have been shown to be synthetically lethal with oncogenic mutated K-Ras (Luo et al. 2009). SAE2 has also been demonstrated synthetically lethal with the Myc oncogene when it is overexpressed in aggressive breast cancers suggesting a potential role of inhibition of sumoylation as a possible therapy for Myc-driven human cancer (Kessler et al. 2012). Moreover expression of SAE1 is upregulated by MYC (Amente et al. 2012) and MYC overexpression in B cell lymphomas is associated with the upregulation of virtually all SUMO pathway components (Hoellein et al. 2014). Furthermore, it has been demonstrated that down-regulation of SAE2 expression inhibited migration and invasion in small cell lung cancer (SCLC) cells (Liu et al. 2015). Inhibition of either SAE1/SAE2 or E2-conjugating enzyme Ubc9 impaired the growth of NOTCH1-activated breast cancer epithelial cells (Licciardello et al. 2015).

Ubc9 transcription is upregulated by oestrogen (17β-oestradiol) treatment in MCF7 breast cancer cells (Ying et al. 2013). Evidence for post-transcriptional regulation of Ubc9 was provided by the demonstration that the microRNAs (miRNAs) miR-30e and miR-214 negatively regulate UBC9 expression and, more importantly, are downregulated in some cancers (Wu et al. 2009). Moreover, Ubc9 is necessary for Ras/Raf-driven oncogenesis in colon cancer cells (Yu et al. 2015). Because it is difficult to drug KRAS itself and the limited efficacy of inhibitors targeting Ras effector kinase, it could be valuable to target sumoylation pathway. Increased Ubc9 expression contributes to tumorigenesis in multiple cancer types (Han et al. 2010; Mattoscio et al. 2015).

In breast cancer, apart from Ubc9, SUMO E3 enzymes were also shown to be deregulated. This is the case for PIAS1, PIAS4 and this impacts DNA-damage repair. PIAS3 is deregulated in glioblastoma. Overexpression of PIAS3 changes cell shape and inhibits cell migration. Expression of the SUMO E3 ligase PIAS1 suppresses TGFβ-induced activation of the matrix metalloproteinase MMP2 in human breast cancer cells (Dadakhujaev et al. 2014). In the same publication, the authors show that knockdown or inhibition of endogenous PIAS1 stimulates the ability of TGFβ to induce an aggressive phenotype in breast cancer cell organoids and promote metastases in mice. For Cbx4 SUMO E3 ligase, it was shown that its expression is significantly correlated with VEGF expression, negatively affecting both overall survival of hepatocellular carcinoma (HCC) patients and mice xenografted with HCC tumor cells, showing that Cbx4 plays a critical role in tumor angiogenesis and progression of cancer (Li et al. 2014).

- **SUMO proteases in cancer**

The SENPs are also subject to multiple regulatory mechanisms. SENP1 was shown to be involved in cancer cell growth *in vitro* and *in vivo*. Overexpression of SENP1 is observed in thyroid oncocytic adenoma (Jacques et al. 2005) and in transgenic mouse model; its expression can lead to the development of prostatic adenocarcinoma (Cheng et al. 2006). Its overexpression in this model correlates with hypoxia-inducing factor 1 (HIF-1) expression, which is associated with an increase in P-glycoprotein expression and the occurrence of multi-drug-resistance in tumor cells (Wartenberg et al. 2003). SENP1 is upregulated by androgen receptor (AR) in prostate cancer cells (Bawa-Khalfe et al. 2007) and by hypoxia (via a hypoxia response element) in endothelial cells. SENP2 is a direct transcriptional target of nuclear factor-κB (NF-κB) (M. H. Lee et al. 2011) and SENP3 is upregulated by low-level of reactive oxygen species (ROS) (Han et al. 2010) and inhibited by heat shock (Pinto et al. 2012). SENP1 overexpression correlated with prostate cancer aggressiveness and recurrence (Wang et al. 2013). SENP6 was shown to promote gastric cancer cell growth via desumoylation of FoxM1 (Song et al. 2015) while high level of SAE2 in these cells promotes malignant phenotype and predicts worse outcome, showing crucial role of the SUMO pathway in the aggressiveness of gastric cancer (Shao et al. 2015). The NF-κB family members including p65 and inhibitor protein IkBα play important roles in the regulation of Multiple Myeloma (MM) cell survival and proliferation. Xu J et al. demonstrated that SENP1 inhibition decreased IL-6-induced p65 and IkBα phosphorylation, leading to the inactivation of NF-κB signaling in MM cells. These results delineate a key role for SENP1 in IL-6 induced proliferation and survival of MM cells (Xu et al. 2015). For USPL1 SUMO isopeptidase, it was shown that its depletion impairs proliferation of HeLa cells and causes loss of Cajal bodies (Schulz et al. 2012) and is embryonic lethal in zebrafish (Amsterdam et al. 2004). In a study of grade 3 breast cancers (poorly differentiated cells with high morphological changes and fast growing rate) Bermejo et al. showed that USPL1 expression is increased with the number of specific USPL1 gene alleles containing defined single nucleotide polymorphism (SNP), confirming the association between higher USPL1 expression and severity of the tumor (Bermejo et al. 2013).

Several cancers do display enhanced levels of both sumoylation and desumoylation enzymes. This suggests a requirement for an accelerated SUMO cycle otherwise increased modification and demodification and SUMO turnover. To give an example, in prostate cancer, increases in levels of SUMO enzyme (Ubc9 and PIAS1) as well as SUMO proteases (SENP1 and SENP3) have been reported (Bawa-Khalfe et al. 2007).

3.5.2 SUMO in AML

3.5.2.a Sumoylation in APL

PML is the organizer of nuclear domains known as PML nuclear bodies (NBs) (Koken et al. 1994). It constitutes the outer shell of the NB sphere and is the organizer of these domains spread-out in nucleus, into which it recruits SP100, Daxx and multiple other proteins, especially in stress conditions (Lallemand-Breitenbach et al. 2001). In APL, the fusion protein PML/RARα has a dual dominant-negative activity on signaling of both of its partners (Melnick and Licht 1999) by repressing nuclear hormone receptor signaling and disrupting PML-NBs.

The key point in APL is the degradation of PML/RARα in order to reactivate RAR signaling. This degradation occurs in two distinct steps:

In a first step, sumoylation intervenes in the formation of PML-NBs. PML is known to be sumoylated on three different lysine residues and contains a SIM domain (Müller et al., 1998). Sumoylation on the critical K160 residue is the key factor that controls recruitment of most partner proteins into the NBs (Lallemand-Breitenbach et al. 2001). Recently it was determined that polySUMO-5 conjugation of PML at lysine K160 also facilitates its recruitment of PML-NB components (Liang et al. 2016) (Figure 20). As PML contains a SIM, which mediates interaction with SUMOs, PML-NBs mature through SUMO-SIM interaction networks that recruit PML-NB components, causing PML shells to enlarge (Shen et al. 2006) and to recruit other SIM-containing proteins in the PML-NBs (Sahin et al. 2014). Moreover ATRA and ATO induce apoptosis through the mitochondrial pathway and by the formation of reactive oxygen species (ROS), which lead to the cross-linking of PML proteins by disulfide bonds (Jeanne et al., 2010). Consequently PML aggregates at the outer shell of NBs to be finally massively sumoylated.

The second step is the disruption of PML-NBs through the intervention of sumoylation. Hypersumoylated PML/RARα recruits SUMO-dependent ubiquitin ligase RNF4, which ubiquitinates PML allowing its recruitment to the proteasome and, ultimately, PML degradation (Lallemand-Breitenbach et al. 2008, Tatham et al. 2008) (Figure 20).

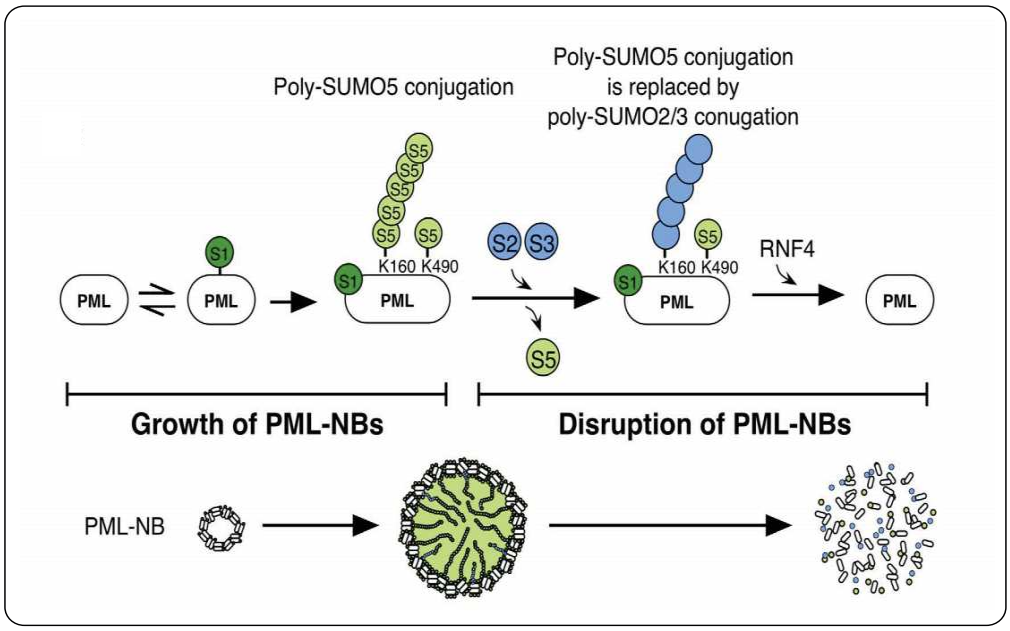


Figure 20: SUMO conjugation regulates the formation and disruption of PML-NBs. (Liang et al., 2016)

3.5.2.b SUMO in non-APL AML

Few studies have addressed the role of sumoylation in non APL-AML. Recently, in the team, we demonstrated that sumoylation plays an important role in AML response to chemotherapeutic drugs (Figure 21). We could show that chemotherapeutic drugs (Ara-C, DNR and Etoposide) treatment generates reactive oxygen species (ROS), which induce the formation of a disulfide bond between the catalytic cysteins of the SUMO E1 and E2 enzymes, owing to a mechanism previously described by G.Bossis (Bossis and Melchior 2006). This inactivation of the enzymes induces a progressive desumoylation in chemosensitive cells. In particular, this desumoylation participates in the activation of proapoptotic genes such as DDIT3. By contrast, in chemoresistant cell, the chemotherapeutic drugs do not induce this ROS/SUMO axis. However, it can be reactivated by pro-oxidants or by inhibition of the SUMO pathway, either using an inhibitor of sumoylation (Anacardic acid) or RNA interference targeting the different SUMO isoforms. Moreover, Anacardic acid limited the growth of chemoresistant AML cells xenografted to immunodeficient mice (Bossis et al. 2014). Altogether, this suggested that targeting the SUMO pathway could be a way to overcome chemoresistance in AML.

C/EBPα is a critical regulator of early myeloid differentiation and it is mutated in 10% of AMLs, where the transcription of the gene produces the p30 C/EBPα form instead of the p42 isoform, which acts as a dominant-negative isoform (Geletu et al. 2007). Overproduction of the p30 C/EBPα isoform leads to an increase in Ubc9 gene expression. Increased Ubc9 activity is then

responsible for the inactivation of p42 C/EBPα factor through its sumoylation, which limits its pro-differentiation potential, making the disease phenotype more aggressive (Hankey et al. 2011).

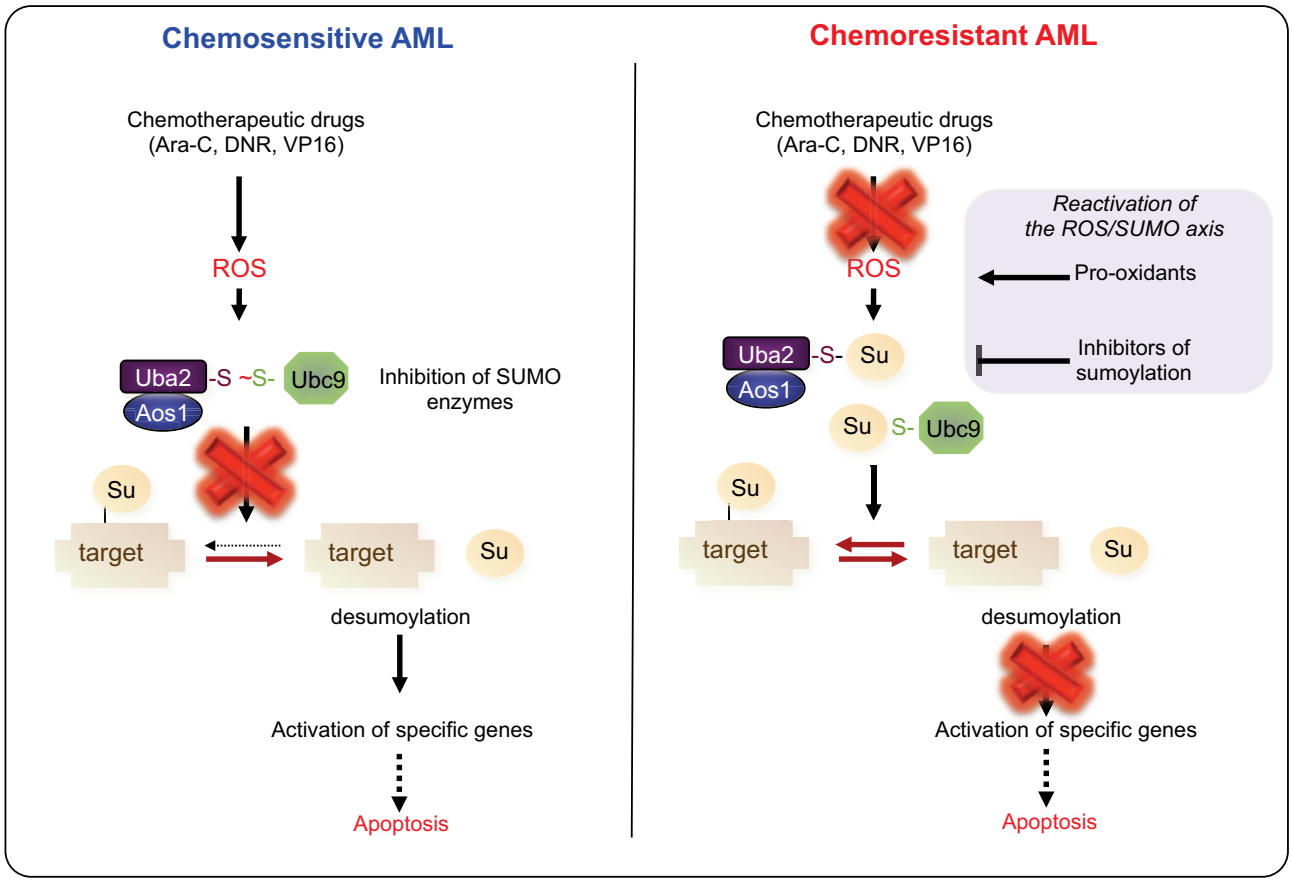


Figure 21: ROS/SUMO pathway is involved in the chemoresistance AML cells. In chemosensitive AML cells, standard chemotherapeutic drugs induce ROS production, which inhibit E1/E2 SUMO enzyme by forming disulfide bond. This leads to desumoylation and activate genes involved in apoptosis. However in chemoresistance AML cells, chemotherapeutic drugs cannot induce ROS production and the ROS/SUMO pathway leading to apoptosis is inhibited. This pathway can be reactivated by using either pro-oxidants or inhibitors of sumoylation.

Sumoylation was also found to play an important role in IGF-1R (insulin growth factor like receptor 1) protein activity, which was found to be upregulated in AML. The proliferation of AML cells was inhibited either by inhibiting Ubc9 or mutating the SUMOylation sites on IGF- 1R, even though cell apoptosis was not affected (Zhang et al. 2015).

An involvement of SUMO in the leukemogenic phenotype was also suggested by the analysis of transcriptomic data showing a significantly repressed expression of SENP5 in AML patients compared to healthy donors neutrophils. Induction of differentiation by ATRA increased the expression of SENP5 and knocking down SENP5 significantly attenuated it, suggesting an important role for SENP5 in AML differentiation (Federzoni et al. 2015). However in contradiction, sumoylation was also shown to have a positive effect on myeloid differentiation. Andrade et al., showed that sumoylation favors GFI1-LSD1/CoREST binding and MYC repression to induce hematopoietic differentiation using HL60 cell lines (Andrade et al., 2016). Moreover it was also

reported that ATRA-induced AML differentiation is impaired by the depletion or inhibition of SUMO-1. The authors could show that sumoylation of RARα increased its stability and promoted differentiation (Zhou et al. 2014).

Finally, positive regulatory domain I-binding factor 1 and retinoblastoma-interactering zinc finger protein-1 (PRDM16) is a transcription factor and the overexpression of one isoform sPRDM16 is oncogenic in leukemia (Shing et al., 2007) promoting proliferation, enhancing self-renewal capacity and inhibiting differentiation of THP-1 AML cell line. Mutation of the sPRDM16 sumoylation site at K568 partially abolished the capacity of sPRDM16 to promote proliferation and inhibit differentiation of AML cells both *in vitro* and in mouse xenografts. Importantly, differentiation-related genes induced by PMA are differentially expressed between THP-1 cells stably expressing sPRDM16-WT and sPRDM16-K568R (Dong and Chen 2015).

In conclusion, the sumoylation and desumoylation machinery is important at different steps of leukemogenesis and AML response to treatments and might thus be a promising target.

II Results

Manuscript 1

Inhibition of the SUMO pathway activates All-*Trans* Retinoic Acid differentiation in Acute Myeloid Leukemias.

