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## Targeting NPM1c in Acute Myeloid Leukemia

Rita Hleihel

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Thèse de doctorat de l'Université Sorbonne Paris Cité  
Préparée à l'Université Paris Diderot  
Ecole doctorale Hématologie, Oncogénèse, et Biothérapies  
U944 / Chaire d'Oncologie Cellulaire et Moléculaire

# **NPM1c, une cible dans le traitement des leucémies aiguës myéloïdes (LAM)**

Par **RITA HLEIHEL**

## **Doctorat Hématologie et Oncologie**

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# ABSTRACT

Acute myeloid leukemia (AML) is a genetically heterogeneous disease. Despite improvements in understanding the biology of AML, survival rates remain quite low. Prognosis of AML patients largely depends on acquired cytogenetic and molecular abnormalities. *Nucleophosmin-1 (NPM1)* is an essential gene encoding for a nucleocytoplasmic shuttling protein mainly localized to the nucleolus. Among several functions, NPM1 plays major roles in stabilization of the p14<sup>ARF</sup> tumor suppressor protein, regulation of ribosome biogenesis, control of centrosome duplication, response to stress stimuli and P53 activation. *NPM1* is one of the most frequently mutated genes in AML accounting for around one third of patients (*NPM1c* AML). In mutant *NPM1c* proteins, critical tryptophan residues in the C-terminus are lost and a *de novo* nuclear export signal is created. This leads to ectopic and aberrant accumulation of *NPM1c*, along with normal NPM1, in the cytoplasm of AML blasts, thus playing a major role in leukemogenesis. The promyelocytic leukemia protein (PML) tumor suppressor organizes nuclear bodies (NB) domains that control proteolysis and P53-driven senescence. PML NBs are disorganized in *NPM1c* AML.

Retinoic acid (RA), a hormone favoring differentiation of myeloid cells, increases survival of chemotherapy-treated *NPM1c* AMLs. We and others previously reported that RA and Arsenic trioxide (ATO) synergize to induce *NPM1c* degradation and to inhibit growth and induce apoptosis of *NPM1c* cells. Importantly, combined RA/ATO treatment significantly reduced bone marrow blasts in some *NPM1c* AML patients and restored the subnuclear localization of both NPM1 and PML. The main objective of the current work was to unravel the biochemical pathways driving *NPM1c* catabolism and to elucidate the molecular mechanisms of RA/ATO anti-leukemic activities.

We demonstrate that PML is required for RA/ATO-induced *NPM1c* degradation. In primary *NPM1c*-AML blasts, RA rapidly upregulates the initial low basal PML expression through Pin-1 inhibition prior to *NPM1c* clearance. This RA-induced PML stabilizes P53 and primes blasts for ATO-driven nuclear body reformation, yielding hyper-activation of P53. RA/ATO combination elicits PML-dependent responses, associated with *in vivo* P53 activation in *NPM1c* AML xenografts. Importantly, RA/ATO-initiated a transient complete remission in a *NPM1c*-AML patient demonstrating clinical relevance of this combination. We also demonstrate that ATO-driven *NPM1c* degradation happens *via* the PML/SUMO/RNF4/Proteasome pathway, which cooperates with direct RA-induced ubiquitination. By establishing the mechanisms underlying RA and ATO sensitivity of *NPM1c* AMLs, our studies identify a striking parallelism with APL, paving the way to the crafting of curative targeted therapies in this category of AML patients.

# RÉSUMÉ

La leucémie aiguë myéloïde (LAM) est une maladie très hétérogène du point de vue génétique. Malgré les avancées dans la caractérisation moléculaire de la LAM, les taux de survie des patients restent assez modestes. Le pronostic de ces patients dépend en grande partie des anomalies cytogénétiques et moléculaires acquises. La *nucléophosmine-1 (NPM1)* est un gène essentiel codant pour une protéine nucléocytoplasmique, qui fait la navette entre le nucléole, le noyau et le cytoplasme mais demeure localisée principalement dans le nucléole. NPM1 joue plusieurs rôles dont la stabilisation de la protéine suppresseur de tumeur p14<sup>ARF</sup>, la régulation de la biogenèse des ribosomes, le contrôle de la duplication des centrosomes, la réponse aux stimuli de stress et l'activation de P53. *NPM1* est considéré comme un des gènes les plus fréquemment mutés dans la LAM, représentant environ un tiers des patients (LAM avec *NPM1c*). Dans les protéines mutées *NPM1c*, deux résidus de tryptophane à l'extrémité C-terminale sont perdus et un signal d'exportation nucléaire est créé. Cela conduit à une accumulation ectopique et aberrante de la protéine *NPM1c*, ainsi que la protéine normale *NPM1*, dans le cytoplasme des blastes. Ceci joue un rôle majeur dans la leucémogénèse et l'établissement de la LAM. La protéine de la leucémie promyélocytaire (PML) organise des corps nucléaires (CN) qui contrôlent la protéolyse et la sénescence induite par la voie P53. Les CN PML sont désorganisés dans la LAM avec *NPM1c*.

L'acide rétinoïque (AR) est une hormone favorisant la différenciation des cellules myéloïdes. L'addition de l'AR à la chimiothérapie augmente la survie des patients LAM avec mutation *NPM1c*. Nous avons précédemment démontré que l'AR et le trioxyde d'Arsenic (ATO) fonctionnent en synergie pour induire la dégradation de la protéine *NPM1c*, inhiber la croissance et induire l'apoptose des cellules leucémiques exprimant *NPM1c*. D'une manière importante, l'association AR/ATO a entraîné la baisse significative du nombre de blastes dans la moelle osseuse chez certains patients atteints de LAM avec *NPM1c*, et a conduit à la restauration de la localisation nucléaire de *NPM1* et de PML. L'objectif principal du travail en cours était de dévoiler les voies biochimiques conduisant au catabolisme de *NPM1c* et d'élucider les mécanismes moléculaires de l'activité anti-leucémique de l'association AR/ATO.

Nous démontrons que PML est nécessaire pour la dégradation de *NPM1c* induite par l'association AR/ATO. Dans les blastes primaires de patients LAM avec *NPM1c*, l'AR augmente rapidement le taux d'expression basal de PML, *via* l'inhibition de Pin-1, et avant la dégradation de la *NPM1c*. L'induction de PML par l'AR conduit à la stabilisation de P53 et prépare pour la reformation des CN par l'ATO, pour induire par la suite une hyper-activation de P53. En outre, l'association AR/ATO induit des réponses dépendantes de PML, associées à l'activation de P53 *in vivo* dans les souris xénogreffées avec des cellules LAM *NPM1c*. D'une manière très importante, l'association AR/ATO a induit une rémission complète, quoique

transitoire, chez un patient LAM avec *NPM1c* démontrant l'efficacité clinique de cette association. Nous démontrons également que la dégradation de *NPM1c* induite par l'ATO se produit par le biais de la voie PML/ SUMO/RNF4/Protéasome, qui coopère avec une ubiquitination directe induite par l'AR. Nos études identifient un parallélisme flagrant avec la leucémie aiguë promyélocytaire, et ouvrent des horizons pour les applications cliniques de thérapies ciblées contre les LAM avec *NPM1c*. De plus, l'activation de Pin-1 et la perte de PML dans plusieurs types de tumeurs suggèrent qu'au-delà des LAM avec *NPM1c*, l'axe AR/ATO/Pin-1/PML/P53 pourrait être plus largement exploité au niveau thérapeutique, surtout dans les tumeurs malignes sensibles à l'association AR/ATO.

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# **LIST OF ABBREVIATIONS**

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**A**

AML	Acute Myeloid Leukemia
APMF	Acute Panmyelosis with MyeloFibrosis
APL	Acute Promyelocytic Leukemia
ASXL1	Additional Sex Combs-Like 1
RA	All Trans Retinoic Acid
allo-SCT	Allogeneic Stem Cell Transplant
APE1	Apurinic/apyrimidinic Endonuclease 1
ATO	Arsenic TriOxide

---

**B**

BER	Base Excision Repair
BAALC	Brain And Acute Leukemia Cytoplasmic protein
BRCA1-BARD	BRCA1-associated RING domain protein
BET	Bromodomain and Extra Terminal
BTK	Bruton tyrosine kinase

---

**C**

CK2	Casein kinase 2
CBL	Casitas B-lineage Lymphoma
Cbx4	ChromoBoX 4
CEBPA	CCAAT/Enhancer Binding Protein
CENPA	CENtromere Protein A
CAR-T	Chimeric Antigen Receptors
Cbx4	Chromobox 4
Crm1	Chromosome Region Maintenance 1
CR	Complete Remission
CBF $\beta$	Core-binding factor, subunit beta
CDKs	Cyclin-Dependent Kinases

---

**D**

DUB	DeUBiquitinating enzymes
DFS	Disease-Free Survival
DBS	DNA double-strand break
DNMT3A	DNA MethylTransferase 3A
DDB2	DNA-binding protein 2

---

**E**

EVI1	Ectopic Viral Integration site1
EZH2	<i>Enhancer of zeste homolog 2</i>
ERG	ETS-related gene
ELN	European LeukemiaNet
EFS	Event-Free Survival

---

**F**

FDA	Federal Drug Administration
FLT3	Fms-Like Tyrosine kinase 3
FAB	French American British

---

**G**

GIST	GastroIntestinal Stromal Tumor
GO	Gemtuzumab Ozogamicin
GVL	Graft-Versus-Leukemia

---

**H**

HSCs	Hematopoietic Stem Cells
HATs	Histone AcetylTransferases
HDAC	Histones DeACetylases
HECT	Homologous to E6-associated protein C-Terminus
HCSM	Hydrophobic Cluster SUMO Motif

---

**I**

ITS2	Internal Transcribed Region 2
ICM	Inverted Consensus Motif
IDHs	Isocitrate De-Hydrogenases

---

**L**

LANA-1	Latency Associated Nuclear Antigens LANA-1
LANA-2	Latency Associated Nuclear Antigens LANA-2
LIC	Leukemia Initiating Cells
LCSs	Leukemic stem cells
LEF1	Lymphoid Enhancer Binding Factor 1

---

**M**

MN1	Meningioma 1
MRD	Minimal Residual Disease
MAPK	Mitogen Activated Protein Kinase
MLLT3	Mixed lineage leukemia gene T3
MLL	Mixed lineage leukemia,
MDM2	Mouse double minute 2
MDS	MyeloDysplastic Syndrome
MDP	Myeloproliferative Disease
MPN	MyeloProliferative Neoplasms

---

**N**

NP	Nanoparticules
NGS	Next Generation Sequencing
NSCLC	Non-Small Cell Lung Carcinoma
NOS	Not otherwise specified
NB	Nuclear Bodies
NES	Nuclear Export Signals
NLS	Nuclear Localization Signal
NUMA	NUclear Mitotic Apparatus protein
NPC	Nuclear Pore Complex
NOD-SCID	Nonobese diabetic/severe combined immunodeficiency
NoLS	Nucleolar Localization Signal
NDF	Nucleolus-Derived Foci
NPM1	Nucleophosmin
Np	NucleoPlasmins
NER	Nucleotide Excision Repair

---

**O**

ORR	Objective Response Rate
OS	Overall Survival

---

**P**

PI3K	PhosphoInositide 3-Kinase
PDSM	Phosphorylation-Dependent SUMO Motif
Plk	Polo-Like Kinases
PC2	Polycomb 2 protein
PTM	Post-Translational Modification

PEL	Primary Effusion Lymphoma
PML	Promyelocytic Leukemia
PIAS	Protein Inhibitor of Activated STAT
PTPN11	Protein Tyrosine Phosphatase, Non-Receptor Type 11

---

**R**

ranBP2	ran Binding Protein 2
RanGAP1	Ran GTPase-activating protein 1
RING	Really Interesting New Gene
RPA14	Replication protein A 14
RB	RetinoBlastoma
RAR	Retinoic Acid Receptor
RARA	Retinoic Acid Receptor $\alpha$
RXR	Retinoid X Receptor
RPN1-EVI1	Ribophorin1 gene-ecotropic virus integration 1 gene
RBCC	RING finger, B-box, Coiled-Coil motif
RBM15-MKL1	RNA binding motif protein 15-megakaryoblastic leukemia 1,
RP1	RNA polymerase I
RUNX1-RUNX1T1:	Runt-related transcription factor 1

---

**S**

SENP	SENtrin-specific Protease
SCID	Severe combined immunodeficiency
SUMO	Small Ubiquitin-like Modifiers
SWOG	Southwest Oncology Group
SAE	SUMO Activating Enzyme
SCM	SUMO Consensus Motif
SIM	SUMO Interacting Motif

---

**T**

TRF1	Telomeric Repeat-binding Factor 1,
TET2	Ten–Eleven Translocation oncogene family member 2
TTF1	Transcription Termination Factor
TAM	Transient abnormal myelopoiesis associated with Down syndrome
TLS	TransLesion Synthesis
TRIM	TRIpertite Motif-containing protein
TRMN	Ttherapy-Related Myeloid Neoplasms
TKIs	Tyrosine Kinase Inhibitors

---

**U**

Ub	Ubiquitin
UBA2	UBiquitin-Like Activating enzyme subunit 2

---

**V**

v-Cyc	Viral Cyclin
v-FLIP	Viral FLICE inhibitory protein

---

**W**

WT1	Wilms tumor 1
WHO	World Health Organization

---

**X**

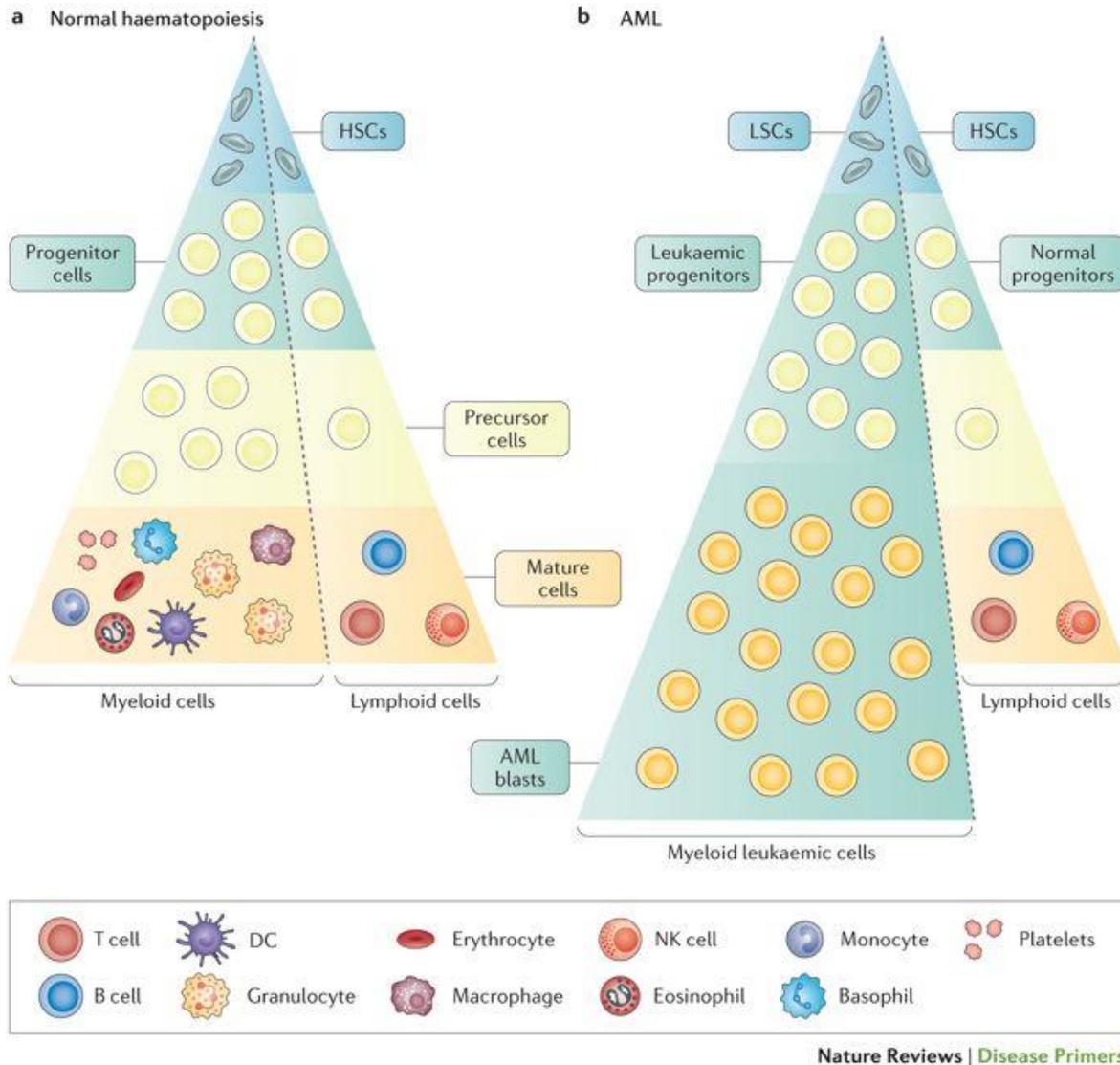
XPC	Xeroderma pigmentosum, complementation group C
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# INTRODUCTION

# Chapter I: Acute Myeloid Leukemia (AML)

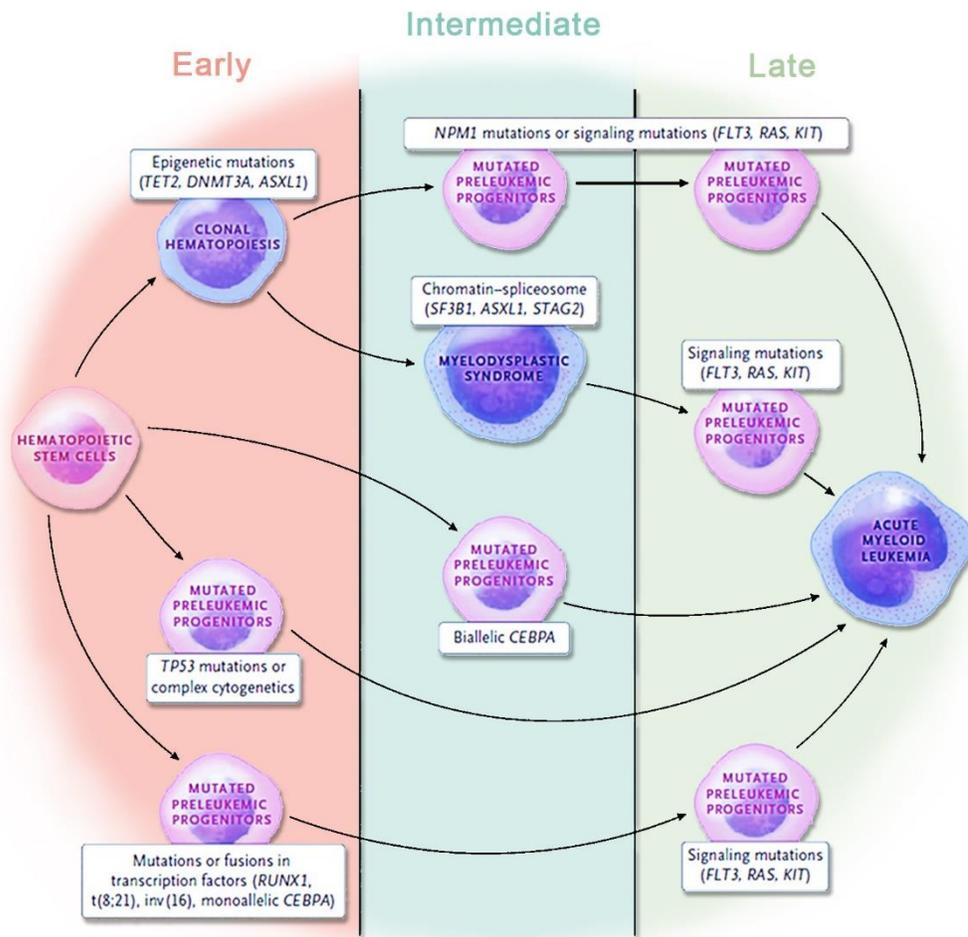
## 1.1 Overview of AML

Acute myeloid leukemia (AML) is a heterogeneous group of aggressive hematological neoplasms characterized by a clonal proliferation of myeloid precursors (Figure 1). These precursors have an increased proliferation rate and a reduced capacity to differentiate, resulting in the decreased production of normal mature blood cells (Lowenberg, Downing et al. 1999). AML is one of the most common acute leukemia in adults, accounting for about 80 percent of cases (Yamamoto and Goodman 2008). In the majority of cases, AML appears as a *de novo* malignancy, most likely due to the acquisition of somatic mutations in hematopoietic progenitors of healthy individuals (De Kouchkovsky and Abdul-Hay 2016). In most cases, AML is caused by the accumulation of various mutations in hematopoietic stem cells. Early mutations can stimulate clonal hematopoiesis and increase the fitness of stem cells. Additional mutations in genes encoding transcription factors and tyrosine kinase receptors, are acquired and lead to the development of the leukemic cells (Figure 2). AML prognosis and treatment largely depend on the patients' age. About 40–50% of young adults with AML (age 18-60 years) can be cured using conventional chemotherapy (Dohner, Weisdorf et al. 2015). However, the incidence of the disease sharply increases with age (Deschler and Lubbert 2006). Patients older than 60 mostly have a poor prognosis, with an overall survival (OS) at 2-years of less than 10 percent (Lowenberg and Sonneveld 1998). In addition, AML is highly polyclonal and evolves over time. It can present at diagnosis with multiple clones and multiple mutations. In response to treatment, a clone becomes dominant, acquires new mutations and expands leading to relapse and resistance to therapy (Figure 3).



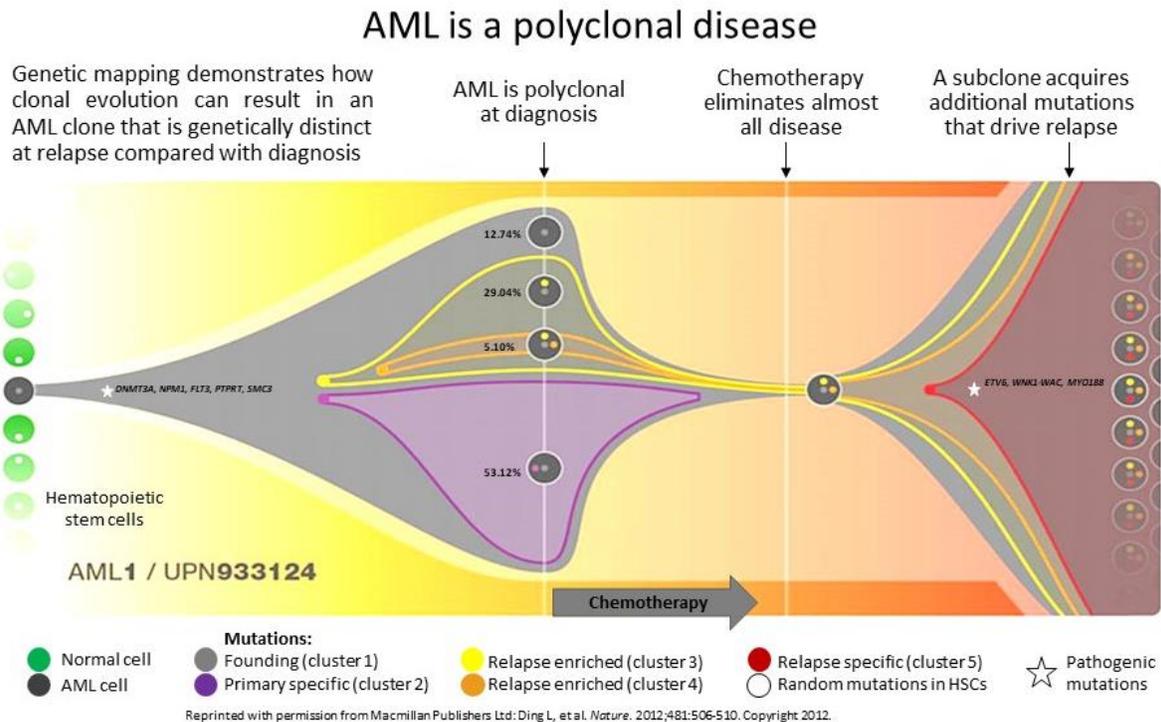
**Figure 1: Normal versus leukemic hematopoiesis (Khawaja, Bjorkholm et al. 2016)**

(a) General hierarchical structure of normal hematopoiesis: The long-term hematopoietic stem cells (HSCs), give rise to various hematopoietic progenitor cells. Progenitors produce various precursor cells and then mature hematopoietic cell types as indicated. (b) Aberrant hematopoiesis observed in acute myeloid leukemia (AML): Leukemic stem cells (LSCs) reside at the top of the developmental pyramid, giving rise to AML progenitor cells and the more mature (but still morphologically primitive) myeloid blast cells that make up the bulk of the neoplasm.



### Figure 2: Model for Leukemogenesis

The Road from normal blood cell to leukemia begins with sentinel acquisition of driving mutations (epigenetic mutations, P53 mutation or mutations in transcription factors). These mutations increase the fitness of stem cells and induce clonal hematopoiesis leading to the acquisition additional mutations (e.g. NPM1 mutations, signaling mutations or CEBPA mutation), all required to the development of AML or other myeloid cancer.



**Figure 3: AML, a polyclonal disease (Ding, Ley et al. 2012)**

At diagnosis, the primary AML cells (UPN933124) harbored multiple clones, with pathogenic mutations (*FLT3*, *DNMT3A*, *NPM1*, *PTPRT*, and *SMC3*). During treatment with chemotherapy, one clone became dominant. At relapse, this clone then expanded and acquired additional mutations (*ETV6* and *MYO18B*, and a *WNK1-WAC* fusion gene).

## 1.2 History of AML classifications

Classification of leukemia is based on the acute or chronic progressions as well as on the lymphoid or myeloid cell origins. In AML, the complexity and heterogeneity of the disease led to the adoption of several classifications. These AML classifications were continuously updated based on the progress in sequencing technologies leading to the discovery of new mutations. The French-American-British (FAB) classification represents the first attempt dividing AML into 8 subtypes (M0 to M7) (Bennett, Catovsky et al. 1976) (Table 1). This classification relied on the origin of the leukemia and the maturity of the cells. Indeed, the subtypes M0 to M5 start in precursors of white blood cells. M6 AML originates in very early forms of red blood cells and M7 AML starts in early megakaryoblasts that will form the platelets.

FAB subtype	Name	% of adult AML patients
<b>M0</b>	Undifferentiated acute myeloblastic	5%
<b>M1</b>	Acute myeloblastic leukemia with minimal maturation	15%
<b>M2</b>	Acute myeloblastic leukemia with maturation	25%
<b>M3</b>	Acute promyelocytic leukemia (APL)	10%
<b>M4</b>	Acute myelomonocytic leukemia	20%
<b>M4 eos</b>	Acute myelomonocytic leukemia with eosinophilia	5%
<b>M5</b>	Acute monocytic leukemia	10%
<b>M6</b>	Acute erythroid leukemia	5%
<b>M7</b>	Acute megakaryoblastic leukemia	5%

**Table 1. The French-American-British (FAB) classification of AML (Bennett, Catovsky et al. 1976).**

This historical FAB classification does not take into consideration the karyotype of AML patients, which is key for the progression of the disease and its response to therapy. Accordingly, in 2001, the World Health Organization (WHO) introduced a new classification for AML followed by a revised one in 2008. Indeed, AML was classified into several broad groups including: AML with recurrent cytogenetic abnormalities such as AML with a translocation between chromosomes 8 and 21, AML with a translocation or inversion in chromosome 16, AML with changes in chromosome 11, and acute promyelocytic leukemia (APL) (M3), which usually has a translocation between chromosomes 15 and 17; AML with multi-lineage dysplasia; AML related to previous chemotherapy or radiation, unspecified AML including those that do not fall into any of the above groups. This last group includes undifferentiated AML (M0), AML with minimal 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, and Myeloid sarcoma (Table 2).

**AML with recurrent genetic abnormalities**

AML with t (8;21) (q22;q22); RUNX1-RUNX1T1  
 AML with inv (16) (p13.1q22) or t (16;16) (p13.1;q22); CBFβ-MYH11  
 Acute promyelocytic leukemia with t (15;17)(q22;q12); PML-RARα  
 AML with t (9;11) (p22;q23); MLLT3-MLL  
 AML with t (6;9) (p23;q34); DEK-NUP214  
 AML with inv (3) (q21q26.2) or t (3;3) (q21;q26.2); RPN1-EVI1  
 AML (megakaryoblastic) with t (1;22) (p13;q13); RBM15-MKL1  
 AML with mutated *NPM1*\*  
 AML with mutated *CEBPA*\*

**AML with myelodysplasia-related changes****Therapy-related myeloid neoplasms****AML NOS**

AML with minimal differentiation  
 AML without maturation  
 AML with maturation  
 Acute myelomonocytic leukemia  
 Acute monoblastic and monocytic leukemia  
 Acute erythroid leukemia  
 Acute megakaryoblastic leukemia  
 Acute basophilic leukemia  
 Acute panmyelosis with myelofibrosis

**Myeloid sarcoma****Table 2: WHO classification of acute myeloid leukemia 2008**

\*These are provisional entities. WHO: World Health Organization, AML: Acute myeloid leukemia, RUNX1-RUNX1T1: Runt-related transcription factor 1; translocated to, 1 (cyclin D-related), CBFβ: Core-binding factor, subunit beta, RARα: Retinoic acid receptor α, MLL: Mixed lineage leukemia, MLLT3: Mixed lineage leukemia gene T3, RPN1-EVI1: Ribophorin1 gene-ecotropic virus integration 1 gene, RBM15-MKL1: RNA binding motif protein 15-megakaryoblastic leukemia 1, NPM1: Nucleophosmin member 1, CEBPA: CCAAT/enhancer-binding protein alpha, NOS: Not otherwise specified

Recent and massive advances in sequencing technologies led to the discovery of new genetic mutations including: *KIT*, *Fms-Like Tyrosine kinase 3 (FLT3)*, *Nucleophosmin-1 (NPM1)*, *CCAAT/Enhancer Binding Protein (CEBPA)*, *RAS*, *Wilms tumor 1 (WT1)*, *brain and acute leukemia cytoplasmic protein (BAALC)*, *ETS-related gene (ERG)*, *Meningioma 1 (MN1)*, *DNA Methyl Transferase (DNMT)*, *Ten–Eleven Translocation oncogene family member 2 (TET2)*, *Isocitrate De-Hydrogenases (IDH)*, *additional Sex Combs-Like 1 (ASXL1)*, *Protein Tyrosine Phosphatase, Non-Receptor Type 11 (PTPN11)* and *Casitas B-lineage Lymphoma (CBL)* associated with AML. This led to further revise AML classification.

In 2010, the European LeukemiaNet (ELN) classification attempted to standardize the risk stratification in adult AML patients, by incorporating recurrent somatic mutations such as *NPM1*, *FLT3* and *CEBPA* (Dohner, Estey et al. 2010). Patients were thus classified according to their baseline cytogenetics into three major risk sub-categories: favorable, intermediate and adverse (Grimwade, Hills et al. 2010) (Table 3). Favorable prognosis is associated with acute promyelocytic leukemia (APL) t (15; 17) (q22; q12), balanced abnormalities of t (8; 21) (q22; q22), inv (16) (p13.1q22), t (16; 16) (p13.1; q22), mutated *NPM1* without *FLT3-ITD* (normal Karyotype) and biallelic mutated *CEBPA*. The intermediate risk subgroup was divided into Intermediate 1 and 2. The intermediate 1 risk subgroup includes mutated *NPM1* with *FLT3-ITD* (normal karyotype), wild-type *NPM1* with or without *FLT3-ITD*; whereas the intermediate-2 risk category includes t(9;11), MLLT3-MLL and cytogenetic abnormality that are neither favorable nor adverse. Last, the adverse risk group include complex karyotype, inv (3) (q21q26)/t (3; 3) (q21; q26), RPN1-EVI1; DEK-NUP214 t (6,9) (p23; q34); t (v;11); -5 or del(5q), -7 or abnormal (17p) and monosomal karyotype and associates with poor prognosis.

Genetic group	Subsets
Favorable	t(8;21)(q22;q22); RUNX1-RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFβ-MYH11 Mutated NPM1 without FLT3-ITD (normal karyotype) Mutated CEBPA (normal karyotype)
Intermediate-I*	Mutated NPM1 and FLT3-ITD (normal karyotype) Wild-type NPM1 and FLT3-ITD (normal karyotype) Wild-type NPM1 without FLT3-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); MLLT3-MLL Cytogenetic abnormalities not classified as favorable or adverse <sup>‡</sup>
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1 t(6;9)(p23;q34); DEK-NUP214 t(v;11)(v;q23); MLL rearranged

-5 or del(5q); -7; abn(17p); complex karyotype<sup>‡</sup>

**Table 3: 2010 ELN risk stratification of molecular, genetic and cytogenetic alterations (Dohner, Estey et al. 2010).**

\* Includes all AMLs with normal karyotype except for those included in the favorable subgroup; most of these cases are associated with poor prognosis, but they should be reported separately because of the potential different response to treatment.

† For most abnormalities, adequate numbers have not been studied to draw firm conclusions regarding their prognostic significance.

‡ Three or more chromosome abnormalities in the absence of one of the WHO designated recurring translocations or inversions, that is, t(15;17), t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23), t(6;9), inv(3) or t(3;3); indicate how many complex karyotype cases have involvement of chromosome arms 5q, 7q, and 17p.

However, both the Intermediate-I and intermediate-II were indistinguishable in terms of prognosis in elderly patients, who constitute the majority of cases of AML (Mrozek, Marcucci et al. 2012). Therefore, the panel decided to simplify the ELN system by employing a three group classification (favorable, intermediate, adverse) rather than the previous four group system (Dohner, Estey et al. 2017) (Table 4).