To be submitted to 'The Journal of Experimental Medicine'

Inhibition of the SUMO pathway activates All-*Trans* Retinoic Acid differentiation in Acute Myeloid Leukemias

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Abstract (160 words max)

Differentiation therapies are a promising alternative to genotoxic-based chemotherapies in the treatment of many cancers. In particular, All-*trans*-retinoic acid (ATRA) is successfully used for Acute Promyelocytic Leukemias, a subtype of Acute Myeloid Leukemias. However, its clinical efficiency is very limited in the other AML subtypes, in particular because of epigenetic repression of ATRA-responsive genes. We show here that sumoylation represses ATRA-induced differentiation in many AML cell lines and primary patient samples, including those resistant to chemotherapies. Inhibition of sumoylation with pharmacological inhibitors or overexpression of desumoylases markedly increased their differentiation by ATRA and increasing sumoylation by overexpression of SUMO or its conjugating enzyme Ubc9 strongly reduce ATRA efficiency. Inhibition of sumoylation synergize with ATRA to arrest AML cells proliferation both *in vitro* and *in vivo*. Mechanistically, inhibition of sumoylation primes AML cells for differentiation by facilitating the expression of master genes of the myeloid differentiation. Targeting the SUMO pathway thus constitute a promising approach to sensitize AML to differentiation therapies.

Acute Myeloid Leukemia (AML) is a heterogeneous group of severe hematological malignancies that arise through the acquisition of oncogenic mutations by hematopoietic stem or progenitor cells. Instead of differentiating in the normal blood constituent, leukemic cells are blocked at a specific stage of the differentiation process, proliferate, and populate the bone marrow, which gives rise to the disease (Estey, 2012). The standard treatment for AML is generally based on the use of two genotoxics, one anthracyclin and the nucleoside analog Ara-C, which aim at eliminating hyper-proliferative leukemic blasts. However, the relapse rates are really high, between 40 and 70% of the patients depending on their age and the overall survival is still really low (Dombret and Gardin, 2016). Differentiation therapies constitute an alternative to chemotherapies (Ma et al., 2017). They aim at restoring the differentiation of the leukemic blasts, resulting in an arrest in their proliferation and their subsequent death. All-trans-retinoic acid (ATRA) is used as a differentiation agent in various cancers. In the case of AML, it is successfully used in combination with Arsenic tri-oxide (As_20_3) for the treatment of Acute Promyelocytic Leukemias, a minor subtype of AML involving the PML-RARα fusion (Lo-Coco et al., 2013). However, many studies have shown that ATRA poorly induces the differentiation of non-APL AMLs. This lack of effect is, at least in part, due to the inability of ATRA to induce the expression of a transcriptional program of differentiation (Ma et al., 2017). Recently, epigenetic reprogramming has been shown to be a promising approach to restore ATRA-induced differentiation of non-APL AML (Brown and Hughes, 2012). This is for example the case for the inhibition of the LSD1 $H3K4me_2$ demethylase, which activates a differentiation gene expression program and strongly sensitizes AML cells to ATRA (Schenk et al., 2012). Finally, some AML subtypes, such as those carrying IDH1/IDH2 mutations, might be more sensitive to ATRA-induced differentiation, in particular because of deregulated epigenetic state, which can prime ATRA-induced gene expression (Boutzen et al., 2016).

SUMO is a post-translational modifier of the ubiquitin family. Its covalent conjugation to its target proteins regulates their function and fate. SUMO has been therefore involved in many cellular process and increasing evidence link deregulations of the SUMO pathway to cancer (Seeler and Dejean, 2017). SUMO has been largely involved in the regulation of gene expression through the modification of numerous transcription factors and co-regulators and can thus be viewed as an epigenetic mark. In general, sumoylation limits transcription efficiency by providing a platform for the recruitment, via their SUMO-interacting motifs of co-

repressors such as HDAC or histone demethylases, including the COREST-LSD1 complex and SETDB1 (Raman et al., 2013; Cubeñas-Potts and Matunis, 2013; Chymkowitch et al., 2015).

We have recently shown that sumoylation is involved in AML response to chemotherapeutic drugs (Bossis et al., 2014). The genotoxics used induce the production of Reactive Oxygen Species, which inactivate the SUMO activating and conjugating enzymes owing to a mechanism that involve the formation of a disulfide bond between their catalytic cysteines (Bossis and Melchior, 2006; Stankovic-Valentin et al., 2016). This leads to a progressive desumoylation in chemosensitive but not in chemoresistant AML cells. This desumoylation is instrumental for the activation of genes involved in drug-induced apoptosis (Bossis et al., 2014). Most studies related to SUMO and ATRA are linked to APL where the SUMO pathway plays a critical role in the clearance of the oncogenic PML-RARα protein upon ATRA+As_2O_3 treatment through the recruitment of the RNF4 SUMO-dependant ubiquitin ligase (Tatham et al., 2008; Lallemand-Breitenbach et al., 2008).

We show here that sumoylation participates in the epigenetic silencing of ATRA-induced gene expression programs in AML and its targeting strongly activates the differentiation of non-APL AMLs cell lines, patient cells and mouse models and lead to an arrest in their proliferation. Mechanistically, sumoylation repress the expression of critical genes involved in the differentiation process. Our work thus opens new perspectives in the treatment of AML with differentiation therapies.

Results and discussion

The SUMO pathway represses AML differentiation

To determine if sumoylation could restrict the pro-differentiating effects of ATRA in AML, we first resorted to the well-characterized AML cell lines U937, THP1, HL-60 and MOLM14, which belong to different AML subtypes and differentiate with different efficiencies in the presence of ATRA *in vitro*. Inhibition of the SUMO E2 conjugating enzyme with 2D08 (Kim et al., 2013, 2014), resulted in a strong increase in the expression of myeloid differentiation markers such as CD11b and CD15 on U937, CD14 on THP1 and CD11b on MOLM14 (Figure 1A). The inhibition of sumoylation also induced morphological changes associated with terminal differentiation, in particular an increase in the cell size, an enlargement of the cytosol and the appearance of numerous and large cytosolic granules as shown for U937 and HL60 (Figure 1B). Interestingly, 2D08 alone was able to induce an increase in the differentiation of the AML cell lines, both at the level of the expression of differentiation markers and morphological changes. Anacardic Acid, an inhibitor of the SUMO E1 activating enzyme (Fukuda et al., 2009), also increased the differentiation, as shown for HL60 and MOLM14 cell lines (Figure 1C), confirming the results obtained with the 2D08 inhibitor. A feature of differentiated myeloid cells is their ability to produced ROS. Both inhibitors of sumoylation increased ATRA-induced NADPH-oxidase derived ROS production by HL-60 cells differentiated for 5 days (Figure 1D), further confirming their pro-differentiating effect. Since pharmacological inhibitors can have off-target effects, we also resorted to genetic approaches to modulate the SUMO pathway and confirm its role in ATRA induced differentiation of non-APL AML cell lines. We thus overexpressed the SENP2 desumoylase to induce a hypo-sumoylated state in the U937 cell line. This led to a significant increase in their ATRA-induced differentiation, as measured by the appearance of the CD11b marker (Figure 2A-2B). The same results were obtained in the U937 cell line overexpressing SENP5 (Figure 2B). Conversely, overexpression of SUMO-1 or SUMO-2 in the THP-1 cell line led to a significant decrease in their ATRA-induced differentiation (Figure 2C). Altogether, these data suggested that sumoylation participates in the repression of ATRA-induced differentiation of non APL AML cells and its pharmacological targeting can prime AML cells for differentiation.

Inhibition of the SUMO pathway allows ATRA-induced differentiation of primary AML cells and chemoresistant AML cells.

We then tested if inhibition of the SUMO pathway could affect the *in vitro* differentiation of primary AML cells. AML cells from 11 patients (non-APL AML), taken at diagnosis from bone marrow aspirate were culture *in vitro* for 7 to 9 days. ATRA alone, at clinically relevant doses (1µM), had no significant effect on their differentiation. Both 2D08 and Anacardic acid induced a slight, although not statistically significant, increase in their differentiation. Interestingly, their combination with ATRA induced a significant increase the expression of the CD15 differentiation-associated marker compared to mock treated conditions and to ATRA alone (Figure 3A). Combining ATRA and 2D08 or Anacardic acid also increased the morphological changes associated with differentiation compared to ATRA alone (Figure 3B), including nuclear lobulation, cytosol enlargement and the appearance of multiple cytosolic granules. Although some patients cells were more sensitive to the differentiating properties of the ATRA + inhibitors of sumoylation combination than others (Figure 3A), we could not identify specific cytogenetic or cellular characteristics within this group. In line with our results on all the AML cell lines tested, this suggests that the repressive function of the SUMO pathway on AML differentiation is not restricted to specific AML subtypes. Interestingly, we could also show that inhibitors of sumoylation increased the ATRA-induced differentiation of primary AML cells from one patient that was not responsive to induction chemotherapies and for 2 out of 3 patients at relapse (Figure 3C). The same results were obtained on a U937 cell line resistant to Ara-C that we generated (Figure 3D). This suggested that inhibitors of sumoylation could increase the efficacy of ATRA and offer a new therapeutical perspective, including in case of treatment failure after conventional chemotherapies.

Inhibition of sumoylation potentiates the anti-leukemic effect of ATRA

The rationale for differentiation therapies is that differentiated cells stop proliferating and have a shorter half-life than undifferentiated cells. We could show that the combination of ATRA and 2D08 rapidly stops the proliferation of U937 (Figure 4A) and their chemoresistant version (Figure 4B) without inducing a significant increase in cell death, at least after 5 days of treatment (Figure 4C). Along the same line, overexpression of SENP2 and SENP5 in U937 cells led to a significant decrease in their proliferation upon ATRA-treatment (Figure 4D). Finally, the combination of 2D08 with ATRA was more efficient than ATRA alone at inducing

the death of primary AML cells treated in vitro (Figure 4E). Altogether, these results suggest that inhibition of the SUMO pathway synergize with ATRA to activate the differentiation of AML cells, stop their proliferation and ultimately lead to their death. We then wondered if ATRA + 2D08 would have an anti-leukemic effect *in vivo*. To this aim, NOD-Scid-IL2Rg^{null} mice were xenografted subcutaneously with the U937 cell line. After engraftment, mice were treated or not with ATRA, 2D08 or the combination. Although both ATRA and 2D08 slightly decreased tumor growth, only the combination induced a statistically significant decrease in tumor growth (Figure 4F). However, it could not completely stop U937 proliferation *in vivo*, as it did for the same cell line *in vitro* (Figure 4A). This can be due to the poor pharmacological properties of the 2D08, which is quite insoluble and precipitates when in aqueous solutions. Anacardic acid, another inhibitor of the SUMO pathway is also highly hydrophobic with poor bio-availability (Bossis et al., 2014). Chemical modifications of these molecules or identification of new inhibitors of the pathway are thus required for further *in vivo* analysis of the therapeutical benefit of the association between ATRA and inhibition of the SUMO pathway and potential clinical use.

Inhibition of sumoylation activates the expression of differentiation-related genes

Sumoylated proteins are highly enriched at gene promoters (Neyret-Kahn et al., 2013; Seifert et al., 2015; Liu et al., 2012) and many transcription factors, co-regulators, histone-modifying enzymes the polymerase and even histones are known SUMO targets (Raman et al., 2013; Hendriks et al., 2015). Sumoylation has been associated with transcriptional activation, in particular of RNAPolIII-dependant tRNA synthesis (Chymkowitch et al., 2017). However, in most cases, sumoylation at promoters has been found to negatively regulate gene expression (Nayak et al., 2014; Decque et al., 2016; Hendriks et al., 2015; Stielow et al., 2008; Tempé et al., 2014). We thus analysed the role of sumoylation on the expression ATRA-responsive genes. Compared to ATRA alone, treatment of U937 cells with the ATRA + 2D08 combination induced a significant increase in the expression of different known ATRA-responsive genes. This is for example the case for *RARa*, which encodes for the receptor for ATRA, *ITGAX* (CD11c), *IL1B*, a cytokine involved in the myeloid differentiation (Jacobsen et al., 1994) and *TNFSF10,* which encodes for the pro-apoptotic TRAIL cytokine, which is known to be involved in ATRA-induced cell death (Altucci et al., 2001) (Figure 5A). Along the same line, overexpression of the SENP1 desumoylase strongly activated the expression of these genes in THP-1 cells (Figure 5B). Interestingly, treatment with 2D08 or overexpression of SENP1 was

sufficient to induce the expression of some of the genes tested, further suggesting that inhibition of sumoylation primes AML cells for ATRA-induced differentiation. We could then show that the combination ATRA + 2D08 led to a significant increase in the level of $H3K4me_3$, a histone mark associated with active transcription, on the promoters of some of these genes (Figure 5C). Altogether, this suggests that sumoylation represses AML differentiation through the modification of chromatin-bound proteins and silencing of ATRA-responsive genes. Its inhibition is able to reactivate the ATRA-induced expression of genes that play key roles in both the differentiation and cell death. Various transcription factors that play key roles in the myeloid differentiation are sumoylated. This includes CEBPα and CEBPε, which sumoylation was shown to respectively repress and activate their transcriptional capacities (Subramanian et al., 2003; Kim et al., 2005). This is also the case for RARα, the receptor for ATRA, which undergoes a dynamic process of sumoylation and desumoylation that was shown to be involved in its ATRA-induced activation (Zhou et al., 2014; Zhu et al., 2009). Although changes in the sumoylation of some specific transcription factors, such as CEBP or RARα, could explain the increased ATRA-induced transcriptional activation of differentiation-associated genes upon sumoylation inhibition, it is more likely that it is due to a coordinated desumoylation of multiple proteins bound to the promoters of these genes. This concept of group sumoylation was first demonstrated for the regulation of DNA-damage repair (Psakhye and Jentsch, 2012). It states that SUMO can exert its function by either recruiting SUMO-binding proteins or stabilizing protein-protein interactions, wherever it is conjugated within a supramolecular complex comprising several sumoylatable constituents. Thus, desumoylation of multiple transcription factors and co-regulators might account for the pro-differentiating role of the inhibition of sumoylation.

In conclusion, our work demonstrate that sumoylation limits ATRA-induced differentiation in AML, in particular by preventing the transcriptional reprogramming required for the cells to differentiate in the myeloid lineage. Global inhibition of the SUMO pathway would prime AML cells for differentiation by removing co-repressor complexes from the promoters of these genes. Importantly, we did not observe overt toxicity of the SUMO inhibitors in mice treated with 2D08, similar to what we observed previously with Anacardic acid (Bossis et al., 2014). Accordingly, even though complete KO of the SUMO E2 enzyme Ubc9 is embryonic lethal, hemizigous mice expressing only 50% of Ubc9 are viable and show no overt phenotype (Nacerddine et al., 2005). This suggests that controlled inhibition of the SUMO pathway could,

in combination with ATRA, target leukemic cells without affecting normal cells. This could also, in ATRA-sensitive APL, allow a decrease in the clinical doses to limit the occurrence of differentiation syndromes. Moreover, inhibition of sumoylation could also favor the differentiating capacities of other molecules such as Vitamin D3, which is highly efficient at differentiating AML cells. However, it causes hypercalcemia, which can have life-threatening effects on cardiac function and strongly limit is clinical use (Gocek and Marcinkowska, 2011). Its combination with inhibitors of the SUMO pathway could be a way to decrease the doses used and limit the side effects. These findings could pave the way to novel therapeutical strategies in the treatment of AML and potentially other cancers treated with retinoids.

Figures and Legends

Figure 1: The SUMO pathway limits non-APL AMLs differentiation

(A) U937 or THP1 cells were treated for 5 days (U937), 9 days (THP1) or 2 days (MOLM14) with ATRA (1µM for U937 and 0,5µM for THP1 and 0.1µM for MOLM14), 2D08 (50µM) or the combination and the expression of differentiation markers CD14 or CD15 was measured by flow cytometry. The median fluorescent intensities (MFI) are indicated on the graph (n=3 to 6, mean =/-sem). (B) U937 and HL60 were treated with ATRA (1µM), 2D08 (50µM for U937 and 10µM for HL-60) or the combination for 5 days and stained with May-Grumwald-Giemsa (MGG). All photographs have the same scale (C) MOLM14 and HL-60 cells were treated for 5 days with ATRA (1µM), Anacardic acid (25µM for MOLM14 and 10µM for HL-60) or the combination and the expression of differentiation markers CD11b was measured by flow cytometry. Representative profiles are shown. (D) HL-60 were treated for 5 days with with ATRA (1µM), 2D08 (10µM), Anacardic acid (10µM) or the combination and NADPH-oxidase derived ROS production was measured by luminometry with or without PMA (100nM). No ROS production was detected in all the conditions without ATRA, ie DMSO (vehicle, 2D08, Anacardic acid) and in the absence of PMA (all curves are on the X axis).

Figure 2: Modulation of the SUMO pathway affects ATRA-induced differentiation of non-APL AMLs

(A) U937 cells were infected with a control virus or a virus encoding for SENP2. Both virus also expressed GFP. Infected cells were sorted and treated or not with ATRA (1µM) for 5 days and their differentiation was analysed by the expression of the CD11b marker. A representative histogram is shown (n=3). (B) Similar to A with quantification of the MFI for CD11b on U937 expressing control, SENP2 and SENP5 (n=3, mean +/- sem). (C). THP1 cells were infected with a control virus or viruses encoding for Ubc9, SUMO-1 or SUMO-2. All viruses also expressed GFP. Infected cells were sorted and treated or not with ATRA (1µM) for 5 days and their differentiation was analysed by the expression of the CD14 marker (n=3, mean +/- sem).