Risk Category	Genetic Abnormality
Favorable	t(8;21)(q22;q22.1); RUNX1-RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFβ-MYH11 Mutated NPM1 without FLT3-ITD or with FLT3-ITD <sup>low*</sup> Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITD <sup>high</sup> Wild type NPM1 without FLT3-ITD or with FLT3-ITD <sup>low*</sup> (w/o adverse risk genetic lesions) t(9;11) (p21.3;q23.3); MLLT3-KMT2Ad Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214 t(v;11q23.3); KMT2A rearranged t(9;22)(q34.1;q11.2); BCR-ABL1 inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM(EVI1) -5 or del(5q); -7; -17/abn(17p) Complex karyotype, monosomal karyotype Wild type NPM1 and FLT3-ITD <sup>high*</sup> Mutated RUNX1 <sup>†</sup> Mutated ASXL1 <sup>†</sup> Mutated TP53h

**Table 4: 2017 ELN risk stratification by Genetics (Dohner, Estey et al. 2017).**

\*Low, low allelic ratio (<0.5); high, high allelic ratio (>0.5); † these mutations should not be used as an adverse prognostic marker if they co-occur with favorable-risk AML subtypes.

On the other hand, the WHO classification reduced the blast percentage from 30% to 20% in peripheral blood or in bone marrow for the diagnosis of AML. However, patients harboring clonal recurring cytogenetic abnormalities such as t(8;21) (q22; q22), inv (16) (p13q22) or t(16;16) (p13; q22), and t(15;17) (q22; q12) would be diagnosed as AML even if they have lower blast percentages (Vardiman, Harris et al. 2002, Arber, Orazi et al. 2016). The 2016 edition of the WHO classification represents a revision rather than a new classification (Arber, Orazi et al. 2016) (Table 5).

Acute myeloid leukemia (AML) and related neoplasms

#### **AML with recurrent genetic abnormalities**

AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1  
 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);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  
 Provisional entity: AML with BCR-ABL1  
 AML with mutated NPM1  
 AML with biallelic mutations of CEBPA  
 Provisional entity: AML with mutated RUNX1

#### **AML with myelodysplasia-related changes**

#### **Therapy-related myeloid neoplasms**

#### **AML, NOS**

AML with minimal differentiation  
 AML without maturation  
 AML with maturation  
 Acute myelomonocytic leukemia  
  
 Acute monoblastic/monocytic leukemia  
 Pure erythroid leukemia  
 Acute megakaryoblastic leukemia  
 Acute basophilic leukemia  
 Acute panmyelosis with myelofibrosis

#### **Myeloid sarcoma**

#### **Myeloid proliferations related to Down syndrome**

Transient abnormal myelopoiesis (TAM)  
 Myeloid leukemia associated with Down syndrome

**Table 5: 2016 WHO classification of AML (Arber, Orazi et al. 2016)**

### 1.3 Genetic alterations in AML

Exome sequencing in AML patients led to the identification of more than 20 driver recurrent mutations (Weinstein, Collisson et al. 2013). The main biological and clinical features of most frequent mutations underlying AML with normal cytogenetics are shown in Table 6.

	CLASS I	CLASS II	CLASS III	Other
Pathways	Signal Transduction	Differentiation	Epigenetic regulation	Tumor suppression
Genes	FLT3	RUNX1 (AML1)	TET2	WT1
	KIT	CBF	IDH1, IDH2	TP53
	NRAS, KRAS	CEBPA	DNMT3A	
	JAK2	NPM1	ASXL1	
	PTPN11	PU1	EZH2	
		MLL		
		RARA		

**Table 6: Gene mutations in AML (reviewed in (Dombret 2011)).**

*Abbreviations: FLT3: Fms-Like Tyrosine kinase 3; PTPN11: Protein Tyrosine Phosphatase, Non-Receptor Type 11; RUNX1: runt-related transcription factor 1; CBF $\beta$ : core-binding transcription factor; CEBPA: CCAAT/Enhancer Binding Protein; Nucleophosmin-1 (NPM1); MLL: Mixed Lineage Leukemia; RARA: Retinoic acid receptor alpha; TET2: Ten–Eleven Translocation oncogene family member 2; Isocitrate De-Hydrogenases (IDH); DNMT:DNA Methyl Transferase; ASXL1:additional Sex Combs-Like 1; EZH2: Enhancer of zeste homolog 2; WT1: Wilms tumor 1.*

*Nucleophosmin-1 (NPM1)* gene represents one of the most frequent genetic aberrations in AML; It is highly mutated in *de novo* AML (Verhaak, Goudswaard et al. 2005) and is identified in about 30% of AML patients, especially in those who have a normal karyotype (Falini, Mecucci et al. 2005). Clinically, the mutation is associated with monocytic morphology. In the absence of *FLT3-ITD*, patients present with favorable prognosis and better OS (Falini, Mecucci et al. 2005).

The second most common genetic aberrations in *de novo* AML patients occur in the *FLT3-ITD* gene on chromosome 13. These associate with poor prognosis and short OS (Gilliland and Griffin 2002).

Mutations in the *DNMT3A* gene occurs in 18%–22% of all AML cases and in about 34% of normal karyotype-AML (Ley, Ding et al. 2010). The prognostic significance of *DNMT3A* mutations

is thought to be adverse. Mutations in the transcription factor *CEBPA*, which plays a central role in normal development of granulocytes, are also observed in about 10% of AML patients (Pabst and Mueller 2009). Mutations in *IDH1* and *IDH2* are detected in approximately 20% of patients with AML (Montalban-Bravo and DiNardo 2018). These enzymes are normally involved in multiple metabolic and epigenetic cellular processes. *IDH* mutations affect the epigenetic state in AML by inducing increase in histone methylation (Lu, Ward et al. 2012) as well as global DNA hyper-methylation (Rakheja, Konoplev et al. 2012). In AML patients with normal karyotype having *NPM1* mutations and wild type *FLT3 ITD*, these mutations, in particular those in *IDH1*, are associated with lower disease-free survival (DFS) and lower OS (Marcucci, Maharry et al. 2010).

*TET2* mutations occur in 9%–23% of AML and associate with poor prognosis, in AML patients with intermediate-risk cytogenetics, especially when present along with other adverse molecular markers (Chou, Chou et al. 2011).

#### **1.4 Clinical manifestations of AML**

AML symptoms result mainly from a shortage of normal blood cells. Nonspecific symptoms in AML patients include fatigue, loss of appetite, thrombocytopenia, anemia and/or neutropenia (Meyers, Albitar et al. 2005). As a result, AML patients experience others symptoms including: bruising, weakness, anxiety, dizziness, depression, bleeding, shortness of breath, and general lack of wellness (Tomaszewski, Fickley et al. 2016). Infections of variable severity can also occur because of leukopenia and neutropenia. Splenomegaly and hepatomegaly are seen in approximately one third of patients, especially in those with a monocytic or monoblastic morphologic subtype. Adenopathy is rare, except in the monocytic variant of AML where the frequency of adenopathy reaches more than 30% of cases (Burns, Armitage et al. 1981, Hu, Wu et al. 2011). Hemorrhagic manifestations including gingival bleeding, ecchymoses, epistaxis, or menorrhagia can also occur (Nebgen, Rhodes et al. 2016). Bleeding in other organs such as stomach, intestine, genital-urinary, lung or central nervous system is less common but may also occur. In some patients, a serious bleeding diathesis can occur, particularly in the early phase of treatment, because the activation of the coagulation cascade by the leukemic blasts leading

to hyper-fibrinolysis. The most serious complication is the intracranial bleeding, and can occur in 5% of patients (Kim, Lee et al. 2004). Moreover, chloroma, also called granulocytic sarcoma or myeloid sarcoma, is an extramedullary manifestation of AML which was also reported as a rare manifestation of AML (King 1853) with an incidence of 2.5–9% (Singh, Kumar et al. 2017).

## 1.5 Treatment of AML

For more than three decades, the standard treatment of AML remained unchanged. It combines an anthracycline, usually daunorubicin, given for 3 days with continuous infusion of cytarabine for 7 days (3+7). The last decade witnessed a better understanding of the molecular pathogenesis of AML. This linked different prognosis and response to therapy with different mutations and karyotypes, and offered the discovery of potential new therapeutic targets. As a result, selective treatment approaches and personalized therapeutic strategies targeting driving mutations could arise. New strategies are nowadays adopted in newly diagnosed or relapsing/refractory patients and others are object of clinical investigation (Ferrara and Vitagliano 2019). The following section will focus on the standard therapeutic regimen and the newly introduced agents that were implemented in the last couple of years.

### 1.5.1 Induction therapy

The standard chemotherapy typically consists of an anthracyclin in combination with high-dose of cytarabine, and is called the “7 + 3” induction therapy regimen. The typical dose and schedule include either idarubicin (10–12 mg/m<sup>2</sup> on days 1, 2 and 3) or daunorubicin (60 or 90 mg/m<sup>2</sup> on days 1, 2 and 3) given with 7 days of nonstop cytarabine infusion (daily dose of 100 mg/m<sup>2</sup> per day for 1 week) (Saultz and Garzon 2016). Young, *de novo*, AML patients achieve CR in 65%–73% using standard induction with “7 + 3” while only 38%–62% of patients over 60 years of age with AML achieve CR (Estey and Dohner 2006). CR is defined as less than 5% leukemic blasts in the bone marrow and recovery of neutrophil count. Several trials have shown that higher dose of anthracycline (90 versus 45mg/m<sup>2</sup>) in both younger and older fit adults (from 60 to 65) results in higher CR rates (Lowenberg, Ossenkoppele et al. 2009). However, this outcome is heavily influenced by various cytogenetic and molecular characteristics of AML blasts. Many

trials were conducted to improve standard therapy. A meta-analysis of randomized studies demonstrated that Gemtuzumab ozogamicin (GO), humanized monoclonal antibody anti-CD33 conjugated with a toxin, can be safely added to conventional induction therapy and provides a significant survival benefit for patients with intermediate, and particularly favorable, cytogenetics (Hills, Castaigne et al. 2014, Amadori, Suciú et al. 2016).

Moreover, a large randomized international study suggested that the addition of the multikinase inhibitor midostaurin to induction and consolidation therapy and its single-agent use during maintenance improved OS and event-free survival (EFS), in patients 18–60 years of age with *de novo* *FLT3*-positive AML (Stone, Mandrekar et al. 2017).

### **1.5.2 Consolidation therapy**

Consolidation and maintenance therapy are given after achieving the first complete remission (CR1), in the ultimate aim of maintaining CR and eradicating Minimal Residual Disease (MRD) in the bone marrow (Mayer, Davis et al. 1994). MRD gives evidence for the presence of residual malignant cells that cannot be found by routine means. Assessment of MRD using real-time PCR (Ivey, Hills et al. 2016), multicolor flow cytometry (Jaso, Wang et al. 2014) or Next Generation Sequencing (NGS) (Grimwade, Ivey et al. 2016, Bullinger, Dohner et al. 2017) has become part of routine clinical practice in the management of patients. It is widely used to track response to treatment and predict impending relapse (Sudhindra and Smith 2014, Ravandi, Walter et al. 2018, Selim and Moore 2018).

Consolidation therapy often includes repeated courses with intermediate/high-dose cytarabine (Ara-C) with or without an anthracycline. It is difficult to determine whether re-induction therapy should be attempted alone or along with hematopoietic cell transplantation. These consolidation treatment strategies are decided based on the outcomes associated with each treatment, the fitness of AML patients to each treatment, the type of AML and the availability of an HLA-matched sibling stem cell donor (Devillier, Legrand et al. 2018).

Post-induction chemotherapy using intermediate-dose of cytarabine (1.5 g/m<sup>2</sup> twice daily on days 1, 3 and 5 over three to four cycles) is an effective and established regimen to prolong

remission and improve survival in favorable risk young adults (<60 year of age) (Byrd, Ruppert et al. 2004, Dombret and Itzykson 2017). These patients are treated with chemotherapy alone; transplantation is performed only at relapse (Estey and Dohner 2006). Higher dose of cytarabine (3 g/m<sup>2</sup>) is still used for patients with *Core binding factor* AML [e.g., t(8:21); or inv (16)] and *NPM1* mutated AML (Burnett, Russell et al. 2013). In elderly patients, there was no benefit with high dose cytarabine with increased and sometimes irreversible neurotoxicity (Schiffer 2014).

Allogeneic hematopoietic stem cell transplantation remains the most effective long term therapy for AML for patients with intermediate risk or high risk disease after achieving CR (Popat, de Lima et al. 2012).

### **1.5.3 Therapy in relapsed/refractory AML patients**

Following AML treatment, the majority of patients eventually relapse and die. Of the relapsing patients, only a small fraction achieves successful second remission (CR2) using salvage chemotherapy followed by stem cell transplantation (Estey and Dohner 2006). Salvage regimens include intermediate dose cytarabine (500–1500 mg/m<sup>2</sup> intravenously every 12 h on days 1–3); MEC (Mitoxantrone 8 mg/m<sup>2</sup> on days 1–5, Etoposide 100 mg/m<sup>2</sup> on days 1–5, and cytarabine 100 mg/m<sup>2</sup> on days 1–5) or I, FLAG-IDA (Fludarabine 30 mg/m<sup>2</sup>, intravenously on days 1–5 (20 mg/m<sup>2</sup> in patient >60 years old), cytarabine 1500 mg/m<sup>2</sup> (500–1000 mg/m<sup>2</sup> in patients >60 year) intravenously, 4h after fludarabine infusion, on days 1–5; Idarubicin 8 mg/m<sup>2</sup>, intravenously, on days 3–5; Granulocyte colony-stimulating factor 5g/kg, subcutaneously, from day 6 to white-cell count >1 g/L (FLAG-IDA) (Dohner, Weisdorf et al. 2015). The likelihood of achieving a second CR is best in patients with a long first remission, younger age and in those with favorable cytogenetics (Breems, Van Putten et al. 2005).

### **1.5.4 Targeted therapies**

AML remains a very aggressive leukemia with severe and complex prognosis. Increased survival among younger AML patients, with further intensification of chemotherapy, is limited by toxicity and compromised by reduced compliance. Older patients face several challenges, including an increased incidence of comorbidities, frequent functional impairment, higher

mortality, and more aggressive disease biology that confers chemotherapy resistance. Recent advances in sequencing techniques led to the discovery of relevant mutations dictating both prognosis and treatment outcome in AML patients. Thus, AML treatment is witnessing more personalized approaches with specific targeting of driving mutations. These include targeted therapies triggering oncoprotein degradation, hypomethylating agents, FLT-3 ITD inhibitors, IDH Inhibitors and monoclonal antibodies.

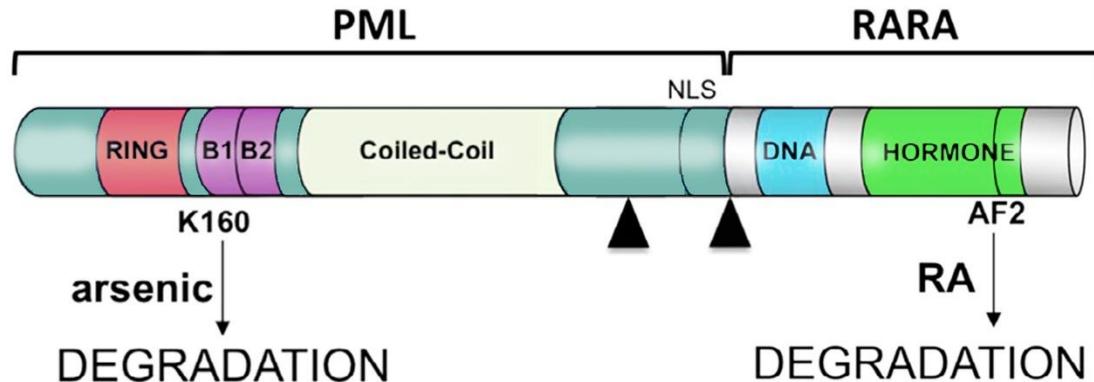
#### **1.5.4.1 Arsenic trioxide (ATO) and All-Trans Retinoic acid (RA)**

APL remains the best example of how targeted therapies on their own can trigger definitive cures in both preclinical models and in patients (Shen, Shi et al. 2004, de The, Pandolfi et al. 2017). APL is an AML subtype, mainly driven by a translocation t (15,17) whose product, the promyelocytic leukemia (PML)/retinoic acid receptor  $\alpha$  (RARA) fusion protein, affects both nuclear receptor signaling and PML body assembly (de The, Chomienne et al. 1990).

Historically, the use of RA (Huang, Ye et al. 1988) or ATO (Chen, Zhu et al. 1996) in the treatment of APL was proved to induce complete remission. Moreover, the addition of RA and ATO to conventional chemotherapy was shown to improve the outcome of patients with APL (Fenaux, Chastang et al. 1999, Fenaux, Chomienne et al. 2001, Ades, Guerci et al. 2010, Abaza, Kantarjian et al. 2017, Ades, Thomas et al. 2018). Furthermore, in *PML/RAR $\alpha$*  transgenic mice, the combination of RA and ATO induced tumor clearance through differentiation and apoptosis (Lallemand-Breitenbach, Guillemain et al. 1999). Recent studies showed that combining ATO and RA including some DNA-damaging chemotherapy cured most patients (Shen, Shi et al. 2004, Hu, Liu et al. 2009, Ravandi, Estey et al. 2009). Finally, a randomized study comparing the efficacy of RA plus chemotherapy with RA plus ATO, showed that the combination of RA and ATO may be superior to RA and anthracycline-based chemotherapy for APL patients with low-to-intermediate-risk APL (Lo-Coco, Avvisati et al. 2013).

In this context, RA synergize with ATO to degrade PML–RAR $\alpha$  fusion protein (Zhu, Koken et al. 1997, Zhu, Gianni et al. 1999). In particular, RA targets the RARA moiety, and ATO targets the PML moiety, both leading to PML/RARA degradation (de The, Le Bras et al. 2012) (Figure 4). This degradation is followed by PML/P53 activation and APL eradication. This provided the first

proof of concept highlighting the curative effect of therapy-induced-oncoprotein degradation in cancers driven by oncoproteins.



**Figure 4: Mechanism of leukemia eradication by ATO and RA**

The PML/RARA fusion protein with its main functional domains. RA targets the RARA moiety, while ATO targets the PML moiety, both leading to PML/RARA degradation. The N terminal contains a RING domain (Pink), two B-boxes (B1 and B2 in purple) and a coiled-coil domain (Gray).

#### 1.5.4.2 Gemtuzumab ozogamicin (GO)

Recently, targeting malignant cells by monoclonal antibodies and immune-conjugates was developed to increase the therapeutic effect and decrease morbidity and mortality in cancer patients. Gemtuzumab ozogamicin (Mylotarg<sup>®</sup>) is a targeted therapy that consists of a humanized monoclonal antibody (immunoglobulin G4, (hP67.6)) directed against CD33. CD33 is a cell surface antigen expressed on immature myelomonocytic lineage cells in healthy bone marrow. In AML, CD33 is expressed on the surface of leukemic blasts, in approximately 90% of all AML patients (Linenberger 2005). The clinical efficacy of GO was first established in AML patients with first relapse (Larson, Sievers et al. 2005). These adult patients ( $\geq 18$  years old) received monotherapy with two doses of GO at  $9 \text{ mg/m}^2/\text{dose}$  administered as a 2h intravenous infusion, and at 2 weeks of interval. As a monotherapy, GO proved a clear anti-leukemic activity in approximately 30% of these treated patients. Accordingly, GO was approved in 2000 by the US Federal Drug Administration (FDA). In line with this FDA accelerated-approval process, the Southwest Oncology Group (SWOG) initiated a randomized trial (SWOG-S0106) comparing GO at a dose of

6 mg/m<sup>2</sup> combined with induction chemotherapy vs. chemotherapy alone in newly diagnosed (ND)-AML adult patients. Patients randomized to the GO arm received daunorubicin at the dose of 45 mg/m<sup>2</sup> for 3 days, combined with infusional cytarabine and GO at the dose of 6 mg/m<sup>2</sup> on day 4. The control group of patients received a higher daunorubicin dose of 60 mg/m<sup>2</sup> for 3 days combined with cytarabine. GO was also added to day 1 of high-dose cytarabine consolidation. The trial was stopped prematurely because of a higher early treatment-related mortality, with no clinical benefit of adding GO (Petersdorf, Kopecky et al. 2013).

Later on, re-approval of GO was made in newly diagnosed AML patients following four completed randomized studies and due to acceptable toxicity (Ravandi and Kantarjian 2012, Burnett, Hills et al. 2013, Rowe and Lowenberg 2013, Lambert, Pautas et al. 2019). In the APL subtype of AML, GO was effective, both as a monotherapy or and in combination with RA, likely because of high surface expression of CD33 in APL cases (Jurcic, DeBlasio et al. 2000, Ravandi, Estey et al. 2009).

#### **1.5.4.3 Fms-like Tyrosine Kinase 3 (FLT3)**

*FLT3* mutations are commonly found in AML. About 25% of AML patients harbor *FLT3-ITD* mutations which are characterized by insertions of repeated base pairs ranging 3 to > 400 base pairs in size, within the juxtamembrane region (kinase domain) of the receptor. *FLT3* point mutations in the tyrosine kinase domain (*FLT3-TKD*) result in single amino acid substitutions within the activation loop, and these are found in 5-10% of AML patients (Shen, Zhu et al. 2011).

*FLT3* mutations associate with poor prognosis and poor clinical response. Indeed, AML patients (>60 years old) with normal karyotype but with *FLT3-ITD* have shorter remission duration and shorter OS compared to those having a normal karyotype but without *FLT3* mutation (Frohling, Schlenk et al. 2002). Given the mutation frequency and poor prognosis associated with *FLT3-ITD*, several small-molecule *FLT3* tyrosine kinase inhibitors (TKIs) arose in active clinical development (Table 7). Midostaurin was the first-in-class approved *FLT3* inhibitor (Levis 2017). Indeed, several trials showed that the addition of midostaurin to standard chemotherapy prolonged overall and event-free survival in *FLT3-ITD* AML patients (Fischer,

Stone et al. 2010, Stone, Fischer et al. 2012, Maziarz, Patnaik et al. 2016, Stone, Mandrekar et al. 2017).

Among the first generation promising FLT3 inhibitors, sorafenib proved potent against AML (Zhang, Konopleva et al. 2008, Zhao, Zhang et al. 2011). Many clinical trials have evaluated adding sorafenib to standard chemotherapy in the first-line induction therapy in AML or using it as maintenance therapy after allo-HCT (Chen, Li et al. 2014, Antar, Kharfan-Dabaja et al. 2015, Antar, Otrrock et al. 2017, Battipaglia, Ruggeri et al. 2017, Burchert, Bug et al. 2018, Bazarbachi, Labopin et al. 2019, Chappell, Geer et al. 2019).

Among the second generation of FLT3 inhibitors, Giltertinib was FDA approved for the treatment of adult relapsing patients or in refractory AML with a *FLT3* mutation (Dhillon 2019).

<b>Name of the drug</b>	<b>Kinase inhibitory profile</b>	<b>Disease under evaluation</b>	<b>Notes</b>
Sorafenib (Nexavar)	<i>CRAF</i> and <i>BRAF</i> <i>KIT</i> , <i>FLT3</i> , <i>VEGFR-2</i> , <i>VEGFR-3</i> and <i>PDGFR-β</i> <i>RAF/MEK/ERK</i> pathway	AML Hepatocellular carcinoma Renal cell carcinoma Thyroid carcinoma	Most of these kinases are involved in angiogenesis.
Quizartinib (AC220)	<i>FLT3/STK1</i> <i>CSF1R/FMS</i> <i>SCFR/KIT</i> <i>PDGFRs</i>	AML	It is the most potent <i>in vitro</i> FLT3 inhibitor.
Midostaurin (PKC412)	<i>FLT3</i> <i>KIT</i> , <i>PDGF-Rβ</i> , <i>VEGFR-2</i> <i>PKC</i>	AML MDS Aggressive systemic mastocytosis and mast cell leukemia	Inhibits FLT3 at very low doses, generally in the nanomolar range.
Lestaurtinib (CEP701)	<i>FLT3</i> <i>JAK2</i> <i>TRK A/TRK B/TRK C</i>	AML and MPN	—
Crenolanib (CP868596)	<i>FLT3-ITD</i> <i>FLT3-D835</i> <i>PDGFR-α</i> <i>PDGFR-β</i>	AML GIST Glioma	—

Gilteritinib (ASP2215)	<i>FLT3</i> <i>AXL</i> <i>ALK</i>	AML	—
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**Table 7: Most frequently used FLT3 inhibitors (Antar, Otrrock et al. 2017).**

Abbreviations: FLT3=FMS-like tyrosine kinase 3; GIST=gastrointestinal stromal tumor; ITD=internal tandem duplications; MDS=myelodysplastic syndrome; MPN=myeloproliferative neoplasms.

#### 1.5.4.4. Isocitrate Dehydrogenase (IDH) Inhibitors

IDH1 and IDH2 are normally homodimeric NADP-dependent enzymes catalyzing the oxidative decarboxylation of isocitrate to alpha-ketoglutarate during the Krebs cycle. NADP<sup>+</sup> will thus be reduced to NADPH and CO<sub>2</sub> will be released. IDH1 exerts its activity in the cytoplasm and peroxisomes, whilst IDH2 acts in the mitochondrial matrix. These enzymes are involved in the metabolism of glucose, fatty acids and glutamine; they regulate the cellular redox status. Mutations of *IDH1* or *IDH2* genes occur in 15–20% of all AML and mostly cluster with normal karyotype AML (Mardis and Wilson 2009, Marcucci, Maharry et al. 2010).

The IDH1 inhibitor AG-120 (Ivosidenib) and the IDH2 inhibitor AG-221 (Enasidenib) showed promising response rates in patients with AML in several phase I/II clinical trials (Yen, Travins et al. 2017, DiNardo, Stein et al. 2018, Pollyea, Tallman et al. 2019). The objective response rate (ORR) was 40% with AG-221 and 31% with AG-120 in relapsed/refractory AML patients. Overall, 76% of responses lasted longer than six months. In addition to standard responses, several studies have reported that IDH inhibition induces cellular differentiation in primary *IDH2*<sup>+</sup> AML *ex-vivo* and in xenograft mouse models (Wang, Travins et al. 2013, Yen, Travins et al. 2017, Abou Dalle and DiNardo 2018).

Recently, these two drugs were approved by the FDA for the treatment of adults with relapsed or refractory AML with *IDH1* or *IDH2* mutations (Kim 2017, Dhillon 2018). However, acquired resistance to IDH inhibition was reported in two *IDH2*-mutant AML patients who achieved a clinical response to enasidenib (Intlekofer, Shih et al. 2018).

#### 1.5.4.5 Demethylating agents

Demethylating agents incorporate into DNA and trap DNMTs, leading to their proteosomal degradation. Vidaza (5-azacytidine), decitabine (5-aza-2-deoxy-cytidine), and zebularine (2(1 H)-pyrimidinone riboside) belong to a class of cytosine analogs that were developed as inhibitors of DNA methylation. 5-azacytidine and decitabine improve survival in AML patients. Indeed, a phase III trial in elderly patients showed that azacytidine was associated with improved OS compared to patients who received common AML treatments including intensive chemotherapy (Dombret, Seymour et al. 2015).

On the other hand, a large phase III study showed a significant improvement of the CR rates but not in the OS in elderly patients who received decitabine compared to those who received supportive care and cytarabine (Malik and Cashen 2014). Several studies have reported that cytogenetic abnormalities may predict the outcome of patients treated with decitabine (Metzeler, Walker et al. 2012, Bejar, Lord et al. 2014, DiNardo, Patel et al. 2014, Welch, Petti et al. 2016). These mutations may correlate with better or poorer drug response in AML patients.

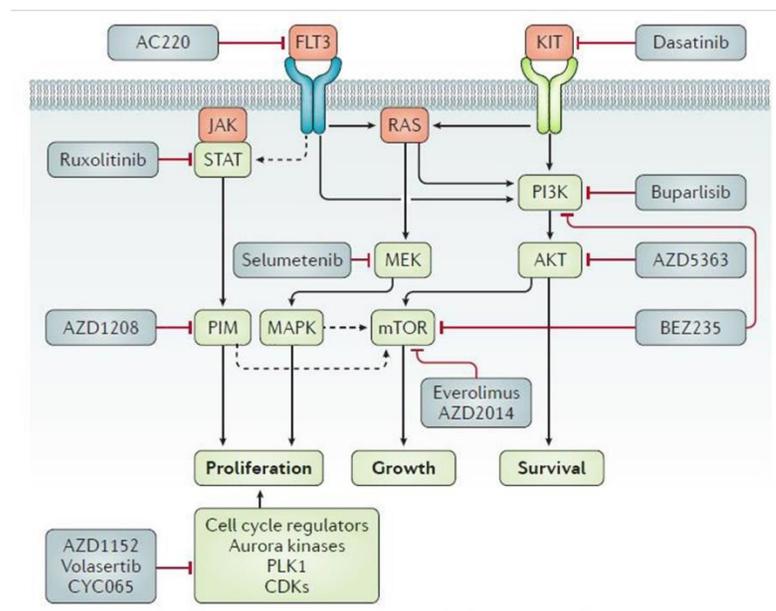
#### 1.5.4.6 Targeting abnormal signaling

Identification of deregulated signaling pathways and kinase mutations that sustain the growth, proliferation and survival of AML cells, herald a potentially new treatment era (Figure 5). The phosphoinositide 3-kinase (PI3K)–AKT–mammalian target of rapamycin (mTOR) axis is frequently activated in AML. Pan-PI3K, PI3K $\delta$ , dual PI3K–mTOR and AKT inhibitors show anti-leukemic activity *in vitro* (Chapuis, Tamburini et al. 2010, Allegretti, Ricciardi et al. 2015). Buparlisib (a pan-PI3K inhibitor) and BEZ235 (a dual PI3K–mTOR inhibitor) are the most advanced of these agents, and are currently in Phase I/ II trials where they demonstrate an acceptable toxicity profile but modest clinical activity (Ragon, Kantarjian et al. 2017, Herschbein and Liesveld 2018). Rapamycin-induced mTOR complex 1 (mTORC1) inhibition has some activity in patients with relapsed or refractory AML and induced partial response in four out of nine treated patients (Recher, Beyne-Rauzy et al. 2005). A Phase I study of everolimus in patients with relapsed AML in combination with chemotherapy demonstrated good tolerability and high rates of response

(Park, Kang et al. 2013). In addition, a phase II study of patients with relapsed/refractory AML, revealed that the combination of everolimus with azacitidine is tolerable, with promising clinical activity in advanced AML (Tan, Tiong et al. 2017).

The use of the mTOR kinase inhibitors (targeting mTORC1 and mTORC2) or PI3K–AKT inhibitors in combination with cytotoxic chemotherapy, mitogen-activated protein kinase (MAPK) pathway inhibitors or BCL-2 inhibitors may improve their efficacy (Vachhani, Bose et al. 2014, Su, Li et al. 2018). In addition, venetoclax, a bcl-2 inhibitor, was used in a phase II study and showed an improved ORR in elderly AML patients with previously limited treatment options and poor clinical outcome (Konopleva, Pollyea et al. 2016). In combination with demethylating agents such as decitabine or azacitidine (DiNardo, Pratz et al. 2018) or low dose cytarabine (Lin, Strickland et al. 2016), venetoclax showed tolerable safety and favorable ORR in elderly AML patients. Bases on these two studies, the FDA granted accelerated approval to venetoclax in combination with azacitidine or decitabine or low-dose cytarabine for the treatment of newly-diagnosed elderly AML patients.

Moreover, ibrutinib, an inhibitor of the Bruton tyrosine kinase (BTK) protein which is constitutively activated in most AML samples has some anti-leukemic activity (Rushworth, Murray et al. 2014). Ibruinib is selectively active in *FLT3*-ITD positive AML (Wu, Hu et al. 2016). The PLK1 inhibitor volasertib has significant activity in relapsed or refractory AML, with 12% of patients achieving CR with incomplete blood count recovery in AML, alone or in combination with cytarabine (Gjertsen and Schoffski 2015). Constitutive NF- $\kappa$ B signaling detected in leukemia stem cells can be reduced by proteasomal inhibitors and parthenolide (Guzman, Rossi et al. 2005).



**Figure 5: Targeting cell signaling to treat AML (Khawaja, Bjorkholm et al. 2016)**

Targeting deregulated signaling pathways represent new treatment strategies in AML. AC220 (also known as quizartinib), dasatinib and ruxolitinib inhibit the kinase activity of proteins encoded by mutated *FLT3*, *KIT* and *JAK2*, respectively. Phosphoinositide 3-kinase (PI3K; buparlisib), AKT (AZD5363), mammalian target of rapamycin (mTOR; everolimus and AZD2014), dual PI3K–mTOR (BEZ235) and MEK (selumetinib) inhibitors have been or are also in trials. Overexpressed PIM1 or PIM2 serine/threonine kinases are targeted by AZD1208. Alternatively, inhibitor therapy can be directed against kinases that control mitosis and cell cycle progression, including Aurora A and Aurora B kinases (AZD1152), polo-like kinase 1 (PLK1; volasertib) or cyclin-dependent kinases (CDK1 and CDK2; CYC065). Dashed arrows indicate the indirect pathway. MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription.

### 1.5.5 Allogeneic Stem Cell Transplant (allo-SCT)

Allogeneic stem cell transplantation (allo-SCT) is the preferred therapy in most young AML patients who are in first CR (Cornelissen, van Putten et al. 2007). Allo-SCT cures AML by both cyto-reduction of the conditioning regimen and the immunologic graft-versus-leukemia (GVL) effect (Hamilton and Copelan 2012).

In fact, only a minority of AML patients undergo transplantation because of older age, comorbidities, toxicity of prior therapy, inability to achieve a remission, and early relapse or refractory leukemia (Juliussen, Karlsson et al. 2011). The decision to perform allogeneic HCT

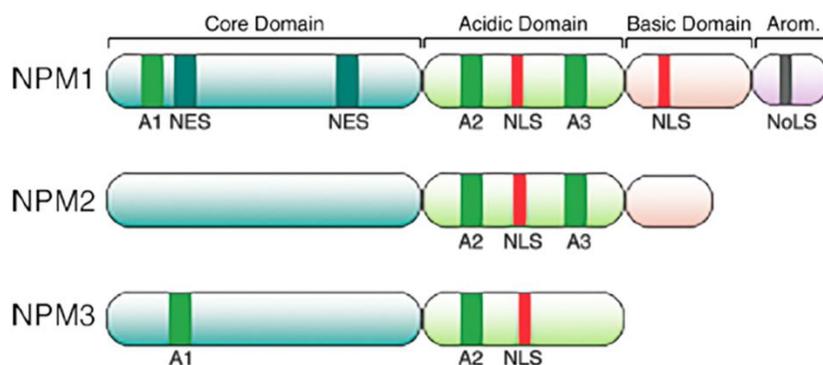
depends on the assessment of the risk-benefit ratio, based on cytogenetic and molecular genetic features as well as patient, donor, and transplant factors (Cornelissen and Blaise 2016).

Allogeneic HSCT is usually recommended if the relapse is expected to be >35% to 40%. It is considered the standard care in patients with intermediate II-risk and adverse-risk AML in first CR, but is not indicated for favorable-risk AML (Schlenk, Kayser et al. 2014, Ho, Schetelig et al. 2016). Indeed, it is considered as the only curative for patients with primary refractory disease (Cornelissen, Gratwohl et al. 2012, Cornelissen and Blaise 2016). However, in patients with intermediate I-risk AML, the role of allogeneic HSCT is still a debate (Cornelissen, Gratwohl et al. 2012). Therefore, establishing definitive and clear recommendations to benefit from transplant is needed.

## Chapter II: Nucleophosmin (NPM)

### 2.1 NPM family

The nucleophosmin (NPM)/nucleoplasmin of histone chaperones family consists of three major functional members (NPM1, NPM2, NPM3) with NPM1 being the prevalent form in all tissues (Chang and Olson 1990) (Figure 6). At a glance, all members of this family exhibit conserved structural motifs; an N-terminal core domain, an acidic domain and a nuclear localization signal, associated with a less conserved, disorganized C-terminus region (Box, Paquet et al. 2016) (Figure 6).



**Figure 6: Domain representation of human NPM1, NPM2 and NPM3 proteins (Box, Paquet et al. 2016).**

All proteins share a core, hydrophobic domain (*blue*) responsible for oligomerization and chaperone activity, followed by an acidic domain (*light green*) required for ribonuclease activity. A basic domain (*light orange*) implicated in nucleic acid binding is common to NPM1 and NPM2, but absent in NPM3. Finally, only NPM1 exhibits a C-terminal aromatic stretch (*purple*) required for its nucleolar localization. In addition, NPM members harbor nuclear-localization signals (NLS) (*red*), nucleolar-localization signal (NoLS, *gray*), nuclear export signal (NES) (*blue cyan*) and acidic clusters (A1, A2 and A3, *dark green*).

Striking differences in expression patterns, intracellular localization and functions exist between the three members of NPM family (Frehlick, Eirin-Lopez et al. 2007). The main functions of the three members are summarized in Table 8. NPM1 (also known as NO38, numatrin or B23) is a ubiquitously expressed nucleolar phosphoprotein that constantly shuttles between the nucleus and the cytoplasm (Federici and Falini 2013). NPM1 is directly implicated in human tumorigenesis (Grisendi, Mecucci et al. 2006). It is overexpressed in various tumors such as colon (Nozawa, Van Belzen et al. 1996) ovarian (Shields, Gercel-Taylor et al. 1997) and prostate (Subong, Shue et al. 1999) carcinomas.

Member	Location	Properties and functions
NPM1	Mainly nucleolar; wide tissue distribution (Spector, Ochs et al. 1984)	<p>Ribosome biogenesis (Herrera, Savkur et al. 1995)</p> <p>Nucleo-cytoplasmic transport (Valdez, Perlaky et al. 1994, Szebeni, Mehrotra et al. 1997)</p> <p>Centrosome duplication (Compton and Cleveland 1994)</p> <p>Embryonic development and genome stability through regulation of p53 and p14ARF (Colombo, Bonetti et al. 2005)</p> <p>DNA duplication (Okuwaki, Iwamatsu et al. 2001)</p> <p>Transcriptional regulation (Weng and Yung 2005)</p> <p>Histone chaperoning (Okuwaki, Matsumoto et al. 2001)</p> <p>Binding and folding of denatured proteins (Szebeni, Hingorani et al. 2003) and nucleic acid binding (Dumbar, Gentry et al. 1989)</p>
NPM2	Nuclear; only found in eggs and oocytes (Laskey, Honda et al. 1978)	<p>Binds histones and promotes chromatin assembly (Shackleford, Ganguly et al. 2001)</p> <p>Paternal chromatin decondensation (Philpott, Leno et al. 1991)</p>
NPM3	Mainly nucleolar (Huang, Negi et al. 2005) wide tissue distribution (Shackleford, Ganguly et al. 2001)	<p>Ribosomal RNA biogenesis (Huang, Negi et al. 2005) and potentially paternal chromatin decondensation in mammals (McLay and Clarke 2003)</p>

**Table 8: NPM family members**

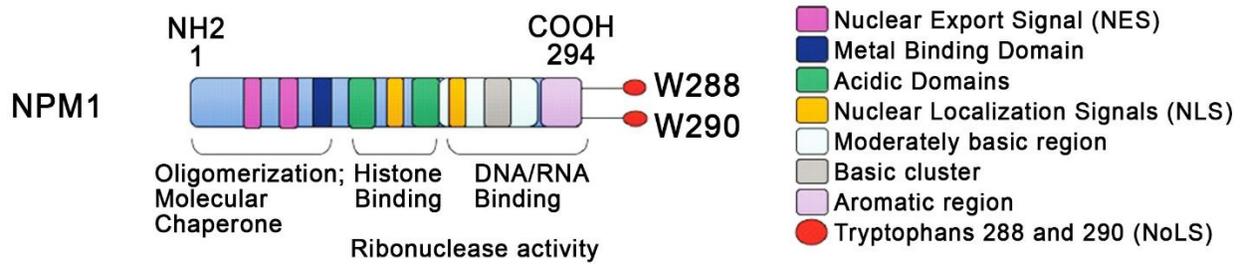
## 2.2 Structural and functional domains of NPM1

The *NPM1* gene, mapping to chromosome 5q35 in humans, contains 12 exons. *NPM1* encodes for a nucleolar phosphoprotein composed of 294 amino acids, with the molecular weight of 37 kDa. The protein has three structural and functional domains, an N amino-terminal oligomerization domain, a carboxy-terminal nucleic acid binding domain, and a histone binding middle domain (Okuwaki 2008) (Figure 7).

The N-terminus portion contains an oligomerization domain (1- 110 aa); It is involved in the chaperone activities toward proteins, nucleic acids, and histones. It contains two nuclear export signals (NES) responsible for nuclear-cytoplasmic shuttling (Okuwaki, Iwamatsu et al. 2001). The first NES (42–49 aa) associates with ribosomal protein L5 and 5S rRNA chaperone and moves the complex to the cytoplasm (Herrera, Savkur et al. 1995). The second NES sequence (94–102 aa) is required for centrosome localization (Falini 2010) (Figure 6).

The middle portion of NPM1 contains 2 acidic stretches that are required for the binding of basic histone (Okuwaki, Iwamatsu et al. 2001), and ribosomal proteins to facilitate nucleosome assembly and chromatin remodeling (Hingorani, Szebeni et al. 2000) (Figure 6). Between the two acidic regions, a ribonuclease activity motif which is critical for ribosome biogenesis and one nuclear localization signal (NLS 190–197 aa) are present (Bolli, De Marco et al. 2009).

The C-terminal domain contains basic regions (189-243 aa) which are involved in nucleic acid binding and ribonuclease activity (Hingorani, Szebeni et al. 2000). At the C-terminal end, NPM1 (244–294 aa) has the nucleolar localization signal (NoLS), and two tryptophan residues at positions 288 and 290; these residues are critical for retaining NPM1 in the nucleolus (Figure 7). The shuttling of NPM1 between different compartments (nucleolus-nucleus-cytosol) is a highly regulated process and is made possible through the various protein motifs (NES, NLS, and NoLS) (Falini, Martelli et al. 2011).



**Figure 7: Structure and functional domains of wild type NPM1 (Falini, Nicoletti et al. 2007).**

Starting from the N-terminus, the protein displays two nuclear export signal (NES) motifs (residues 42–49 and 94–102), a metal binding domain, two acidic regions (residues 120–132 and 160–188), a bipartite nuclear localization signal (NLS) motif (residues 152–157 and 190–197), a basic cluster inside a moderately basic region, and an aromatic region at the C-terminus unique to NPM isoform 1 containing the nucleolar localization signal (NLS) with tryptophan residues 288 and 290.

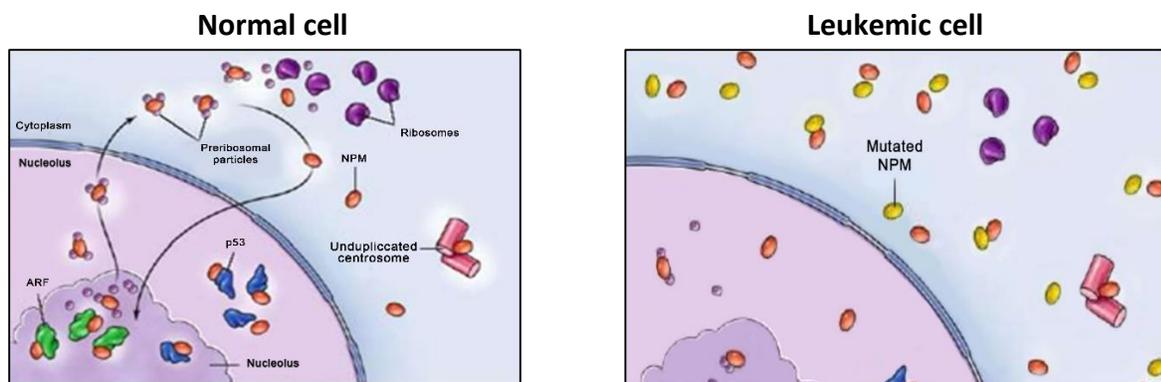
## 2.3 NPM1 functions

NPM1 is a multifunctional nuclear chaperone. It is an abundant and highly conserved phosphoprotein that normally resides in nucleoli, but continuously shuttles between the nucleus and cytoplasm (Yun, Chew et al. 2003). NPM1 contributes to various cellular processes. These include the transport of pre-ribosomal particles and ribosomal biogenesis, the response to stress stimuli such as UV irradiation and hypoxia, the regulation of crucial tumor suppressors such as P53 and p14<sup>ARF</sup>, the maintenance of genomic stability through the control of centrosome duplication, the participation in DNA-repair processes, and the regulation of DNA transcription through modulation of chromatin condensation and de-condensation events.

### 2.3.1 Ribosome biogenesis and histone chaperoning

Ribosomes play a central role in protein synthesis, growth and development. NPM1 is implicated in the processing and assembly of ribosomes due to its nucleocytoplasmic shuttling properties, its intrinsic RNase activity (Herrera, Savkur et al. 1995), its ability to bind nucleic acids (Wang, Baumann et al. 1994), to process pre-RNA molecules (Savkur and Olson 1998) and to act as a chaperone (Szebeni and Olson 1999). NPM1 provides all the necessary export signals and chaperoning capabilities that are required for the transport of maturing pre-ribosomal particles

from the nucleus to the cytoplasm (Figure 8). A major role of NPM1 is to mediate, through a chromosome region maintenance 1 (Crm1) -dependent mechanism, nuclear export of the ribosomal protein L5/5S rRNA subunit complex (Yu, Maggi et al. 2006). Indeed, blocking NPM1 nucleocytoplasmic shuttling inhibits the export of the 28S and 18S rRNA subunits (Yu, Maggi et al. 2006). Moreover, NPM1 interacts directly with a subset of ribosomal proteins RPS9 (Lindstrom and Zhang 2008), RPL23 (Wanzel, Russ et al. 2008), RPL5 (Yu, Maggi et al. 2006). NPM1 associates with rDNA and depletion of NPM1 or expression of a dominant negative *NPM1* mutant lacking histone chaperone activity leads to a decrease in rDNA transcription (Murano, Okuwaki et al. 2008). NPM1 facilitates cleavage of rRNA *in vitro* and acts as an endoribonuclease for the maturing rRNA transcript. Indeed, when tested on rRNA fragments synthesized from rDNA transcripts, NPM1 showed preferential cleavage of a short region in the middle of the internal transcribed region 2 (ITS2) of rRNA, which is removed during ribosome maturation (Savkur and Olson 1998). Knock down of NPM1 inhibits the processing of pre-ribosomal RNA into the mature 28S form and induces cell death (Itahana, Bhat et al. 2003).



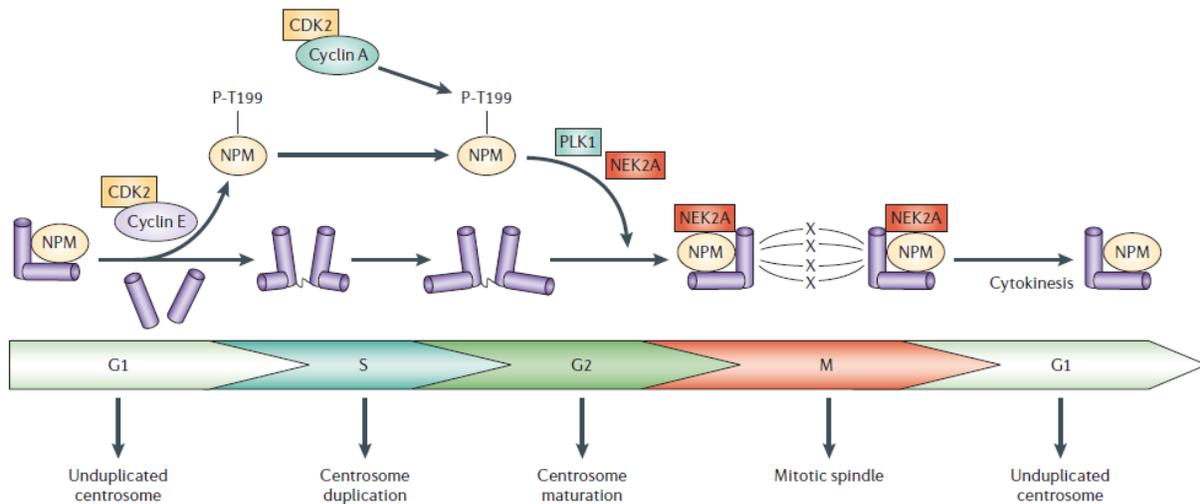
**Figure 8: NPM1 function in ribosome biogenesis**

NPM is a nucleolar phosphoprotein that shuttles between the nucleus and cytoplasm. Shuttling plays a fundamental role in ribosome biogenesis, since NPM transports preribosomal particles. In cytoplasm, NPM binds to the unduplicated centrosome and regulates its duplication during cell division. Furthermore, NPM interacts with p53 and its regulatory molecules (ARF, Hdm2/Mdm2) influencing the ARF-Hdm2/Mdm2-p53 oncosuppressive pathway

## 2.3.2 Maintenance of genome stability

### 2.3.2.1 Centrosome duplication

During mitosis, the nucleolus undergoes reversible disassembly, and many nucleolar proteins translocate from the nucleolus to the cytoplasm (Hernandez-Verdun and Gautier 1994). Specifically, NPM1 relocates at the chromosome periphery and in cytoplasmic entities called nucleolus-derived foci (NDF). NPM1 migrate then to the poles of the mitotic spindle, where it co-localizes with the nuclear mitotic apparatus protein (NUMA) (Zatsepina, Rousselet et al. 1999), that is associated with centrosomes in pro-metaphase and with the mitotic poles in metaphase (Compton and Cleveland 1994). The spindle pool bound form of NPM1 is modified, and presumably phosphorylated (Yao, Fu et al. 2004). NPM1 is identified as one of the substrates of CDK2/cyclin E; NPM1 phosphorylation at Threonine 199 (Thr199) plays a role in centrosome duplication (Figure 9) (Okuda, Horn et al. 2000).



**Figure 9: NPM1 involvement in centrosome duplication (Grisendi, Mecucci et al. 2006)**

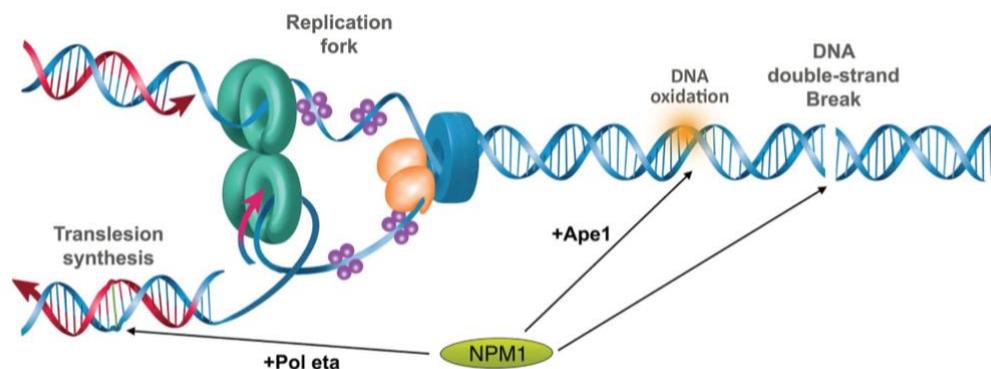
In early G1, centrosome-bound nucleophosmin (NPM) dissociates from centrosomes after phosphorylation on Threonine 199 by cyclin-dependent kinase 2 (CDK2)–cyclin E, which in turn triggers centriole separation and the initiation of centrosome replication. During centrosome duplication and maturation (S and G2 phases), cytoplasmic NPM is prevented from re-associating with centrosomes, an event that is probably mediated by the phosphorylation activity of CDK2–cyclin A. During mitosis, the re-association of NPM with the centrosome at the mitotic spindle depends on the phosphorylation activity of polo-like kinase 1 (PLK1) and never in mitosis gene A-related kinase 2 (NEK2A) mitotic kinases, the functions of which have been implicated in the control of correct spindle formation and chromosome segregation.

Centrosome amplification is also observed in the presence of hyper-phosphorylated NPM1 (Zhang, Shi et al. 2004). A proteomic analysis revealed that the phosphorylation of NPM1 is increased in melanoma cell lines, and is a marker of melanoma progression and aneuploidy (Bernard, Litman et al. 2003). Moreover, NPM1 is implicated in the control of chromosomal ploidy and DNA repair. NPM1 also associates with Centromere protein A (CENPA), a centromere protein which contains a histone H3 related histone fold domain, required for targeting to the centromere. This interaction implicates NPM1 also in centromere control (Foltz, Jansen et al. 2006).

### 2.3.2.2 DNA repair

NPM1 is required for DNA integrity (Colombo, Bonetti et al. 2005) and its overexpression is associated with improved DNA-repair process (Wu, Chang et al. 2002). *NPM1*<sup>-/-</sup> cells display an increased phosphorylation of histone  $\gamma$ -H2AX, a downstream target of the DNA repair kinases, along with formation of  $\gamma$ -H2AX and ATM positive DNA repair foci (Colombo, Bonetti et al. 2005).

Loss of NPM1 function is associated with increased genome instability (Wang, Budhu et al. 2005). NPM1 plays a critical role in the maintenance of genome stability through its interaction with unduplicated centrosomes (Wang, Budhu et al. 2005). Recently, a direct role of NPM1 in DNA repair mechanism was demonstrated (Figure 10).



**Figure 10: Overview of NPM1 functions in various DNA damage response pathways (Box, Paquet et al. 2016).**

Overview of NPM1 functions in various DNA damage response pathways. NPM1 modulates the BER pathway as well as the translesion synthesis by modulating the levels of apurinic/aprimidinic endonuclease 1 (APE1) and polymerase eta. In the absence of NPM1 or with the expression of non-

phosphorylated NPM1, double-strand break repair by homologous recombination fails to be completed, however the exact mechanism of NPM1 function in these pathways remain to be fully elucidated.

Following DNA double-strand break induction, NPM1 is recruited from the nucleolus into the nucleoplasm where it binds to the chromatin (Lee, Park et al. 2005). Phosphorylated NPM1 on Thr199 is recruited to the site of DNA double-strand breaks, and co-localizes with other DNA repair proteins such as  $\gamma$ H2AX and BRCA1 (Koike, Nishikawa et al. 2010). When NPM1 is depleted or non-phosphorylated, the repair of DNA DSBs is not completed. Moreover, increased DNA lesions were detected in cells expressing a mutant of Thr199.

NPM1 is also a key player of the translesion synthesis (TLS) pathway (Ziv, Zeisel et al. 2014). NPM1 regulates TLS by binding to and stabilizing DNA Polymerase Eta (POLH, pol $\eta$ ). NPM1 transcriptionally regulates the Nucleotide Excision Repair (NER) protein PCNA (Wu, Chang et al. 2002). NPM1 has been also shown to modulate the BER (base excision repair) pathway through control of the apurinic/apyrimidinic endonuclease 1 (APE1) protein levels and modulation of the AP-site incision activity of APE1, required for BER (Poletto, Lirussi et al. 2014, Vascotto, Lirussi et al. 2014). Finally, dephosphorylation of NPM1 on Thr199, 234 and 237 residues enhance the interaction between NPM1 and the retinoblastoma tumor suppressor protein (pRB), which then allows the release of E2F1 from pRB. E2F1 subsequently functions to transcriptionally activate several downstream DNA repair genes, including *Xeroderma pigmentosum*, complementation group C (XPC), DNA-binding protein 2 (DDB2) and Replication *protein A 14* (RPA14), favoring DNA repair (Lin, Tan et al. 2010).

### **2.3.3 Regulation of the tumor suppressor proteins P53 and p14<sup>ARF</sup>**

#### **2.3.3.1 NPM1 and p14<sup>ARF</sup>**

*p14<sup>ARF</sup>* is a tumor suppressor gene encoding for a nucleolar protein that is involved in cell-cycle arrest and apoptosis. p14<sup>ARF</sup> inhibits MDM2 (Mouse double minute 2), the negative regulator of P53, by re-localizing it to the nucleolus (Weber, Taylor et al. 1999); this leads to P53 stabilization and enables its transcriptional response (Kamijo, Zindy et al. 1997).

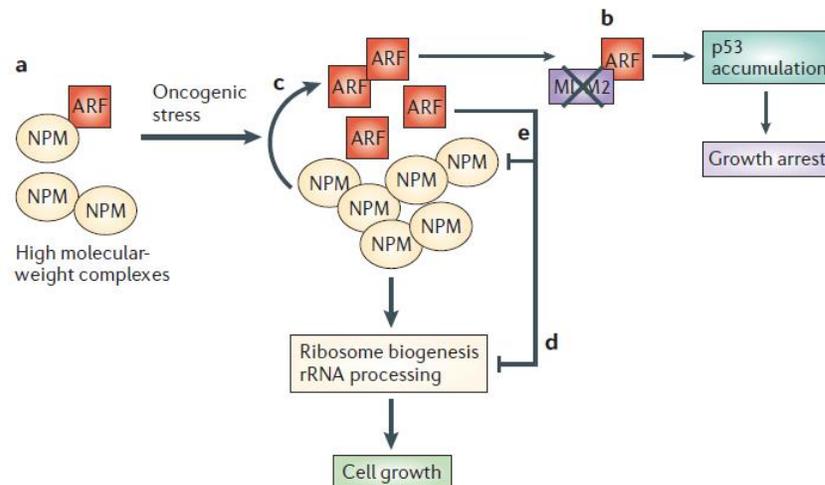
In response to stress stimuli, p14<sup>ARF</sup> accumulates within the nucleolus where it associates in high-molecular-mass complexes with NPM1 (Itahana, Bhat et al. 2003). This leads to the stabilization of p14<sup>ARF</sup> by retarding its turnover (Kuo, den Besten et al. 2004) (Figure 11 a, c). Recently, a NPM1-interacting motif was described in the C-terminal region of p14<sup>ARF</sup> (Luchinat, Chiarella et al. 2018). This motif binds to a region in the N-terminal domain of NPM1 and form soluble supramolecular complexes upon binding. p14<sup>ARF</sup> mutants that are unable to bind to NPM1 are unstable and functionally impaired. Inhibition of the proteasome machinery only partially restores the stability of these mutants. This indicates that NPM1 protects p14<sup>ARF</sup> from both proteasome-dependent and proteasome-independent degradation (Kuo, den Besten et al. 2004). In addition, in cells that lack both P53 and NPM1, p14<sup>ARF</sup> is unstable and excluded from the nucleolus. These cells grow faster than control cells which makes them more susceptible to transformation (Colombo, Bonetti et al. 2005). All together, these findings indicate that NPM1 protects p14<sup>ARF</sup> from degradation and localizes it to the nucleolus. This is responsible for protecting p14<sup>ARF</sup> from degradation, and for its nucleolar compartmentalization. Cells that lack NPM1 show decreased stability of the p14<sup>ARF</sup> protein and susceptibility to transformation. Therefore, NPM1 might participate in the ARF-mediated response to oncogenic stress.

Reciprocally, when upregulated in response to oncogenic stimuli, p14<sup>ARF</sup> can inhibit the production of rRNA by retarding the processing of 47S, 45S and 32S precursors, which in turn inhibits ribosome assembly and therefore proliferation (Sugimoto, Kuo et al. 2003). This means that the interaction between p14<sup>ARF</sup> and NPM1 in the nucleolus facilitates the contact between ARF and the ribosomal processing machinery (Figure 11d). Moreover, p14<sup>ARF</sup> directly regulates ribosome biogenesis by binding to the RNA polymerase I (RP1) transcription termination factor (TTF1) and inhibiting its nucleolar import (Lessard, Morin et al. 2010).

ARF also promotes ubiquitination of NPM1 and accelerated turnover (Itahana, Bhat et al. 2003) (figure 11e). However, p14<sup>ARF</sup> does not possess a ubiquitylating activity, indicating that ARF-induced NPM1 degradation is triggered indirectly in response to ARF-dependent cell-cycle arrest.

Moreover, several reports have shown that p14<sup>ARF</sup> induces SUMOylation of many nucleolar proteins including NPM1 (Xirodimas, Chisholm et al. 2002, Chen and Chen 2003, Woods, Xirodimas et al. 2004, Tago, Chiocca et al. 2005). More recently, it has been demonstrated that ARF induces Tripartite motif-containing protein (TRIM28) -mediated SUMOylation of NPM1 which contributes to its centrosomal localization and suppression of centrosome amplification (Neo, Itahana et al. 2015).

In conclusion, NPM1 plays an important role in the regulation of p14<sup>ARF</sup> function especially in the cellular response to oncogenic stress, while p14<sup>ARF</sup> controls NPM1 function through the inhibition of rRNA processing, transport of pre-ribosomal particles, and inhibition of protein synthesis.



**Figure 11: NPM1 and p14<sup>ARF</sup> regulate cellular growth and proliferation through the control of each other's stability and/or activity (Grisendi, Mecucci et al. 2006).**

(a) NPM associates with ARF in the nucleolus. Increased expression of both ARF and NPM occurs in response to oncogenic stimulation. ARF activates and promotes both P53-dependent (b) and P53-independent growth-arrest pathways (c–e). (b) ARF inhibits MDM2, which leads to P53 activation and the suppression of cell proliferation. (c–e) higher levels of NPM facilitate the accumulation of ARF by stabilizing it, whereas ARF negatively regulates ribosomal RNA processing (d), and even opposes NPM nucleocytoplasmic shuttling activity (e).

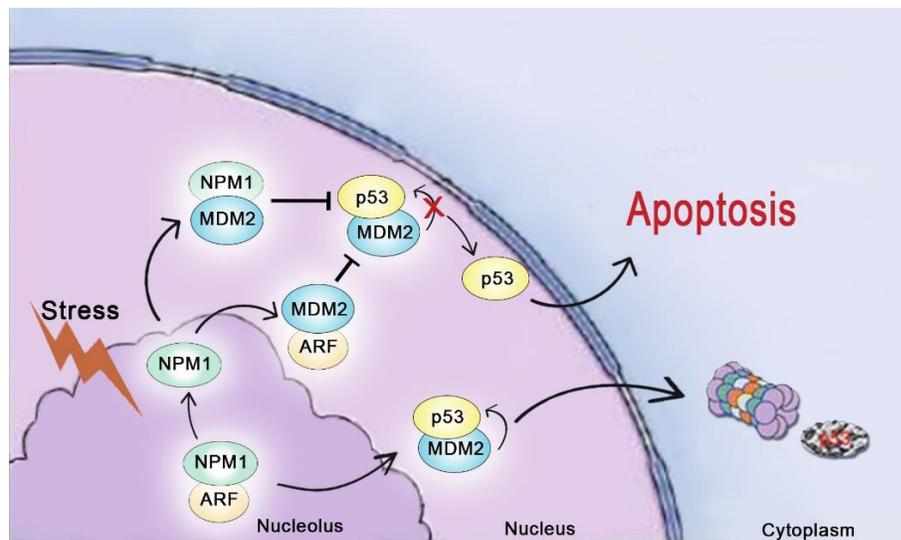
### 2.3.3.2 NPM1 and P53

*TP53* is a tumor suppressor gene regarded as the “guardian of the genome”. It encodes for the P53 protein playing a fundamental role in cycle arrest and apoptosis through transcriptional regulation (Freed-Pastor and Prives 2012). Activation of P53 can be triggered by a

series of stress signals such as DNA damage, ribosome dysfunction, hypoxia and activation of an oncogene (Brady and Attardi 2010). The level of P53 in the cell is tightly regulated by MDM2 (Oren and Prives 1996). A link between P53 stability and nucleolar integrity was demonstrated (Rubbi and Milner 2003). For instance, the interference with rRNA processing creates a state of nucleolar stress and consequently a P53-dependent cell-cycle arrest, revealing that the nucleolus functions as a stress sensor to maintain a low level of P53 in normal cells. When NPM1 is overexpressed, a P53 response is triggered, perhaps by directly binding to and stabilizing P53 (Colombo, Marine et al. 2002, Itahana, Bhat et al. 2003). Reciprocally, in response to P53, several nucleolar proteins translocate from to nucleolus and participate in the initiated P53-responses (Horn and Vousden 2004).

NPM1 interacts with P53 regulatory molecules (p14<sup>ARF</sup>, Hdm2/Mdm2) influencing the p14<sup>ARF</sup> -HDM2/MDM2-P53 oncosuppressive pathway (Figure 12). Indeed, NPM1 interacts with MDM2 and thus protects P53 from degradation (Jin, Itahana et al. 2004, Kurki, Peltonen et al. 2004). Interestingly, NPM1 was also reported to associate with P53 (Colombo, Marine et al. 2002). NPM1 also regulates both intrinsic and extrinsic apoptosis pathways. When NPM1 is overexpressed, it prevents P53 translocation to the mitochondria, thus protecting the cells from apoptosis (Dhar and St Clair 2009).

These findings indicate that NPM1 is a key player in the cellular response to stress through the potentiation of the P53 pathway in favor of DNA repair. In this context, NPM1 would once again function as a tumor-suppressor molecule. However, P53 stabilization is observed in cells that lack NPM1 which mean that NPM1 is not required for P53 activation (Colombo, Bonetti et al. 2005).



**Figure 12: P53/ p14<sup>ARF</sup> /mdm2 axis and NPM1**

Regulation of apoptosis by NPM1. In unstressed cells, p14<sup>ARF</sup> and NPM1 form a dimer in the nucleoli, allowing MDM2 to target P53 for proteasomal degradation. Following a stress, such as DNA damages, p14<sup>ARF</sup> and NPM1 dissociate and relocate to the nucleus where they sequester MDM2, leading to the stabilization and activation of P53. P53 then induces the transcription of various genes involved in cell-cycle arrest, DNA repair and apoptosis.

## 2.4 Post-translational modifications of NPM1

Post-translational modification (PTM) are referred to by the covalent addition of functional groups on one or several amino acids. These modifications can regulate the subcellular localization, activate or inhibit signaling pathways, alter interactions with other molecules or contribute to the regulation of expression and stability of modified proteins. Many studies highlighted the role of SUMOylation, ubiquitination and phosphorylation, in regulating the activity of many proteins including NPM1.

### 2.4.1 SUMOylation

The ubiquitin-related SUMO (SUMOylation) system is conserved in all eukaryotes (Han, Feng et al. 2018). It is a reversible post-translational modification that involves the addition of one or more “Small Ubiquitin-like Modifiers” (SUMO) conjugates on a particular lysine of a target protein. SUMOylation requires three-steps involving three enzymes (E1, E2, and E3) through a

process similar to that of the Ubiquitination (Melchior, Schergaut et al. 2003). In humans, four SUMO isoforms were identified (Cappadocia and Lima, 2018; Flotho and Melchior, 2013; Gareau and Lima, 2010). A major difference between the different isoforms of SUMO proteins is a lysine (K11) found on the N-terminal domain of SUMO-2 and 3. This lysine is found within the consensus sequence of SUMOylation which allows the SUMO2-3 proteins to be SUMOylated themselves, leading to a chain of poly-SUMOylation (Tatham, Jaffray et al. 2001). SUMO-1 does not have this lysine and therefore it can be only part of a mono-SUMOylation reaction (Bayer, Arndt et al. 1998). Nonetheless, SUMO1 can also act as a terminator of a poly-SUMO chain allowing the formation of mixed chains of SUMO1, 2 and 3 (Matic, Macek et al. 2008). The SUMO-4 isoform possesses K11 lysine, but its involvement in poly-SUMOylation was not described yet (Bohren, Nadkarni et al. 2004).