Figure 3: Targeting the SUMO pathway induce the differentiation of primary AML cells and chemoresistant AMLs

A. Primary AML cells purified from bone marrow aspirate at diagnosis were treated in vitro with ATRA (1µM), 2D08 (50µM), Anacardic acid (25µM) or the combinations of these drugs. After 7 to 9 days, their differentiation was analysed by flow cytometry using CD15 antibody. The MFI for each conditions were corrected with corresponding isotypic controls and normalized to the mock (DMSO) treated condition. B. Bone marrow aspirate from one AML patient was treated as in A for 5 days and analysed by MGG. C. Primary cells from 4 patients were treated and analysed as in A. AML-1 correspond to a patient refractory to induction chemotherapy and AML-2 to -4 to patients at relapse. D. U937 cells resistant to Ara-C were treated for 5 days with ATRA (1µM), 2D08 (50µM) or the combination. Their differentiation was analysed by flow cytometry using the CD15 marker and normalized to mock (DMSO) condition (n=3, mean +/- sem).

Figure 4: Inhibition of the SUMO pathway limits AML proliferation in vitro and in vivo

(A-B) U937 (A) or Ara-C resistant U937-R (B) were treated for the indicated times with ATRA (1µM), 2D08 (50µM) or the combination of both. The number of viable cells was determined using Trypan blue exclusion and automatic cell counting. The cells were diluted and new drug was added after every counting. The number of cells on Day 0 was set to 1 (n=3, mean +/- sem). (C) U937 cells were treated as in A and cell viability was measured with Sytox after 5 days by flow cytometry (n=3, mean +/- sem). (D) U937 cells were infected with a control virus or a virus encoding for SENP2 or SENP5. Both viruses also expressed GFP. Infected cells were sorted and treated or not with ATRA (1µM). The number of viable cells was determined using Trypan blue exclusion and automatic cell counting at the indicated times. The number of cells on Day 0 was set to 1 (n=4, mean +/- sem) (E) Primary AML cells obtained at diagnosis were treated with ATRA (0.1µM), 2D08 (50µM), Anacardic acid (25µM) for 9 days. The number of viable leukemic cells (CD45/SSC gating) in each condition was then determined by flow cytometry using counting beads. (F) U937 cells were xenografted subcutaneously on each flank of NOD-Scid-IL2Rg^{null} mice (NSG) (7 mice per group). When the tumors reached 100mm^3 (4 days after injection), the mice were treated peritumorally with 2D08 (10mg/kg) and intraperitoneally with ATRA (2.5mg/kg) every two days for 10 days. Tumor volume was

measured every two days. The only significant p values indicated on the graph are calculated between the DMSO and the ATRA+2D08 groups.

Figure 5: the SUMO pathway represses ATRA-induced gene expression

(A) U937 were treated for 2 days with ATRA (1µM), 2D08 (50µM) or the combination and mRNA were purified and the expression of the indicated genes was analysed by qRT-PCR and normalized to the housekeeping gene TBP (n=6, mean =/- sem) (B) THP1 cells were infected with a control virus or a virus encoding for SENP1. Both viruses also expressed GFP. Infected cells were sorted and treated or not with ATRA (1µM) for 2 days. mRNA were purified and the expression of the indicated genes was analysed by qRT-PCR and normalized to the housekeeping gene TBP (n=5, mean +/- sem). (C) U937 were treated as in A and used for ChIP experiments with control, $H3K4Me_3$ and H3 antibodies. Regions in the promoter of RARA and that of IL1B were amplified from the immunoprecipitated fragments and normalized to the input DNA. Results are represented as a ratio between $H3K4Me_3$ and H3 normalized to the mock (DMSO) condition (n=5, mean +/- sem).

Figure 1

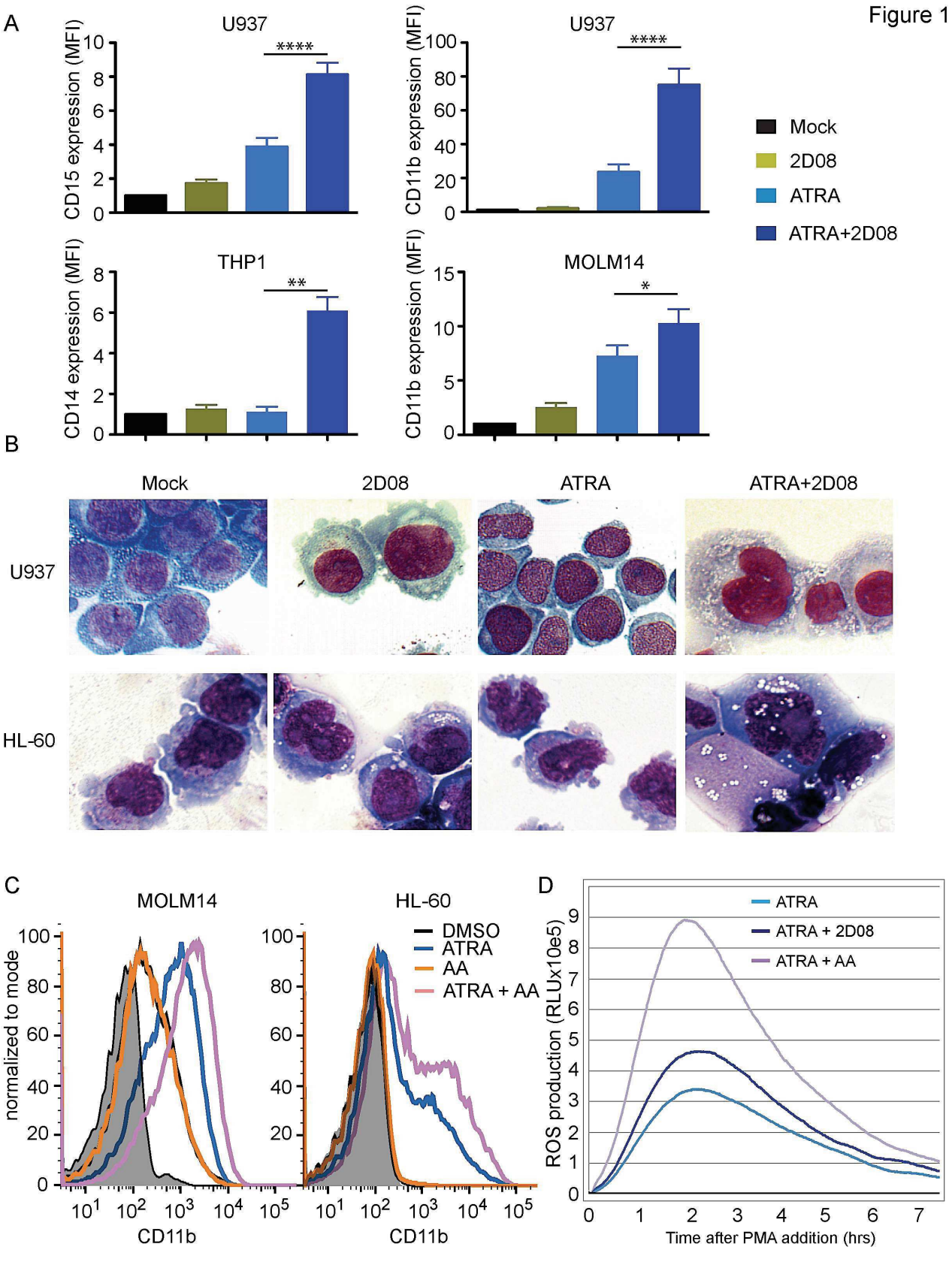
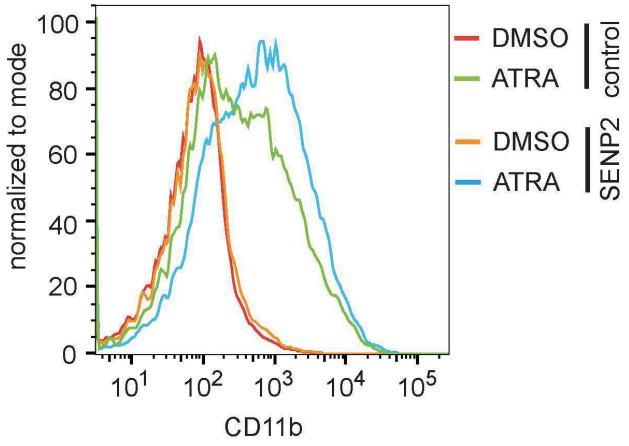
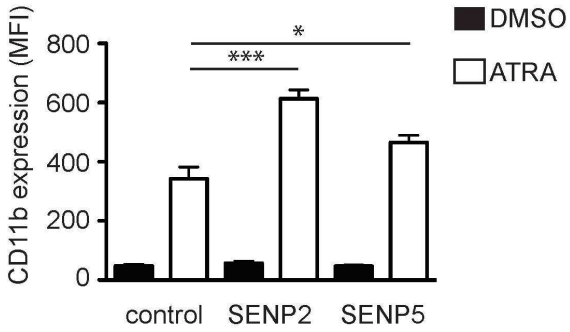

A
U937
CD15 expression (MFI)
****
U937
CD11b expression (MFI)
****
Mock
2D08
ATRA
ATRA+2D08
THP1
CD14 expression (MFI)
**
MOLM14
CD11b expression (MFI)
*
B
Mock
2D08
ATRA
ATRA+2D08
U937
HL-60
C
MOLM14
HL-60
DMSO
ATRA
AA
ATRA + AA
normalized to mode
CD11b
CD11b
D
ATRA
ATRA + 2D08
ATRA + AA
ROS production (RLUx10e5)
Time after PMA addition (hrs)

Figure 2

A


normalized to mode
100
80
60
40
20
0
10^1 10^2 10^3 10^4 10^5
CD11b
DMSO
ATRA
control
DMSO
ATRA
SENP2

B


CD11b expression (MFI)
800
600
400
200
0
*
***
DMSO
ATRA
control
SENP2
SENP5

C

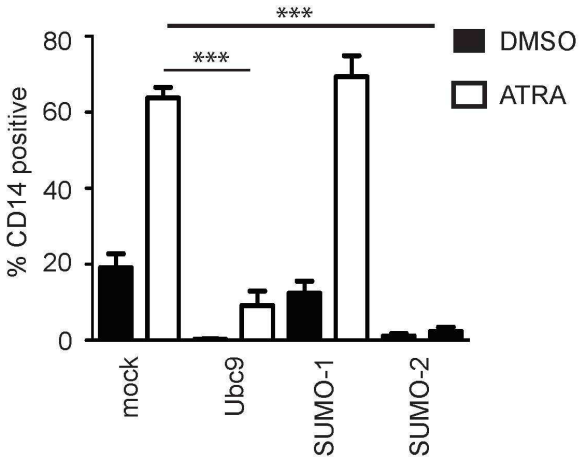
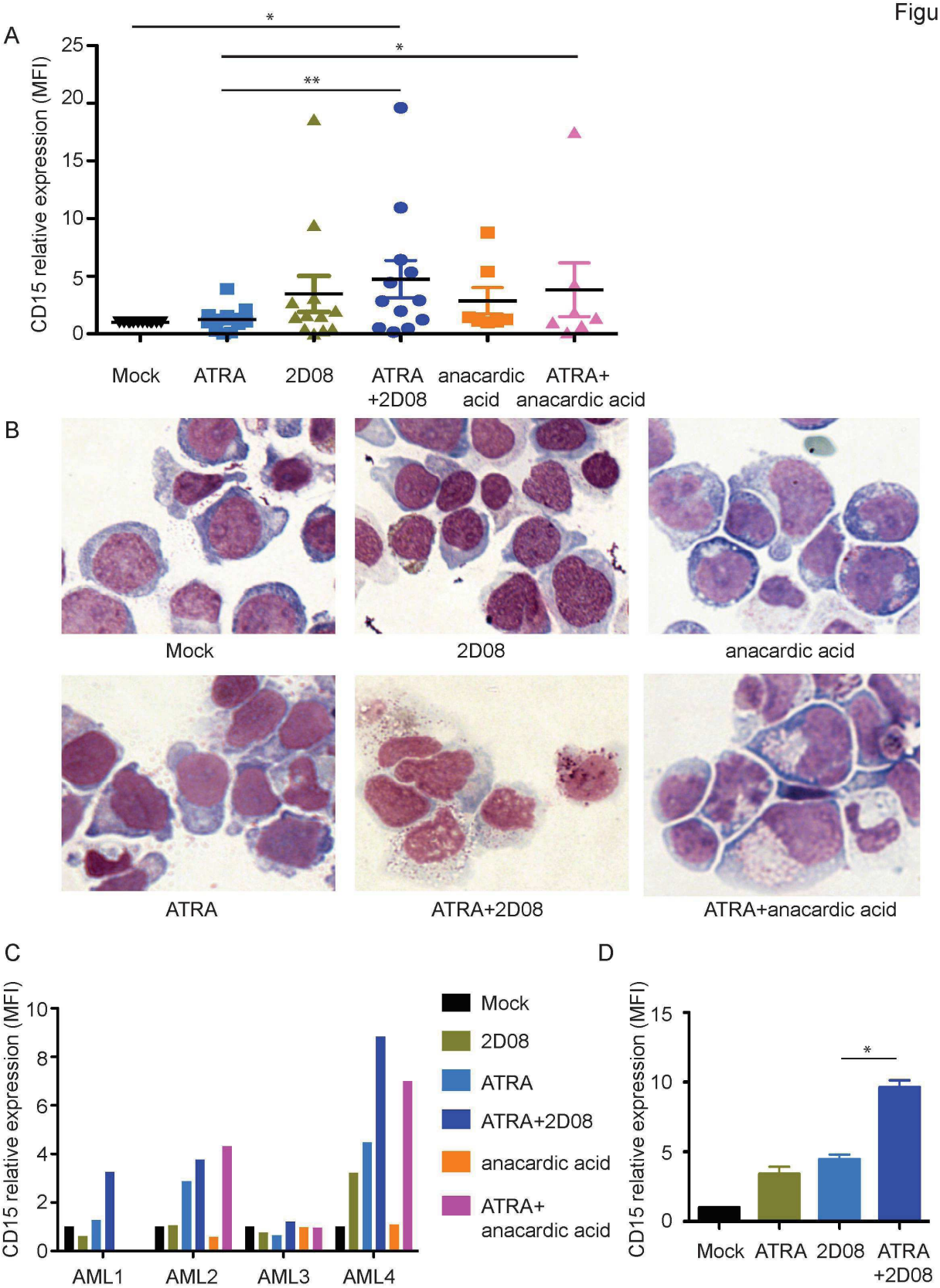

% CD14 positive
80
60
40
20
0
***
***
DMSO
ATRA
mock
Ubc9
SUMO-1
SUMO-2

Figure 3


A
CD15 relative expression (MFI)
25
20
15
10
5
0
*
*
**
Mock
ATRA
2D08
ATRA +2D08
anacardic acid
ATRA+ anacardic acid
B
Mock
2D08
anacardic acid
ATRA
ATRA+2D08
ATRA+anacardic acid
C
CD15 relative expression (MFI)
10
8
6
4
2
0
AML1
AML2
AML3
AML4
Mock
2D08
ATRA
ATRA+2D08
anacardic acid
ATRA+ anacardic acid
D
CD15 relative expression (MFI)
15
10
5
0
*
Mock
ATRA
2D08
ATRA +2D08