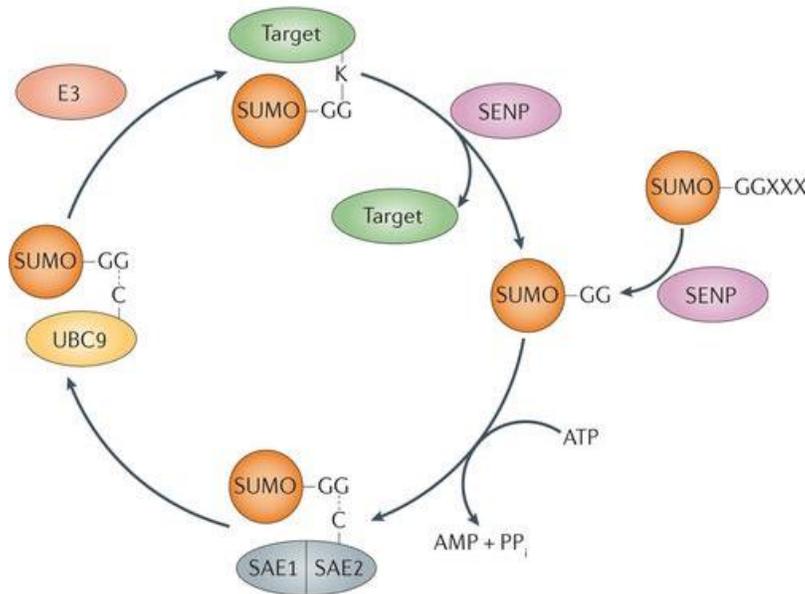
SUMOylation is a heavily used post-translational protein modification that dictates many functions: It regulates a large number of biological processes, including DNA damage repair, immune responses, carcinogenesis, regulation of mitochondrial division, regulation of ion channels, cell cycle progression and apoptosis (Zhao 2007). Indeed, cells lacking UBC9 exhibit major defects in cell cycle progression as well as in chromosome segregation (Nowak and Hammerschmidt 2006).

#### **2.4.1.1 SUMO enzymatic cascade**

SUMO and the ubiquitin cycles are similar, but differ in their function. A ubiquitin-modified target protein is essential for its recognition and degradation by the proteasome, while a SUMO-modified protein is more stable (Gill 2004). For instance, SUMOylation modulates protein-protein interactions triggering the localization and functional regulation of target proteins (Bogachek, Chen et al. 2014, Leidner, Voogdt et al. 2014)

All SUMO proteins undergo the same enzyme catalytic mechanism on substrate proteins for attachment or dissociation. The SUMO cycle consists of maturation, activation, conjugation, and ligation steps (Figure 13). The mature form of SUMO is activated by the E1 enzyme. The activated SUMO is then transferred to the active cysteine site of the E2 conjugating enzyme.

Afterwards, SUMO is attached to a specific lysine residue in the substrate. Of note, all these enzymes are essentially found in the nucleus (Melchior, Schergaut et al. 2003, Han, Feng et al. 2018). In some cases, UBC9 can induce direct SUMOylation without the intervention of E3 ligase (Okuma, Honda et al. 1999, Chu and Yang 2011).



**Figure 13: The catalytic cycle of SUMOylation (Hendriks and Vertegaal 2016).**

The catalytic cycle of SUMOylation. **Maturation:** Small ubiquitin-like modifier (SUMO) precursors are cleaved by members of the SUMO1/sentrin specific peptidase (SENP) family to expose a C-terminal di-glycine motif. **Activation:** The mature form of SUMO is then activated by the E1 enzyme SAE1/SAE2 which is ATP-dependent. **Conjugation:** The activated SUMO is then passed to the active site cysteine of the E2 conjugating enzyme, Ubc9. **Ligation:** SUMO is then attached to specific lysine residue in the substrate which usually requires E3 ligases. **De-modification:** SUMO proteins are removed from substrates by SENP, and free SUMO proteins are available for another catalytic cycle.

#### 2.4.1.2 SUMO-proteases: de-SUMOylation enzymes

SUMOylation is a reversible and highly dynamic process in which a step of de-modification is required and involves the removal of a SUMO terminal glycine from the lysine residues of the target protein. Specific cysteine proteases of the SENP family reverse SUMO conjugation in mammalian cells (Figure 13) (Melchior, Schergaut et al. 2003, Nayak and Muller 2014). Six members of SENP (SENP1, SENP2, SENP3, SENP5, SENP6, and SENP7) were identified in humans (Mukhopadhyay and Dasso 2007). They orchestrate multifaceted de-conjugation events to

coordinate gene expression, DNA damage response and inflammation. Another SENP called SENP8 was described later and is known to process the full length of NEDD8 to its mature form (Enchev RI et al, 2015). SENPs differ by their intracellular distributions and by their substrate specificities. While SENP-3 and SENP-5 localize to the nucleolus, SENP-2 localizes in nuclear-related structures. SENP-1 and SENP-6 present a dual nuclear and cytoplasmic localization, (Hay 2013). SUMO1 and SUMO2/3 proteins can be de-conjugated by SENP1 and SENP2 , while SUMO2/3 protein can be mainly dissociated by SENP3 and SENP5 (Reverter and Lima 2004). SUMO2/3 poly chain is dissociated by SENP6 and SENP7 (Mendes AV et al, 2016).

#### **2.4.1.3 SUMO consensus sequence**

The covalent modification of substrates by SUMO is primarily directed to lysine residues located within the conserved SUMO consensus motif (SCM),  $\psi$ -K-X-E/D, where  $\psi$  is a hydrophobic residue, K is the target lysine, X an amino acid, E is a glutamic acid and D is an aspartic acid (Rodriguez, Dargemont et al. 2001). This sequence is shown to be directly recognized by the catalytic cleft of Ubc9 (Sampson, Wang et al. 2001).

#### **2.4.1.4 SUMO Interacting Motif (SIM)**

SUMO can regulate protein functions *via* non-covalent binding to so-called SUMO-interaction motifs (SIM). SIMs are defined by a short hydrophobic core region consisting of V/I-X-V/I- where V is a valine, I is an isoleucine and X can be any residue (Song, Durrin et al. 2004). This hydrophobic sequence is often flanked by an acidic stretch (glutamic acid residues) and/or serine and threonine residues (Hecker, Rabiller et al. 2006). SIM itself is prone to regulation. For instance, SIM phosphorylation induces a change preference for SUMO1 instead of SUMO2. This is mainly due to lysine residue (lysine 39) on SUMO1, but not SUMO2, capable of interacting with the negative loads of phosphate on the phosphorylated SIM motif (Hecker, Rabiller et al. 2006).

### 2.4.1.5 NPM1 SUMOylation

Regulation of NPM1 through its post translational modifications is critical to study NPM1-associated diseases. The nucleolus is heavily implicated in SUMOylation/de-SUMOylation cycles (Nishida, Tanaka et al. 2000). p14<sup>ARF</sup> binds NPM1 on both lysine 230 and 263 and mediates its SUMOylation, while SENP3 catalyzes de-sumoylation of NPM1–SUMO2 conjugates (Yun, Wang et al. 2008). A novel binding partner of p14<sup>ARF</sup>, TRIM28, is an E3 ligase responsible for p14<sup>ARF</sup> - mediated SUMOylation of NPM1 (Neo, Itahana et al. 2015).

NPM1 SUMOylation on lysine 263 is essential for its localization in the nucleolus and the centrosomes (Liu, Liu et al. 2007). NPM1/K263 mutant shows an abnormal localization, causing altered centrosome duplication and interference with cell cycle regulation (Colombo, Alcalay et al. 2011).

Rb interacts with NPM1, allowing the transcriptional activation of E2F and therefore the activation of DNA repair genes. Interestingly, K263R fails to bind Rb demonstrating that NPM1 SUMOylation on lysine 263 is essential for its binding to Rb (Liu, Liu et al. 2007).

In addition, NPM1 SUMOylation was shown to prevent maturation of 28S rRNA from its precursor 32S rRNA, which in turn affects ribosomal biogenesis and cell cycle. Conversely, the nucleolar de-SUMOylating enzyme, SENP-3, leads to NPM1 de-SUMOylation, promoting rRNA synthesis after processing of the 32S pre-RNA to 28S pre-RNA then to 5.8S pre-RNA (Haindl, Harasim et al. 2008). Depletion of SENP3 by short interfering RNA interferes with nucleolar ribosomal RNA processing and inhibits the conversion of the 32S rRNA species to the 28S form (Haindl, Harasim et al. 2008).

### 2.4.2 Ubiquitination

Ubiquitination is a protein post-translational modification in which an ubiquitin protein is added on one or more lysine in the target protein. Indeed, it occurs by the covalent bonding of the C-terminal glycine of an ubiquitin molecule to a lysine residue on a substrate protein. Ubiquitin contains seven lysines (K6, K11, K27, K29, K33, K48, and K63), which can be conjugated to another ubiquitin to form polyubiquitin chains, or to other target proteins (Hershko 1996,

Hershko 2005). Ubiquitin (Ub) is a small protein modifier of 76 amino acids (8 kDa) whose covalent addition to various proteins in the cell dictates their fate and plays many biological functions. Aside from proteasomal degradation, ubiquitination plays important roles in transcriptional regulation, protein trafficking, including endocytosis and lysosomal targeting, and activation of kinases involved in signaling processes (Neutzner and Neutzner 2012). The enzymes of de-ubiquitination or DUB (Deubiquitinating enzymes) catalyze the cleavage of these precursors to ensure the availability of active ubiquitin protein (Pickart and Eddins 2004).

#### **2.4.2.1 Ubiquitin enzymatic cascade**

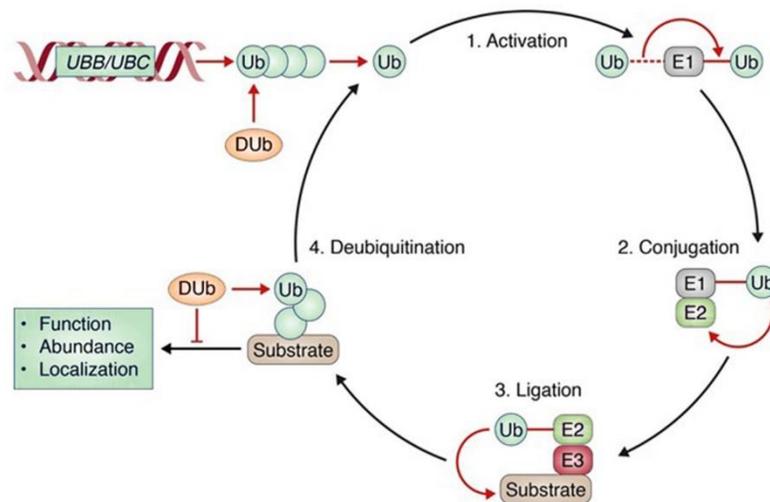
Ubiquitination is a three-step process catalyzed by different enzymes; the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin protein ligase (E3) (Figure 14). Sometimes, it requires the presence of elongation enzymes (Pickart and Eddins 2004, Lanfranca, Mostafa et al. 2014).

#### **2.4.2.2 De-ubiquitination Enzymes**

Like SUMOylation, ubiquitination is a highly dynamic and reversible post translational modification. The removal of ubiquitin chains from substrate proteins is carried by a class of highly specific cysteine proteases termed “de-ubiquitinases” or “DUBs”. Humans have approximately 80 DUBs that remodel ubiquitin modifications and antagonize ubiquitin-driven functional outcomes (Eletr and Wilkinson 2014).

DUBs are categorized into five families based on their catalytic function: Serine proteases, threonine proteases, cysteine proteases, aspartic proteases and metallo-proteases (Eletr and Wilkinson 2014). DUBs play an essential role in the recycling of ubiquitin. Indeed, when a protein is post translationally conjugated to ubiquitin and targeted for proteasomal degradation, DUBs ensure the recycling of ubiquitin proteins (Welchman, Gordon et al. 2005). Therefore, the levels of free and conjugated ubiquitin are generally stable although its expression level varies from a cell to another (Shabek and Ciechanover 2010). Free ubiquitin constitutes 40 to 60% of total

ubiquitin (Haas and Bright 1987, Poneles, Hirsch et al. 2005, Patel and Majetschak 2007), and this ratio is maintained by DUBs (Shabek and Ciechanover 2010).



**Figure 14: The catalytic cycle of Ubiquitination (Heaton, Borg et al. 2016).**

Ubiquitin (Ub) expresses as an inactive polyprotein, encoded by the *UBB* and *UBC* genes. DUBs cleave this polyprotein into monomers that are activated by the E1-activating enzyme, involving the energy-dependent adenylation of the ubiquitin C-terminal glycine. The ubiquitin-adenylate intermediate (dashed line) converts into a covalent thioester bond (solid line). (2) Ubiquitin transfers to the active site cysteine residue of an E2-conjugating enzyme. (3) The E3 directly or indirectly transfers the E2-bound ubiquitin to a substrate acceptor residue, forming an isopeptide bond. (4) DUBs remodel ubiquitin modifications and antagonize ubiquitin-driven functional outcomes.

### 2.4.2.3 NPM1 ubiquitination

In addition to SUMOylation, NPM1 is also ubiquitinated (Itahana, Bhat et al. 2003, Sato, Hayami et al. 2004, Enomoto, Lindstrom et al. 2006). Several studies proposed that the nucleolar interaction between p14<sup>ARF</sup> and NPM1 promotes the ubiquitination of overexpressed NPM1 and its subsequent degradation (Itahana, Bhat et al. 2003, Colombo, Bonetti et al. 2005). Moreover, NPM1 is mono-ubiquitinated by the E3 ubiquitin ligase, BRCA1–BARD1 (BRCA1-associated RING domain protein) (Sato, Hayami et al. 2004) in a process that is not linked to protein degradation (Nishikawa, Ooka et al. 2004). Indeed, NPM1 co-localizes with BRCA1–BARD1 at the centrosome during mitosis, playing an important role in the stabilization and the maintenance of NPM1 at the spindle poles (Sato, Hayami et al. 2004, Joukov, Groen et al. 2006).

USP36 is a de-ubiquitylating enzyme that removes ubiquitin from NPM1 leading to its stabilization and improved nucleolar function. In fact, depletion of USP36 impairs ribosome biogenesis (Endo, Matsumoto et al. 2009). Moreover, USP36-depleted cells have abnormal nucleolar morphology indicating that the nucleolar structure itself is not well developed in USP36-depleted cells. Interestingly, NPM1 itself can target USP36 to nucleoli by direct binding.

### 2.4.3 NPM1 Phosphorylation

Being a nucleolar phosphoprotein, NPM1 protein is extensively modified by phosphorylation at many different consensus sites. Several cyclin-dependent kinases (CDKs) can phosphorylate NPM1. For instance, Cyclin-B/Cdc2 kinase phosphorylates NPM1 at T199, T219, T234 and T237 residues (Peter, Nakagawa et al. 1990). Phosphorylation of all four threonine residues are essential for NPM1 mediated-ribosomal biogenesis (Szebeni, Hingorani et al. 2003). Indeed, Cyclin-B/Cdc2-induced NPM1 phosphorylation helps to inhibit the cleavage of r-RNA (Bolli, De Marco et al. 2009). Moreover, NPM1 is identified as a substrate for Casein kinase 2 (CK2) (Szebeni, Hingorani et al. 2003). The S125 phosphorylation by CK2 during interphase modulates its nuclear-cytoplasmic mobility making NPM1 more mobile in the nucleolus (Negi and Olson 2006). T199 at the C-terminus of the NPM1 is phosphorylated by CDK1 at the start of mitosis which may contribute to its dissociation from the nucleolus during mitosis (Negi and Olson 2006). CDK2/cyclin E also phosphorylate NPM1 at T199. NPM1 dissociates from centrosomes by CDK2/cyclin E-mediated phosphorylation thus initiating centrosome duplication (Okuda, Horn et al. 2000). Polo-like kinases, Plk1 and Plk2 mediate NPM1 phosphorylation at S4 residue. Alterations of Plk1-induced NPM1-S4 phosphorylation *in vitro* resulted in multiple mitotic defects, including aberrant numbers of centrosomes, elongation and fragmentation of nuclei, and incomplete cytokinesis (Zhang, Shi et al. 2004). Plk2-mediated phosphorylation contribute to the regulation of centriole duplication (Krause and Hoffmann 2010). NPM1 undergoes phosphorylation at S125 by Aurora Kinases A and B, which plays a major role in cytokinesis (Shandilya, Senapati et al. 2014).

Finally, it has been shown that phosphorylation of T95 or S125 shifts the thermodynamic equilibrium in favor of monomeric form thereby facilitating kinase accessibility to residues that were structurally occluded in the pentameric fold (Mitrea, Grace et al. 2014).

#### **2.4.4 NPM1 acetylation**

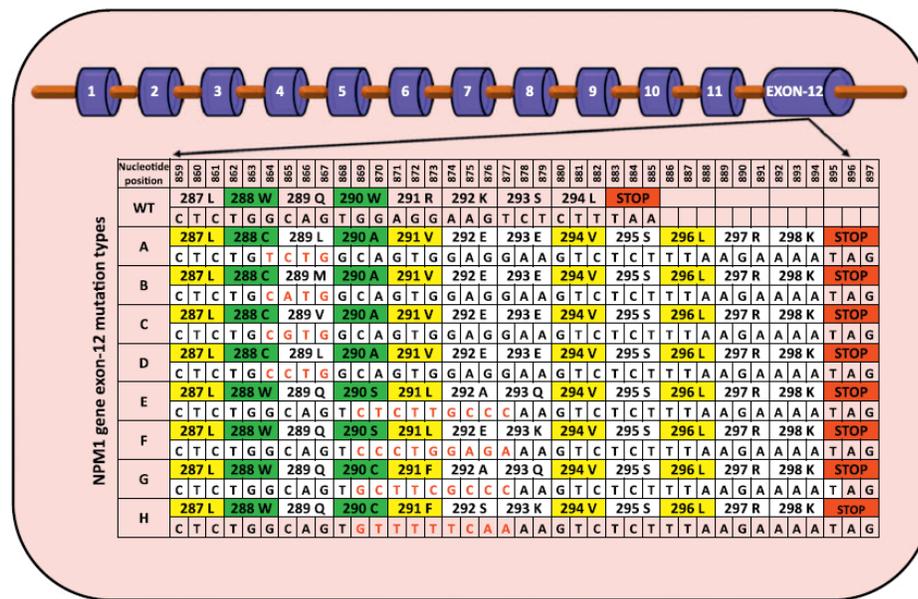
N-acetylation is a post and co-translational modification. It consists of the addition of an acetyl group on the  $\epsilon$ -amine group of a lysine or on the amino-terminal group of the protein (Walsh 2006). This modification was first observed on lysine residues of histones, which associate with DNA in order to regulate gene expression (Lin, Zhou et al. 2014). The transfer of an acetyl group neutralizes the positive charge of lysine residues and abolishes its electrostatic bond with the phosphate group of the DNA, therefore resulting in the decrease of histone-DNA interaction. Thus, the DNA is more available to accommodate the RNA polymerase (Lin, Zhou et al. 2014). The equilibrium between acetylation and deacetylation is very finely regulated. These reactions are catalyzed by histone acetyltransferases (HATs) and histones deacetylases (HDAC), respectively (Vogelauer, Wu et al. 2000). In addition to histones, many other proteins undergo acetylation (Lin, Zhou et al. 2014). Indeed, it had been reported that C-terminus K212, K215, K229, K230, K257, K267, and K292 sites of NPM1 are acetylated by p300 acetyltransferase (Shandilya, Swaminathan et al. 2009). Acetylated NPM1 localizes to the nucleoplasm in association with RNA polymerase II, resulting in the activation of target genes (Shandilya, Swaminathan et al. 2009). Acetylation of NPM1 affects its histone chaperone function and induces transcription of chromatin (Swaminathan, Kishore et al. 2005).

Deacetylation of NPM1 by class III HDAC, SIRT1 significantly reduces transcription of the genes (Shandilya, Swaminathan et al. 2009).

## Chapter III: *NPM1* mutations in AML

### 3.1 Genetic alterations of *NPM1* in AML

*NPM1* is one of the most frequently mutated genes in AML patients, accounting for 30% of cases (Falini, Mecucci et al. 2005). While chromosomal translocations of *NPM1* resulting in fusion oncoproteins (*NPM-ALK*, *NPM-RAR $\alpha$* , *NPM-MLF1*) were reported in some leukemia and lymphoma types (Falini, Nicoletti et al. 2007), mutations in *NPM1* are the most common in AML. These are mostly found at exon 12 in AML patients with normal karyotype (Rau and Brown 2009). These mutations consist in the duplication or insertion of small nucleotide stretches (Falini, Mecucci et al. 2005, Falini, Nicoletti et al. 2007) and were named alphabetically in the order of their discovery (Jeong, Lee et al. 2007). Mutation A is the most frequent *NPM1* mutation; It accounts for about 75-80% of cases of patients with normal karyotype (Falini, Mecucci et al. 2005). Mutation B and D occur in around 10% and 5% of AML cases respectively (Falini, Nicoletti et al. 2007), while other mutations occur in less than 1% of all cases. Type A mutations are characterized by a 'TCTG' tetranucleotide 'tandem' duplication while Types B/C/D mutations carry CATG, CGTG and CCTG tetranucleotide insertions respectively (Figure 15) (Kunchala, Kuravi et al. 2018). Although many other mutations were reported, the consequences at the protein level are similar and the same frameshift is observed in all exon-12 mutations. Indeed, all mutants share common alterations at the C-terminus domain of *NPM1*. These changes result in an aberrant cytoplasmic accumulation of *NPM1* mutants, highly contributing to leukemogenesis (Federici and Falini 2013).



**Figure 15: Various types of NPM1 exon-12 mutations in AML (Kunchala, Kuravi et al. 2018).**

Various types of NPM1 exon-12 mutations in AML: The variable sized nucleotide insertions in exon-12 result in a frameshift mutation and create a nuclear export signal (NES) motif. Red letters indicate nucleotides insertions in each type of mutations.

## 3.2 Cellular and functional consequences of *NPM1* mutations in AML

### 3.2.1 Haplo-insufficiency and *NPM1* localization

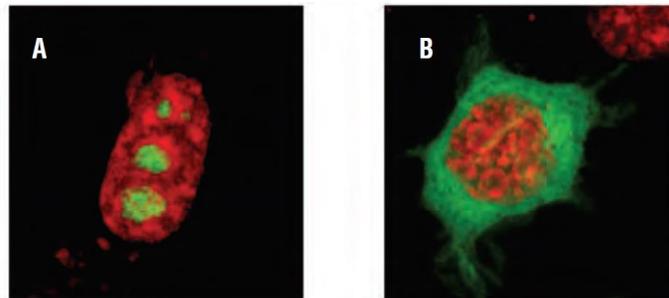
The wild type NPM1 protein (NPM1wt) resides in the nucleolus and binds to the nucleolar membrane through its NoLS containing tryptophan-288 and -290 residues (Falini, Martelli et al. 2006). In *NPM1c*-AML, aberrant cytoplasmic localization of NPM1 is a hallmark of leukemic cells (Falini, Martelli et al. 2006) (Figure 16). All the known mutations of NPM1 in exon-12 result in a shift of the reading frame at the C-terminus; thus abrogating the NoLS critical tryptophan residues 288 and 290 (Federici and Falini 2013). These alterations result in a strong leucine-rich motif creating a NES leading to the abnormal trafficking of NPM1 to the cytoplasm (NPM1c). This NES allows the binding of NPM1c to the nuclear export receptor, exportin 1, and the translocation of the aberrant protein into the cytosol (Falini, Bolli et al. 2006, Grummitt, Townsley et al. 2008).

In *NPM1c*-AML, NPM1 mutations are always heterozygous. Immunohistochemistry staining of blasts from these patients, showed that NPM1wt is dimerized with NPM1c through a

conserved N-terminal dimerization domain and translocated to the cytoplasm along with the mutant protein (den Besten, Kuo et al. 2005, Falini, Bolli et al. 2006).

Some studies suggested the presence of a dose dependent “tug of war” determining the subcellular distribution of NPM1c and NPM1wt proteins (Bolli, De Marco et al. 2009). In more details, when NPM1wt and NPM1c are expressed in transfected cells at equimolar doses, a fraction of NPM1wt was retained in the nucleolus. Importantly, excess of NPM1c drives the total cytoplasmic delocalization of the NPM1wt protein while excess doses of NPM1wt relocated the mutant protein to the nucleolus (Pasqualucci, Liso et al. 2006). This later scenario can also be observed under chronic oxidative stress.

NPM1 is acting as a haplo-insufficient tumor suppressor in blood cells (Sportoletti, Grisendi et al. 2008). *In vitro* and *in vivo* studies showed that inactivation of the nucleolar NPM1 protein results in mitotic spindle defects, aneuploidy, increased centrosome numbers and DNA damage checkpoint activation; Thus enhancing the rate of oncogenic transformation (Grisendi, Bernardi et al. 2005).



**Figure 16: Altered nucleocytoplasmic traffic of wild-type and mutant NPM1 (Falini, Nicoletti et al. 2007).**

(A) Immunofluorescence analysis shows nucleolar localization of NPM in NIH3T3 mouse fibroblasts transfected with eGFP/wild-type NPM. (B) NPM1c mutants accumulate in the cytoplasm

### 3.2.2. NPM1: an oncogene or a tumor suppressor gene?

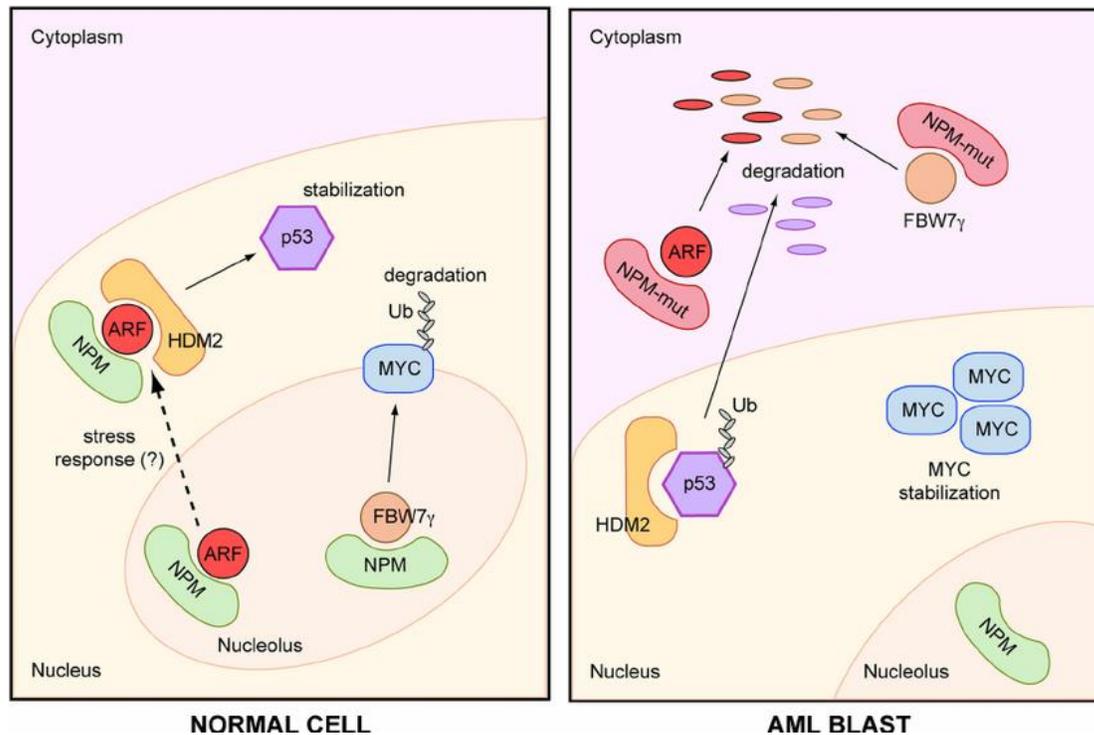
Given its pleiotropic functions, it has been proposed that *NPM1* mutations drive leukemia through a combination of loss of functions and gain of functions in different cellular processes. NPM1 functions both as an oncogene and a tumor suppressor gene (Grisendi, Mecucci et al. 2006), depending on dosage of expression levels, interacting partners, and

compartmentalization. Several studies reported that cytoplasmic expression of NPM1c contribute to AML development by inactivating p14<sup>ARF</sup> and blocking its P53-dependent and P53-independent activities (Gallagher, Kefford et al. 2006, Heath, Chan et al. 2017, Kunchala, Kuravi et al. 2018). In fact, p14<sup>ARF</sup> co-localizes with NPM1 protein in the nucleolus (Bertwistle, Sugimoto et al. 2004), and exhibits a stable structure when bound to NPM1 (Sherr 2006). NPM1-ARF interaction protect p14<sup>ARF</sup> from its rapid proteasomal degradation (Kuo, den Besten et al. 2004, Colombo, Bonetti et al. 2005) and ensure its nucleolar localization (Sherr 2006). Consistently, after various stimuli, NPM1 releases p14<sup>ARF</sup> allowing binding to MDM2, therefore preventing the proteasomal degradation of P53 (Qin, Shao et al. 2011). Lowering NPM1 levels using shRNA accelerated p19<sup>ARF</sup> (the mouse equivalent of the human p14<sup>ARF</sup>) degradation (Kuo, den Besten et al. 2004) and NPM1 inactivation resulted in p19<sup>ARF</sup> destabilization and exclusion from the nucleolus (Colombo, Bonetti et al. 2005). Indeed, in NIH-3T3 fibroblasts engineered to produce a zinc-inducible p14<sup>ARF</sup> protein, NPM1c delocalizes NPM1wt and p14<sup>ARF</sup> from the nucleoli to the cytoplasm, resulting in reduced P53-dependent and P53- independent p14<sup>ARF</sup> activities (den Besten, Kuo et al. 2005). When complexed with NPM1c, p14<sup>ARF</sup> stability was greatly compromised and the P53-dependent cell-cycle arrest at the G1/S boundary was appreciably lower (Colombo, Martinelli et al. 2006).

c-Myc is overexpressed in AML and other leukemia (Hoffman, Amanullah et al. 2002), favoring myeloid leukemogenesis in mice (Luo, Li et al. 2005). While NPM1wt was proven to regulate c-Myc, *via* its interaction with the E3 ligase Fbw7 $\gamma$ , NPM1c protein interacts with Fbw7 $\gamma$  and delocalizes it into the cytoplasm, resulting in stabilization of c-Myc (Figure 17) (Bonetti, Davoli et al. 2008).

NPM1wt binds and activates APE1, a core enzyme in BER pathway, in the nucleolus and nucleoplasm (Poletto, Lirussi et al. 2014). This implicates the role of NPM1 in DNA damage response as mentioned earlier. *NPM1c* AML cells showed a cytoplasmic accumulation of APE1, truncated APE1 products following targeting the protein by granzymes A and K, thus resulting in an impaired BER process (Vascotto, Lirussi et al. 2014).

NPM1c inhibits caspase-6 and caspase-8, both playing a major role in coordinating apoptosis. These results suggest that NPM1c raises the threshold for apoptosis initiation and hinders caspase-activated myeloid cell differentiation (Leong, Tan et al. 2010).



**Figure 17: Mutated NPM attenuates an oncosuppressor pathway and enhances an oncogenic one**

Normal cell: NPM is mainly localized in the nucleolus and is required for nucleolar accumulation and stability of FBW7 $\gamma$  and ARF. This is relevant for the control of MYC turnover and provides an active pool of ARF ready to inactivate the HDM2-mediated p53 degradation in response to cellular stress. AML blast: NPM-mut is mainly localized to the cytoplasm and causes cytoplasmic delocalization and degradation of ARF and FBW7 $\gamma$ . As a consequence, HDM2 can induce ubiquitination/degradation of p53, and MYC accumulates and activates its target genes.

In *NPM1c* AML, homeobox (HOX) genes HOXA and HOXB are consistently overexpressed compared to AML with NPM1wt (Alcalay, Tiacci et al. 2005). These genes play an important role in controlling the self-renewal capacity of leukemic cells. Recently, it has been shown that loss of the cytoplasmic expression of NPM1c results in a significant decrease of acetylated H<sub>3</sub>K<sub>27</sub> mainly at HOX loci. This event was coupled with HOX downregulation and was followed by cell differentiation (Brunetti, Gundry et al. 2018). These results suggest that NPM1c is acting epigenetically upstream of HOX to maintain the undifferentiated state of AML cells.

Moreover, Miz1 is a transcription factor that activates the expression of two important cell cycle inhibitors, p15Ink4b and p21. NPMc delocalizes Miz1 to the cytoplasm, favoring uncontrolled cell cycle progression.

Although *NPM1* gene mutations are the most frequent genetic lesions in AML, there is no mouse model demonstrating their leukemogenic potential *in vivo*. Several *NPM1* transgenic mouse models were generated to model AML carrying *NPM1* mutations (Sportoletti, Varasano et al. 2015). None of these published models recapitulated so far the AML disease. Thus, *NPM1c* is not sufficient to induce AML suggesting the requirement of cooperating mutation to induce clinical AML in mice (Cheng, Sportoletti et al. 2010, Vassiliou, Cooper et al. 2011, Chou, Ko et al. 2012, Mallardo, Caronno et al. 2013). For example, NPM1 and FLT3-TD cooperate powerfully to drive a rapid-onset of leukemogenesis in knock-in mice (Vassiliou, Cooper et al. 2011, Mupo, Celani et al. 2013). Similarly, potent *in vivo* oncogenic cooperation was also demonstrated by crossing the NRAS-G12D knock-in strain with *NPM1c* knock-in mice. In this setting, 95% of mice developed AML (Vassiliou, Cooper et al. 2011).

### 3.3 Targeted therapies of *NPM1c* AML

Since *NPM1c* mutations largely affect the prognosis of AML patients, targeting NPM1c *via* its degradation is an important step towards a personalized therapeutic approach against this category of AML patients. This is further asserted by the outcome of NPM1 mutations on the inhibition of tumor suppressor genes like *P53* and *p14<sup>ARF</sup>* and alteration of apoptosis through inhibition of caspases 6 and 8 (Di Matteo, Franceschini et al. 2016). In the section below, potential ways of targeting NPM1c will be discussed:

#### 3.3.1 Targeting NPM1 oligomerization

NPM1wt exists as dimers or oligomers through its N terminal oligomerization domain (Grisendi, Mecucci et al. 2006). This oligomerization plays an essential role in NPM1wt functions, especially its chaperoning activity. Oligomers of NPM1wt contribute as well to the architecture of the nucleolus (Falini, Martelli et al. 2011). Hence, the nucleolus of cells harboring NPM1c might

be more susceptible for nucleolar-targeted therapies highlighting the relevance for developing drugs that interfere with NPM1wt oligomerization (Falini and Martelli 2011).

NSC348884 (N, N, N', N'-tetrakis [(5- methyl-1H-benzimidazol- 2-yl) methyl]ethane-1,2-diamine) is a small molecule inhibitor of NPM1 oligomerization (Qi, Shakalya et al. 2008). Treatment with NSC348884 resulted in selective P53 activation and induction apoptosis in cells expressing NPM1c (Balusu, Fiskus et al. 2011, Di Matteo, Franceschini et al. 2016). Importantly, NSC348884 displayed a synergistic effect with RA, exclusively in cells expressing NPM1c without FLT3-ITD (Balusu, Fiskus et al. 2011).

Another molecule, the YTR107 ((Z) -5 - ((N-benzyl-1H-indol-3-yl) methylene) pyrimidine-2, 4, 6 (1H, 3H, 5H)- trione), known for its capacity to sensitize cancer cells to radiation (Destouches, Huet et al. 2012), binds to the N-terminal domain of NPM1 and interferes with its oligomerization. *In vitro* and *In vivo* studies suggested that the YTR107-induced radiosensitization is mediated by NPM1 (Sekhar, Benamar et al. 2014).

### 3.3.2 Targeting NPM1 cytoplasmic translocation

NPM1wt shuttles from the nucleolus to the cytoplasm through a XPO1/CRM1 dependent mechanism. The latter is a key regulator of leukemogenesis (Falini, Bolli et al. 2009), making it an ideal therapeutic target for the treatment of *NPM1c* AML.

Selinexor or KPT-330 ((Z)-3-(3-(3, 5-bis (trifluoromethyl)phenyl)- 1H-1,2,4-triazol-1-yl)-N'-(pyrazin-2-yl) acrylohydrazide) is an orally bioavailable selective XPO1 inhibitor. Although it may interfere with other cellular pathways, Selinexor-induced XPO1 inhibition induced anti-leukemic activity in cultured and primary AML cells (Parikh, Cang et al. 2014). Importantly, NPM1c-expressing cells were much sensitive to this inhibitor (Parikh, Cang et al. 2014).

Another molecule, Oridonin, induced nuclear accumulation of CRM1 and P53-mediated apoptosis resulting in NPM1c translocation to the nucleus in OCI-AML3 cells (Li, Yi et al. 2014).

### 3.3.3 Targeting nucleolar assembly

CK2 is a serine/threonine kinase which has numerous substrates including NPM1 (Meggio and Pinna 2003). CIGB-300 is a synthetic peptide that binds the phosphor-acceptor sites of CK2 substrates. NPM1 is identified as a major target for CIGB-300 (Perera, Farina et al. 2009). When CIGB-300 binds NPM1, it inhibits its CK2-mediated phosphorylation resulting in nucleolar disassembly and massive apoptosis (Perera, Farina et al. 2009).

Actinomycin D (ActD) is an antibiotic that showed anticancer activity (Hollstein 1974). It binds DNA at the transcription initiation complex and restrain the activity of RNA polymerase I thus inhibiting ribosome biogenesis (Sobell 1985). NPM1c expressing AML cells are partially depleted of NPM1 because of NPM1 haploinsufficiency and its cytoplasmic accumulation which make their nucleolus more vulnerable to drugs that trigger a nucleolar stress response (Falini and Martelli 2011, Falini, Brunetti et al. 2015). Recently, ActD was shown to induce complete morphological and immunohistochemical remission after two cycles of therapy and complete molecular remission after four cycles in one *NPM1c/FLT3*- patient (Falini, Brunetti et al. 2015). A progressive decrease in the copies of *NPM1c* was also observed until the achievement of MRD negativity. In the same study, six more patients with refractory/relapsed *NPM1c* AML were treated with ActD. Two of them achieved hematologic complete remission after 5 days of treatment (Falini, Brunetti et al. 2015).

### 3.3.4 Targeting NPM1c levels

Cancer cells acquire abnormalities in multiple oncogenes and tumor suppresser genes. Despite this complexity, their growth and survival can often be impaired by the inactivation of a single oncogene. This dependency called “oncogene addiction” represents a rationale for molecular targeted therapies.

In AML, NPMc inactivates relevant tumor suppressor proteins including ARF, Miz1 and Fbw7 $\gamma$  and activates several oncogenes including c-Myc. Collectively, NPM1c represents a culprit that can simultaneously lead to oncogene overexpression (c-Myc) and tumor

suppressor inactivation (ARF, p21, p15Ink4b), (Schmitt et al., 1999), two key events in tumor development. Hence, the need to develop drugs targeting NPM1c.

Addition of RA to chemotherapy was proposed to improve survival of some of NPM1c patients. Our team showed that RA and ATO synergistically induce proteasomal degradation of mutant NPM1, leading to differentiation and apoptosis in AML cell lines or primary samples (El Hajj, Dassouki et al. 2015). We have shown that *NPM-1* mutation, known to delocalize NPM1 from the nucleolus, disorganizes PML nuclear bodies. RA/ATO reduced leukemic blasts in the bone marrow of *NPM1*-mutant AML patients and restored nucleolar localization of NPM1 and PML both *ex vivo* and *in vivo*. Similar results on the effect of ATO and RA were obtained by another Italian group (Martelli, Gionfriddo et al. 2015).

Our team has also demonstrated the potency of EAPB0503, an imiquimod derivative, on *NPM1c* AML. Indeed, EAPB0503 induces selective proteasomal degradation of NPM1c in both *NPM1c* AML cell lines and blasts of *NPM1c* AML patients. Moreover, introduction of *NPM1c* into wt-NPM1-expressing cells sensitizes them to EAPB0503. Consequently, P53 was stabilized and cell growth was inhibited. Finally, EAPB0503 showed promising *in vivo* effect in reducing leukemia burden in *NPM1c* AML-xenograft animals (Nabbouh, Hleihel et al. 2017) (Annex 2).

*NPM1c* AML cells are highly dependent on continued export of NPM1c to proliferate. Indeed, NPM1c knockdown in OCI-AML3 cells by siRNA was associated with induction of p53 and the differentiation markers, p21 and C/EBP $\alpha$ . Importantly, knockdown of NPM1c significantly enhanced RA-induced differentiation of OCI-AML3 cells. The same study showed that Knockdown of NPM1c dramatically inhibited leukemia initiation by cultured AML cells in Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) (Balusu, Fiskus et al. 2011). In the same context, NPM1c was also shown to inhibit myeloid differentiation by modulating miR-10b in AML cells. This was rescued after NPM1c knockdown in AML cells (Zou, Tan et al. 2016).

## Chapter IV: All Trans Retinoic Acid

RA is an active metabolite of vitamin A with multiple effects on development and tissue homeostasis. It belongs to the family of retinoids. RA exerts most but not all its effects by binding to a nuclear receptor family – retinoic acid receptor (RAR) and retinoid X receptor (RXR); these receptors generally form RAR/RXR heterodimers, modulating the transcription of target genes (Schultze, Collares et al. 2018). Accordingly, RA has a dual effect on stem cell fate and differentiation. RA exerts several anti-cancer effects *via* both induction of apoptosis and differentiation (Lotan 1991, Hofmann 1992).

The most prominent and well understood effect of RA was shown against APL. Indeed, RA induces complete remission in a high proportion of APL patients (Huang, Ye et al. 1988, Castaigne, Chomienne et al. 1990). In APL, PML–RAR $\alpha$  disrupts PML nuclear bodies and blocks the transcription of RAR $\alpha$  target genes. PML–RAR $\alpha$  homodimers bind tightly to transcriptional corepressors, which attract HDACs (Figure 18). Treatment with RA dissociates corepressors from the PML–RAR $\alpha$  fusion protein and turns it into an activator, allowing the recruitment of histone acetyltransferases (HATs) and gene transcription to occur (Zhu, Chen et al. 2002). RA also triggers the degradation of the PML/RARA fusion oncoprotein resulting in a PML NB-driven, P53/senescence checkpoint induction and APL eradication (Ablain, Rice et al. 2014, de The, Pandolfi et al. 2017). Some studies have reported unexpected clinical relapses despite continued RA treatment suggesting that resistance to the anti-leukemic effects of RA or additional mutations were acquired during drug therapy (Castaigne, Chomienne et al. 1990, Warrell, Frankel et al. 1991). This, high RA doses are required to achieve durable response in APL patients (Muindi, Frankel et al. 1992, Zhu, Gianni et al. 1999). Similarly, in mouse APL models, high doses of RA are required to achieve PML/RARA degradation and APL clearance (Zhu, Gianni et al. 1999). Moreover, some studies have shown that single-agent liposomal RA induced CR in 79% of APL patients (Tsimberidou, Tirado-Gomez et al. 2006).

In non-APL cells, RA may regulate stem cell fate and normal myeloid differentiation through RAR $\alpha$ -modulated transcription (Sidell 1982, Tocci, Parolini et al. 1996, Zhu, Heyworth et

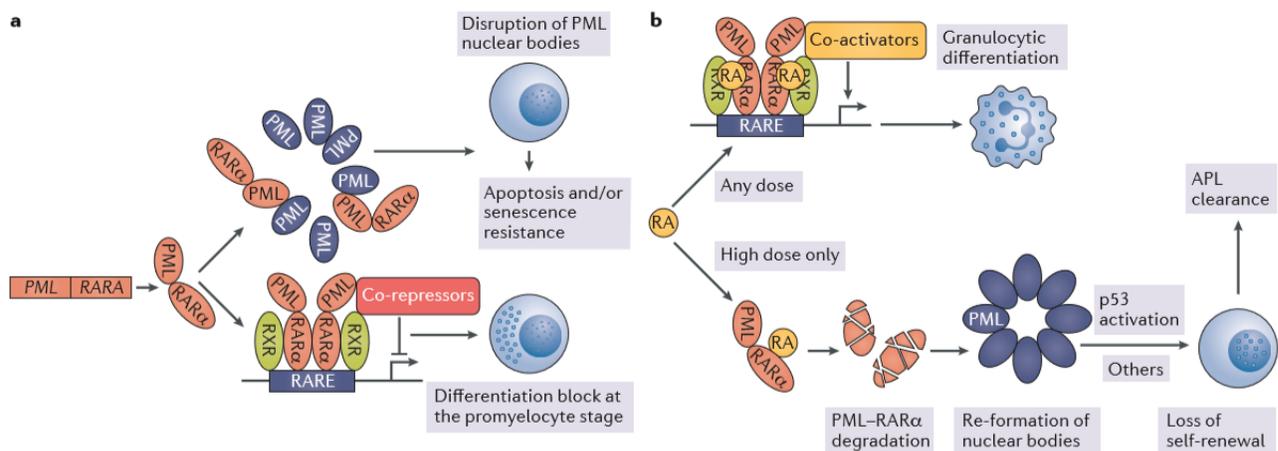
al. 2001, Altucci, Rossin et al. 2005). Some studies suggested that addition of RA to conventional chemotherapy improves survival, selectively in *NPM1c* AML patients (Schlenk, Dohner et al. 2009). Other clinical studies reported negative results (Burnett 2011; Nazha 2013). Indeed, in AML non-APL patients, addition of RA to induction or consolidation therapy, improved CR rate and OS in elderly patients (Schlenk, Frohling et al. 2004). Importantly, the clinical benefit of RA co-administration with chemotherapy appears maximal in AMLs with *NPM1c* mutation (Schlenk and Dohner 2009). For instance, the biochemical basis for oncoprotein degradation remains elusive. Whether RA-associated increased long-term survival in this condition actually reflects differentiation is not known (de The 2018). Moreover, IDH mutations that lead to epigenetic reprogramming were proposed to prime RA-triggered AML differentiation *ex vivo* (Schenk, Chen et al. 2012, Boutzen, Saland et al. 2016).

RA was combined with various chemotherapeutic drugs to improve its anti-tumor effects. In non-small cell lung carcinoma (NSCLC), RA inhibits the proliferation and induces apoptosis and differentiation of tested cell lines (Lokshin, Zhang et al. 1999). In combination with FLT3 TKI, RA reduces the engraftment of primary FLT3/ITD<sup>+</sup> AML cells in mice with evidence of cellular differentiation occurring *in vivo* (Ma, Greenblatt et al. 2016). RA was also proven to have anti-cancer activities in various tumors including Kaposi sarcoma (Saiag, Pavlovic et al. 1998), head and neck squamous cell carcinoma (Park, Gray et al. 2000), ovarian cancer (Lokman, Ho et al. 2019), bladder cancer (Hameed and el-Metwally 2008), neuroblastoma (Chlapek, Redova et al. 2010).

As mentioned earlier, two independent studies showed that RA degrades NPM1c in cell lines and in primary AML cells, suggesting that NPM1c degradation plays a central role in RA-induced differentiation and apoptosis (El Hajj, Dassouki et al. 2015, Martelli, Gionfriddo et al. 2015). In fact, five untreated or relapsed elderly AML patients with normal karyotype and mutated NPM1 were treated with RA and or ATO. Bone marrow blasts were reduced in three patients after 15 days of RA/ATO treatment. Similarly, peripheral blood blasts disappeared at day 21 of RA/ATO. However, blast counts re-increased upon discontinuation of treatment (El Hajj, Dassouki et al. 2015) suggesting that RA and ATO exert transient anti-leukemic activities.

Moreover, NPM1 mutant downregulation by ATO/RA was shown to potentiate response to the anthracyclin daunorubicin in NPM1c AML cells (Martelli, Gionfriddo et al. 2015).

Many oncoproteins and tumor suppressors are regulated by a central signaling mechanism, the pro-directed Ser/Thr phosphorylation (pSer/Thr-Pro). The latter is regulated by many kinases and phosphatases (Zhou and Lu 2016), as well as by a single proline isomerase Pin1 (Lu and Hunter 2014). RA was shown to inhibit and degrade active Pin-1 selectively in cancer cells by directly binding to the substrate phosphate- and proline-binding pockets in the Pin-1 active site. In this context, RA alone (Wei, Kozono et al. 2015) or in combination with ATO (Kozono, Lin et al. 2018) blocks multiple Pin-1-regulated cancer-driving pathways. Indeed, Kozono et al reported that ATO binds the active site of Pin1 and induce its proteasome-dependent degradation resulting in cell growth arrest. Pin1 KO cells were more resistant to ATO, which was rescued by re-expressing Pin1. In addition, RA was shown to induce aquaporin-9 (AQP9) which increased the ATO uptake. Consequently, RA cooperates with ATO to exert potent anticancer activity.



**Figure 18: APL pathogenesis and response to RA therapy (de The 2018)**

a. The fusion protein PML–RAR $\alpha$  blocks basal transcription of a number of targets, notably those involved in differentiation. Dimerization of the PML–RAR $\alpha$  fusion protein and wild-type PML (depicted in blue) impedes PML oligomerization and nuclear body assembly and, among other effects, blunts basal p53 activation. b. Dual effects of RA on APL cells: transcription-based differentiation (top) and PML driven senescence (bottom). RA binding recruits co-activators to the PML–RAR $\alpha$ –RXR complex, making it a transcriptional activator and promoting differentiation. It also initiates proteasome-dependent PML–RAR $\alpha$  degradation, allowing re-formation of nuclear bodies by wild-type PML proteins and subsequent p53 activation, thereby promoting loss of self-renewal and APL clearance.

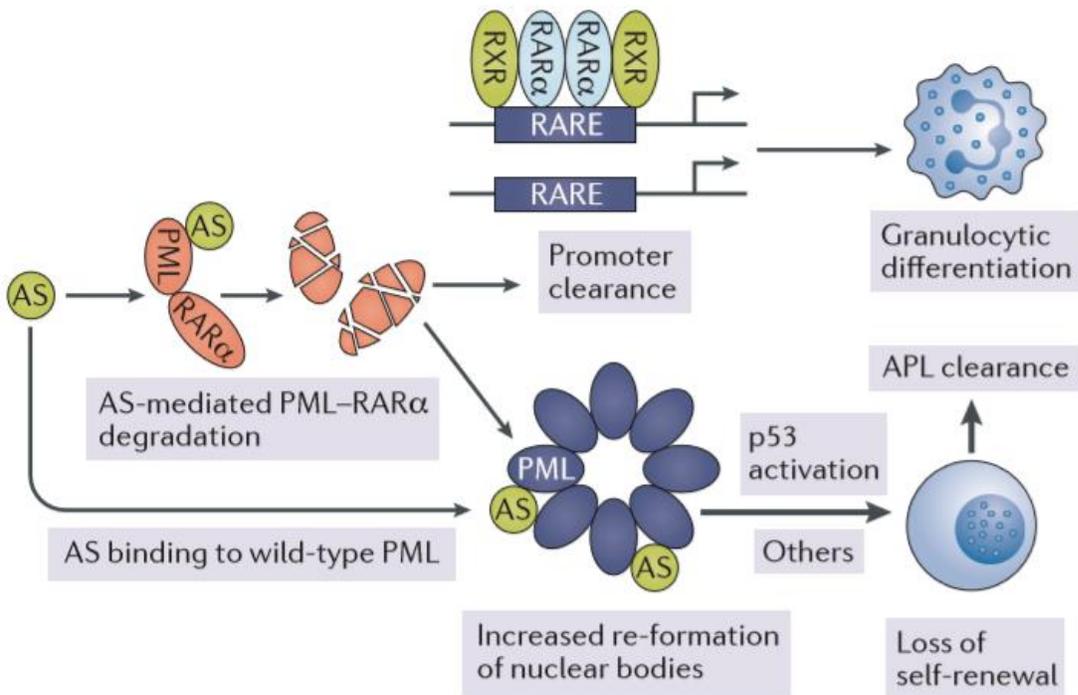
## Chapter V: Arsenic trioxide

Arsenic is commonly known as a poison. It was first used by Greek and Chinese medicine more than 2,000 years ago to treat everything from syphilis to cancer, and was also the favorite poison of the Savellis, the Borgias and Agatha Christie (Zhu, Chen et al. 2002). Arsenic occurs widely in the natural environment, it is present in soil and water, which contributes to its migration to food products.

In the early 1970s, based on the principle in Chinese traditional medicine of “using a toxic agent against a toxic agent”, researchers at Harbin Medical University treated more than 1000 cancer patients with intravenous infusions of dissolved ATO and showed remarkable clinical efficacy in patients with newly diagnosed and relapsed APL (Sun 1992, Zhang, Hu et al. 1996). Later on, clinical trials with ATO were conducted in Shanghai Second Medical University, in collaboration with Harbin. They confirmed the efficacy of pure ATO in relapsed patients with APL after RA plus chemotherapy (Chen, Zhu et al. 1996).

The efficacy of ATO in APL treatment led to its FDA approval as a first-line treatment for APL was granted in September 2000. Low concentration of ATO (0.25–0.50  $\mu\text{M}$ ) triggers differentiation of APL cells, while a high concentration of ATO (1–2  $\mu\text{M}$ ) induces apoptosis of APL cells (Chen, Shi et al. 1997). Moreover, ATO plays a more profound role in the promotion of APL clearance than RA as it targets both the PML–RAR $\alpha$  fusion protein and the wt PML protein. ATO binds to PML–RAR $\alpha$  and initiates its proteasomal degradation, thereby clearing target promoters and allowing RAR $\alpha$  and other nuclear receptors to re-activate transcription and promote differentiation (top). Following ATO-induced PML–RAR $\alpha$  degradation, wt PML oligomerizes and leads to the re-formation of NB. This process drives P53 activation, loss of self-renewal and APL clearance (Nasr, Lallemand-Breitenbach et al. 2009) (Figure 19). Indeed, ATO induce disulfide bond formation between PML proteins resulting in the assembly of PML nuclear bodies (NB) (Jeanne, Lallemand-Breitenbach et al. 2010). In response to oxidative stress, previously diffused PML are transferred to the nuclear matrix where it becomes SUMOylated and assembles by intramolecular covalent bonds to form NB (Figure 19). ATO preferentially binds to cysteine residues in zinc fingers located

within the RBCC domain of PML resulting in a conformational change in PML protein (Jeanne, Lallemand-Breitenbach et al. 2010, Zhang, Yan et al. 2010).



**Figure 19: APL pathogenesis and response to ATO therapy**

ATO targets both the PML-RAR $\alpha$  fusion protein and the wild-type PML protein. AS binding to PML-RAR $\alpha$  initiates its proteasomal degradation, thereby clearing target promoters and allowing RAR $\alpha$  and other nuclear receptors to re-activate transcription and promote differentiation (top). Upon AS-induced PML-RAR $\alpha$  degradation, oligomerization of wild-type PML proteins leads to the re-formation of nuclear bodies.

Similarly, ATO showed efficacy in chronic myeloid leukemia (CML), known to be driven by the translocation t (3;21) (q26;q22) resulting in *AML1/MDS1/EVI1* (*AME*) fusion gene. Indeed, ATO targets *AME* via both myelodysplastic syndrome 1 (MDS1) and Ectopic Viral Integration site1 (EVI1) moieties and degrades EVI1 via the ubiquitin-proteasome pathway and MDS1 in a proteasome-independent manner (Shackelford, Kenific et al. 2006, Nasr, Guillemin et al. 2008). In the same setting, ATO induces differentiation and apoptosis of *AME* positive cells *in vitro* and reduces tumor bulk in *AME* positive cells-xenograft mice (Shackelford, Kenific et al. 2006).

Multiple studies from our lab have demonstrated the efficacy of ATO in combination with interferon  $\alpha$  (IFN) for the treatment of Adult T cell leukemia (ATL). Indeed, this combination has shown potency and specificity against HTLV-1 transformed cells (Bazarbachi, El-Sabban et al. 1999). ATO/IFN treatment targets two critical pathways for the survival of the leukemic cells: proteasome-mediated degradation of Tax and inhibition of an important pathway in cellular proliferation (NF- $\kappa$ B) (El-Sabban, Nasr et al. 2000). Furthermore, using a preclinical model of murine ATL derived from *Tax* transgenic, we have demonstrated that the combination of ATO and IFN cures Tax-driven ATLs through eradication of LIC (El Hajj, El-Sabban et al. 2010). Finally, the triple combination of Arsenic, IFN and zidovudine induce long lasting remissions in chronic ATL patients (Kchour, Tarhini et al. 2009) and reversed the immunosuppressed phenotype of patients to an immunocompetent one (Kchour, Rezaee et al. 2013).

More recently, our team demonstrated that ATO modulates the stability on the HTLV-I viral oncoprotein Tax by promoting its hyper-SUMOylation followed by a SUMO-dependent ubiquitination that require both PML and RNF4 (Dassouki, Sahin et al. 2015). Indeed, upon ATO/IFN treatment, Tax is recruited into PML NB where it undergoes SUMOylation by SUMO2/3 followed by RNF4-dependent ubiquitination and proteasome-dependent degradation.

Another study done by our team has also shown a promising use of ATO in the treatment of primary effusion lymphoma (PEL). ATO treatment induced apoptosis and downregulated the latent viral transcripts of Latency Associated Nuclear Antigens LANA-1 and 2 (LANA-1 and LANA-2), the viral cyclin (v-Cyc), and viral FLICE inhibitory protein (v-FLIP) in PEL cells derived from malignant ascites. An in-vivo response was also noted where administration of ATO, in combination with interferon alpha, decreases the peritoneal volume and increases survival of PEL-xenograft mice (El Hajj, Ali et al. 2013).

Exposure to a high concentration of ATO leads to increased ROS production (Alarifi, Ali et al. 2013), DNA damage (Andrew, Burgess et al. 2006), mitochondrial dysfunction and glutathione depletion. Glutathione, which is known to protect the cells against oxidative stress (Reipa 2004), is one of the most important ligands of ATO.

ATO is also described as an immunomodulatory agent. This effect is heavily studied *in vitro* and *in vivo*. Indeed, ATO induces apoptosis of immune cells through both mitochondrial-

mediated and receptor-mediated pathways (Gupta, Yel et al. 2003). ATO was also described to replace phosphorus of the mitochondria respiratory chain leading to the release of cytochrome C in the cytoplasm of the cell (Larochette, Decaudin et al. 1999).

ATO induces immune cell intracellular ROS accumulation (Lemarie, Bourdonnay et al. 2008). *In vivo* studies showed that ATO have therapeutic efficacy in several mouse models of autoimmune and inflammatory diseases (Bobe, Bonardelle et al. 2006).

Nevertheless, PML remains the major target of ATO. Indeed, its ability to promote PML–RAR $\alpha$  degradation is therefore likely to be an important aspect of its function.

## Chapter VI: PML and nuclear bodies

PML is a phosphoprotein that belongs to the family of TRIM proteins. *PML* gene was discovered in APL where it forms a fusion protein with the *RAR $\alpha$*  gene (de The, Chomienne et al. 1990). This fusion is a result of a chromosomal translocation t (15; 17) leading to the expression of a PML-RAR $\alpha$  chimera protein that blocks cell differentiation at the promyelocytic stage (Borrow, Goddard et al. 1990, de The, Lavau et al. 1991, Goddard, Borrow et al. 1991).

In normal cells, PML is a nuclear protein partly organized in NB that are associated with the nuclear matrix, and partly found diffused in the nucleoplasm (Lallemand-Breitenbach and de The 2010).

Many proteins can be recruited into these NB (Ivanschitz, De The et al. 2013), indicating the crucial role of PML in different cellular processes including apoptosis, senescence, DNA damage response, antiviral defense, transcription, protein catabolism, and post-translational modifications (Koken, Linares-Cruz et al. 1995, Delleire and Bazett-Jones 2004, Bernardi and Pandolfi 2007).

### 6.1 Structure and isoforms of PML

The N terminal of PML contains a RING finger, B-box, Coiled-Coil motif (RBCC). It consists of a RING domain, two B-boxes (B1 and B2) and a coiled-coil domain (Jensen, Shiels et al. 2001) (Figure 20). This motif is preserved in all PML isoforms emphasizing its importance in PML functions. It is rich in cysteines and histidines which bind a zinc ion and form a zinc finger structure. The RBCC motif contains two lysine residues, K65 and K160, located in the RING and in the box B1, respectively (Ishov, Sotnikov et al. 1999).

This domain is associated with UBC9, the SUMO conjugation enzyme E2, hence its role in the SUMOylation process (Duprez, Saurin et al. 1999). The B1 and B2 boxes are adjacent to the RING domain and have two Zinc fingers, rich in cysteines and histidines. These boxes are involved in protein-protein interactions as well as Arsenic binding (Jeanne, Lallemand-Breitenbach et al. 2010). The Coiled-coil domain contains alpha-helices arranged in a coiled-coil rope-like structure (Parry 1982). This domain plays an important role in the heterodimerization with the fusion

protein PML-RAR $\alpha$  and in the multimerization of PML (Perez, Kastner et al. 1993, Grignani, Testa et al. 1996).

*PML* gene is composed of 9 exons. The first three encode for the RBCC motif. Alternative splicing of exons 4 to 9 results in the expression of seven different PML isoforms (Chelbi-Alix, Quignon et al. 1998, Wang, Ruggero et al. 1998). These isoforms adopt different distributions, functions and shapes (Condemine, Takahashi et al. 2006). Indeed, PML I is the most expressed isoform. It has NLS and NES that allows its localization in the cytoplasm and in the nucleus (Condemine, Takahashi et al. 2006). PML II is the second mostly expressed isoform (Condemine, Takahashi et al. 2006). PML III interacts with several proteins, including acetyl transferase TIP60 (Tat-interactive protein) and the telomeric repeat-binding factor 1, TRF1 (Wu, Hu et al. 2009, Yu, Lan et al. 2010). The PML isoform IV has a binding region to P53 (Fogal, Gostissa et al. 2000) and plays a key role in senescence (Pearson, Carbone et al. 2000). PML V forms dense NB (Condemine, Takahashi et al. 2006).



**Figure 20: PML structure**

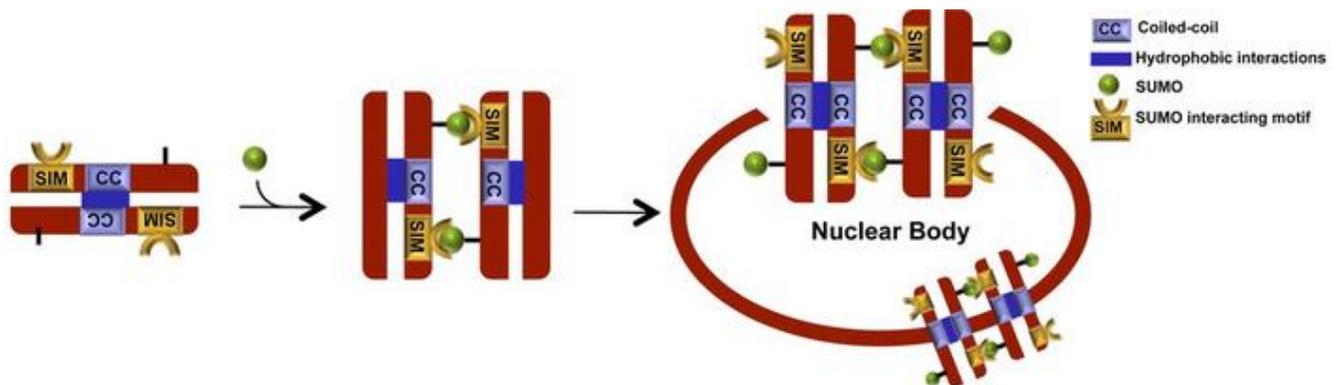
PML structure and SUMO-induced modifications. The N terminal contains a RING finger, B-box, Coiled-Coil motif (RBCC). It consists of a RING domain, two B-boxes (B1 and B2) and a coiled-coil domain.

## 6.2 PML NB biogenesis

Several models were proposed for PML NB biogenesis. A first model proposes that the non covalent binding between the SIM domain of PML with a SUMO protein of another SUMOylated PML induce the formation of NB (Shen, Lin et al. 2006) (Figure 21). Another mechanism indicates that PML SUMOylation is important for the maturation of NB and not for their biogenesis (Lallemand-Breitenbach, Zhu et al. 2001) (Figure 22). As a first step, PML binds to the nuclear matrix to form the NB's envelope. The disulfide bond formed between PML and the nuclear matrix will further stabilize this interaction (Jeanne, Lallemand-Breitenbach et al. 2010). Indeed,

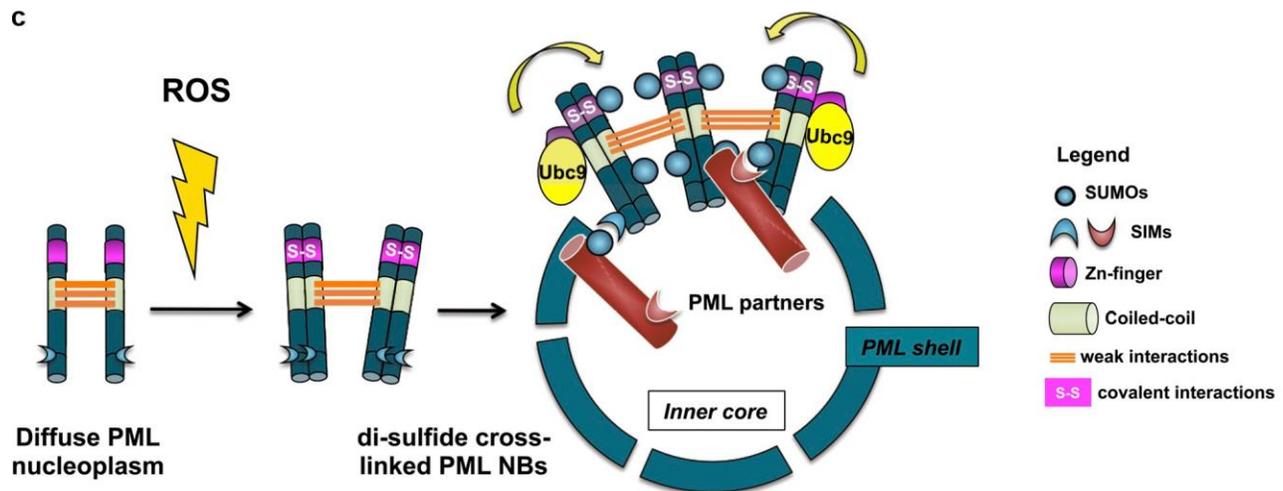
PML3KR mutant that lacks the three major SUMOylation sites of PML, and PML3KR $\Delta$ SIM mutant that further lacks the SIM domain, are both able to form NBs (Sahin, Ferhi et al. 2014). However, the loss of Coiled-Coil domain inhibits the binding of PML to the nuclear matrix, the formation of NBs and their SUMOylation (Jeanne, Lallemand-Breitenbach et al. 2010, Sahin, Ferhi et al. 2014).