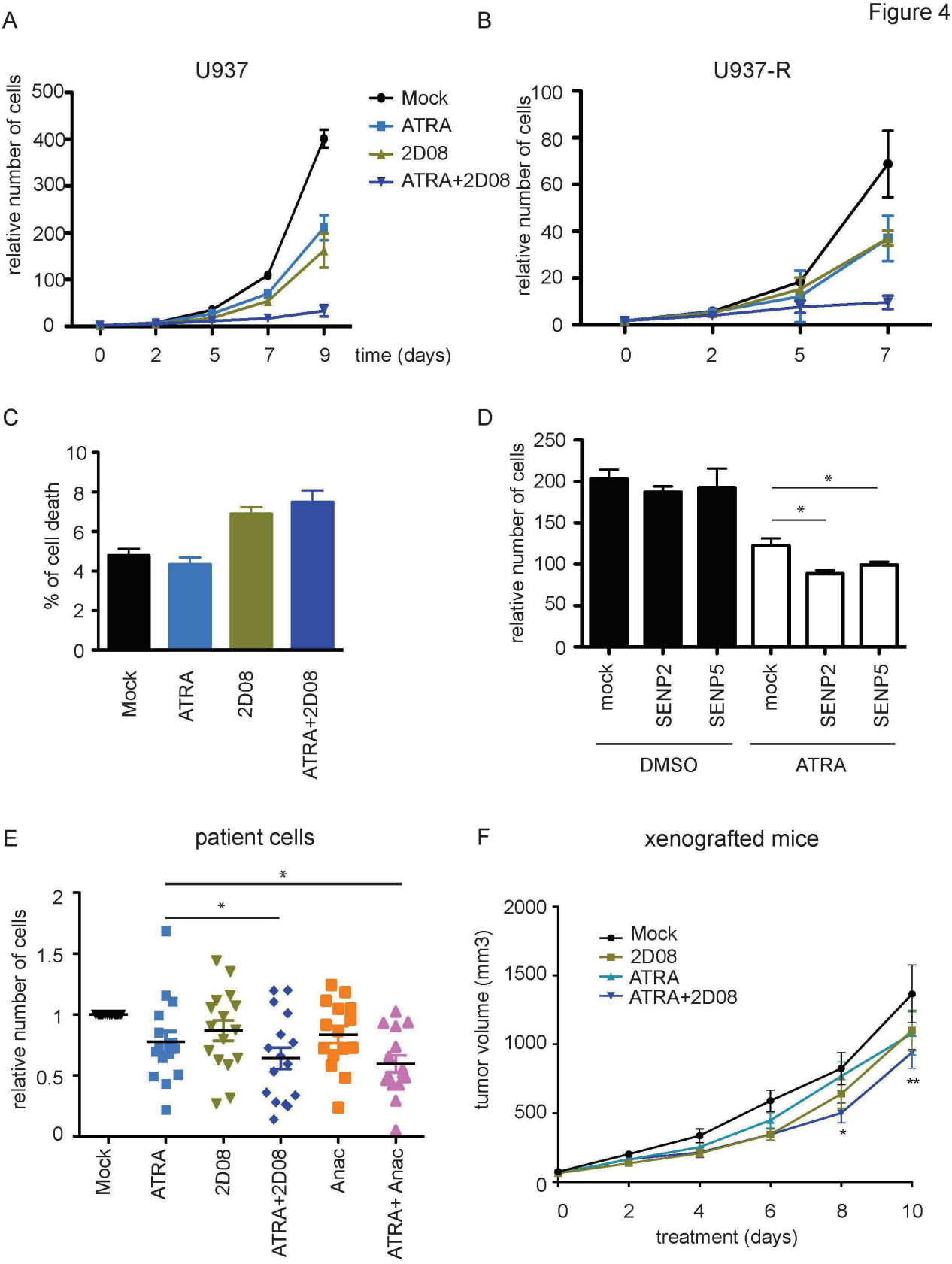
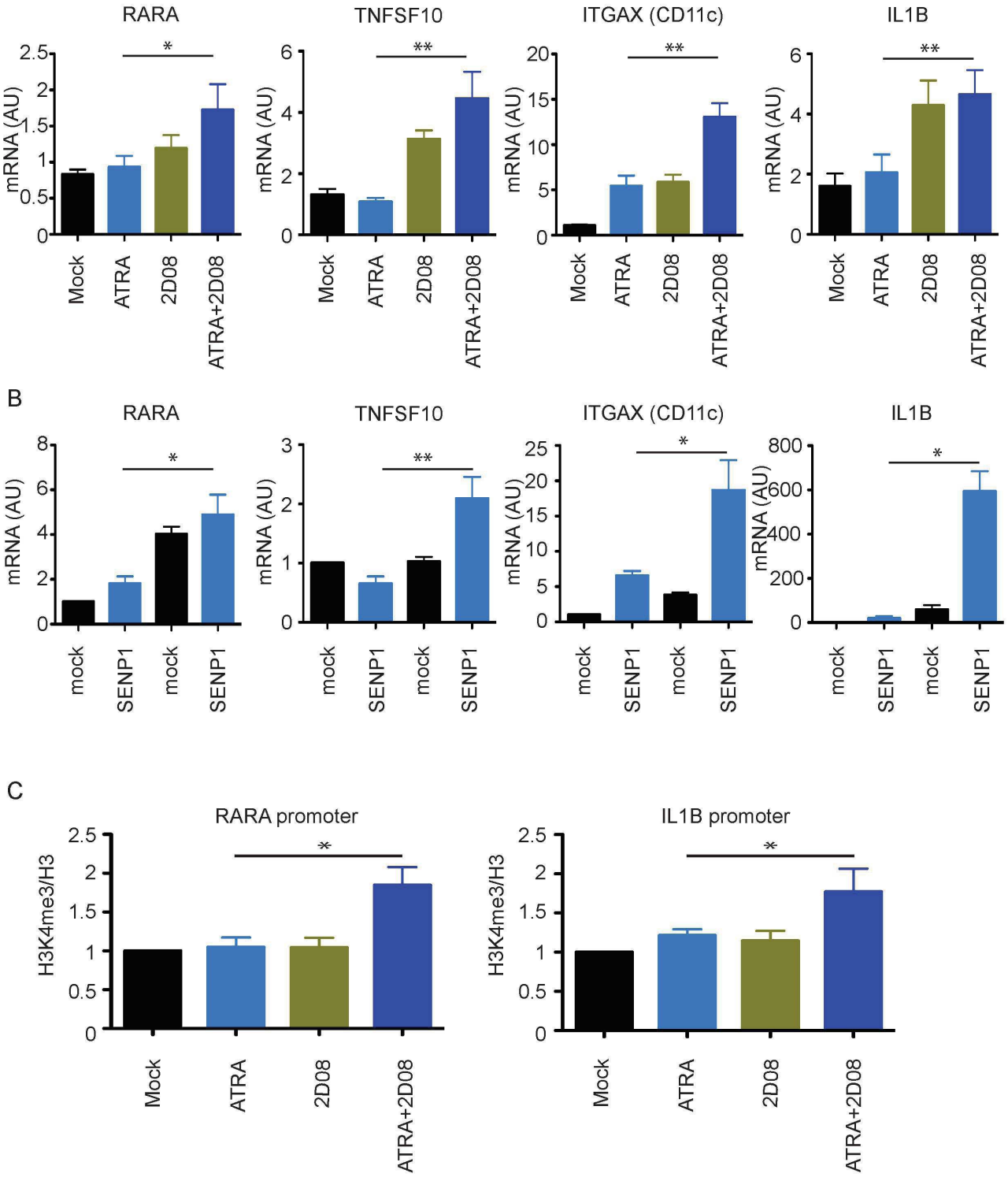

A
U937
relative number of cells
500
400
300
200
100
0
0
2
5
7
9
time (days)
Mock
ATRA
2D08
ATRA+2D08
B
U937-R
relative number of cells
100
80
60
40
20
0
0
2
5
7
C
% of cell death
10
8
6
4
2
0
Mock
ATRA
2D08
ATRA+2D08
D
relative number of cells
250
200
150
100
50
0
*
*
mock
SENP2
SENP5
mock
SENP2
SENP5
DMSO
ATRA
E
patient cells
relative number of cells
2
1.5
1
0.5
0
*
*
Mock
ATRA
2D08
ATRA+2D08
Anac
ATRA+ Anac
F
xenografted mice
tumor volume (mm3)
2000
1500
1000
500
0
Mock
2D08
ATRA
ATRA+2D08
*
**
0
2
4
6
8
10
treatment (days)

Figure 5


A
RARA
TNFSF10
ITGAX (CD11c)
IL1B
mRNA (AU)
Mock
ATRA
2D08
ATRA+2D08
B
mock
SENP1
C
RARA promoter
IL1B promoter
H3K4me3/H3

Material and methods

Cell line and primary AML patient cell Culture:

U937, HL60, THP1, KG1a and NB4 cells (DSMZ, Germany) were cultured in RPMI with 10% fetal bovine serum (FBS) and streptomycin/penicillin at 37°C and 5% CO_2 incubator.

Fresh bone marrow aspirates were collected after obtaining informed consent from patients (Ethical Committee « Sud Méditerranée 1 », ref 2013-A00260-45) and stored in the HemoDiag collection. Leukocytes were purified using a density-based centrifugation (Histopaque 1077, SIGMA) and resuspended at a concentration of 10^6/mL in IMDM (SIGMA) complemented with BSA 1.5mg/ml, insulin 4.4µg/ml, transferrin 60µg/ml, 5% antibiotic (streptomycin/penicillin) 5% FBS, 5 µM ß mercaptoethanol, pyruvate, MEM NEAA (Life Technologies), 10 ng/ml IL-3, 40 ng/ml SCF, 10 ng/ml TPO (PeproTech).

Pharmacological inhibitors, Reagents, and antibodies:

All-*trans* retinoic acid (ATRA) was from Sigma. It was prepared in DMSO and stored at -20°C for 2 weeks maximum. Anacardic acid was from Santa Cruz Biotechnologies and 2D08 from Merck-Millipore. SUMO-1 (21C7) and SUMO-2 (8A2) hybridomas were from the Developmental Studies Hybridoma Bank. H3K4Me3 and H3 antibodies were from Abcam.

Flow Cytometry:

Cells were washed in PBS with 2% FBS, and incubated at 4°C during 30 minutes in presence of cell surface antibodies: CD45-Pacific Blue (A74763, Beckman Coulter), CD14-PE (130-091-242, Miltenyi), CD15-PE-Vio770 (130-100-425, Miltenyi), CD11b-APC (130-109-286) or the corresponding isotype controls for each treatment condition. After washing, cells were analyzed by flow cytometry using a LSR Fortessa Becton Dickinson using FacsDiva software. Data were analyzed using FlowJo software (version 10). Leukemic cells were selected using CD45/SSC gating (Brahimi et al., 2014) and the MFI for each differentiation marker was analyzed on this population. The MFI for the isotype controls in each treatment condition was subtracted to the corresponding sample.

Viability and cell proliferation assay:

For proliferation assays on cell lines, cells were seeded at 3.10^5/ml and viable cells were counted every today using Trypan-blue exclusion with EVE automatic cell counter. For patient cells, an equal number of CountBright™ absolute counting beads (C36950, life technologies) was added to each samples. Viable cells were selected using SSC/FSC gating and their number was normalized to the number of beads counted in the same sample. For measuring viability, cells were stained using the SYTOX® Dead Cell Stain (S34862, Molecular Probes ™) according to manufacturer protocol and analysed by flow cytometry.

Retroviral Infections:

Retroviral constructs expressing SUMO-1, SUMO-2, Ubc9, SENP2, SENP5 were constructed by inserting human cDNA into the pMIG retroviral vector (Van Parijs et al., 1999) by Gateway recombination. Viruses were produced in HEK293T cells by tranfection using Lipofectamine-2000 (Invitrogen) of viral construct together with gag-pol and env (VSVG) expression vectors. Viral supernatants were collected 48hr after transfection, 0,45µm filtered and used to infect AML cell lines. Then only GFP-positive cells were considered in the flow cytometry analysis. Where indicated, the GFP positive cells were sorted using a FACS-Aria cell sorter (Beckton Dickinson)

qRT-PCR:

Total mRNA was purified using the GenElute Mammalian Total RNA kit (Sigma). After DNase I treatment, 1µg of total RNA was used cDNA synthesis with the Maxima First Strand cDNA (Thermo Scientific) and used for qPCR with taq platinium (Invitrogen). Data were normalized to S26 or TBP mRNA levels.

Chromatin immunoprecipitation assay (ChIP):

Cells were first cross-linked for 8 minutes by adding formaldehyde directly to tissue culture medium to a final concentration of 1% and then 10 minutes of neutralization using Glycine at 125mM. Cross-linked cells were then washed with cold PBS. Cells were resuspended in cell lysis buffer (5mM PIPES, 85mM KCL, 0.5% NP40; 20mM N-ethyl maleimide, protease

inhibitors) and incubated at 4°C during 10 minutes. Then nuclei were lysed in nuclei lysis buffer (50mM Tris pH 7.5, 1% SDS, 10mM EDTA, 20mM N-ethyl maleimide, protease inhibitors) during 2-3hours at 4°C. the lysates were then sonicated for 30 cyccles of 30s each at 4°C using Bioruptor Pico (Diagenode) in standard conditions. After sonication, samples were centrifuged and the supernatants diluted 10 fold in IP buffer (1.1% Triton, 50mM Tris pH 7.5, NaCL 167mM, 5mM N-ethyl maleimide, 1mM EDTA, 0.01% SDS, Protease inhibitors) with 2mg of antibodies in presence of magnetic Dynabeads Protein G. Immunoprecipitation is performed overnight at 4°C. After extensive wahing in Low salt buffer, High salt buffer, LiCl salt and TE buffer and eluted in 200µl of elution buffer (100mM $NaHCO_3$, 1% SDS). Cross-linked chromatins were reversed by overnight incubation at 65°C after adding 12µl of 5M NaCl. Samples are purified using Nucleospin Gel and PCR cleanup (Macherey-Nagel). The immunoprecipitated DNA and inputs taken from samples before immunoprecipitation were analysed using the Roche LightCycler 480 with specific primers

Tumor xenografts:

Xenografts tumors were generated by injecting $2*10^6$ U937 cells (in 100µl of PBS) subcutaneously on both flanks of NOD-Scid-IL2Rg^{null} mice (adult males and females, 25g, Charles River Laboratories). When tumors reached $100mm^3$, mice received intra-peritoneal injections of ATRA (2.5mg/kg/day) and peritumoral injections of 2D08 (10mg/kg/day) or both or vehicle (DMSO) every 2 days. Tumor dimensions were measured with a caliper and volumes calculated using the formula: $v = \frac{\pi}{6 \times A \times B^2}$, where A is the larger diameter and B is the smaller diameter.

Statistical analysis:

Statistical analyses were performed by Student's t test with Prism 5 software. For patient samples the Wilcoxon matched-pairs signed rank test was used. For xenografts experiments in animals, we used the Mann-Whitney test. Differences were considered as significant for p values of <0.05. *p<0.05; **p<0.01; ***p<0.001. ****p<0.0001

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III Discussions

The chemotherapy based on anthracyclin (daunorubicin or idarubicin) and nucleoside analogue (cytarabine) is the main therapy of AML but the relapse rate is high and no major improvement of this treatment was reached over the past 40 years. All-*trans* retinoic acid (ATRA) has been introduced as a successful agent in the reactivation of retinoic acid signaling to induce differentiation of leukemic blasts in a minor subgroup of AML, promyelocytic leukemia (APL). Thus, there has been great interest in the release of the differentiation block in non-APL AML as well as in many other cancers. Many clinical trials have thus been performed. However ATRA as single agent is not efficient to restore the retinoic acid signaling. However, various recent works suggest that epigenetic drugs could be used in combination with ATRA to activate differentiation of non-APL AMLs. Indeed because the proteins involved in retinoic acid signaling are not disrupted in non-APL AML, we assume that there are other mechanisms, which impair this signaling. In this context, we investigated the role of sumoylation, which is known to negatively regulate gene expression, in ATRA-induced myeloid differentiation and the relevance of its targeting to re-activate non-APL AML differentiation.

In the first step, using 2D08, an inhibitor of SUMO E2 conjugating enzyme (Kim et al. 2014) , I showed that sumoylation represses ATRA-induced differentiation in many AML cell lines U937, THP1, HL-60 and MOLM14. Interestingly, 2D08 alone was also enough to differentiate cells. I could confirm this result with another SUMO E1 activating enzyme, Anacardic acid (Fukuda et al. 2009) on U937 and MOLM-14. To validate that the sensitization to ATRA is really due to the desumoylation, I generated SENP1 (THP-1), SENP2 and SENP5 (U937) overexpressing cells. I could observe an increased expression of differentiating markers upon ATRA treatment of these cells. This suggested that sumoylation plays a role in the repression of the differentiation program of AML cells and inhibition of this modification could prime AML cells to differentiate.

Importantly, I also demonstrated that the combination between ATRA and sumoylation inhibitors enhance the differentiation of primary patient samples taken at diagnosis as well as on patient who were even not responsive to induction chemotherapies or relapsed patient samples.

Unlike progenitors, differentiated cells stop to proliferate. In this context, we could demonstrate that ATRA+2D08 rapidly stops the proliferation and induced cell death of U937, including chemoresistant U937 that I generated. Accordingly, SENP2 and SENP5 overexpressing U937 decreased their ability to proliferate upon ATRA+2D08 treatment compare to normal U937. The same result was also confirmed on primary AML cells. This underlines that inhibition of sumoylation synergizes with ATRA to induce AML differentiation and to stop their proliferation.

The next step was to prove the anti-leukemic effect *in vivo*. Thus, NOD-Scid-IL2Rg^{null} mice were xenografted subcutaneously with U937 cell line and treated with ATRA or ATRA+2D08. This experiment revealed a slight decreased tumor growth upon ATRA or 2D08 treatments and significant decrease in tumor growth when combining both of them.

Mechanistically, we demonstrated that inhibition of sumoylation primes AML cells for differentiation by facilitating the expression of master genes of the myeloid differentiation RARA, ITGAX and IL1B but also TNFSF10, which encodes for the pro-apoptotic TRAIL cytokines known to be involved in ATRA-induced cell death. In the same way, overexpression of SENP1 desumoylase strongly activated the expression of these genes in THP-1 cells. Interestingly, 2D08 or overexpression of SENP1 were sufficient to induce the expression of these genes. Finally, I could show that ATRA+2D08 led to increased levels of the H3K4Me$_3$ histone activation mark on the promoter region these genes. Altogether, this suggests that sumoylation represses AML differentiation through modification of chromatin-bound proteins and silencing of ATRA-responsive genes.

Altogether, these results suggest that targeting the SUMO pathway could constitute a promising approach to sensitize AML to differentiation therapies.

How does sumoylation regulate ATRA-induced differentiation in AML?

One open question in this project is to understand the molecular mechanisms explaining how desumoylated transcription factors and co-regulators activate ATRA-induced gene expression. The inhibitor of histone demethylase LSD1 in combination with ATRA in AML treatment was shown to differentiate non-APL AML and has now reached now the Phase I/II clinical trials (NCT02717884). LSD1/CoREST1/HDAC co-repressor complex actively repress gene transcription by changing methylation and acetylation. Interestingly, its recruitment on the chromatin is enhanced by CoREST1, which recognizes SUMO-2 chains via its SIM (Ouyang et al. 2009). In this context, one hypothesis could be that inhibition of sumoylation might suppress the recruitment of the LSD1/CoREST1/HDAC co-repressor complex on the promoters of the genes involved in ATRA-induced differentiation (Figure 22). Inhibitor of sumoylation could thus potentiate the pro-differentiating effects of LSD1 inhibitors in non-APL AML treatment. This could also allow the use of lower doses of each molecule and limit the toxicity as well as the side effect of each compound. To test this hypothesis, I performed preliminary CHIP-qPCR experiment with LSD1 antibody on the locus of RARα, ITGAM, ITGAX and Il1B. However I could not observe any significant decrease in the binding of LSD1 recruitment on these promoters upon 2D08 treatment. The desumoylation could affect the recruitment of other chromatin regulators that are recruited to

promoters by SUMO-depending mechanisms. This includes HDAC2, the histone methyl transferases SETDB1 and SUV4-20h, the ATP-dependent remodeler Mi2, and chromatin-associated proteins HP1 and L3MBTL1 and -2 (Ivanov et al. 2007 ; Stielow et al. 2008a ; 2008b ; Yang and Sharrocks, 2004).