On the other hand, Shen's model suggests that the PML SIM domain is needed to recruit partners to NBs (Shen, Lin et al. 2006). However, DE THE team showed that the NBs of PML $\Delta$ SIM co-localize with Daxx and Sp100 (Ishov, Sotnikov et al. 1999, Lallemand-Breitenbach, Zhu et al. 2001, Sahin, Ferhi et al. 2014). Finally, Sahin et al. showed that the treatment of cells with Arsenic and IFN induces the recruitment of diffuse Ubc9 to PML NBs and enhances the SUMOylation of PML and partners. Thus, due to this SUMO-SIM interaction, the partner proteins, SUMOs and PML are sequestered in the soluble core forming the mature NBs (Sahin, Ferhi et al. 2014). A descriptive model of the PML NBs is agreed upon; these are formed of an insoluble envelope associated with the nuclear matrix and a soluble core in which accumulates Ubc9, the SUMOs and the partners having a SIM and a SUMOylation site.



**Figure 21: First model of NBs biogenesis**

Schematic representation of the model in which NB biogenesis relies on noncovalent intermolecular interactions between PML-attached SUMO and the PML SIM.



**Figure 22: PML and NBs.**

PML cross-linking by disulfide bounds underlies formation of the matrix-associated shell, and polarized SUMO–SIM interactions recruit partner proteins within NBs. PML multimers recruit Ubc9, leading to sumoylation of PML and possibly its partners.

### 6.3 PML SUMOylation

SUMOylation is one of the most important post-translational modifications of PML. The modification of PML by SUMO-1 and SUMO-2/3 is carried out on three target lysines: K65 in the RING domain, K160 located in the box B1 and K490 located in the NLS domain (Kamitani, Kito et al. 1998, Muller, Matunis et al. 1998, Duprez, Saurin et al. 1999) (figure 18). K160 SUMOylation plays a major role in the formation of mature NBs and in the recruitment of partner proteins (Lallemand-Breitenbach, Zhu et al. 2001).

PML also has a SIM domain located in its C-terminal part (Figure 18). SIM / SUMO interactions between PML and its partners participate in the maturation of NBs (Matunis, Zhang et al. 2006, Shen, Lin et al. 2006, Sahin, Ferhi et al. 2014). PML can be SUMOylated by the E3 ligase RanBP2 (Tatham, Kim et al. 2005). However, PML has an E3 ligase activity that could be assumed by its RING domain and is sufficient to induce SUMOylation of both PML and NBs-associated proteins (Quimby, Yong-Gonzalez et al. 2006, Shen, Lin et al. 2006).

## 6.4 Nuclear bodies function

Through NBs, PML regulates several key processes such as apoptosis, senescence, DNA damage response, antiviral defense, transcription, protein catabolism, and post-translational modifications (Koken, Linares-Cruz et al. 1995, Dellaire and Bazett-Jones 2004, Bernardi and Pandolfi 2007).

### 6.4.1 PML: a tumor suppressor gene

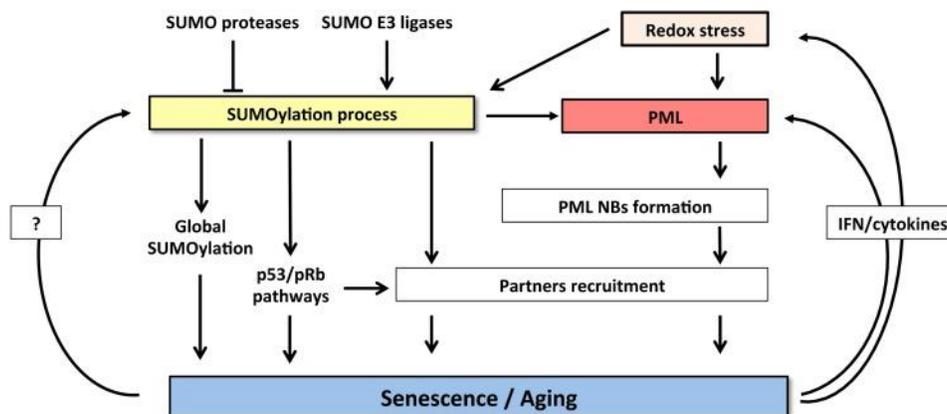
PML can be considered as a tumor suppressor due to its pro-senescent, pro-apoptotic and anti-proliferative capabilities (Ivanschitz, De The et al. 2013). In many pathologies, such as viral infections and cancers, NBs lose their integrity (Koken, Linares-Cruz et al. 1995). Indeed, several studies reported an alteration of NBs formation in malignant tumors rather than a loss of PML expression (Gurrieri, Capodiecici et al. 2004, Scaglioni, Yung et al. 2006). For instance, PML is deregulated in APL where the oncoprotein PML/RAR, by its dominant negative effect, relocates PML from NBs (Koken, Puvion-Dutilleul et al. 1994, Stadler, Chelbi-Alix et al. 1995). Other pathways of oncogenes-induced PML degradation were described (Ivanschitz, De The et al. 2013). These oncogenes include the ubiquitin ligase E6AP, the E2F transcription factor, the transcriptional E2FBP1 regulator, and the isomerase Pin1 (Ivanschitz, De The et al. 2013). PML<sup>-/-</sup> mice are more susceptible to the development of several types of cancers including papillomas, carcinomas and lymphomas (Wang, Ruggero et al. 1998). It has been proposed that the tumor suppressive properties of PML are primarily associated with the ability of NBs to induce senescence thus preventing malignant transformation (Vernier, Bourdeau et al. 2011).

From a transcriptional point of view, PML promoter contains elements of response to IFN and P53 (Stadler, Chelbi-Alix et al. 1995, de Stanchina, Querido et al. 2004). IFN-increased PML transcription results in a PML/P53-dependent senescent phenotype (Chiantore, Vannucchi et al. 2012).

Finally, through the regulation of P53 (Ferbeyre, de Stanchina et al. 2000, Bischof, Kirsh et al. 2002) and Rb (Malette, Goumard et al. 2004, Bischof, Nacerddine et al. 2005), PML isoform IV plays a leading role in senescence. Like all PML isoforms, PML IV is capable of recruiting P53 to

NBs (Ivanschitz, De The et al. 2013). However, PML IV has the ability to stabilize P53 (Ivanschitz, De The et al. 2013). Indeed, Ras-induced senescence results in the recruitment of acetyl transferase CPB to PML NBs leading to the acetylation and stabilization of P53 (Pearson, Carbone et al. 2000). Furthermore, in response to cell stress, the acetyl transferase MOZ is also recruited to the NBs leading to P53 acetylation and P21 activation (Rokudai, Laptenko et al. 2013). Finally, following PML IV overexpression, PRB and E2F are both sequestered in PML NBs. The latter could induce senescence through these two factors (Vernier, Bourdeau et al. 2011). All these data highlight the major role of PML in the control of cell growth and the induction of senescence.

Several reports proposed a role of PML SUMOylation in senescence. Indeed, the overexpression of PML increases the recruitment of SUMOylation elements machinery thus inducing premature senescence (Ivanschitz, De The et al. 2013). In addition, PML IV, playing the role of a SUMO ligase, is capable of inducing P53 SUMOylation and subsequently promoting senescence (Chu and Yang 2011). SUMOylation can also induce senescence through modulation of the response to oxidative stress (Kim, Yun et al. 2011, de la Vega, Grishina et al. 2012). Treatment with Arsenic and H<sub>2</sub>O<sub>2</sub>, both inducing oxidative stress, induces the formation of disulfide bonds and triggers the biogenesis of NBs (Jeanne, Lallemand-Breitenbach et al. 2010) (Figure 23).



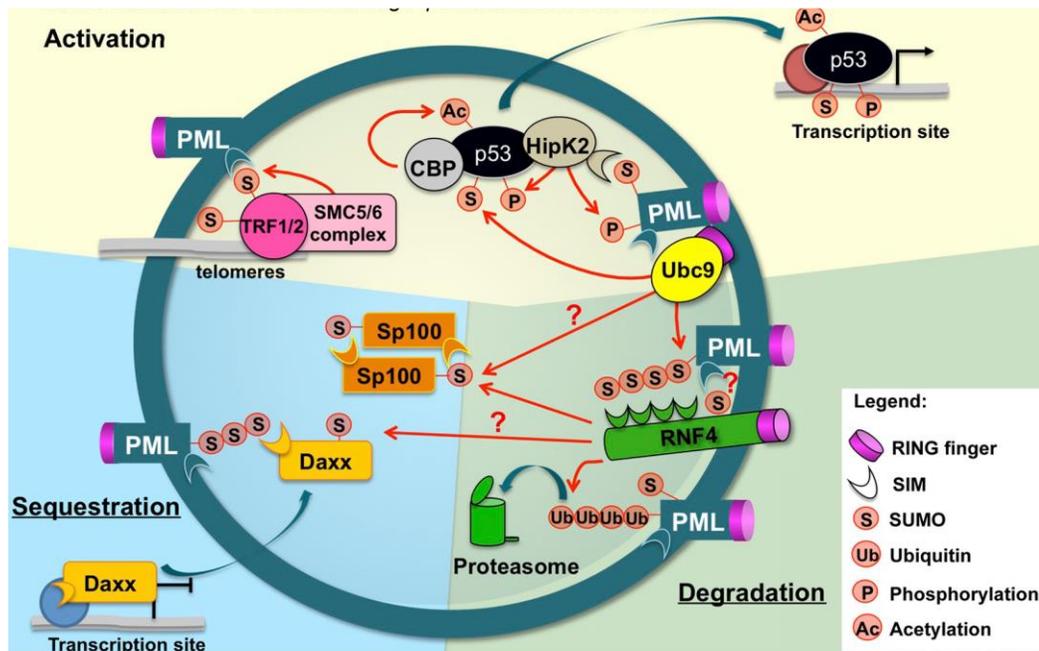
**Figure 23: Crosstalk between SUMOylation machinery and PML pathway controls senescence induction (Ivanschitz, De The et al. 2013).**

SUMOylation process and PML NBs functions are highly cross-connected. Increase of both SUMO and PML levels induces senescence. SUMOylation enzymes regulate NBs formation and partners' recruitment. Conversely, NBs could potentiate SUMOylation process and partner's modification. Finally, senescent cells express specific cytokines (IFNs or IL-6) that, in a positive feedback loop, enhance PML expression and also induce oxidative stress further enforcing NBs formation.

### 6.4.2 PML: a platform for post-translational modifications

An *in silico* study studying the functions of PML NBs has associated these structures with "hot spots" for protein SUMOylation (Van Damme, Laukens et al. 2010). In these NB, all the machinery of SUMOylation is present: The Sumo paralogues, the UBC9, the Sumo E3 ligases and the enzymes of de-SUMOylation SENP1 and 2 (Ivanschitz, De The et al. 2013). In addition, PML interacts directly with UBC9 (Duprez, Saurin et al. 1999). Thus, the majority of the proteins associated with these NBs are modified by SUMO (Figure 24).

The presence of the SIM motif is considered to be an addressing signal to PML NBs, as is the case for Daxx and Sizn1 proteins (Lin, Huang et al. 2006, Cho, Lim et al. 2009). The most widely studied factor, modulating post-posttranslational changes in PML and its partner proteins, is Arsenic. Indeed, Arsenic-induced oxidative stress increases PML SUMOylation as well as the recruitment of ubiquitin E3 ligase RNF4 to PML NBs resulting in the polyubiquitination and the proteasomal degradation of PML (Lallemand-Breitenbach, Jeanne et al. 2008). Arsenic and IFN promote the recruitment of UBC9 to NBs and significantly decrease its nucleoplasmic distribution (Sahin, Ferhi et al. 2014). In addition, the SUMOylation of SP100, which is PML-dependent, was significantly increased following treatment with Arsenic and IFN (Sahin, Ferhi et al. 2014). Other post-translational modifications also occur in PML NBs. Indeed, many P53 regulators have been identified in NBs, such as CBP, HIPK2, HDM2, and PIAS proteins. These proteins modify P53 by acetylation, phosphorylation, ubiquitination and SUMOylation respectively (Lain, Midgley et al. 1999, Pearson, Carbone et al. 2000, Hofmann, Moller et al. 2002).



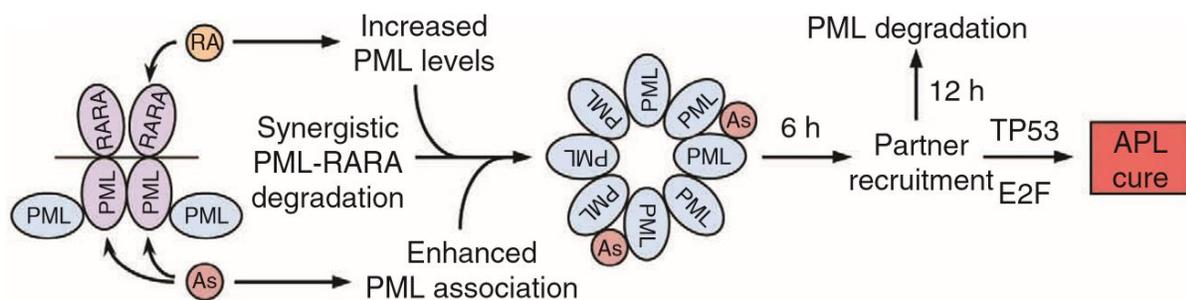
**Figure 24: NBs are redox-regulated hubs of sumoylation and SUMO-initiated, RNF4-mediated ubiquitination**

PML oxidation elicits PML NB formation. Enzymes, such as SUMO E2 Ubc9, SUMO E3 SMC5/6 subunits, ubiquitin E3 RNF4, the acetyltransferase CBP, or the kinase HIPK2, are recruited onto NBs. Concentrating enzymes and their substrates supports posttranslational modifications of partner proteins, such as SP100, the structural maintenance or chromosome (SMC) complex involved in alternative lengthening telomeres, DAXX, or p53. These modifications affect activity and stability of PML partners and/or lead to their sequestration within NBs, converging into quiescence (stem cells) or a senescence program.

## 6.5 Role of PML NB in the treatment of APL

Treatment with the combination of RA and ATO cured the disease in almost 90% of cases. Indeed, RA/ATO induces CR in mouse models of APL (Lallemand-Breitenbach, Guillemain et al. 1999) as well as in treated patients (Shen, Shi et al. 2004). As previously mentioned, in APL, RA induces granulocyte differentiation, activates the transcription of RAR $\alpha$  and triggers the degradation of the RAR $\alpha$  moiety of the PML / RAR $\alpha$  chimeric protein (Zhu, Gianni et al. 1999, Licht 2006). ATO induces PML SUMOylation on lysine K160 resulting in the degradation of the PML moiety (Lallemand-Breitenbach, Zhu et al. 2001). In addition, ATO binds directly to PML and PML / RAR- $\alpha$  inducing their oxidation and promoting the biogenesis of PML NBs (Jeanne, Lallemand-Breitenbach et al. 2010). Indeed, it was shown that PML/RARA degradation is

followed by PML NBs reformation resulting in P53 activation and loss of LIC self-renewal, therefore eradicating the disease (Ablain, Rice et al. 2014) (Figure 25). Moreover, they showed that treatment with high or moderate doses of RA induces the activation of differentiation gene. However, only high doses of RA activate genes involved in both differentiation and cell cycle arrest. Finally, this study has shown that the PML NBs are re-formed as early as 6 hours of treatment following the degradation of PML-RARA. This seems necessary for the recruitment of partner proteins as well as for P53 activation, loss of LIC self-renewal and therefore, the eradication of the disease (Ablain, Rice et al. 2014). These data support a model in which PML NBs are not only "Hot spots" of post-translational modifications and proteasomal degradation, but also an activation site of P53.



**Figure 25: Model by which targeting normal Pml contributes to APL cure by the RA-Arsenic combination (Ablain, Rice et al. 2014).**

# RESULTS

# **Results part 1: Manuscript to be submitted to Nature Medicine**

## **A Pin1/PML/P53 axis activated by retinoic acid and arsenic in *NPM1c*-AMLs**

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¶,\* H.D.T and A.B. contributed equally to this study.

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**ABSTRACT**

Retinoic acid (RA) was proposed to increase survival of chemotherapy-treated Nucleophosmin-1 mutated Acute Myeloid Leukemia patients (NPM1-mutant AMLs). Ex vivo, RA or Arsenic (ATO) trigger NPM1c degradation, P53 reactivation and growth arrest. The promyelocytic leukemia protein (PML) tumor suppressor organizes domains that control proteolysis and P53-driven senescence. Here we show that PML is required for RA/ATO-induced NPM1c degradation. In primary NPM1c-AML patient blasts, RA also rapidly upregulates the initial low basal PML expression through Pin-1 inhibition prior to NPM1c clearance. This RA-induced PML stabilizes P53 and primes blasts for ATO-driven nuclear body reformation, yielding hyper-activation of P53. RA/ATO combination elicits PML-dependent responses, associated with in vivo P53 activation of in NPM1c AMLs xenografts. in vivo associated to early? P53 activation. Finally, RA/ATO-initiated a transient complete remission in a NPM1c-AML patient demonstrating clinical relevance of this combination. PIN1 activation and PML loss in multiple tumor types suggests that, beyond NPM1c-AMLs, this the RA/ATO/Pin1/PML/P53 axis may be more broadly therapeutically exploited.

Nucleophosmin 1 (NPM-1) is a chaperone implicated in multiple processes, notably ribosomal biogenesis and growth control. *NPM-1* alterations were directly implicated in cancer development, through a variety of mechanisms, including chromosomal translocations or recurrent mutations (Grisendi, Mecucci et al. 2006). In acute myeloid leukemia (AML) subtypes, the most prevalent one is a short nucleotide insertion that induces a frame shift in the C-terminus

of the protein, yielding NPM1c (Falini, Nicoletti et al. 2007). Multiple properties were demonstrated for NPM1-c, including P53 inhibition or cytoplasmic sequestration of key regulatory proteins (Heath, Chan et al. 2017, Kunchala, Kuravi et al. 2018). NPM-1-associated AMLs represent a major unmet medical need, particularly in relapsed patients or in aged patients unfit for chemotherapy.

Retinoic acid (RA) is a hormone with multiple effects on development and tissue homeostasis. RA has a dual effect on stem cell fate and differentiation following Retinoic Acid Receptors (RARs and RXRs)-mediated modulation of transcription. Recently, high doses of RA were also shown to inhibit Pin-1, an enzyme involved in the modulation of multiple growth suppressive pathways (Wei, Kozono et al. 2015, Kozono, Lin et al. 2018). Hence, Pin-1 inhibition could be implicated in some of the growth suppressive properties of RA. RA demonstrated unambiguous clinical efficacy in a variety of conditions including neuroblastoma and acute promyelocytic leukemia (APL) (de The 2018). In APL, RA directly targets the driving PML/RARA oncoprotein for degradation and yields complete remissions (CR) (de The, Pandolfi et al. 2017) through activation of a PML/P53 senescence checkpoint (Ablain, Rice et al. 2014, de The, Pandolfi et al. 2017). Genetic analysis from historical therapy-resistant APL patients point to a central role of PML/RARA degradation and PML activation in clinical responses (Lehmann-Che, Bally et al. 2014, de The, Pandolfi et al. 2017, Lehmann-Che, Bally et al. 2018). In other AMLs, RA may exert some clinical activity in combination with chemotherapy (Schlenk, Frohling et al. 2004). Whether this reflects RA-induced AML differentiation, as observed in some non-APL AML primary patient cells or models (Altucci, Rossin et al. 2005, Boutzen, Saland et al. 2016) (de The 2018), remains to

be elucidated. Intriguingly, the clinical benefit of RA co-administration with chemotherapy appears maximal in AMLs bearing an *NPM1-c* mutation (Schlenk, Dohner et al. 2009). We reported that *NPM1-c* is degraded upon RA administration in cell-lines, suggesting that loss of *NPM1-c* expression may underlie, or at least contribute to, RA-driven differentiation and apoptosis *ex vivo* (El Hajj, Dassouki et al. 2015, Martelli, Gionfriddo et al. 2015). This degradation was accelerated by co-administration of arsenic trioxide (ATO). Intriguingly, a recent study suggested that similar to RA, ATO may also targets Pin-1 and proposed that the RA/ATO combination could have broad therapeutic impacts in multiple cancers with high Pin-1 activity (Kozono, Lin et al. 2018). Yet, the actual mechanism(s) of RA-enhancement of chemotherapy response in *NPM1c*- AMLs and the downstream targets of the RA/ATO-mediated Pin-1 inhibition remain (s) to be established.

PML (TRIM19) nucleates nuclear bodies (NBs) which are stress-responsive domains that exert growth suppressive properties (Lallemand-Breitenbach and de The 2018). *In vivo*, PML NBs are oxidative stress sensors controlling P53 activation (Niwa-kawakita, Ferhi et al. 2017). PML plays a key role in the therapeutic response of APL and is the direct target of ATO therapy (Zhu, Koken et al. 1997, Jeanne, Lallemand-Breitenbach et al. 2010, Ablain, Rice et al. 2014, Lehmann-Che, Bally et al. 2014, Lehmann-Che, Bally et al. 2018). PML expression is altered in multiple tumor types, most often through PML protein loss upon activation of various degradation pathways (Koken, Linares-Cruz et al. 1995, Gurrieri, Capodiecici et al. 2004, Scaglioni, Yung et al. 2006, Yuan, Lee et al. 2011, Wu, Lin et al. 2014).

Here, we demonstrate that NPM1c-expressing AMLs exhibit a dramatic response to RA and/or ATO *in vivo*. Exploring the basis for this response, we unravel an unexpected key role of PML. PML is required to initiate RA and/or ATO-driven NPM1c degradation. RA sharply stabilizes the initially low PML through Pin-1 inhibition; ATO subsequently enforces PML NB-formation, P53 activation and loss of clonogenic activity. Our studies enlighten a dual and synergistic impact of RA and ATO to promote, not only NPM1c loss, but also PML NB reformation and P53 activation in AMLs.

## Results

### PML-dependent NPM1c degradation activates P53

RA and ATO trigger NPM1c degradation in NPM1c-expressing AML cells. Several studies reported ATO-induced degradation of oncoproteins, some of them PML-dependent (Goussetis, Gounaris et al. 2012, Lo and Kwong 2014, Dassouki, Sahin et al. 2015, Piao, Chau et al. 2017). In OCI-AML3 cell-line in which PML expression was abrogated by CRISPR-mediated excision (OCI-AML3<sup>pml<sup>-/-</sup></sup>) cells, RA/ATO mediated NPM1c degradation was impeded (Fig. 1a) and cell death abrogated (Fig. 1b). To assess any *in vivo* relevance of these observations, we compared xenografts from OCI-AML3 or OCI-AML3<sup>pml<sup>-/-</sup></sup>. Four weeks of treatment with RA/ATO combination led to a sharp decrease of human leukemic cells in the bone marrow of treated mice xenografted with OCI-AML3, but not OCI-AML3<sup>pml<sup>-/-</sup></sup> cells (Fig. 1c), demonstrating *in vivo* efficacy of this strategy and suggesting that RA/ATO response may be mediated though PML-facilitated NPM1c degradation. We then treated mice xenografted with OCI-AML3 or OCI-AML3<sup>pml<sup>-/-</sup></sup> cells with 7 days of RA and ATO. Therapy led again to a rapid decrease of human cells in the bone marrow of

treated mice (data not shown), associated with NPM1c degradation and P53 induction *in vivo*, only in cells harboring intact PML (Fig. 1d). Remarkably, the RA/ATO combination induced massive cell death *in vivo* (data not shown). This result is in line with the *ex vivo* demonstration that NPM1c controls P53 signaling (Cheng, Grisendi et al. 2007, El Hajj, Dassouki et al. 2015, Heath, Chan et al. 2017, Kunchala, Kuravi et al. 2018). To assess any role of P53 in cell death upon RA/ATO exposure, we also generated a CRISPR P53 OCI-AML3 (OCI-AML3<sup>p53<sup>-/-</sup></sup>) cell line. *Ex vivo*, RA/ATO failed to initiate cell death (Fig. 1e), although it efficiently degraded NPM1c (Fig. 1f). Thus, RA/ATO-triggered, PML-facilitated, NPM1c degradation activates P53 to impede growth of AML *in vivo*.

To directly explore the RA/ATO-triggered pathways involved in growth inhibition, we assessed the transcriptional effects of RA and/or ATO treatment in OCI-AML3 and control OCI-AML2 cells. As expected a clear P53 signature was found in AML3, compared to AML2 (Fig. 1g). Other pathways were also identified (data not shown) and their contribution to response will require further analysis.

### **PML-dependent P53 activation prior to NPM1c loss**

Further investigating the response to the RA/ATO combination, we unexpectedly obtained evidence for rapid P53 stabilization prior to any significant NPM1c loss or restoration of ARF expression (Fig. 2a, b). P53 was stabilized upon exposure to RA and/or ATO and its activation required PML (Fig. 2c). Thus, NPM1c loss is not the sole contributor to P53 activation. Remarkably, similar data was obtained upon *ex vivo* treatment of primary blasts derived from NPM1c AML patients (Fig. 2d-f). Complete NPM1c loss by the RA/ATO combination was only

obtained after 48h (Fig. 2e, f), while P53 stabilization was generally observed as soon as 2h after RA or ATO exposures. Critically, such P53 activation was solely observed in *NPM1c* AMLs (Fig. 2d and data not shown). As expected, *NPM1c* degradation was associated to restoration of normal ARF levels (Fig. 2e). Nevertheless, ARF restoration is most unlikely to explain the early P53 activation.

There are suggestions for altered PML NB in OCI-AML3 cells (El Hajj, Dassouki et al. 2015, Martelli, Gionfriddo et al. 2015), which in principle may affect basal P53 signaling (de The, Le Bras et al. 2012, Niwa-kawakita, Ferhi et al. 2017). We confirmed these differences in PML NBs in OCI-AML3 and AML2 cells, using low amounts of antibodies to highlight the contrast between normal and faint NBs (Fig. 3a). To investigate the basis for impaired NB-formation, we analyzed PML expression in primary AML patients' blasts by Western blot. *NPM1c* expression was tightly correlated with low levels of PML expression while PML transcription was not affected (Fig. 3b and data not shown).

We then examined the effects of RA or ATO on PML expression and NB formation in AML blasts. As expected, ATO induced NB-formation and hyper-sumoylation of PML (Fig. 3c) (Zhu, Koken et al. 1997, Lallemand-Breitenbach, Zhu et al. 2001). Unexpectedly, RA also rapidly stabilized PML levels, solely in *NPM1c*-positive patient cells (Fig. 3c, d). The kinetics of PML up-regulation closely paralleled that of P53 stabilization (Fig. 3d) and was accompanied by appearance of normal PML NBs, contrasting with faint ones observed pre-treatment (Fig. 3e). PML is required for RA-triggered P53 stabilization (Fig. 2c), so that RA-mediated PML up-regulation most likely contributes to P53 stabilization. Then, in RA-primed cells, ATO promotes

NB formation (Zhu, Koken et al. 1997, Jeanne, Lallemand-Breitenbach et al. 2010) to further activate P53 prior to NPM1c loss. Functionally, RA lead to PML- and P53-dependent loss of clonogenic activity of AML3 cells in methyl-cellulose, demonstrating that PML stabilization and P53 activation impede self-renewal and clonogenic activity *ex vivo* (Fig. 3f).

### **RA targets PML through Pin-1 inactivation**

RA inconsistently enhanced *PML* gene expression, possibly through enhanced interferon production (Stadler, Chelbi-Alix et al. 1995), questioning the basis for increased PML expression. RA inhibits the Pin1 enzyme and the latter regulates PML stability (Reineke, Lam et al. 2008, Yuan, Lee et al. 2011, Wei, Kozono et al. 2015). We thus compared the effects of RA and a Pin1 inhibitor (AG17724) on PML abundance NB formation and P53 activation. Strikingly, RA or AG17724 similarly promote NB formation and stabilize PML or P53 levels in OCI-AML3 and *NPM1c*-AML patient cells (Fig. 4a-e, data not shown). In contrast, *NPM1*-WT AML cells were unresponsive to AG17724, as previously shown for RA. These results strongly support a model wherein RA inactivates Pin-1, to stabilize PML, to restore NB and activate P53.

To directly demonstrate this model, we generated an OCI-AML3 cell-line with stable Pin-1 down-regulation by ShRNA. Remarkably, PML and p53 or p21 activation by RA was abrogated following down-regulation of Pin-1 (Fig. 4f) Loss of clonogenic activity by RA or Pin-1 inhibition and induction of apoptosis by RA/ATO were also abrogated (Fig. 4g, h). Since Pin-1 inhibition does not affect NPM1c stability (Fig. 4i), these results imply a key role for the Pin-1/PML/P53 axis, in response to the RA/ATO association.

### **PML is the primary target of RA and Pin1 inhibitors**

Pin-1 directly controls both PML stability and P53 signaling (Mantovani, Zannini et al. 2015). PML and P53 are also highly cross-regulated: PML controls P53 activation, but P53 transcriptionally induce PML expression (Pearson, Carbone et al. 2000, de Stanchina, Querido et al. 2004). To decipher the respective roles of PML and P53 in response to Pin-1 and RA, we compared RA and AG17724 response in OCI-AML3 and its *pml*<sup>-/-</sup> and *p53*<sup>-/-</sup> derivatives. Both drugs upregulated PML levels in *P53*<sup>-/-</sup> cells, and no induction of P53 was observed in OCI-AML3<sup>*pml*<sup>-/-</sup></sup> cells (Fig. 4j). These results establish that PML is the primary target of RA and Pin1 inhibitors in NPM1c expressing cells, allowing subsequent P53 activation. This does not exclude the possibility that P53 constitute a feed-forward amplification loop on PML expression.

### **The RA/ATO combination has clinical activity in an AML patient**

The combination of RA/ATO is a broadly used and very well-tolerated therapeutic association in APL (Lo-Coco, Di Donato et al. 2016). Strikingly, in an *NPM1c* AML patient, unfit for conventional therapy who received this RA/ATO combination on a compassionate basis, a complete molecular remission in the bone marrow was reached after 3 weeks (Fig. 5a). Longer follow-up of the patient, after 2 months showed appearance of slowly growing AML cells. Thus, the RA/ATO combination has the ability to transiently clear AML cells in some *NPM1c* patients.

### **RA and Actinomycin D cooperate to clear NPM1c-expressing cells**

Actinomycin D (ActD) triggered a long-lasting clinical remission in an *NPM1c*-AML patient (Falini, Brunetti et al. 2015). We thus examined the possibility of cooperation between these two

active drugs. Both RA and ActD crippled *ex vivo* clonogenic activity in OCI-AML3 cells, but not in OCI-AML2 ones (Fig. 5b). Again, RA or ActD failed to exert any effect, even in combination, in OCI-AML3<sup>pml<sup>-/-</sup></sup> or AML3<sup>P53<sup>-/-</sup></sup> cells (Fig. 5b). To assess the *in vivo* relevance of these cooperation, we used xenografts from OCI-AML3 or OCI-AML3<sup>pml<sup>-/-</sup></sup>, in immuno-deficient mice. RA and ActD synergized and led to a sharp decrease of human cells in the bone marrow of treated mice (Fig. 5c). This was accompanied by human P53 stabilization (Fig. 5c).

## DISCUSSION

We report that PML constitutes an unsuspected actor downstream of RA/ATO, a combination active in *NPM1c*-AMLs *in vivo*. The basis for RA response of non-APL AMLs was initially believed to be RA-induced differentiation. Here, we observed RA-induced AML clearance without evidence for terminal differentiation in xenograft models. Note that even if differentiation occurs, it may not necessarily be the driving force underlying long-term AML clearance (de The 2018). Previous *ex vivo* studies suggested that RA/ATO-driven *NPM1c* degradation was the molecular basis of their therapeutic activity, at least in part through upregulation of ARF which become comparable to other AML cells and contribute to restoration of P53 signaling. *NPM1c* degradation should also correct multiple other phenotypes associated with *NPM1c*, including sequestration of key regulators in the cytoplasm or transcriptional deregulation (Haindl, Harasim et al. 2008, Kuo, den Besten et al. 2008, Yun, Wang et al. 2008, Gu, Ebrahim et al. 2018, Kunchala, Kuravi et al. 2018). Kinetic analysis of P53 activation revealed that it actually preceded significant *NPM1c* loss, suggestive for the existence of at least another activation pathway. Recent reports found evidence for RA/ATO synergism in multiple tumor types through Pin-1 inhibition (Wei,

Kozono et al. 2015, Kozono, Lin et al. 2018). We indeed found that in *NPM1c*-AML cells, RA has an essential role for growth arrest through Pin-1 inhibition. Downstream of Pin1, we identify the PML growth suppressor and P53 (Reineke, Lam et al. 2008, Lim, Liu et al. 2011), as essential downstream effectors of ATO or RA in AML cells. Analysis of *pml*<sup>-/-</sup> and *p53*<sup>-/-</sup> AML3 cells demonstrate that P53 is downstream of PML-triggered Pin1/RA responses. Synergism between RA and ATO for growth arrest was proposed to rely on Pin-1 inhibition by ATO, RA also promoting ATO intake through upregulation of the ATO transporter AQ9 (Kozono, Lin et al. 2018). Our discovery of the key role of PML downstream of RA-initiated Pin-1 inhibition suggests a novel complementary mechanism (at least in AML cells) wherein RA-initiated Pin-1 inhibition would upregulate PML and ATO would promote PML NB-formation (Zhu, Koken et al. 1997), both ultimately driving P53/senescence (Fig. 5d). In *NPM1c*-positive AMLs, the respective contributions of PML NB-reformation and *NPM1c* degradation in the *in vivo* long-term response require further investigations. Yet, the absence of RA effect on clonogenic activity of Pin-1 down-regulated cells favors an important role of Pin-1 inhibition in biological response and not only P53 activation (Fig. 4g). Similarly, the mechanism through which *NPM1c*-AMLs consistently exhibit very low basal levels of PML and faint NBs requires further studies. The dual and key implication of PML in both processes was not anticipated. This model presents a number of feed-forward loops all favoring anti-proliferative responses: RA-induced PML stabilization should facilitate ATO-induced/PML-facilitated *NPM1c* degradation and P53 activation will enhance PML expression. Our results unravel a striking parallelism with the APL model: both involve oncoproteins that alter NBs and down-regulate basal P53 signaling. In both, therapy response involves degradation of the driving oncogene, PML NB reformation and P53 activation (de The,

Pandolfi et al. 2017). The co-existence of other major oncogenes (key epigenetic regulators, FLT3) in *NPM1c*-positive AMLs, most likely explains why RA/ATO is not curative on its own. Yet, both are likely to favor the action of standard chemotherapy by reverting P53 inhibition. In that respect, we demonstrate a dramatic synergy between RA and ActD, a drug which alone allows a substantial number of remissions (Falini, Brunetti et al. 2015). The excellent tolerance of the RA/ATO combination *in vivo* could promote clinical trials in other malignancies, notably those where Pin1 and/or PML are deregulated (Koken, Linares-Cruz et al. 1995, Gurrieri, Capodieci et al. 2004). In that respect, unexpected clinical responses to solid tumors were observed in some RA/ATO-treated APL patients who presented a synchronous another malignancy (Alsafadi, Even et al. 2013, Jain, Konoplev et al. 2018), possibly reflecting activation of the ATO-enhanced RA/Pin-1/PML/P53 axis unraveled by this study.

## **METHODS**

### **Cell lines, patient blasts and *ex vivo* treatments**

OCI-AML3 or OCI-AML2 AML cells (harboring the *NPM1c* mutation without *FLT3*-ITD or wild type (wt) *NPM-1* respectively) were grown in minimum essential medium- $\alpha$  (MEM $\alpha$ ) supplemented with 20% fetal bovine serum (FBS) and antibiotics. Cells were seeded at the density of  $2 \times 10^5$ /ml.

Primary bone marrow blasts from AML patients were extracted following Ficoll separation and cultured in MEM- $\alpha$  supplemented with 20% FBS and antibiotics. Patients' samples were collected following approval by the American University of Beirut Institutional Review Board and after patients provided written informed consent in accordance with the declaration of Helsinki.

ATO (Sigma Aldrich) or RA (Sigma Aldrich) were used at 1  $\mu$ M final concentration. The Pin-1 inhibitor AG17724 (Sigma Aldrich) was used at 20  $\mu$ M. ActinomycinD (Sigma Aldrich) was used at 5 nM. Cell growth was assessed using the CellTiter® 96 cell proliferation assay kit (Promega Corp., Madison, WI) or by trypan blue dye assay.

### **Short hairpin RNA (shRNA)**

In knock-down experiments, the following shRNA Pin1 (CCACCGTCACACAGTATTTAT) and control scrambled were used. Lentiviruses were produced by transient transfections of HEK-293T cells. Infection of OCI-AML2 or OCI-AML3 cells with different lentiviruses was performed by spinoculation for 3h at 1500rpm and at 32°C.

### **CRISPR AML3 cell lines**

PML expression was abrogated by CRISPR-mediated excision. A guide RNA targeting *PML* (Forward: 5'-GTCGGTGTACCGGCAGATTG; Reverse: 5'-AATCTGCCGGTACACCGAC) was designed and cloned into pLAS5w.Ppuro-Cas9 plasmid for viral packaging. OCI AML-3 cells were infected with the corresponding viruses. Stable selection of knock-out cells was performed in the presence of 1 $\mu$ g/ml of puromycin, over a period of 2 weeks. Similarly, P53 extinction was performed using a guide RNA targeting *P53* (Forward: 5'-CCATTGTTCAATATCGTCCG; Reverse: 5'-CGGACGATATTGAACAATGG). Recombinant Cas9 protein was synthesized from IDT to form Alt-R CRISPR/Cas9 RNP. OCI AML-3 cells were transiently transfected with Alt-R CRISPR/Cas9 RNP by using Nucleofactor kit T (Amaxa) and applied program number X-01 in the nucleofactor device (Lonza). The stable CRISPR knock-out clones were cloned by serial dilution to generate a single-cell separation. DNA from individual clones was extracted and the region surrounding the Cas9

cutting site was amplified by PCR and verified by sequencing to ensure the deletion of the target genes.

### **Colony Formation Assay**

AML cells were pretreated for 3hrs with 1 $\mu$ M for RA before treatment with 5 nM ActinomycinD for additional 3hrs. Cells were then embedded, at a density of 250 cells/well in 6-well plates, into methylcellulose (Stem Cell Technologies) supplemented with 20% FBS. After 10 days, colonies were counted using an inverted microscope device.

### **Microarray Analysis**

cDNA microarray was conducted at the Curie Institute. The heatmap was composed of top 40 differentially expressed P53 target gene in NPM1c mutant cells. P53 target gene were analyzed based on reports from the literature {Fischer, M, 2017}.

### **Immunoblotting**

For immunoblotting, cells were solubilized in 2x laemmli buffer. 50  $\mu$ g of proteins were separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Blots were incubated with the following specific monoclonal antibodies: anti-NPM1 recognizing both WT and mutated NPM1c (Abcam), and ARF (p14<sup>arf</sup>) (Abcam), anti-p21(cell signaling), anti-P53 (Santa Cruz), and polyclonal antibodies: anti-NPM-1 recognizing specifically the mutated NPM1c (Invitrogen), a homemade chicken anti-PML, and anti-Pin1 (Cell signaling). Proteins were then visualized using the enhanced chemi-luminescence system (Bio-Rad).

### **Immunofluorescence and confocal microscopy**

OCI-AML2, OCI-AML3 or patients' derived AML blasts were fixed with ice cold methanol at -20°C for 20 minutes and cytospun onto glass slides. Immunostaining was performed with rabbit polyclonal antibody against NPM1c (Invitrogen) and a mouse monoclonal antibody against human PML (Santa Cruz) or a homemade rabbit anti-PML antibody. Primary antibodies were revealed by Alexa Fluor 488- or Fluor 594-labeled secondary antibodies (Abcam). Staining of nuclei was performed with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen).

Images were acquired by confocal microscopy using a Zeiss LSM710 confocal microscope (Zeiss, Oberkochen, Germany) with a Plan Apochromat 63/1.4 numeric aperture oil-immersion objective, using Zen 2009 (Carl Zeiss).

### **Xenograft Animal Studies**

NOD/Shi-scid IL2 $\gamma$ <sup>-/-</sup> (NSG) mice were obtained from Jackson Laboratories (United States). All mouse protocols were approved by the Institutional Animal Care and Utilization Committee of the American University of Beirut. Three million OCI-AML3 or OCI-AML3<sup>pml<sup>-/-</sup></sup> cells were injected into the tail vein of 8-week-old mice (5 mice per group) 7 days post AML cells' injection, mice were treated intraperitoneally with RA (2.5mg/kg) or ActinomycinD (Cosmegen® Lyovac) (60 $\mu$ g/kg) every other day over a period of 4 weeks or daily for 7 consecutive days. RA was dissolved in dimethyl sulfoxide and diluted in 1x PBS supplemented with 5% Cremophor/5% Ethanol before its intraperitoneal administration to the mice.

### Human CD45 staining

Bone Marrow (BM) from the femurs and tibias of euthanized engrafted NSG animals with OCI-AML3 or OCI-AML3<sup>pml<sup>-/-</sup></sup> cells, was flushed at the end of different treatments. To assess the percentage of human engrafted cells following treatment with RA, ActD or their combination, cell surface staining was performed using an anti-human CD45 Peridinin Chlorophyll Protein (PerCP) conjugated antibody (Becton Dickinson). Labeled samples were analyzed on a Guava flow cytometer. BM cells were also used to assess protein human P53 and NPM1c levels by western blot, upon *in vivo* treatment using the monoclonal anti-human P53 (Abcam) and the polyclonal anti-NPM1c (Invitrogen) antibodies. PML nuclear bodies and NPM1c localization were also analyzed in BM of different xenograft NSG, by confocal microscopy.

### Statistical analysis

Data was reported as the average  $\pm$  standard deviations. Statistical analysis was done using Student's t test p-value of less than 0.05 was considered as significant.

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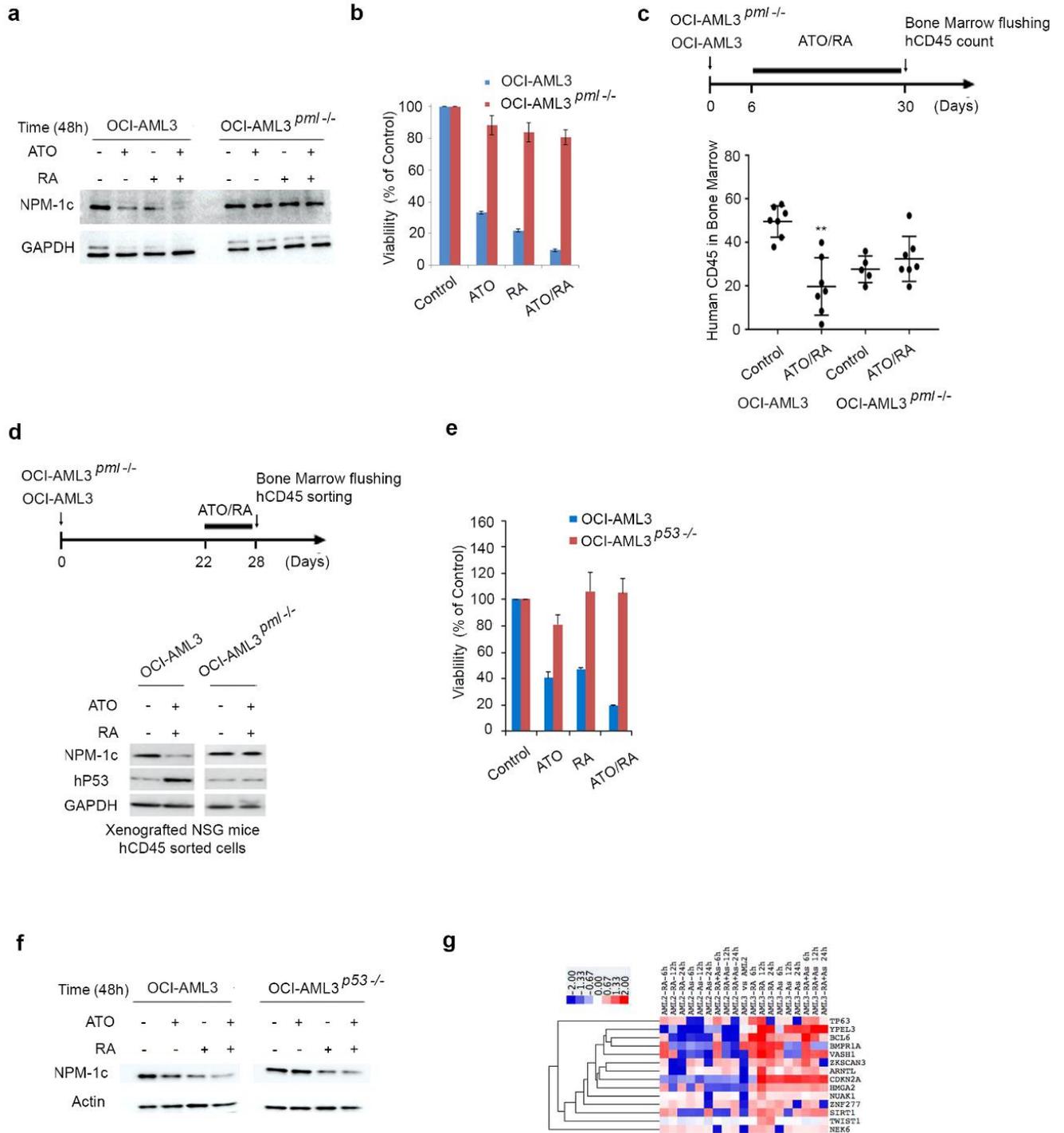
### **Author contribution**

R.H, H.C.W, and C.B. performed experiments; Hong Hu ZHU treated patients; R.M. collected bone marrow samples; R.H, H.E.H., H.C.W, C.B., Z.C., M.E.S., H.d.T. and A.B. analyzed results; R.H, H.E.H., H.C.W, C.B. made the figures; H.E.H, A.B. and H.d.T. designed the research and wrote the paper.

### **Competing interests**

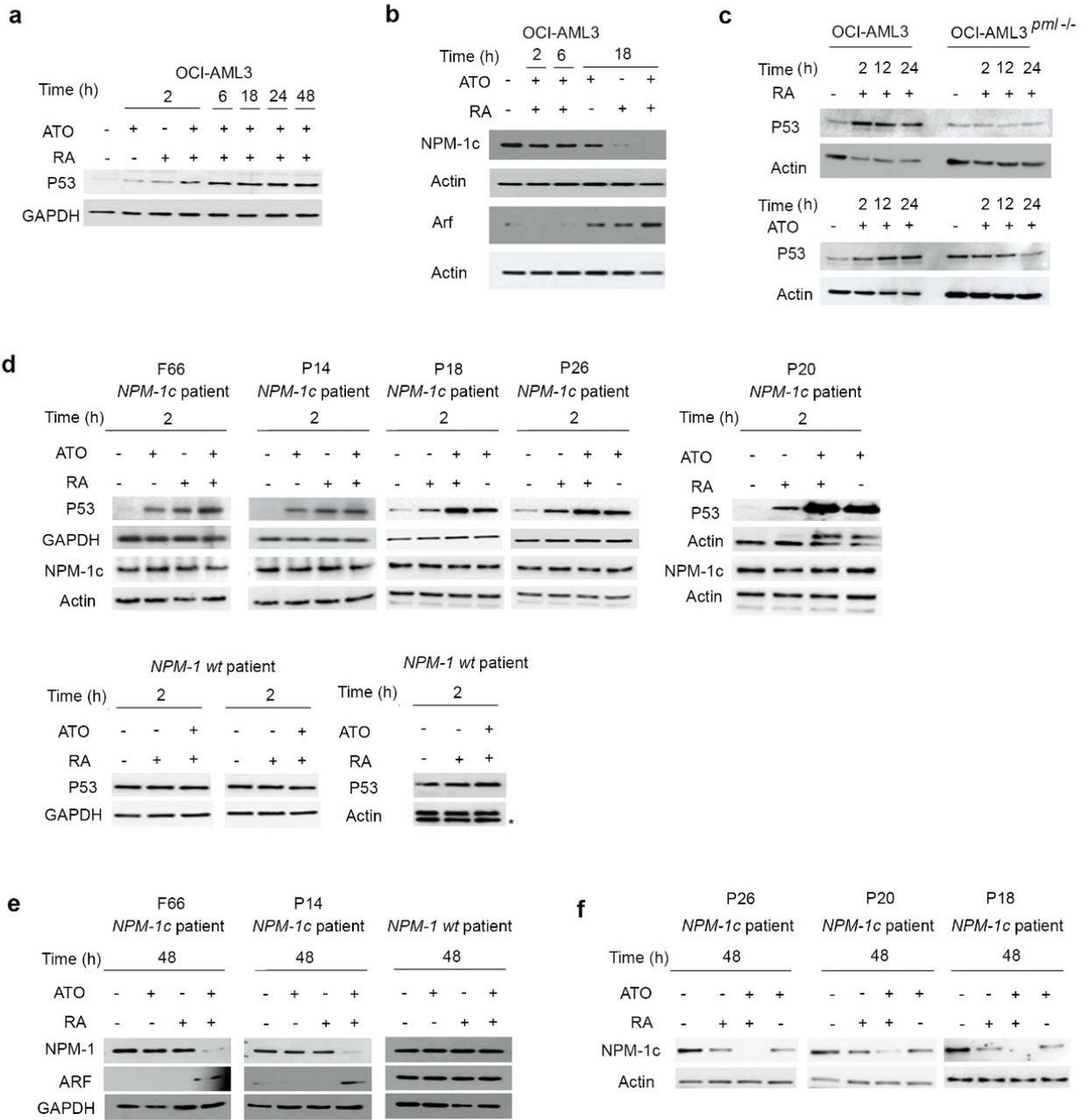
The authors declare no competing interests.

**Figure 1**



**Fig. 1. RA and/or ATO activate p53 and its downstream signaling.** (A) Western blot analysis of NPM1c was performed on extracts of OCI-AML3 and OCI-AML3<sup>pml<sup>-/-</sup></sup> cells after treatment with ATO, RA or their combination for 48 hours. (B) Cell growth (percent of control) was assessed in triplicate wells in OCI-AML3 and OCI-AML<sup>pml<sup>-/-</sup></sup> cells following treatment with ATO, RA and their combination for 48 hours. (C) ATO/RA combination reduces the leukemia bone marrow burden in OCI-AML3 xenograft NSG mice in a PML dependent manner. Eight-week-old NSG mice were injected with 3 million OCI-AML3 or OCI-AML3 PML<sup>-/-</sup> cells intravenously. At day 7 post-leukemic cells injection, ATO and RA were administered every other day, over a period of 4 weeks intraperitoneally. At the end of week 5, bone marrow was harvested from femurs and tibias of xenograft mice and then stained with the anti-hCD45 antibody. Graphs show the hCD45 PerCP percentage of xenograft animals (7 mice per group). (D) Western blot of human P53 and NPM1c in hCD45 positive BM cells from NSG mice xenografted with OCI-AML3 or OCI-AML3<sup>pml<sup>-/-</sup></sup> cells, after *in vivo* treatment with RA and ATO for 6 days. (E) Cell growth (percent of control) was assessed in triplicate wells in OCI-AML3 and OCI-AML3<sup>p53<sup>-/-</sup></sup> cells following treatment with ATO, RA and their combination for 48 hours. (F) Western blot for NPM1c in OCI-AML3 and OCI-AML3<sup>p53<sup>-/-</sup></sup> cells after treatment with ATO, RA or their combination for 48 hours. (G) Transcriptome microarray analysis of OCI-AML2 and OCI-AML3 upon treatment with ATO, RA or their combination for 6, 12 or 24h as indicated.

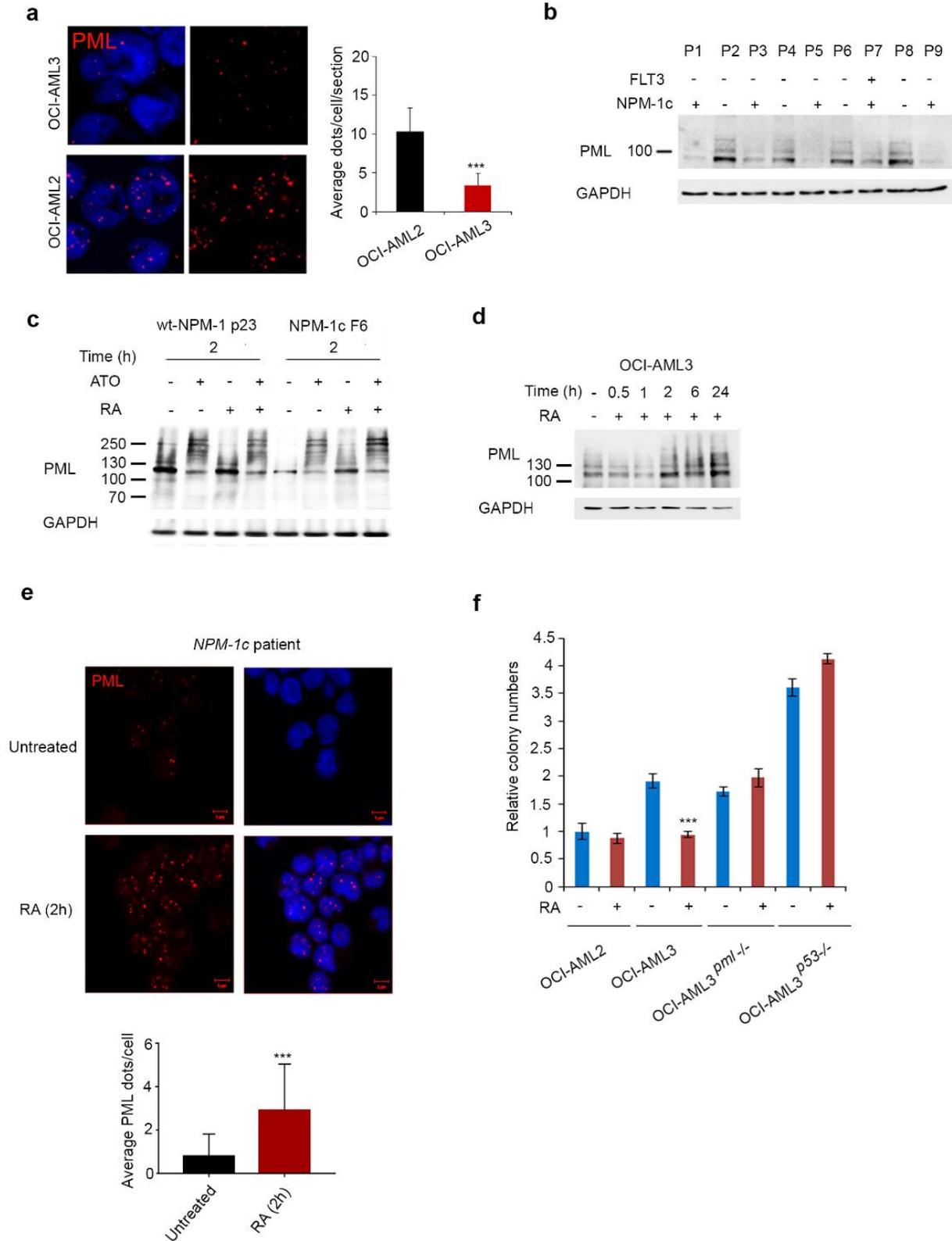
**Figure 2**



**Fig. 2: ATO/RA induce early p53 stabilization**

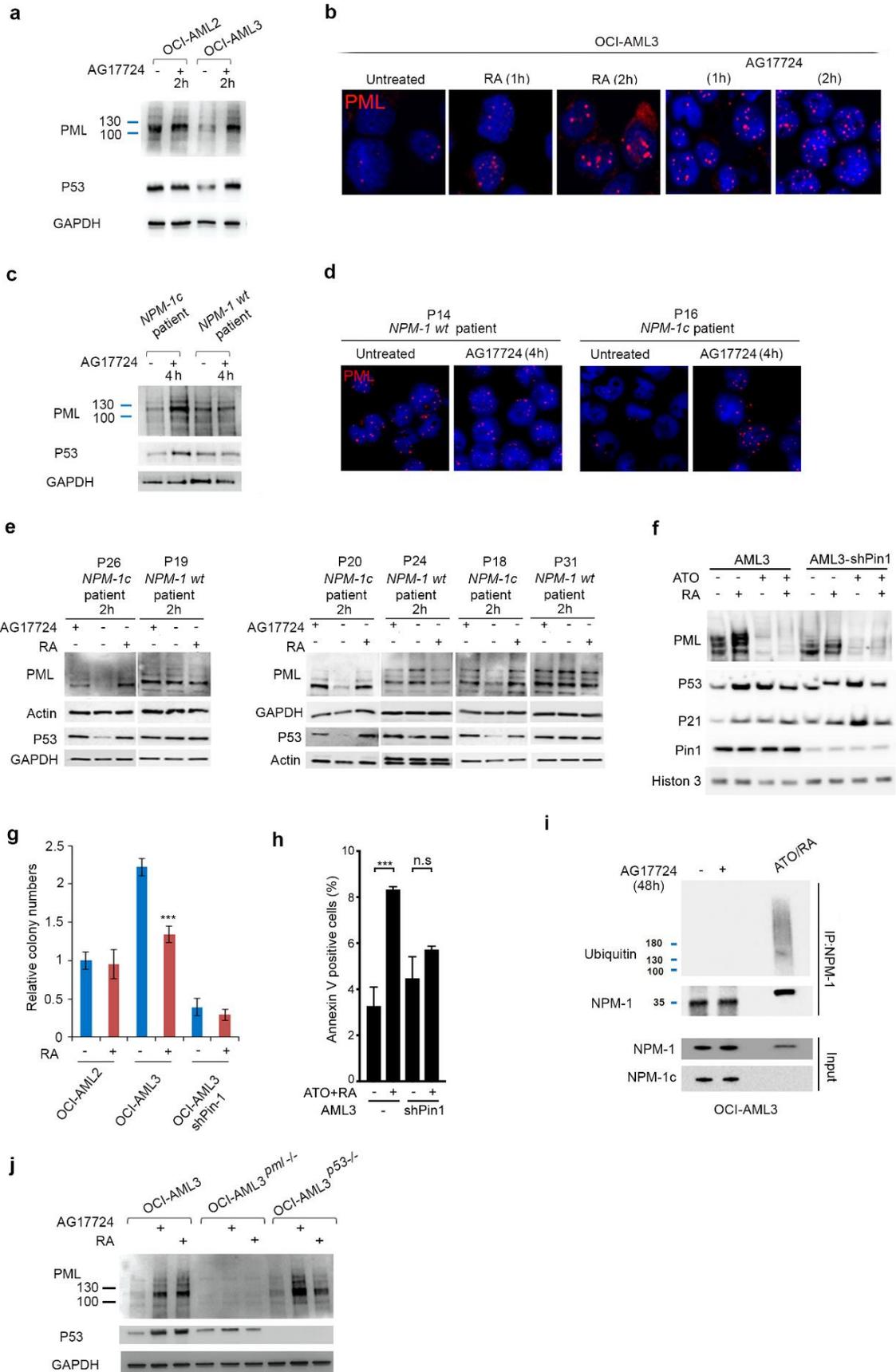
(A-B) Early stabilization of P53 in response to RA/ATO prior to NPM1c loss or ARF restoration. Western blot of p53, NPM1c and ARF in OCI-AML3 cells after treatment with ATO, RA or their combination for 2, 6, 18h, 24h and 48h as indicated. (C) PML-dependent P53 stabilization in OCI-AML3 upon exposure to RA or ATO. Western blot of P53 in OCI-AML3 and OCI-AML3<sup>pml<sup>-/-</sup></sup> after treatment with ATO or RA for 2, 12 and 24 hours. (D) Western blot using anti- NPM1c and P53 antibodies after *ex-vivo* treatment of primary blasts from patients with NPM1c or NPM-1 wild type (wt) AML, with ATO, RA and their combination for 2h. (E) Western blot using anti- NPM1 (wt+c) and anti-ARF antibodies after *ex-vivo* treatment of primary blasts from patients with NPM1c or NPM1wt AML, with ATO, RA and their combination for 48h. (F) Western blot using anti- NPM1c antibody after *ex-vivo* treatment of primary blasts from patients with NPM1c or NPM1wt AML, with ATO, RA and their combination for 48h.

**Figure 3**



**Fig. 3. RA treatment stabilizes PML.** (A) Altered PML nuclear bodies (NB) in OCI-AML3 cells. Immunofluorescence analysis of PML-NBs in OCI-AML3 and OCI-AML2 cells following 1:10000 dilutions of the PML antibody. (B) Low protein levels of PML in primary blasts from NPM1c AML patients. Western blot of PML in primary blasts from 7 AML patients (3 patients expressing *wt-NPM-1* and 4 patients expressing *NPM1c*) as indicated. (C) RA induced stabilization of PML in NPM1c-positive cells. Western blot of PML in primary blasts from AML patients after *ex-vivo* treatment with ATO, RA or their combination for 2 hours. (D) RA-induced rapid stabilization of PML in NPM1c positive cells. Western blot of PML in OCI-AML3 after treatment with RA for 0.5, 1, 2, 6 or 24h. (E) RA rapidly promote PML-NB formation in *NPM1c* positive cells. Confocal microscopy of PML-NBs in primary NPM1c blasts following treatment with RA for 2h. (F) RA abrogates the clonogenic activity of OCI-AML3 cells in a PML and P53 dependent manner. Colony formation assays in methylcellulose of OCI-AML2, OCI-AML3, OCI-AML3<sup>*pml*<sup>-/-</sup></sup> and OCI-AML3<sup>*p53*<sup>-/-</sup></sup> cells after pre-treatment for 3 h with RA as indicated.

Figure 4

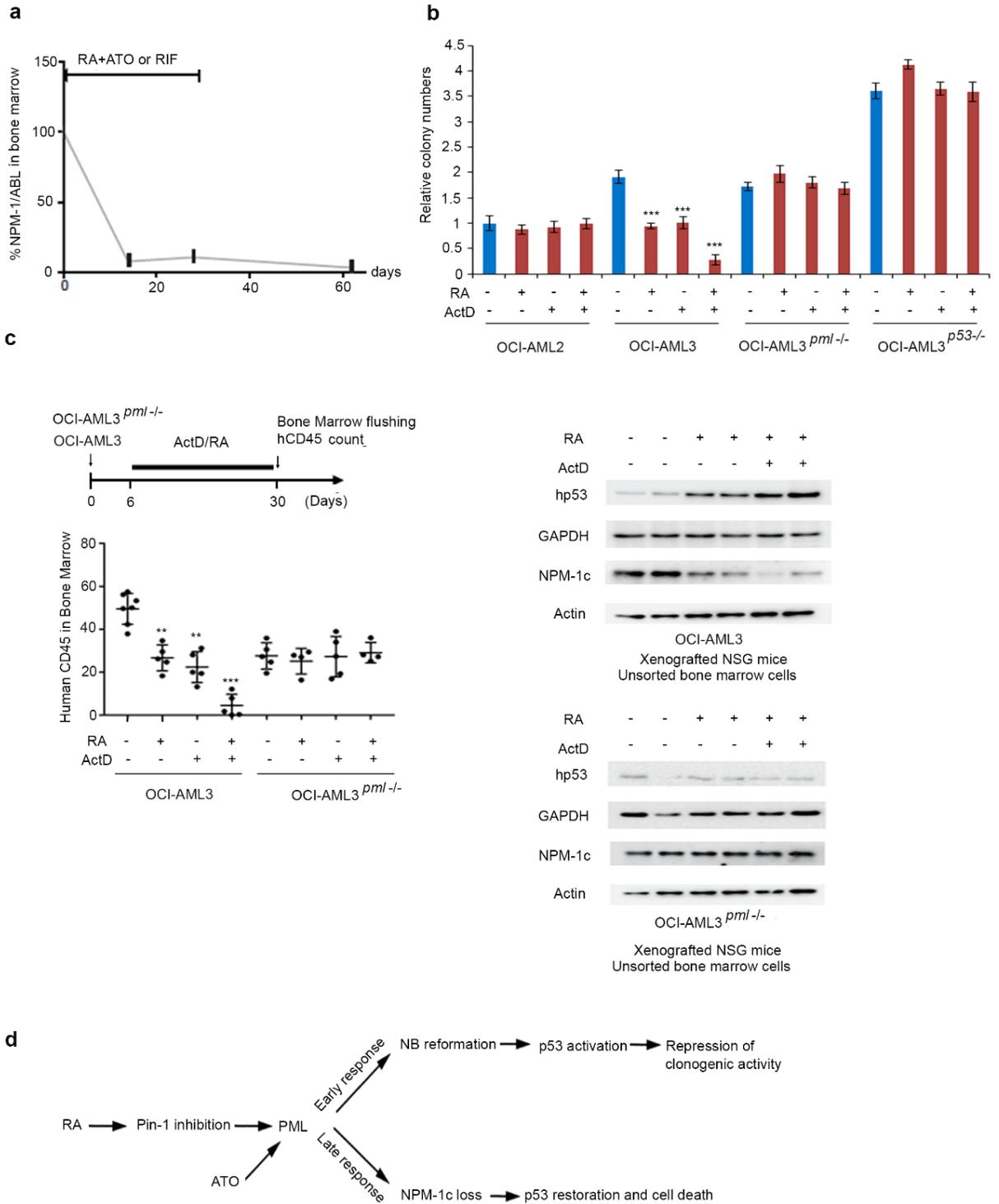


**Fig. 4. RA treatment stabilizes PML through Pin1 inactivation**

(A) Treatment with Pin-1 inhibitor AG17724 stabilizes PML and P53 protein levels in OCI-AML3. Western blot of PML and p53 in OCI-AML3 and OCI-AML2 cells treated with 20 $\mu$ M of AG17724 for 2h. (B) RA or AG17724 rapidly promote PML-NB formation in OCI-AML3 cells. Confocal microscopy of PML-NBs in OCI-AML3 cells following treatment with RA or AG17724 for 1 or 2h as indicated. (C) Western blot of PML and p53 in primary blasts from patients with AML *NPM-1 wt* or *NPM1c* after *ex-vivo* treatment with 20 $\mu$ M of AG17724 for 4h. (D) AG17724 rapidly promote PML-NB formation in *NPM1c*-primary AML cells. Confocal microscopy of PML-NBs in *NPM1wt* and *NPM1c* primary AML cells following treatment with AG17724 for 4h as indicated. (E) AG17724 stabilizes PML and P53 levels in AML *NPM1c* patient derived blasts. Western blot of PML and p53 in primary blasts from patients with AML *NPM-1 wt* or *NPM1c* after *ex-vivo* treatment with 20 $\mu$ M of AG17724 for 2h. (F) Pin-1 down-regulation abrogates RA-induced PML and p53 activation. OCI-AML3 and OCI-AML3 shPin1 cells were treated with ATO, RA or their combination. Cell extracts were analyzed by western blot using antibodies against PML, p53, p21 and Pin1. (G) RA-induced loss of clonogenic activity is abrogated upon Pin-1 downregulation. Colony formation assays in methylcellulose of OCI-AML2, OCI-AML3 and OCI-AML3 shPin1 cells, pre-treated for 3 hours with RA. (H) RA/ATO-induced apoptosis is abrogated upon Pin-1 downregulation. Annexin V staining of non-transduced or OCI-AML3 cells transduced with shPin-1 after treatment with ATO and RA for 48h. (I) RA/ATO-induced apoptosis is rescued upon Pin-1 downregulation. Annexin V staining of non-transduced or OCI-AML3 cells transduced with shPin-1 after treatment with ATO and RA for 48h (J) PML is the primary target of RA and Pin-1 inhibitors.