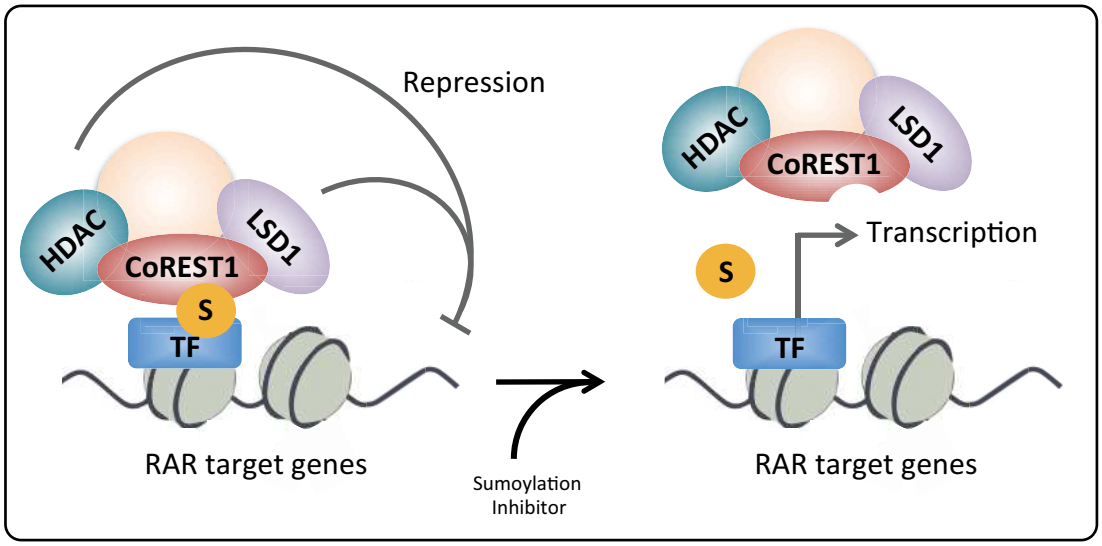

Figure 22: SUMO-interacting motif (SIM) in CoREST1 is required for transcriptional repression of RAR target genes. CoREST1 through its SIM recruits co-suppressor complex and repress RAR-target genes. When inhibitor of sumoylation is present, CoREST1 is released and RAR-target genes and this initiate gene transcription.

My results suggest that global desumoylation primes AML cells for ATRA-induced differentiation. More precisely, inhibition of sumoylation facilitates the expression of genes involved in this process. However, inhibition of sumoylation itself is not sufficient to significantly increase their expression. Rather, inhibition of sumoylation likely creates an environment on the chromatin that favors transcription but this requires other specific activating signals to be brought by ATRA. It is thus expected that inhibition of sumoylation will not activate genes randomly but will favor the expression of ATRA-responsive genes. In line with this idea our team could show that inhibition of sumoylation favors daunorubicin-induced gene expression, in particular of genes involved in apoptosis and inflammatory response in AML (Boulanger et al, manuscript in preparation). This suggests that inhibiting sumoylation should not lead to major deregulations in global gene expression but rather create permissive condition favoring the effects of drugs/compounds on their specific target genes.

Although I focused my work on the role of SUMO on ATRA-induced gene expression, it is possible that sumoylation affects other ATRA-regulated processes that are not taking place on the chromatin. For example, cellular retinoic acid-binding protein 2 (CRABP-2) is an important regulator of RAR activation. CRABPs are known to be sumoylated and this modification is crucial for ATRA-induced dissociation of CRABPs from the endoplasmic reticulum membrane (Majumdar

et al. 2011). Modulation of its sumoylation in combination of ATRA could therefore participate in the activation of differentiation.

Linking ATRA treatment and the regulation of global sumoylation

I observed in different AML cell lines that ATRA treatment decreases global sumoylation (**Figure 23**). This effect of ATRA was also reported in a previous publication (Zhou et al. 2014). My results suggest that this desumoylation is involved in the induction of differentiation through the activation of gene expression and enhancing this desumoylation accelerates differentiation. How ATRA regulates sumoylation remains an open question.. Interestingly, ATRA activates several kinase cascades such as p38 mitogen-activated protein kinase (p38MAPK) in epithelial cells and fibroblasts as well as p42/p44 extra-cellular signal resulted kinases (ERKs) and classical MAPKs in neuronal cells (di Masi et al. 2015). Protein kinases constitute important mechanisms, which transmit signals to downstream cytosolic or nuclear machineries. In particular, ATRA-activated MAPKs translocate to the nucleus where they phosphorylate several targets including mitogen-and stress-activated protein kinase (MSK1) (Piskunov and Rochette-Egly, 2011). MAPKs and MSK1 can thus phosphorylate several nuclear proteins involved in the transcription of the ATRA target genes such as histones, RARs, and their co-regulators. ATRA treatment can also decrease significantly the activity of serine/threonine phosphatases 2A, B and C (Sanli et al. 2003). Contradictorily, other studies indicated that ATRA inhibits cell proliferation by reducing phosphorylation of ERK1/2 in human scleral fibroblasts (HSFs) (Huo et al. 2013). sumoylation can be affected by phosphorylation of the target proteins. In particular, phosphorylation increases the sumoylation of protein carrying a phopho-dependent SUMO sites (PDSMs). However, phosphorylation of certain SUMO-targets decreased its sumoylation such as PML (Müller et al. 1998) or c-Fos (Bossis et al. 2005). Regulation of specific kinases by ATRA could thus affect the sumoylation level of specific substrates. However, since we see a global effect of ATRA on sumoylation, it could also be that changes in signaling pathways activation regulate the activity of enzymes of the SUMO pathway. Phosphorylation of Ubc9 by CDKA/CyclinB at Ser71 (Su et al. 2012) or by Akt at Thr 35 (Lin, Liu, and Lee 2016) was shown to increase its activity. Another way to globally regulate sumoylation is through the production of reactive oxygen species (ROS), which induce the formation of a disulfide bond between the catalytic cysteines of the SUMO E1 and E2 enzymes (Bossis and Melchior 2006). Interestingly, ATRA was shown to induce the production of ROS in neuroblastoma cells, which participate in its differentiating effect (Silvis et al. 2015). In addition, it was shown that NADPH oxidases are involved in ATRA-induced differentiation of neuroblastoma cells (Nitti et al. 2010). In AML, our team has shown that NAPDH-oxidase derived ROS are inhibiting the SUMO pathway, through the inactivation of the SUMO E1 and E2, in the

context of their response to chemotherapeutic drugs, in particular daunorubicin treatment (Bossis et al. 2014). In addition, I could show that differentiated AML cell lines show a massive increase in NADPH oxidase activity. One future direction will therefor consist in determining if the inhibition of sumoylation by ATRA is linked to the production of ROS via the activation of NADPH oxidase and how this affects the differentiation of AML. Interestingly, I could show that inhibition of sumoylation enhances NADPH oxidase activity. This could be part of an amplification loop, which induce more ROS production and thus less sumoylation, which would result in an increased activation of the expression of genes involved in the differentiation process.

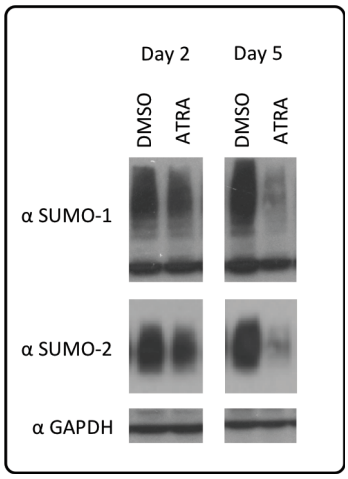

Figure 23: ATRA treatment decreases global sumoylation in AML cells. THP1 cells were treated with ATRA (1µM) for 2 or 5 days. Immunoblotting were performed using SUMO-1, SUMO-2 or GAPDH antibodies.

Combining ATRA with inhibitors of sumoylation for efficient AML treatment

My results on both patient samples and mouse models suggest that combining ATRA with inhibitors of the SUMO pathway could constitute a new therapeutic approach in the treatment of AML. Interestingly, this combination is also efficient on chemoresistant AML cells, suggesting that this treatment could help overcome chemoresistance, which is the main issue in AML treatment. This approach could also be useful for ATRA-resistant patients or even APL patients to diminish side effect of ATRA/ATO treatment and overcome differentiation syndrome described in the introduction. In addition, I performed preliminary experiments with Vitamin D3 (VD) and could show that inhibitor of sumoylation in combination with VD increased differentiation compared to VD alone in AML cell lines. This combination treatment could allow the use of lower doses of VD and thus prevent its strong cardiotoxicity, which prevents its clinical use.

As for every therapeutic approach, a fine balance between efficacy and toxicity has to be found and, considering the high number of sumoylated proteins and their roles in almost all cellular processes, inhibition of sumoylation could have severe side effects. However, although sumoylation is an essential process, hemizygote deletion of Ubc9 in mice has proven that deletion of 50% of Ubc9 and decreased sumoylation doesn't impact viability (Nacerddine et al. 2005). In addition, global and transient desumoylation is observed in conditions of stress such as UV, Ionizing Radiations and Oxidative stress (Denis Tempé, Piechaczyk, and Bossis 2008), further suggesting that transient inhibition of the SUMO pathway could be tolerated by normal cells. This is confirmed by the fact that we didn't detect over toxicity of both Anacardic acid (Bossis et al., 2014)

and 2D08 (my work) when used in mice. Various alteration of sumoylation and desumoylation enzyme have been described and it has been suggested that cancer cells could have an accelerated SUMO cycle (Seeler and Dejean 2017). Therefore, a slight decrease in SUMO enzymes activity could be enough to disturb the SUMO cycle in cancer cells and not in other cells. This is quite similar to the effect of proteasome inhibitor, which was expected to have massive side effects. Nevertheless, bortezomib, an inhibitor of proteasome, has been approved by FDA as an anti-cancer drug in the treatment of multiple myeloma and Mantle cell lymphoma. Since multiple myeloma cells require high protein turnover, a modest inhibition of the proteasome is sufficient to induce their death without have overt toxicity.

Our *ex vivo* study on primary AML patients samples suggests that most patient cells differentiate better upon combination treatment. However not all samples had the same behavior and some patients did not respond to the treatment. Because AML is a very heterogeneous disease with various genetic alterations, this suggests that the combination of ATRA and inhibitors of sumoylation could be more useful in specific AML subtypes. However, I could not identify a specific subgroup of patients that respond better to this treatment and more patients should thus be analyzed. Moreover, the sequencing for all common mutations is not available for all patients, which limits the possibility to stratify them.

Although my work could demonstrate the potential of the inhibition of sumoylation in the treatment of AML, its clinical application will require the development of new inhibitors of the SUMO pathway. Few inhibitors of sumoylation are known. One of the first described inhibitor of sumoylation was Anacardic acid, which binds covalently to the SUMO E1 and inhibit its activity (Fukuda et al. 2009). However, Anacardic acid is known to have other targets, in particular histone acetyltransferases. 2D08 is a newer inhibitor that was selected to inhibit Ubc9 (Kim et al. 2014). It is less potent than Anacardic acid and its potential other targets have not been investigated so far. A main limitation for these inhibitors is that they are poorly soluble. This strongly limits their bioavailability *in vivo*. This is why we opted for peritumoral treatment on xenograft model instead of intravenous treatment after intravenous injection of the mice with the AML cell lines. The team has thus started to develop new inhibitors of the SUMO pathway that would be more potent and soluble than those existing so far and be used in clinical trials.

IV Other Projects

During the course of my PhD, I participated in other projects with the same objective of improving AML treatment. As mentioned in my introduction, standard AML treatment is based on a chemotherapy comprising genotoxics and is related with high relapse rate. When I arrived in the team, I participated in a study aiming at understanding the role of sumoylation and its regulation by ROS in AML chemosensitivity/chemoresistance. In addition, I participated in a study aiming at determining the role of ROS in AML chemoresistance.

1 The ROS/SUMO axis Contributes to the Response of Acute Myeloid Leukemia Cells to Chemotherapeutic Drugs (Manuscript 2)

Bossis G, Sarry JE, Kifagi C, Ristic M, Salland E, Vergez F, Salem T, Boutzen H, Baik H, Brockly F, Pelegrin M, Récher C, Manenti S and Piechaczyk M.

Cell Reports, 2014. 7(6) 1815-23

No major improvement in the treatment of AMLs has been done for more than 40 years and the prognosis of this disease remains particularly poor. Current chemotherapeutic drugs used in clinic to treat AMLs such as anthracycline and cytarabine are thought to induce cancer cell death mostly through the generation of DNA double-strand breaks. However we could show that one of their early effects is the loss of conjugation of the ubiquitin-like protein SUMO from its targets via reactive oxygen species (ROS)-dependent inhibition of the SUMO-conjugating enzymes. Desumoylation regulates the expression of specific genes, such as the proapoptotic gene DDIT3, and helps induce apoptosis in chemosensitive AMLs. In contrast, chemotherapeutics do not activate the ROS/SUMO axis in chemoresistant cells. However, pro-oxidants or inhibition of the SUMO pathway by Anacardic acid restores DDIT3 expression and apoptosis in chemoresistant cell lines and patient samples, including leukemic stem cells. Finally, inhibition of the SUMO pathway decreases tumor growth in mice xenografted with AML cells.

In this work, my contribution mostly consisted in analyzing the effects of the treatments with chemotherapeutic drugs (Ara-C, DNR and VP16) on the sumoylation levels in both chemosensitive and chemoresistant AML cell lines by immunoblotting techniques. I could show that desumoylation was induced by drugs in chemosensitive cell lines and no significant difference was observed in chemoresistant KG1a cell line. I also compared the sensitivity to Anacardic acid, an inhibitor of sumoylation, of both normal and AML cells. For that I measured IC50 to Anacardic acid on PBMC compared to chemosensitive HL60 and chemoresistant TF1 and could show that PBMC are much less sensitive to Anacardic acid than AML cell lines. These results contributed to conclude that

targeting the ROS/SUMO axis might constitute a therapeutic strategy for AML patients resistant to conventional chemotherapies.

Manuscript 2

The ROS/SUMO Axis Contributes to the Response of Acute Myeloid Leukemia Cells to Chemotherapeutic Drugs

Cell Reports
Report

The ROS/SUMO Axis Contributes to the Response of Acute Myeloid Leukemia Cells to Chemotherapeutic Drugs

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SUMMARY

Chemotherapeutic drugs used in the treatment of acute myeloid leukemias (AMLs) are thought to induce cancer cell death through the generation of DNA double-strand breaks. Here, we report that one of their early effects is the loss of conjugation of the ubiquitin-like protein SUMO from its targets via reactive oxygen species (ROS)-dependent inhibition of the SUMO-conjugating enzymes. Desumoylation regulates the expression of specific genes, such as the proapoptotic gene *DDIT3*, and helps induce apoptosis in chemosensitive AMLs. In contrast, chemotherapeutics do not activate the ROS/SUMO axis in chemoresistant cells. However, pro-oxidants or inhibition of the SUMO pathway by anacardic acid restores *DDIT3* expression and apoptosis in chemoresistant cell lines and patient samples, including leukemic stem cells. Finally, inhibition of the SUMO pathway decreases tumor growth in mice xenografted with AML cells. Thus, targeting the ROS/SUMO axis might constitute a therapeutic strategy for AML patients resistant to conventional chemotherapies.

INTRODUCTION

Acute myeloid leukemias (AMLs) are severe hematological malignancies induced by the oncogenic transformation of hematopoietic stem and myeloid progenitor cells. It leads to bone marrow failure and related complications, including infections, anemia, or bleeding. Despite recent progress in the molecular characterization and prognosis refinement of this disease (Cancer Genome Atlas Research Network, 2013), treatments have not significantly changed during the past 30 years. The standard induction chemotherapy relies on a combination of the nucleoside analog cytarabine (Ara-C) with an anthracyclin, such as daunorubicin (DNR) or idarubicin, sometimes in association with other drugs, such as etoposide (VP16). Although most patients reach the complete remission after initial chemotherapeutic treatment, relapses are frequent, and the global prognosis remains poor with an overall survival of 40% in young patients and much less in old ones (Estey, 2012). Relapses are largely due to the persistence of leukemic stem cells (LSCs), which are refractory to chemotherapeutic drug-induced cell death (Vergez et al., 2011).

Generally, the mechanisms of action of the chemotherapeutic drugs used for AMLs treatment rely on the inhibition of DNA synthesis and the induction of DNA double-strand breaks in highly replicating cancer cells, which in fine lead to their apoptosis. However, these drugs can induce cell death by other mechanisms. In particular, reactive oxygen species (ROS) have been known as critical mediators of genotoxics-induced cell death for long (Matés et al., 2012). They are also responsible for certain side effects of chemotherapeutic drugs, such as anthracyclin cardiotoxicity (Gewirtz, 1999; Hole et al., 2011). However, their cellular effectors have not been clearly identified (Matés et al., 2012).

SUMO is a family of three related ubiquitin-like peptidic post-translational modifiers, SUMO-1, -2, or -3, the latter two being almost identical (referred to as SUMO-2/3). SUMO is conjugated to ε-amino groups of lysines of numerous target proteins by a heterodimeric SUMO-activating E1 enzyme (AOS1/UBA2), a SUMO-conjugating E2 enzyme UBC9 (encoded by *UBE2I*) and various E3 factors facilitating its transfer from the E2 onto substrates. Most sumoylated proteins go through constant cycles of conjugation/deconjugation due to various desumoylases. Sumoylation changes substrate protein properties, in particular by favoring the recruitment of SUMO-binding partners (Flotho and Melchior, 2013). Sumoylation is sensitive to various stresses that regulate the activity of the SUMO pathway's enzymes. In particular, ROS can inactivate SUMO conjugation by inducing

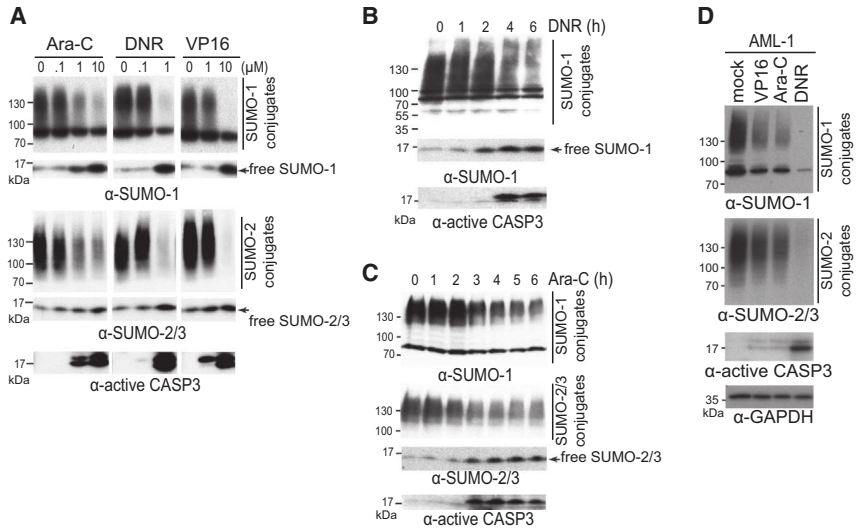

Figure 1. Chemotherapeutic Drugs Induce Desumoylation in AML Cells

(A) HL60 cells were treated with Ara-C, VP16, or DNR for 7 hr and immunoblotted for SUMO-1, SUMO-2, and active caspase-3 (B).

(B and C) HL60 cells were treated with 1 μM DNR (B) or 2 μM Ara-C (C) for the indicated times and immunoblotted for SUMO-1, SUMO-2, or active CASPASE-3.

(D) Primary AML cells were treated in vitro with VP16 (10 μM), Ara-C (2 μM), or DNR (1 μM) for 24 hr and immunoblotted for SUMO-1, SUMO-2, active-caspase-3, and GAPDH.

the formation of a reversible disulfide bridge between UBA2 and UBC9 catalytic cysteines (Bossis and Melchior, 2006). This disrupts the sumoylation/desumoylation cycle, resulting in protein desumoylation. Such global shifts in the cell sumoylome are thought to play critical roles in the cellular response to these stresses (Tempé et al., 2008). Although sumoylation controls many cellular functions, one well-characterized role is the regulation of transcription via the modification of histones, transcription factors and cofactors, chromatin-modifying enzymes, and basal transcription machinery (Raman et al., 2013). Finally, deregulation of the SUMO pathway has been found in various cancers (Bettermann et al., 2012) and is generally associated with an adverse outcome (Driscoll et al., 2010). Moreover, recent evidence suggests that targeting sumoylation could be beneficial for cancer treatment. In particular, inhibition of sumoylation preferentially induces death of Myc-overexpressing cancer cells (Kessler et al., 2012).

Here, we address the role of the SUMO pathway in AMLs apoptotic response to chemotherapeutic drugs. We show that the genotoxics currently used in the clinic induce rapid ROS-dependent protein desumoylation, which participates both in transcriptome alteration and apoptosis of chemosensitive AML cells. Failure to activate this ROS/SUMO axis is associated with AMLs chemoresistance. However, its induction by different means is sufficient to induce death of chemoresistant AML cell lines, as well as that of AML patient cells, including their leukemic stem cells. Furthermore, inhibition of the SUMO pathway reduces AML cell growth in xenografted mice. Overall, our work identifies the ROS/SUMO axis as a novel player in chemotherapeutic drugs-induced apoptosis and a potential target to overcome chemoresistance in AMLs.

RESULTS

Chemotherapeutic Drugs Induce Massive Desumoylation in Chemosensitive AMLs

A chemosensitive AML model cell line, HL60 (Quillet-Mary et al., 1996), was treated with Ara-C, DNR, and VP16 at doses consistent with plasma concentrations in treated AML patients (Gewirtz, 1999; Krogh-Madsen et al., 2010). This induced a dose-dependent decrease in SUMO-1 and SUMO-2/3 (Figure 1A) conjugate levels and the appearance of free SUMO, which did not result from increased *SUMO-1* or *-2* gene transcription (Figure S1). This suggested that these chemotherapeutic drugs induced SUMO deconjugation from its target proteins. Desumoylation rapidly began after drug addition, as indicated by the increase in the free SUMO pool already after 1 hr of treatment. Desumoylation onset preceded mitochondrial membrane potential loss (Figure S2), caspase-3 activation, and a more global disappearance of SUMO conjugates visible after 3–4 hr (Figures 1B and 1C). Importantly, primary chemosensitive AML cells (Figure 1D), as well as two other chemosensitive AML cell lines (U937 and THP1) (Figure S3), also showed massive drug-induced decrease in SUMO conjugates correlating with caspase-3 activation. These data indicated that one of the early effects of chemotherapeutic drugs currently used to treat AMLs is the induction of protein desumoylation.

Chemotherapeutic Drug-Induced Desumoylation Regulates Gene Expression and Apoptosis

Considering the acknowledged role of sumoylation in the control of gene expression, we asked whether desumoylation could alter specific transcriptional program. To this aim, we profiled and compared the transcriptome of HL60 cells treated with anacardic acid, a natural inhibitor of the SUMO E1 enzyme (Fukuda et al., 2009), with that of mock (DMSO) -treated cells. We found 318 significant differentially expressed (SDE) genes (fold change over 2-fold), 200 being upregulated (71 more than 3-fold), and 118 downregulated (ten more than 3-fold) (Table S1). Gene ontology analyses revealed that upregulated genes are involved in cellular processes such as the response to endoplasmic reticulum (ER) stress, transcription control, nucleosome assembly, cell-cycle arrest, and apoptosis (Figure 2A). No specific process was significantly enriched ($p < 0.01$) for the downregulated genes (data not shown). We confirmed the transcriptional activation of six of the most induced genes by RT-qPCR and showed that the expression of these genes was also strongly activated by Ara-C (Figure 2B), suggesting that chemotherapeutic drug-induced desumoylation is involved in their induction. We further studied the DNA Damage-Induced Transcript 3 (*DDIT3*) gene, as it encodes

the CHOP10/GADD153 protein, an activator of apoptosis involved in the ER stress response. CHOP10 has also been implicated in the apoptotic response of AML cells to chemotherapeutic drugs (Eymin et al., 1997). While *DDIT3* mRNA levels increased upon DNR and Ara-C treatment of HL60 cells, SUMO conjugates rapidly decreased in the gene proximal promoter region (Figures 2C and 2D). Sumoylation of promoter-bound proteins is principally associated with transcriptional repression (Cubeñas-Potts and Matunis, 2013) or limitation of transcriptional activity (Rosonina et al., 2010), including in the case of the *DDIT3* gene (Tempé et al., 2014). Consistent with this idea, counteracting protein desumoylation by overexpressing SUMO-2 significantly reduced *DDIT3* induction by Ara-C (Figure 2E). Moreover, overexpression of SUMO-2 delayed Ara-C-induced apoptosis (Figure 2F). Thus, in chemosensitive AML cells, drug-induced desumoylation stimulates genes, such as *DDIT3*, and facilitates the induction of apoptosis.

ROS Are Involved in Chemotherapeutic Drug-Induced Protein Desumoylation in AMLs

Chemotherapeutic drugs induce the production of ROS (Gewirtz, 1999). As shown in Figure 3A, Ara-C, DNR, and VP-16 led to the formation of the ROS-induced disulfide crosslink between UBA2 and UBC9 catalytic cysteines (Bossis and Melchior, 2006). Importantly, this correlated with a strong decrease in the level of the UBC9∼SUMO thioester adduct, the active form of UBC9. Using a mouse retroviral model of AML (Michaud et al., 2010; Moreau-Gachelin, 2006), we showed that the treatment of leukemic animals with Ara-C and, to a lesser extend with DNR, also induced UBC9-UBA2 crosslink in vivo in tumor cells (Figure 3B). Inhibition of NADPH oxidases (NOX), a major source of ROS in cancer cells (Block and Gorin, 2012), by diphenyleneiodonium (DPI) prevented both DNR- and VP16-induced loss of SUMO conjugates, UBC9-UBA2 crosslinking and apoptosis (Figure 3C). Finally, treatments of AML patient cells also led to UBC9-UBA2 crosslinking, the levels of which correlated with cell sensitivity to the different drugs in vitro (Figures 3D and 3E). These data suggest that chemotherapeutic drug-induced protein desumoylation in AMLs is a consequence of ROS production.

The ROS/SUMO Axis Is Not Activated by Chemotherapeutic Drugs in Chemoresistant AMLs

We next asked whether chemoresistance could be associated with impaired activation of the ROS/SUMO axis. In contrast to the chemosensitive U937 and HL60 cells, which exhibit a strong desumoylation upon DNR, Ara-C, and VP16 treatment, the chemoresistant AML cell lines TF1 and KG1a (Quillet-Mary et al., 1996) were resistant to drug-induced desumoylation. This correlated with the absence of ROS-dependent crosslinking of UBA2 to UBC9 (Figures 4A and 4B). We therefore tested whether chemoresistant AML cells were intrinsically resistant to ROS-dependent protein desumoylation and whether forced activation of the ROS/SUMO axis could lead to their death. First, TF1 or KG1a cells were treated with increasing doses of glucose oxidase, which causes sustained production of ROS from the degradation of extracellular glucose. This led to UBC9-UBA2 crosslinking and protein desumoylation (Figures 4C and S4), which correlated with strong induction of *DDIT3* mRNA and massive cell death (Figures 4D and S4). Next, we derived TF1 clones expressing inducible control- or SUMO-1/2/3 miRNAs. SUMO-1/2/3 RNA interference was sufficient to induce massive death of these chemoresistant cells (Figure 4E). Thus, the ROS/SUMO axis is inactive in AML cells that are resistant to chemotherapy-induced apoptosis. However, its reactivation restores a cell death program in these cells.

Inhibition of the SUMO Pathway Targets Chemoresistant AML Cells In Vitro and In Vivo

Finally, we tested the effect of pharmacological inhibition of protein sumoylation on AML cells using anacardic acid. It decreased the amount of SUMO conjugates in chemoresistant TF1 cells (Figure 5A, left panel), activated caspase 3 (Figure 5A, right panel) and induced *DDIT3* mRNA (Figure 5B), whereas Ara-C had no effect. Next, we measured anacardic acid IC_{50} in chemosensitive (HL60, U937) and chemoresistant (TF1, KG1a) cells. All were sensitive to comparable concentrations of the drug (Figure 5C). Importantly, anacardic acid had significantly lower effect on peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T lymphocytes from healthy volunteers, as well as on proliferating mouse embryonic fibroblasts (MEFs) than on AML cells (Figure 4C). Similar to AML cell lines, patient samples showed variable sensitivity to Ara-C (IC_{50} ranging from 2 to >500 μM), but their IC_{50} for anacardic acid was relatively homogeneous with a median concentration of 42 μM (Figure 5D). For seven of the patient samples, we compared the IC_{50} of LSCs ($CD34^+$ $CD38^{low/-}CD123^+$) to the bulk of leukemic cells. Although globally less sensitive to Ara-C-induced cell death, LSCs showed similar sensitivity toward anacardic acid than the bulk of leukemic cells (Figure 5E). Interestingly, anacardic acid led to a strong activation of *DDIT3* mRNA in two primary patient samples, either chemosensitive (Figure 5F, left panel, IC_{50} = 10 μM for Ara-C) or chemoresistant (Figure 5F, right panel, IC_{50} = 250 μM for Ara-C), whereas Ara-C induced *DDIT3* expression only in the chemosensitive sample. Finally, nude mice xenografted with chemoresistant KG1a cells and peritumorally treated with anacardic acid showed a significant delay in tumor growth (Figures 5G–5I). Anacardic acid did however not alter general biological parameters in the treated mice, as assayed by weight control or blood cell counting (Figure S5). These data suggest that targeting sumoylation might overcome chemoresistance in AMLs.

DISCUSSION

Although targeted therapies have strongly improved the treatment of a subset of cancer patients, the classical chemotherapeutic drugs remain the standard therapy in most cancers. This is especially true for acute myeloid leukemia patients whose front-line treatment is generally a combination of an anthracyclin and the nucleoside analog Ara-C. Here, we show that a role of these drugs is the inhibition of the SUMO pathway. They induce a progressive loss of conjugation of SUMO to its targets, gene promoter-bound proteins being among the most rapidly affected. Recent studies reveal that SUMO can be considered as an integral component of chromatin and regulates specific

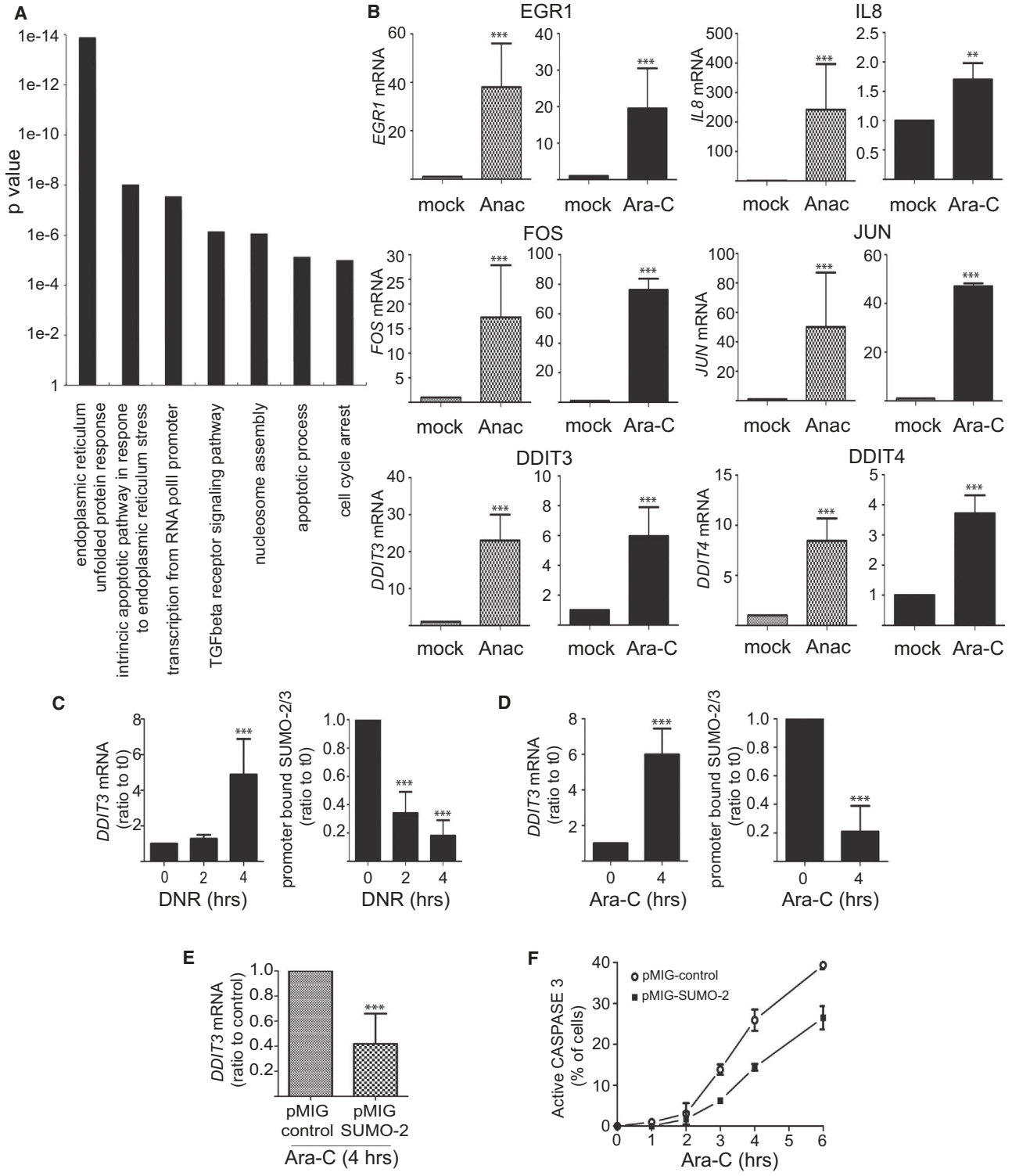

Figure 2. Desumoylation Regulates Specific Transcriptional Programs and Participates in the Induction of Apoptosis

(A) Top categories identified by gene ontologies of genes upregulated (more than 2-fold) in HL60 cells treated with anacardic acid (100 μM) for 5 hr compared to mock (DMSO) -treated cells.

(B) HL60 cells were treated with 100 μM anacardic acid (5 hr) or 2 μM Ara-C (3 hr) or control vehicle and mRNA for the indicated genes were monitored by RT-qPCR (n = 3).

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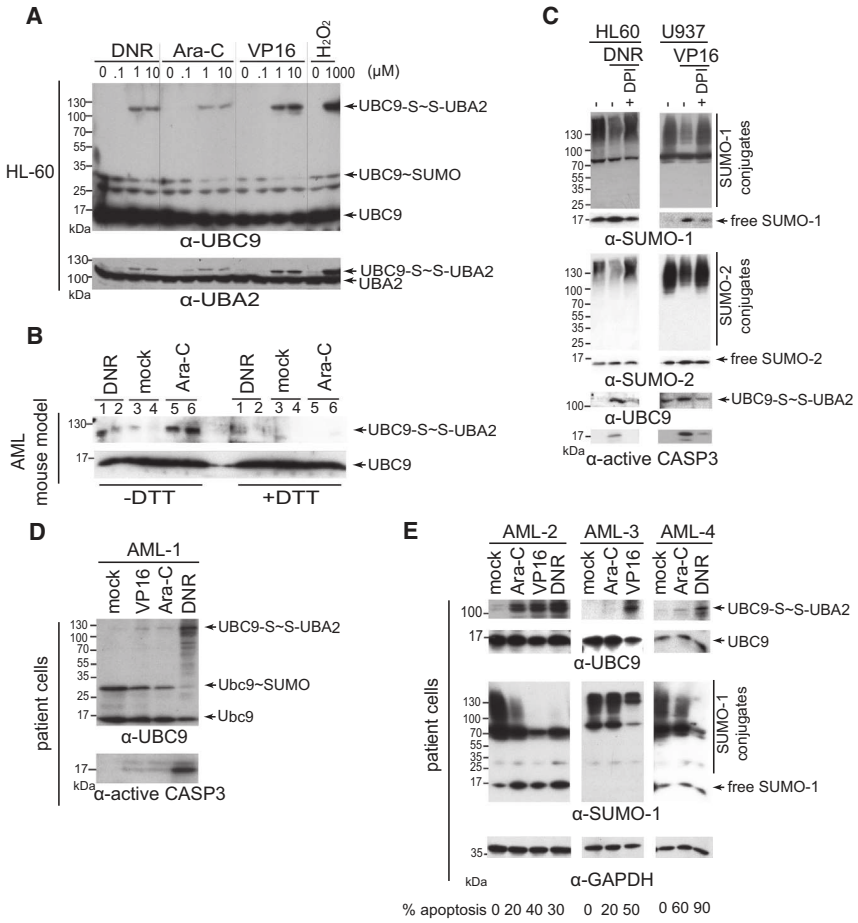

Figure 3. Chemotherapeutic Drug-Induced ROS Inhibit SUMO-E1 and E2 Enzymes

(A) HL60 cells were treated with Ara-C, VP16, or DNR for 7 hr or H_2O_2 (15 min), lysed in a nonreducing sample buffer and immunoblotted for UBC9 and UBA2.

(B) Leukemic FrCasE-infected mice were treated with DNR (10 mg/kg) or Ara-C (50 mg/kg) every 2 days for 2 weeks and sacrificed 4 hr after the last injection. Spleen extracts (in nonreducing or reducing conditions) were immunoblotted for UBC9.

(C) HL60 and U937 cells were treated with DNR (1 μM) or VP16 (10 μM) for 7 hr ± DPI (10 μM) and immunoblotted for SUMO-1, UBC9 (nonreducing gel), or active caspase-3.

(D) Primary leukemic cells (same patient as in Figure 1D) were treated in vitro with VP16 (10 μM), Ara-C (2 μM), or DNR (1 μM) for 24 hr and immunoblotted for UBC9 (nonreducing gel) and active-CASPASE-3.

(E) Same as (C) with three other patient samples immunoblotted for UBC9 (nonreducing gel), SUMO-1, and GAPDH. Viability was assessed and compared to mock-treated cells.

transcriptional programs (Neyret-Kahn et al., 2013). Consistent with this, our gene expression data suggest that desumoylation triggers the expression of genes associated with the ER stress, apoptosis induction, nucleosome remodeling, and cell-cycle arrest. Considering the various roles of sumoylation, in particular, in the control of genome integrity (Jackson and Durocher, 2013), we do not exclude that drug-induced hyposumoylation might also have other consequences, including impairment of genotoxics-induced DNA damage repair. However, our data suggest that one of its important roles is to regulate the expression of specific genes involved in AML cell response to chemotherapeutic drugs.

ROS can no longer be considered solely as toxic molecules causing random damages to biomolecules. They are also essential second messengers regulating numerous signaling pathways (Paulsen and Carroll, 2010). Consistent with this, we show here that they are responsible for drug-induced inhibition of the SUMO pathway in chemosensitive AML cell lines and patient samples. This is due to their ability to promote the formation of a disulfide-bond between the catalytic cysteines of the SUMO E1 and E2 enzymes (Bossis and Melchior, 2006). Although only a fraction of both E1 and E2 are crosslinked upon chemotherapeutic drug treatment, this inactivation involves the active fraction of these enzymes. Given the fact that desumoylases are not inhibited by these ROS concentrations (Feligioni and Nisticò, 2013), this explains the massive protein desumoylation we observed. Importantly, the inhibition of ROS production with an NADPH oxidase inhibitor strongly dampened drug-induced protein desumoylation and delayed entry into apoptosis. This confirms the role of ROS production in drug-induced death of chemosensitive AML cells. An important issue is whether chemotherapeutic drugs can also induce the ROS/SUMO axis in other types of cancer. Our data (data not shown) suggest that this might not always be the case because, even though we could detect the UBC9-UBA2 crosslink in an ALL (acute lymphocytic leukemia) cell line treated with DNR or Ara-C, we could not in epithelial cancer cell lines, such as MCF-7, HEK293, or HeLa. This might reflect differences in antioxidant or ROS production capacities between cancer types.

(C and D) HL60 cells were treated with 1 μM DNR (C) or 2 μM Ara-C (D) for the indicated times before analysis of *DDIT3* mRNA (left panels, n = 4 for DNR, n = 6 for Ara-C). SUMO-2/3 on the *DDIT3* promoter was assayed by ChIP (right panels) and normalized to DNA input. SUMO level in nontreated cells was set to 1 (n = 7 for DNR, n = 3 for Ara-C).

(E) HL60 cells infected with pMIG or pMIG-SUMO-2 lentiviral vectors were treated with Ara-C (2 μM) for 4 hr. *DDIT3* mRNA was RT-qPCR assayed (n = 7).

(F) The same cells as in (E) were treated with 2 μM Ara-C for the indicated times and flow cytometry-analyzed for active-CASPASE-3 (n = 3).

Results are expressed as means ± SD.

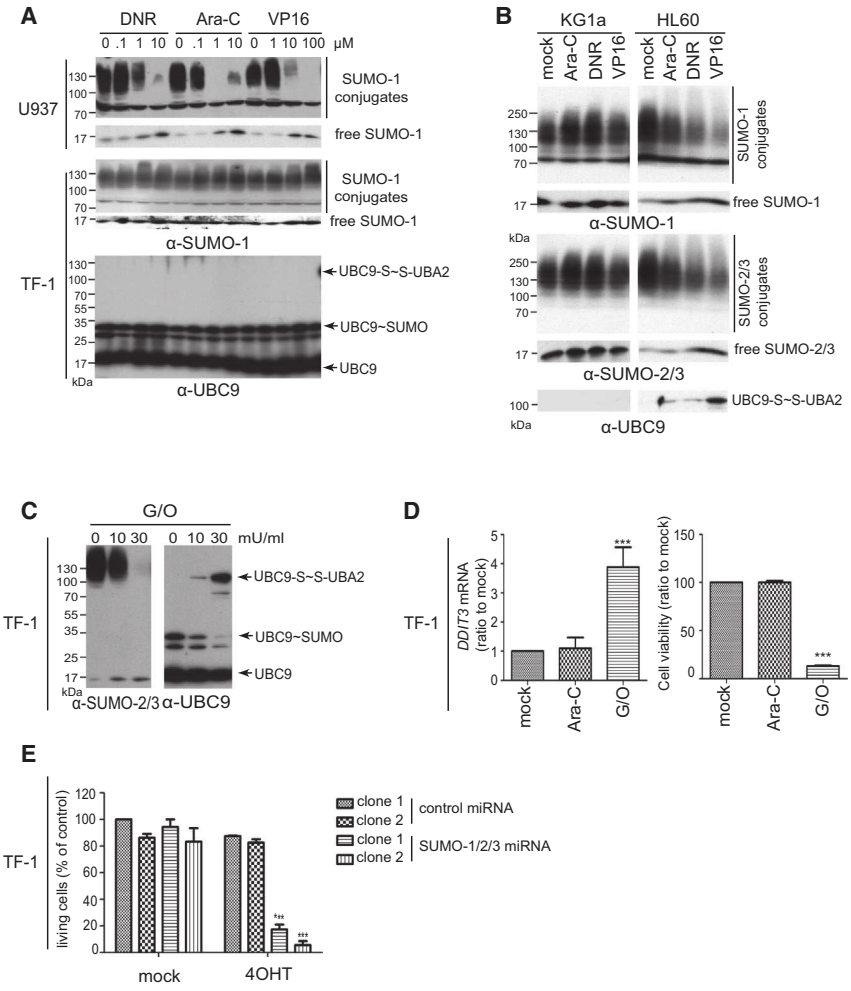

Figure 4. Reactivation of the ROS/SUMO Axis Restores Chemoresistant AML Cells Death

(A) U937 and TF1 cells were treated with Ara-C, VP16 or DNR for 7 hr and immunoblotted for SUMO-1 or UBC9 (nonreducing gel).

(B) HL60 and KG1a cells were treated with Ara-C (2 μM), DNR (1 μM), or VP16 (10 μM) for 7 hr and processed as in (A).

(C) TF1 cells were treated with glucose oxidase (G/O) for 6 hr and immunoblotted for SUMO-2 or UBC9 (nonreducing gel).

(D) TF1 cells were treated with Ara-C (2 μM) or glucose oxidase (10 mU/ml). *DDIT3* mRNA was analyzed after 6 hr of treatment using mock-treated cells as a reference (left panel, n = 3), and cell viability was assessed at 24 hr (right panel, n = 3).

(E) TF1 clones expressing a control or SUMO-1/2/3 miRNAs under the control of 4-OHT-inducible promoter were treated with 4OHT (20 nM) for 5 days and viability was assessed by MTS (n = 3). Results are expressed as means ± SD.

Chemotherapeutic drugs do not activate the ROS/SUMO axis in chemoresistant AML cells. The absence of ROS-induced UBC9-UBA2 disulfide-crosslinking upon treatment suggests that this might be due to lower ROS production and/or higher antioxidant capacity of chemoresistant AML cells. Along this line, LSCs, which are highly resistant to chemotherapeutic drugs and thought to be responsible for relapses, produce less ROS than the bulk of leukemic cells (Lagadinou et al., 2013). At least two lines of evidence suggest that increasing ROS concentration could be of therapeutic value for treating AMLs: (1) the inhibition of antioxidant systems induces primitive $CD34^+$ AML cell death (Pei et al., 2013) and (2) pro-oxidants induce the regression of acute promyelocytic leukemia (a subtype of AMLs characterized by a chromosome translocation fusing the *PML* and *RARA* genes) in mouse models (Jeanne et al., 2010). However, the clinical usefulness of pro-oxidant therapies might be limited by their toxicity (Hole et al., 2011; Matés et al., 2012). An alternative strategy to activate the ROS/SUMO axis in chemoresistant cells may therefore consist of targeting the SUMO pathway. In support of this idea, anacardic acid, a natural molecule of the Chinese pharmacopeia known to trigger apoptosis of various cancer cell lines in vitro (Tan et al., 2012) induced death of chemoresistant AML cell lines in vitro and in vivo as well as that of patient leukemic cells, including LSCs. Moreover, the absence of overt toxicity of anacardic acid on nontransformed cells and in living mice (except local sensitization when injected subcutaneously; data not shown) suggests that inhibiting the SUMO pathway may have less severe side effects than pro-oxidant therapies. Chemical engineering of anacardic acid to improve its solubility and bioavailability or developing novel SUMO pathway inhibitors might therefore offer an avenue to improve the outcome of AMLs patients by targeting leukemic cells, including LSCs resistant to conventional chemotherapies.

EXPERIMENTAL PROCEDURES

Pharmacological Inhibitors, Reagents, and Antibodies

Cytosine-β-D-arabinofuranoside (Ara-C), daunorubicin-hydrochloride (DNR), etoposide (VP-16), glucose-oxidase, and hydrogene-peroxide were from Sigma. Anacardic acid from Merck Millipore. SUMO-1 (21C7) and SUMO-2 (8A2) hybridomas were from the Developmental Studies Hybridoma Bank. Goat anti-SUMO-2 (used for chromatin immunoprecipitation [ChIP]) and anti-UBA2 were previously described (Bossis and Melchior, 2006). Anti-UBC9 (sc-10759) and GAPDH (sc-25778) were from Santa Cruz Biotechnologies; anti-cleaved CASPASE-3 (D175) were from Cell Signaling Technology. Antibodies and gating strategies used to phenotype patient samples were described previously (Vergez et al., 2011).

Cell Lines and Clinical Samples

U937, HL60, THP1, KG1a, and TF1 cells (DSMZ, Germany) were cultured in RPMI or Iscove modifier Dulbecco's medium (IMDM) (for KG1a) with 10% fetal bovine serum (FBS). TF1 were cultured with addition of 2 ng/ml GM-CSF (PeproTech). Mouse embryonic fibroblasts were a kind gift from M. Bialic and were cultured in DMEM with 10% FBS. For treatments, cells were seeded at 0.3×10^6 cells/ml the day before the experiment, and fresh medium was added together with the drugs. PBMC and $CD4^+$ lymphocytes were purified

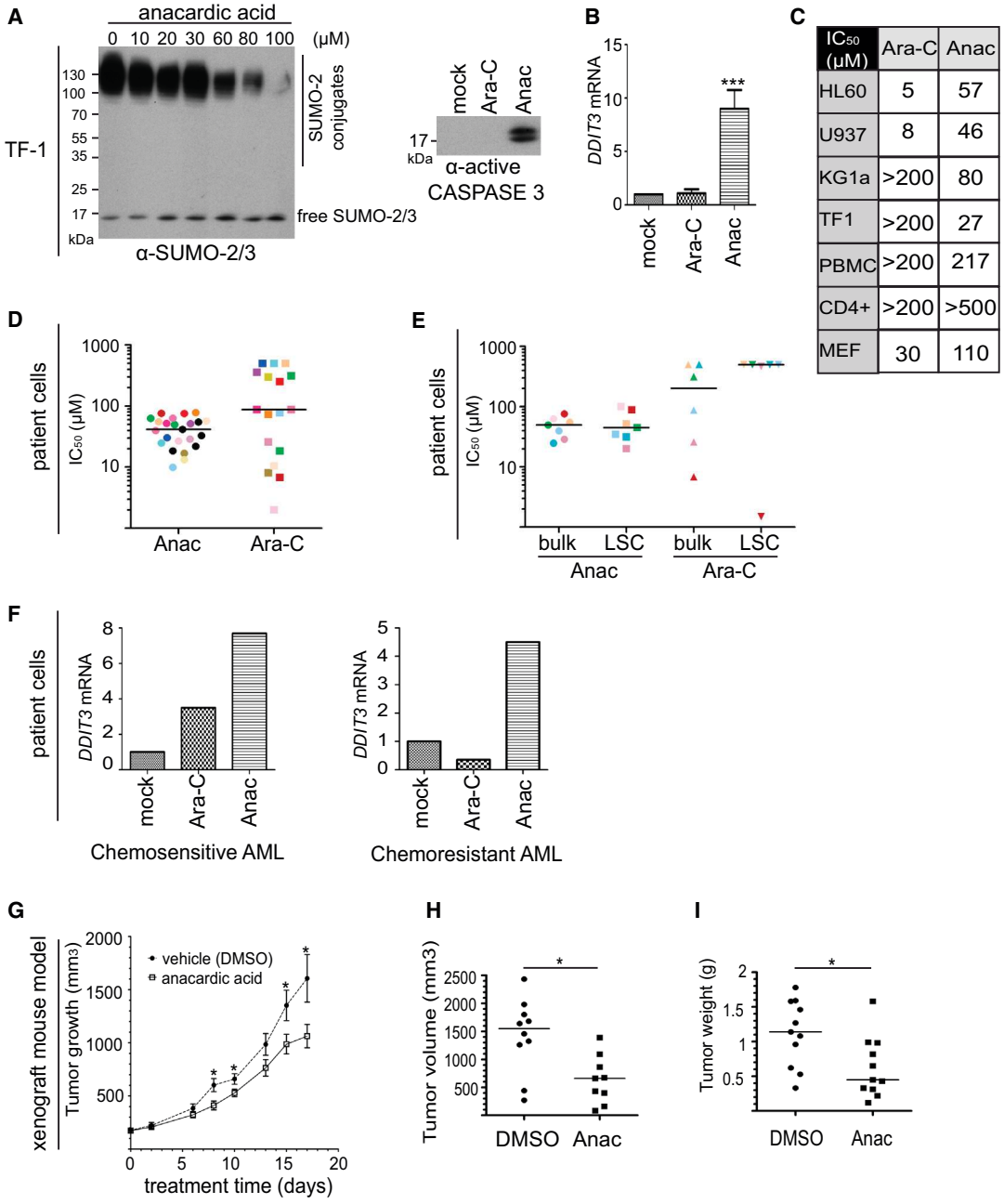

Figure 5. Inhibition of Sumoylation with Anacardic Acid Induces Chemoresistant Cells Death and Reduces Tumor Growth In Vivo

(A) TF1 cells were treated with anacardic acid for 8 hr and immunoblotted for SUMO-2 or active-CASPASE-3.

(B) TF1 cells were treated with Ara-C (2 μM) or anacardic acid (Anac, 100 μM) for 4 hr before *DDIT3* mRNA RT-qPCR assay (n = 3).

(C) HL60, U937, TF1, KG1a, PBMC, $CD4^+$ T lymphocytes, and MEF cells were treated with increasing doses of anacardic acid or Ara-C for 24 hr before viability assay using MTS (n = 3).

(D and E) Primary AML cells IC_{50} of anacardic acid (n = 23) and Ara-C (n = 17) was measured on the bulk of leukemic cells (CD45/SSC gating) at 24 hr (D). For some of the samples (n = 7), IC_{50} of the bulk of leukemic cells was compared to that of LSCs ($CD34^+CD38^{low/-}CD123^+$) (E). IC_{50} >500 μM could not be calculated precisely and were set to 500 μM. The same color is used for data coming from the same patient sample.

(F) AML cells were treated with 50 μM anacardic acid or 10 μM Ara-C for 24 hr before *DDIT3* mRNA RT-qPCR assay.

(G–I) Mice xenografted with KG1a were treated with anacardic acid or the vehicle (DMSO), and tumor growth was measured for 17 days (G). Mice were then sacrificed and tumor volume (H) as well as tumor weight (I) were measured. Results are expressed as means ± SD.

from peripheral blood. Bone marrow aspirates containing leukemic blasts from patients diagnosed with AMLs were obtained as previously described (Vergez et al., 2011) after informed consent and stored at the HIMIP collection (DC-2008-307-collection1). A transfer agreement was obtained (AC-2008-129) after approbation by the "Comité de Protection des Personnes Sud-Ouest et Outremer II" (Ethical Committee). For some experiments, fresh leukemic blasts recovered at diagnosis were immediately treated with the drugs or inhibitors. In most cases, frozen cells were thawed in IMDM with 20% FBS and immediately processed.

Lentiviral and Retroviral Infections

Retroviral constructs expressing SUMO-2 were constructed by inserting His-tagged human SUMO-2 cDNA into the pMIG retroviral vector. The 4-hydroxytamoxifen(4-OHT)-inducible control and SUMO-1/2/3 miRNA (miR-SUMO-1/2/3) lentivirus were a kind gift from Dr. W. Paschen (Yang et al., 2013). Viruses were produced in HEK293T cells by transfection using Lipofectamine-2000 (Invitrogen) of viral constructs together with gag-pol (lentiviral or retroviral) and env (VSVG) expression vectors. Viral supernatants were collected 48 hr after transfection, 0.45 μM filtered and used to infect AML cell lines. For pMIG-infected cells, only GFP-positive cells were considered in the flow cytometry analysis. For the miR-control and miR-SUMO-1/2/3, clones resistant to hygromycin and puromycine were selected and tested for inhibition of SUMO-1/2/3 expression.

Microarray-Based Whole-Transcript Expression Analysis and Profiling

Total RNA was extracted using the GenElute Mammalian Total RNA kit (Sigma) and treated with DNase I according to the manufacturer's specifications. For each condition, three independent batches of RNA were prepared and controlled for purity and integrity using the Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip kits (Agilent Technologies). Only RNA with no sign of contamination or degradation (RIN >9) were further processed to generate amplified and biotinylated sense-strand cDNA targets using the GeneChip WT PLUS Reagent kit from Affymetrix according to the manufacturer's specifications. After fragmentation, cDNA targets were used to probe Affymetrix GeneChip Human Gene 2.0 ST arrays, which were then washed, stained, and scanned according to Affymetrix instructions (user manual P/N 702731 Rev. 3).

Microarrays, Data Analysis, and Gene Ontology

CEL files generated after array scanning were imported into the Partek Genomics Suite 6.6 (Partek) for preprocessing consisting of estimating transcript cluster expression levels from raw probe signal intensities. Analyses were performed using default Partek settings. Resulting expression data were then imported into R (http://www.R-project.org/) for further analysis. First nonspecific filtering was applied to remove transcript clusters with no specified chromosome location. Then, box plots, density plots, relative log expressions (RLEs), and sample pairwise correlations were generated to assess the quality of the data. They revealed no outlier within the series of hybridizations. Principal component analysis (PCA) was also applied to the data set. The first two components of the PCA were able to separate samples according to the treatment. Thus, the treatment was considered as the unique source of variability. Finally, the LIMMA package (Smyth, 2005) (R/Bioconductor) was used to detect differentially expressed genes (DEGs) between treated and nontreated samples. A linear model with treatment as unique factor was fitted to the data before applying eBayes function to calculate the significance of the difference in gene expression between the two groups. p values were adjusted by Benjamin and Hochberg's false discovery rate (FDR) (Benjamini and Hochberg, 1995) and genes with FDR less than 0.05 and absolute linear fold change (FC) greater or equal to 2 were considered as DEG. Gene Ontologies associated with the DEG were obtained with BINGO (Maere et al., 2005).

Chromatin Immunoprecipitation and RT-qPCR

ChIPs were performed as previously described (Tempé et al., 2014). The immunoprecipitated DNA and inputs taken from samples before immunoprecipitation were analyzed using the Roche LightCycler 480 with primers specific for the proximal promoter *DDIT3* gene (forward: 5′-atgactcacccacctcctccgtg-3′; reverse: 5′-ccccgtcgctccctctcgcta-3′). Total RNA was purified using the GenElute Mammalian Total RNA kit (Sigma). After DNase I treatment, 1 μg of total RNA was used for cDNA synthesis with the Maxima First Strand cDNA (Thermo Scientific) and used for qPCR with primers specific for the *DDIT3* mRNA (forward: 5′-gtcacaagcacctcccagagcc-3′; reverse: 5′-tctgtttccgtttc ctggttctcc-3′). Data were normalized to *GAPDH* or *TBP* mRNA levels.

Caspase 3 Activity Assay

Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with digitonin-containing buffer (eBioscience) for 15 min before addition of anti-cleaved CASPASE-3 antibody. After 2 hr, cells were washed and incubated with an anti-rabbit Alexa 647 antibody (Molecular Probes) for 1 hr, washed, and analyzed by flow cytometry.

Viability Assays

Cells were treated with increasing doses of drugs. After 24 hr, MTS assay (Promega) was used to assess the percentage of metabolically active cells according to manufacturer protocol. For primary AMLs, cells were stained with CD45-V450, CD34-PE-Cy7, CD38-APC, CD123-PE, AnnexinV-FITC, and 7-AAD as previously described (Vergez et al., 2011), and viability of the bulk of leukemic cells (CD45/SSC gating) or of LSCs ($CD34^+CD38^{low/-}CD123^+$) was determined by flow cytometry as the percentage of $AnnexinV^-/7\text{-}AAD^-$ cells within each population. IC_{50} were calculated with Prism 4 software (GraphPad).

In Vivo Treatment with Chemotherapeutic Drugs

The mouse AML model used in this study was the erythroleukemia induced by the FrCasE Murine Leukemia Virus (Michaud et al., 2010). Eight-day-old 129S7/SvEvBrdBkl-$Hprt^{b\text{-}m2}$ mice (H-$2D^b$ haplotype) were infected intraperitoneally with 100 μl of a $FrCas^E$ virus suspension containing 5×10^5 ffu/ml. Mice were examined at regular intervals for clinical signs of erythroleukemia (spleen swelling and reduction in hematocrits). Two-month-old leukemic mice were subjected to intraperitoneal administration of DNR (10 mg/kg) or Ara-C (50 mg/kg) every 2 days for 2 weeks and euthanized 4 hr after the last injection. Their spleens, as well as those of mock-treated leukemic mice of the same age, were lysed in 20 mM Na_2HPO_4 pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 5 mM EDTA, 5 mM EGTA, 1 μg/ml of a aprotinin/pepstatin/leupeptin mix, 10 mM N-Ethyl-Maleimide using a Dounce homogeneizer. Homogenates were cleared by centrifugation (20,000 × *g* for 10 min), and supernatants were used for immunoblotting analysis after protein concentration normalization.

Tumor Xenografts

Xenograft tumors were generated by injecting 2×10^6 KG1a cells (in 100 μl of PBS) subcutaneously on both flanks of NU/NU Nude mice (adult male and females, 25 g, Charles River Laboratories). Mice were given peritumoral injections of anacardic acid (2 mg/kg/day in 30 μl) or vehicle (DMSO). Tumor dimensions were measured with a caliper and volumes calculated using the formula: $v = \pi/6 \times A \times B^2$, where A is the larger diameter and B is the smaller diameter. At the end of the experiment, tumors were dissected, measured and weighed. Animal experiments were approved by the Ethical Committee from the UMS006 (approval number 13-U1037-JES-08).

Statistical Analyses

Results are expressed as means ± SD. Statistical analyses were performed by Student's t test with Prism 4 software. Differences were considered as significant for p values of <0.05. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

ACCESSION NUMBERS

The ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) accession number for the microarray expression data reported in this paper is E-MATB-2382.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.05.016.

AUTHOR CONTRIBUTIONS

G.B. and M.P. designed the study. G.B., JE.S., C.K., M.R., E.S., F.V., T.S., H.B., H.B., and F.B. performed the experiments. T.K. and L.V. performed the transcriptomic analysis. JE.S., S.M. and C.R. brought essential advice and resources. G.B. and M.P. wrote the manuscript.

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2 Role of NADPH oxidases in Acute Myeloid Leukemias chemoresistance

As demonstrated in the above-mentioned, chemotherapeutic drugs do not activate the ROS/SUMO axis in chemoresistant cells, which is necessary to induce their apoptosis. Still in this context, our laboratory continued further to understand the alteration of ROS pathway in AML cells and link it to chemoresistance. In this context, I generated AML chemoresistant cells lines from sensitive ones (U937 and HL60) to mimic the relapse upon chemoresistance acquisition by increasing gradually the concentration of chemotherapeutic drug used in clinic (Ara-C) in the medium of culture. Then I measured the level of ROS in these cells and the results showed that ROS levels are higher in chemoresistant cells compared to chemosensitive cells (Figure 24). Moreover my contribution in this project was to collect AML patient bone marrow aspirate from Saint Eloi Hospital, Montpellier in order to measure ROS level in leukemic blasts, I analysed around 50 AML patients at diagnosis and 5 healthy donors during the first 3 years of my Ph.D. This showed that AML cells generally produce for ROS than normal bone marrow CD34+ cells (Figure 25 **A**). In addition, preliminary analysis revealed that patients with high ROS level have a lower survival rate compare to patients with low ROS level (Figure 25 **B**). This suggests that chemoresistant cells are in a persistent high level of ROS and this could explain why chemoresistant cells are not sensitive to drug-mediated ROS production in order to induce desumoylation and then apoptosis. This project was continued by Tamara Salem and now Rosa Paollilo, post-docs in the team, who could show that NADPH oxidase (NOX)-derived ROS are critical player in AML resistance to chemotherapeutic drugs and that their targeting may represent a novel therapeutical option to overcome chemoresistance.

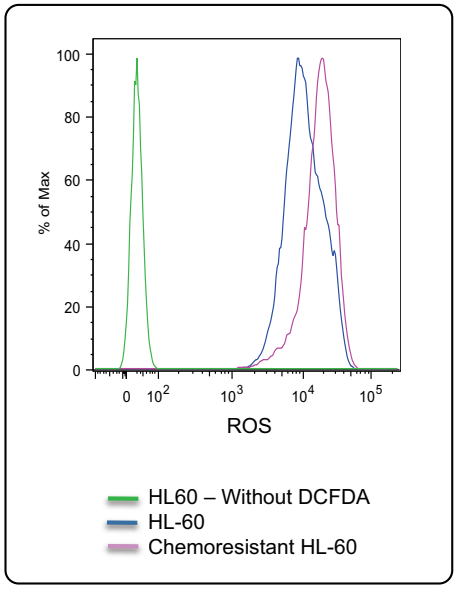

Figure 24: Measurement of ROS. ROS level of parental HL-60 and generated chemoresistant HL-60 is measured using DCFDA by cytometer. ROS level in chemoresistant is higher than in parental HL-60.

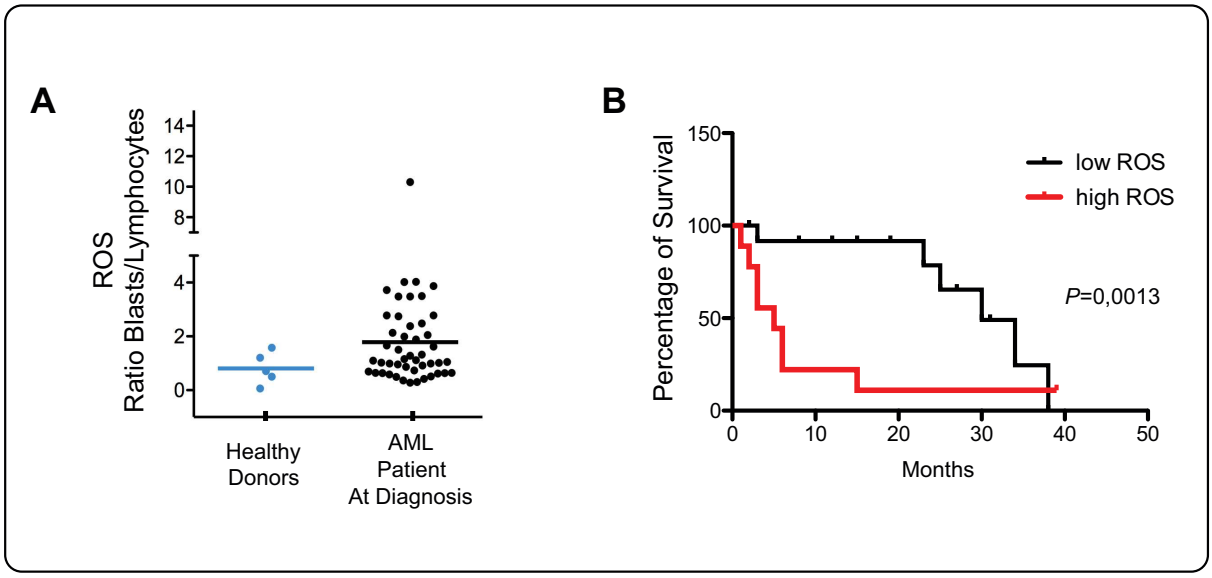

Figure 25: Level of ROS in AML diagnosis patients. A/ Measurement of ROS on diagnosis AML patients blasts. ROS level in primary AML cells are higher than in healthy donor cells. **B**/ Survival curve of AML patients according to their ROS level. Patients with high ROS have lower prognosis than patients with low ROS level at the diagnosis.

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Summary

Titre / Résumé en français

Rôle de la sumoylation dans la réponse aux traitements des leucémies aiguës myéloïdes. Les thérapies de différenciation sont une alternative prometteuse aux drogues génotoxiques utilisées en chimiothérapie pour le traitement de nombreux cancers. En particulier, l'acide tout-*trans* rétinoïque (ATRA) est utilisé avec succès pour traiter la leucémie aiguë promyélocytaire, un sous-type des leucémies aiguës myéloïdes (LAM). Malheureusement, son efficacité clinique est limitée dans les autres sous-types des LAM. Cela est en particulier du à une répression épigénétique des gènes de réponse à l'ATRA. Les SUMO constituent une famille de modificateurs post-traductionnels apparentés à l'ubiquitine dont la conjugaison sur de nombreuses protéines, appelée sumoylation, est impliquée dans la régulation de nombreux processus cellulaires, dont la transcription. Dans ce contexte, l'objective de ma thèse a été de comprendre le rôle de la sumoylation dans la réponse des LAM aux thérapies de différenciation. Nous avons pu montrer que la sumoylation réprime la différenciation induite par ATRA dans plusieurs lignées cellulaires, des cellules primaires de patients y compris celles résistantes à la chimiothérapie. L'inhibition de la sumoylation par les inhibiteurs pharmacologiques ou la surexpression des désumoylases augmente de façon remarquable la différenciation par ATRA et, à l'inverse l'augmentation de la sumoylation suite à une surexpression de SUMO ou son enzyme de conjugaison Ubc9 réduit fortement l'efficacité d'ATRA. L'ATRA synergise avec l'inhibition de la sumoylation pour limiter la prolifération des cellules de LAM *in vitro* et *in vivo*. D'un point de vue mécanistique, l'inhibition de la sumoylation favorise la différenciation des cellules de LAM en facilitant l'expression des gènes responsables de la différenciation myéloïde. Ainsi, cibler la sumoylation constitue une approche prometteuse pour sensibiliser la LAM aux thérapies de différenciation.

Title / Abstract

Role of the SUMO pathway in acute myeloid leukemias response to treatments. Differentiation therapies are a promising alternative to genotoxic-based chemotherapies in the treatment of many cancers. In particular, All-trans-retinoic acid (ATRA) is successfully used for Acute Promyelocytic Leukemias, a subtype of Acute Myeloid Leukemias. However, its clinical efficiency is very limited in the other AML subtypes, in particular because of epigenetic repression of ATRA-responsive genes. SUMOs are a family of post-translational modifiers related to ubiquitin and their conjugation, sumoylation, to their substrate proteins regulate many processes including gene transcription. The aim of my thesis was to understand the role of sumoylation in AML responses to treatments. I showed that sumoylation represses ATRA-induced differentiation in many AML cell lines and primary patient samples, including those resistant to chemotherapies. Inhibition of sumoylation with pharmacological inhibitors or overexpression of desumoylases markedly increased their differentiation by ATRA and increasing sumoylation by overexpression of SUMO or its conjugating enzyme Ubc9 strongly reduce ATRA efficiency. Inhibition of sumoylation synergize with ATRA to arrest AML cells proliferation both *in vitro* and *in vivo*. Mechanistically, inhibition of sumoylation primes AML cells for differentiation by facilitating the expression of master genes of the myeloid differentiation. Targeting the SUMO pathway thus constitute a promising approach to sensitize AML to differentiation therapies.