Western blot analysis of PML and p53 in OCI-AML3, OCI-AML3<sup>pml<sup>-/-</sup></sup> and OCI-AML3<sup>p53<sup>-/-</sup></sup> cells after treatment with AG17724 or RA for 2h.

Figure 5



**Fig. 5. RA and Actinomycin D cooperate to clear NPM1c-expressing cells.**

(A) Leukemic burden, assessed by NPM1c expression, in one patient treated with RA and ATO.

(B) RA/ActD combination selectively abrogates the clonogenic activity of OCI-AML3 cells in a PML and P53 dependent manner. Colony formation assays in methylcellulose of OCI-AML2, OCI-AML3, OCI-AML3<sup>pml<sup>-/-</sup></sup> and OCI-AML3<sup>p53<sup>-/-</sup></sup> cells after pre-treatment for 3 h with RA, ActD or ATO as indicated. (C) RA/ActD reduces the leukemia bone marrow burden in OCI-AML3 xenograft NSG mice in a PML dependent manner. Eight-week-old NSG mice were injected with 3 million OCI-AML3 or OCI-AML3<sup>pml<sup>-/-</sup></sup> cells intravenously. At day 7 post-leukemic cells injection, ActD and RA were administered every other day, over a period of 4 weeks intraperitoneally. At the end of week 5, BM was harvested from femurs and tibias of xenograft mice and then stained with the anti-hCD45 antibody. Graphs show the hCD45 PerCP percentage of xenograft animals (5 mice per group). RA and ActD induce P53 protein in NPM1c cells *in vivo*. Western blot of human P53 and NPM1c in BM harvested from NSG mice xenografted with OCI-AML3 or OCI-AML3<sup>pml<sup>-/-</sup></sup> cells, after *in vivo* treatment with RA, ActD or their combination as indicated. (E) Proposed model on the molecular mechanisms dictating NPM1c AML response to RA, ATO or RA/ATO combination.

# **Results part 2: Manuscript in preparation**

## **ATO and RA therapeutic response in AML is triggered by SUMO/PML/RNF4-dependent NPM1c degradation**

To unravel the effect of ATO/RA on NPM1c degradation, we studied NPM1c post-translational modifications following treatment. Indeed, in APL, RA directly targets the driving PML/RARA oncoprotein to drive complete remissions (de The, Pandolfi et al. 2017). This effect is due to the activation of a PML/P53 senescence checkpoint following PML/RARA degradation (Ablain, Rice et al. 2014). In some other AMLs, RA exerts clinical activity in combination with classic chemotherapy (Schlenk, Frohling et al. 2004). Whether this proposed activity reflects RA-induced AML differentiation, as observed in some non-APL AML primary patient cells or models (Altucci, Rossin et al. 2005, Boutzen, Saland et al. 2016), is yet to be elucidated. Intriguingly, the clinical benefit of RA co-administration with chemotherapy appears maximal in AMLs bearing an NPM1-c mutation (Schlenk and Dohner 2009). In that respect, we observed that NPM1-c was degraded by RA administration in cell-lines, suggesting that loss of NPM1-c expression may underlie RA-driven differentiation and apoptosis (El Hajj, Dassouki et al. 2015, Martelli, Gionfriddo et al. 2015). Furthermore, this degradation was accelerated by co-administration of ATO, drawing an unexpected similarity with the APL model.

PML plays a key role in the therapeutic response of APL and is the direct target of ATO therapy (Ablain, Rice et al. 2014, Lehmann-Che, Bally et al. 2014, Lehmann-Che, Bally et al. 2018). Our previous studies detected some alterations of PML NBs in NPM1c-positive cell-lines (El Hajj, Dassouki et al. 2015, Martelli, Gionfriddo et al. 2015).

We demonstrated that NPM1c-expressing patients and mouse models exhibit a dramatic response to RA and/or ATO *in vivo* (Part 1 of our results, submitted manuscript). Exploring the basis for this response, we unravel an unexpected key role of PML in these processes. Indeed, PML is required to initiate arsenic-driven NPM1c degradation. This happens *via* the SUMO/RNF4/Proteasome pathway, which cooperates with direct RA-induced ubiquitination. By establishing the mechanisms underlying RA and ATO sensitivity of NPM1c-positive AMLs, our

studies identify a striking parallelism with APL, paving the way to the crafting of curative targeted therapies in this category of AML patients.

## Results

### ATO enhances rapid SUMOylation of NPM1c

RA and ATO trigger apoptosis and NPM1c degradation in *NPM1c*-expressing AML cell lines (Figure 1a) (El Hajj, Dassouki et al. 2015). Degradation was also observed in transiently transfected HeLa cells, demonstrating that NPM1c loss is not the consequence of cell death induction (Figure 1b).

ATO modulates stability of a number of oncoproteins (PML/RARA, EVI-1 or Tax), at least in part by promoting their SUMOylation, followed by SUMO-dependent ubiquitination and proteasomal degradation (Shackelford, Kenific et al. 2006, Lallemand-Breitenbach, Jeanne et al. 2008, Dassouki, Sahin et al. 2015). NPM-1 is efficiently SUMO conjugated (Haindl, Harasim et al. 2008). This prompted us to investigate whether ATO may enhance NPM1c SUMOylation. Using two complementary strategies (NPM1c immunoprecipitation or direct purification of His-tagged SUMO-2 conjugates), we indeed found that ATO rapidly enhances SUMO-2 (Figure 1c, 1d) but not SUMO-1 conjugation of NPM1c (Figure 1e), while NPM1wt remained unaffected (1c). This rapid SUMOylation of NPM1c triggers its polyubiquitylation later (Figure 1f, 1g). These results were further asserted by Proximity ligation assay (PLA) demonstrating ATO-enhanced cytoplasmic interactions between NPM1 and SUMO2 (2h) or ubiquitin (24h) (Figure 1h).

### **NPM1c is degraded by ATO *via* the PML/RNF4 axis.**

ATO enhancement of NPM1c SUMOylation suggested that the conjugation process might be PML-facilitated and followed by RNF4-mediated poly-ubiquitination. Indeed, a longer exposure to ATO allowed NPM1c ubiquitination (Figure 2a). PML extinction sharply diminished both NPM1c SUMO2-conjugation and ubiquitination (Figure 2a), while that of RNF4 abolished ubiquitination only (Figure 2a). Moreover, PML or RNF4 extinction reversed ATO-induced NPM1c degradation (Figure 2A-right panel). That ATO-initiated NPM1c degradation involves SUMO2-

initiated, RNF4-mediated ubiquitination was directly demonstrated by expressing tagged SUMO2 and tagged ubiquitin, followed by dual tag purification (Figure 2b).

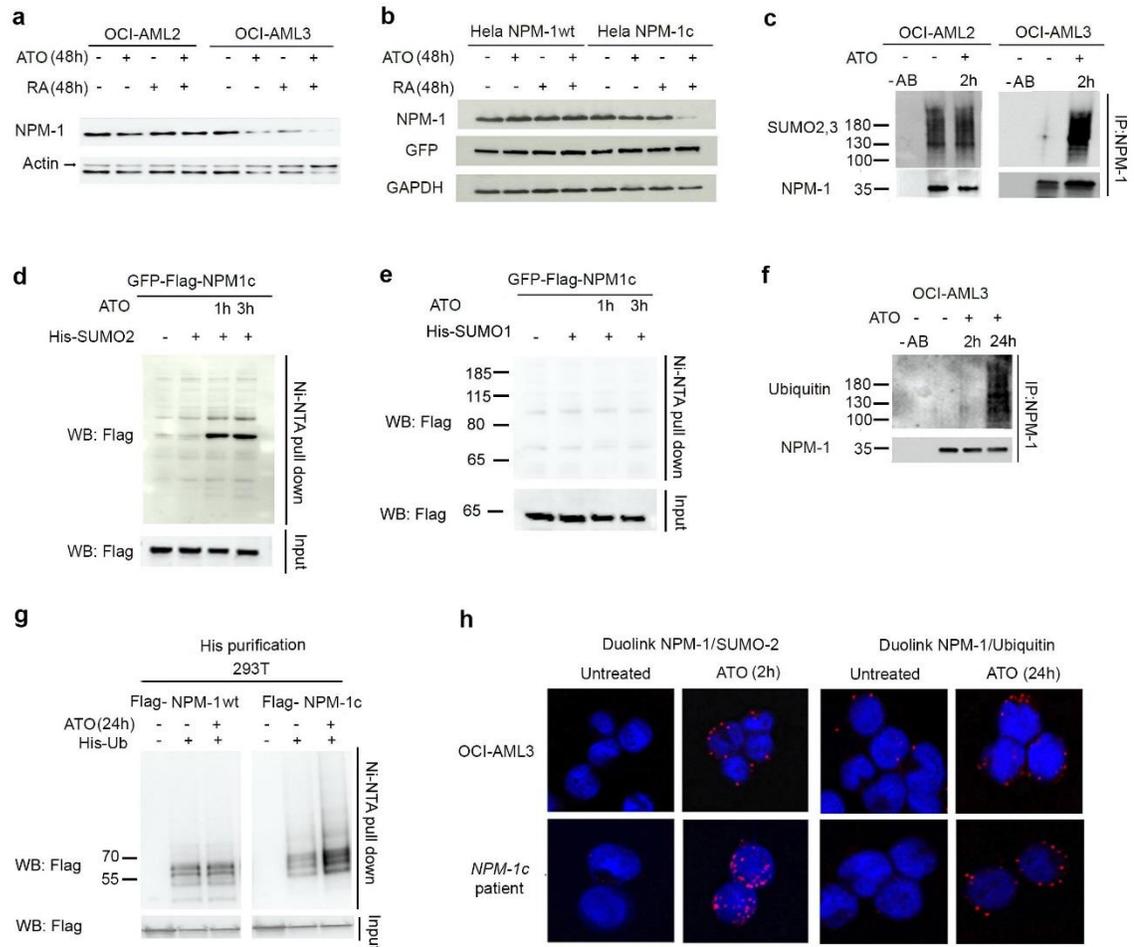
### **RA enhances late SUMO2/Ubiquitin NPM1c conjugates**

Following characterization of ATO mediated NPM1c post-translational modifications, we aimed at understanding RA effect on both SUMOylation and ubiquitylation of NPM1c. Unexpectedly, RA also promoted formation of dual SUMO2/Ubiquitin/NPM1c conjugates (Figure 2b) suggestive for their implication in RA-initiated NPM1c degradation. Indeed, a 24h RA-treatment increased SUMO2 conjugation of NPM1c, but not NPM1 (Figure 3a). Direct conjugation of His-ubiquitin was also promoted by RA exposure (Figure 3b). Moreover, in OCI-AML3 cells or NPM1c-positive AMLs, RA induced SUMO2 and ubiquitin interactions by PLA (Figure 3a and 3b-Right panels).

### **PML and RNF4 down-regulation partially abolished RA-induced NPM1 degradation**

The unexpected effect of RA on NPM1c SUMOylation prompted us to study PML and RNF4 involvement. Importantly, extinction of PML or RNF4 expression blunted not only SUMOylation and/or ubiquitination, but also RA-induced NPM1c degradation (Figure 4a). Similarly, in OCI-AML3 cell-line in which PML expression was abrogated by CRISPR-mediated excision (OCI-AML3<sup>pml<sup>-/-</sup></sup>) cells, NPM1c SUMOylation, ubiquitination and degradation upon RA-exposure were abolished (Figure 4b, 4c). Collectively, RA-induced NPM1c degradation is delayed, but unexpectedly resembles the one initiated by ATO in its PML-dependence.

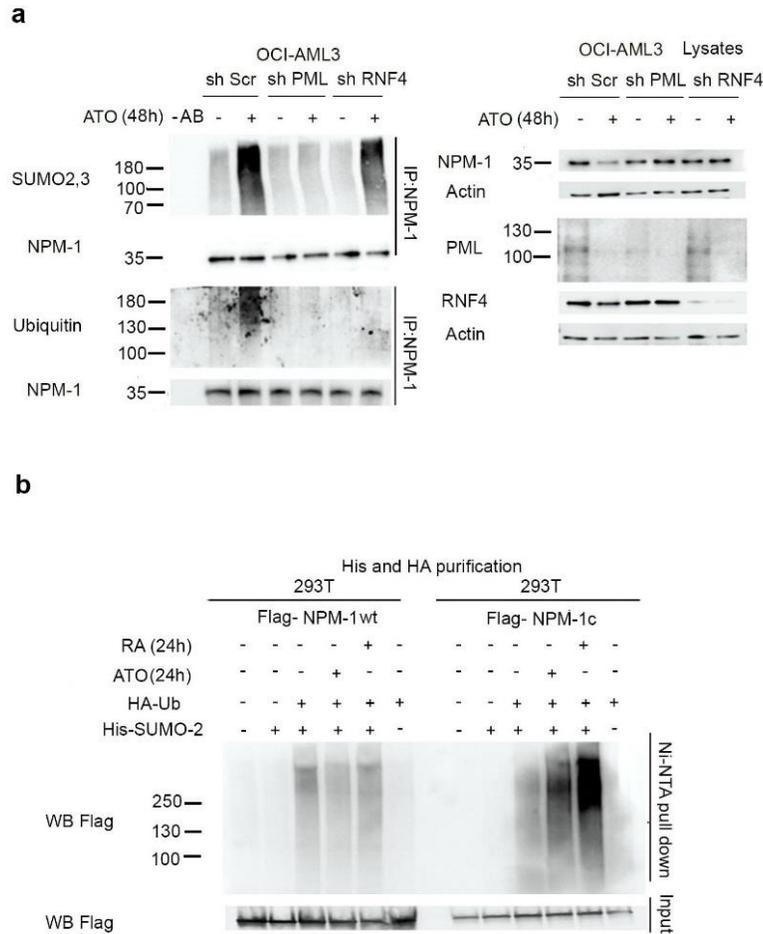
Figure 1



**Figure 1. ATO induces rapid SUMO-2 conjugation of NPM1c leading to its poly-ubiquitylation.**

(a) ATO/RA induced NPM1c degradation: AML cells with mutant *NPM1c* (OCI-AML3) or wild-type *NPM-1* (OCI-AML2) were treated with arsenic trioxide (ATO) (1 $\mu$ M), All-trans-retinoic acid (RA) (1 $\mu$ M), or their combination for 48h. Western blot analysis using an anti-NPM1 antibody recognizing both NPM1 (wt+c). (b) ATO/RA induced NPM1c degradation in HeLa cells transiently transfected with NPM1c but not NPM1wt. Western blot analysis using an anti-NPM1 recognizing both NPM1 (wt+c), after treatment of cells with ATO (1 $\mu$ M), RA (1 $\mu$ M), or their combination for 48h. (c) ATO treatment rapidly induced NPM-1 SUMO2,3 poly-sumoylation in OCI-AML3: OCI-AML3 and OCI-AML2 were treated with ATO for 2h. Immunoprecipitation of NPM-1 followed by

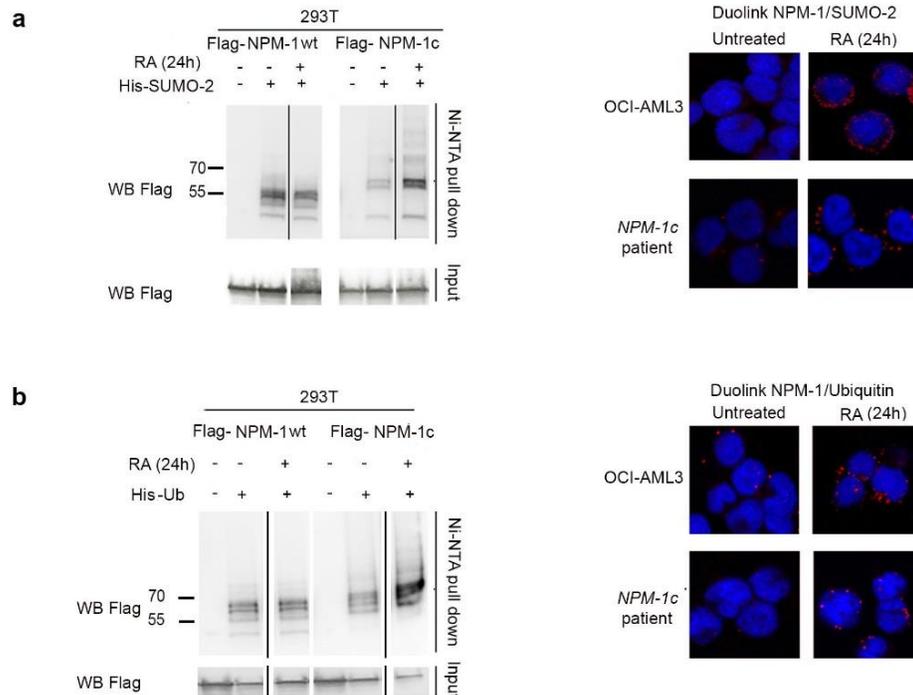
western blot of SUMO2/3 and NPM-1 (wt+c). (d-e) ATO induced NPM-1 SUMO-2 (d) but not SUMO-1 (e) modification. HEK-293T cells were transiently co-transfected with 6-Flag-NPM1 (wt or c) and 10-His-SUMO2 or 10-His-SUMO1 respectively. After 1h or 3h of treatment with ATO, His-purified extracts were precipitated using Ni-NTA pull down assay. Western blot was performed using an anti-flag antibody. (f) Prolonged ATO treatment triggered NPM-1 poly-ubiquitylation in OCI-AML3 cells. Cells were treated with ATO for up to 24h. Cell extracts were immunoprecipitated with anti-NPM1 antibody. Western blot was performed using anti-ubiquitin and anti-NPM1 antibodies. (g) ATO triggered NPM1c but not NPM1wt poly-ubiquitylation in HEK-293T cells transiently co-transfected with 6-Flag-NPM1 (wt or c) and 10-His-ubiquitin. After 24h of treatment with ATO, His-purified extracts were precipitated using Ni-NTA pull down assay. Western blot was performed using an anti-flag antibody. (h) Endogenous NPM1-SUMO2/3 and NPM1-ubiquitin interactions in OCI-AML3 or AML NPM1c patient derived blasts, after ATO treatment (treatment duration is indicated) as detected by Duolink Proximity Ligation Assay (PLA assay). Nuclei were stained with DAPI (blue).

**Figure 2**

**Figure 2. ATO induced NPM1c degradation is PML and RNF-4 dependent.** (a) OCI-AML3 cells were transduced with nontargeting control shRNA (shCTRL) or shRNA against PML (shPML) or shRNA against RNF4 (shRNF4) and treated with ATO for up to 48 hours. Cell extracts were immuno-precipitated with anti-NPM1 (wt+c) antibody, and western blot was performed using anti-NPM1 (wt+c), anti-SUMO2/3 and anti-ubiquitin antibodies. Corresponding control cell lysates are shown as indicated (right panel). (b) ATO and RA triggered sequential NPM1c SUMO-2 sumoylation, followed by ubiquitylation in HEK-293T cells transiently co-transfected with 6-Flag-NPM1, 10-His-SUMO2 and HA-ubiquitin. Extracts of transfected HEK-293T cells were double

purified using anti-HA-ubiquitin and Ni-NTA pull down assay. NPM1 conjugates were analyzed by western blot using anti-Flag antibody.

**Figure 3**

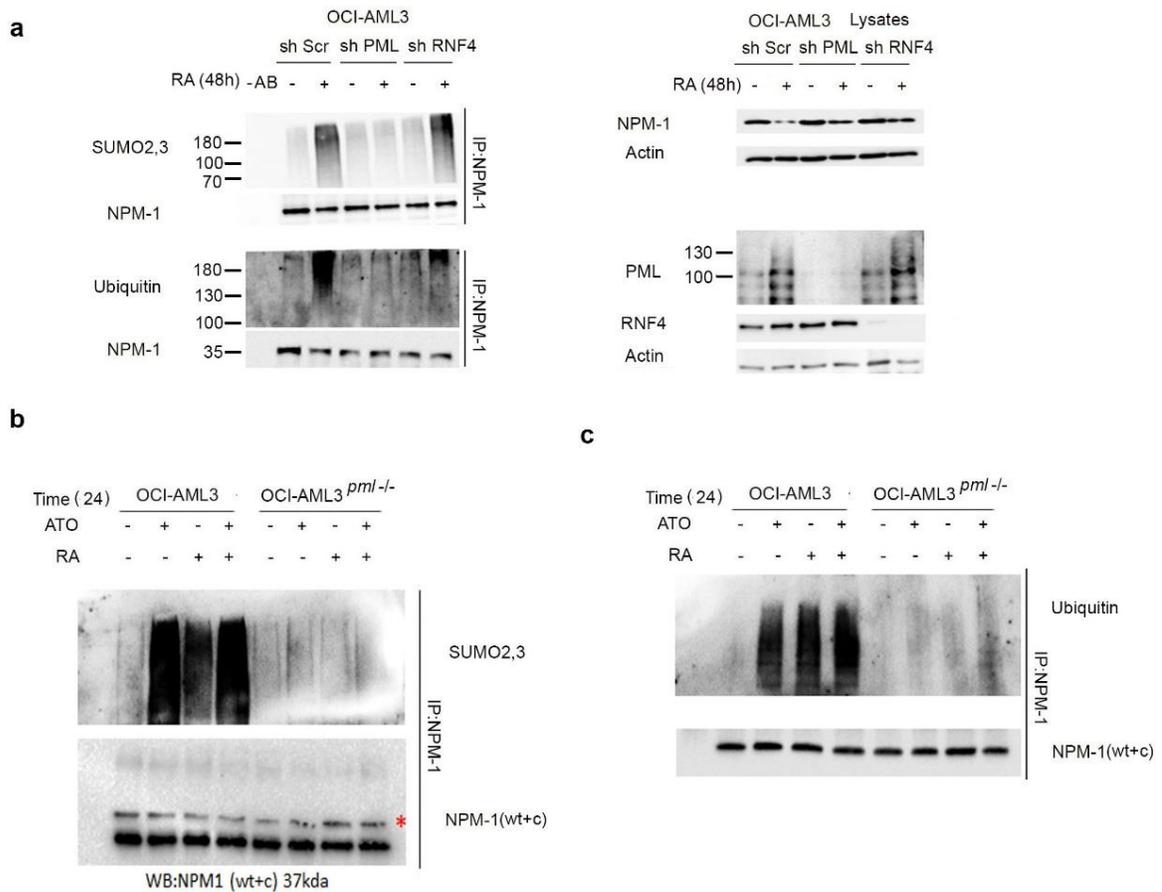


**Figure 3. RA triggers late SUMO2 and ubiquitine modification of NPM1c.**

(a) RA triggered NPM1c but not NPM1wt SUMO2 sumoylation in HEK-293T cells transiently co-transfected with 6-Flag-NPM1 (wt or c) and 10-His-ubiquitin. Cells were treated with RA for 24h. (Left panel). His-purified extracts were precipitated using Ni-NTA pull down assay, western blot was performed using anti-Flag antibody. (Right panel) Endogenous NPM1-SUMO2 interactions in OCI-AML3 or AML *NPM1c* patient-derived blasts, after RA treatment for 24h, as detected by Duolink PLA assay. Nuclei were stained with DAPI (blue). (b) RA-triggered NPM1c but not NPM-1 wt ubiquitylation in HEK-293T cells transiently co-transfected with 6-Flag-NPM1 (wt or c) and 10-His-ubiquitin. (Left panel) After 24h of treatment with RA, His-purified extracts were precipitated using Ni-NTA pull down assay, western blot was performed using anti-Flag antibody. (Right panel) Endogenous NPM1- ubiquitin interactions in OCI-AML3 or AML *NPM1c* patient-derived blasts,

after RA treatment for 24h as detected by Duolink PLA assay. Nuclei were stained with DAPI (blue).

**Figure 4**



**Figure 4: PML and RNF4 down-regulation partially abolished RA-induced NPM1 degradation**

(a) RA-induced NPM1c degradation is partially dependent on PML and RNF4. OCI-AML3 cells were transduced with nontargeting control shRNA (shCTRL), or shRNA against PML (shPML) or shRNA against RNF4 (shRNF4) and treated with RA for up to 48 hours. Cell extracts were immunoprecipitated with anti-NPM1 (wt+c) antibody. Western blot was performed using anti-NPM1 (wt+c), anti-SUMO2/3 and anti-ubiquitin antibodies (left panel). Corresponding control cell lysates are shown as indicated (right panel). (b, c) PML inactivation abrogates SUMO2/3 or

ubiquitin- conjugation to NPM1c. OCI-AML3 and OCI-AML3<sup>pml<sup>-/-</sup></sup> cells were treated with ATO, RA or their combination for up to 24h. Cell extracts were immunoprecipitated with anti-NPM1 antibody. Western blot was performed using anti-SUMO2/3 (b), anti-ubiquitin (c) and anti-NPM1 antibodies. Red star in (b) indicates NPM1 (wt+c).

## Materials and methods

### Cell lines, patient blasts and *ex vivo* treatments

OCI-AML3 or OCI-AML2 AML cells (harboring the *NPM1c* mutation without *FLT3*-ITD or wild type (wt) *NPM-1* respectively) were grown in minimum essential medium- $\alpha$  (MEM $\alpha$ ) supplemented with 20% fetal bovine serum (FBS) and antibiotics. Cells were seeded at the density of  $2 \times 10^5$ /ml. ATO (Sigma Aldrich) or RA (Sigma Aldrich) were used at 1  $\mu$ M final concentration.

### Cell culture, plasmids and transfection

HeLa and HEK-293T were grown in DMEM supplemented with 10% FBS, 2mM glutamine, and antibiotics. Transfection with different DNA constructs was performed using Lipofectamine 2000<sup>®</sup> (Gibco, Invitrogen) according to the manufacturer's recommendations. HEK-293T cells were also transfected using the calcium phosphate procedure. Vectors encoding for His-ubiquitin, His-SUMO1 and His-SUMO2 were used as previously described in (Lallemand-Breitenbach, Jeanne et al. 2008).

### Short hairpin RNA (shRNA)

In knock-down experiments, the following shRNAs *PML* (CACCCGCAAGACCAACAACA), *RNF-4* (CATACTCCCAGAAACGCCAGG), and control scrambled were used. Lentiviruses were produced by transient transfections of HEK-293T cells. Infection of OCI-AML2 or OCI-AML3 cells with different lentiviruses was performed by spinoculation for 3h at 1500rpm and at 32°C.

### **CRISPR AML3 cell lines**

PML expression was abrogated by CRISPR-mediated excision. A guide RNA targeting *PML* (Forward: 5'-GTCGGTGTACCGGCAGATTG; Reverse: 5'-AATCTGCCGGTACACCGAC) was designed and cloned into pLAS5w. Ppuro-Cas9 plasmid for viral packaging. OCI AML-3 cells were infected with the corresponding viruses. Stable selection of knock-out cells was performed in the presence of 1µg/ml of puromycin, over a period of 2 weeks. Similarly, P53 extinction was performed using a guide RNA targeting *P53* (Forward: 5'-CCATTGTTCAATATCGTCCG; Reverse: 5'-CGGACGATATTGAACAATGG). Recombinant Cas9 protein was synthesized from IDT to form Alt-R CRISPR/Cas9 RNP. OCI AML-3 cells were transiently transfected with Alt-R CRISPR/Cas9 RNP by using Nucleofector kit T (Amaza) and applied program number X-01 in the nucleofactor device (Lonza). The stable CRISPR knock-out clones were cloned by serial dilution to generate a single-cell separation. DNA from individual clones was extracted and the region surrounding the Cas9 cutting site was amplified by PCR and verified by sequencing to ensure the deletion of the target genes.

### **Ni-NTA pull down**

Ni-NTA pull-down was performed as described by (Chiari, Lamsoul et al. 2004). Briefly, 24h post-transfection, HEK-293T cells were treated for 3 or 24h with ATO, RA or their combination. For detection of His or HA-tagged conjugates, tagged proteins purification on Ni-NTA resin (QIAGEN or Invitrogen) was performed 24h after transfection with His-ubiquitin, His-SUMO1– or His-SUMO2–encoding vector as described (Lallemand-Breitenbach, Jeanne et al. 2008). Cells were lysed in reducing and highly denaturing conditions with buffer A (6M guanidinium-HCl, 0.1M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 0.01M Tris-Cl, pH 8.0, 5mM imidazole, and 10mM β-mercaptoethanol). Lysates were then incubated with Ni-NTA resin (Qiagen) for 12h at 4°C. Beads were subject to three subsequent washes with decreasing amounts of guanidinium-HCl, before elution in Laemmli buffer supplemented with 200 mM imidazole. Products were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and blotted with a polyclonal anti-Flag antibody (Sigma aldrish).

### **Immunoprecipitation and immunoblotting**

For immunoprecipitation (IP), cells were washed in ice-cold phosphate-buffered saline (PBS) supplemented with 10mM N-ethylmaleimide (NEM) before lysis in 2% SDS and 50 mM Tris(pH 8). Following a brief sonication, lysates were 10-fold diluted in an IP buffer containing 50 mM Tris (pH 8), 200 mM NaCl, 0.1 mM EDTA, 0.5% NP-40, 10% glycerol, and a cocktail of protease inhibitors. Specific antibodies were added for 12h at 4°C, and then proteins A-agarose were added for additional 2h. Beads were washed 3 times in the IP buffer prior to elution of immunoprecipitated proteins.

For immunoblotting, cells were solubilized in 2x laemmli buffer. 50 µg of proteins were separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Blots were incubated with the following specific monoclonal antibodies: anti-NPM1 recognizing both WT and mutated NPM1c (Abcam), anti-SUMO2/3, anti-SUMO1 and anti-p21(cell signaling), FK2 antibody recognizing Poly-ubiquitylated proteins (BIOMOL International), anti-GFP (Roche applied science), and polyclonal antibodies: anti-NPM-1 recognizing specifically the mutated NPM1c (Invitrogen), a homemade chicken anti-PML, anti-RNF4 (Kind gift from J. Palvimo). Proteins were then visualized using the enhanced chemi-luminescence system (Bio-Rad).

### **Proximity ligation assay (PLA) and confocal microscopy**

OCI-AML3 or patients' derived AML blasts were fixed with ice cold methanol at -20°C for 20 minutes and cytospun onto glass slides. Protein-protein interactions were visualized using the Duolink *in situ* proximity ligation assay (PLA) system (Olink Bioscience) following the manufacturer's instructions. Anti-ubiquitin (Santa Cruz), anti-SUMO2/3 (Abcam), anti-NPM-1 (Abcam) monoclonal antibodies were used in PLA assays. Primary antibodies were revealed by Alexa-Fluor 488- or 594-labeled secondary antibodies from Abcam. Staining of nuclei was performed with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen). Images were acquired by confocal microscopy using a Zeiss LSM710 confocal microscope (Zeiss, Oberkochen, Germany) with a Plan Aplanachromat 63/1.4 numeric aperture oil-immersion objective, using Zen 2009 (Carl Zeiss).

## Statistical analysis

Data was reported as the average  $\pm$  standard deviations. Statistical analysis was done using Student's t test p-value of less than 0.05 was considered as significant.

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# **DISCUSSION AND PERSPECTIVES**

The combination of ATO and RA is a very effective treatment for APL (Dos Santos, Kats et al. 2013, de The, Pandolfi et al. 2017). This combination selectively induces differentiation and apoptosis in blasts from *PML/RARA* transgenic mice resulting in tumor clearance (Lallemand-Breitenbach, Guillemain et al. 1999). This approach was transposed in patients, resulting in their definitive cures (Lo-Coco, Avvisati et al. 2013, de The, Pandolfi et al. 2017). In this context, ATO/RA drive the degradation of the oncoprotein PML/RARA resulting in PML/p53 activation and APL eradication (Ablain, Rice et al. 2014). This model provides a striking example of chemically-induced oncoprotein degradation, demonstrating in patients the relevance of oncoprotein dependency, previously well-established in mouse models. It also stresses the key effector role of the PML/P53 senescence pathway.

In non-APL AMLs, clinical studies have reported an improved outcome when RA was added to intensive chemotherapy in AML-patients, particularly in the presence of *NPM1c* mutation (Schlenk and Dohner 2009). Whether this proposed activity reflects RA-induced AML differentiation, as observed in some AML models treated with retinoids, is yet to be elucidated. AMLs with IDH mutations are very sensitive to RA-induced differentiation, notably because of LSD1 inhibition by ROS produced downstream of mutant IDH. Retinoid activity can be enhanced by cAMP or LSD1 inhibitors, suggesting that a RA-sensitive differentiation program persists in many AMLs (Altucci, Rossin et al. 2005, Schenk, Chen et al. 2012, Boutzen, Saland et al. 2016). Our group has demonstrated that RA or ATO -and furthermore their combination- induce differentiation, growth arrest and apoptosis in *NPM1c*-AML cell-lines and that this combination reduced marrow blasts in *NPM1c* AML patients (El Hajj, Dassouki et al. 2015). However, the *in vivo* anti-leukemic effect of RA and ATO was both modest and transient. Here, we report a greater *in vivo* clinical potency of combining ATO and RA in *NPM1c* AML, since ATO and RA induced a complete molecular response in one AML patient after 3 weeks of treatment. We also demonstrate that ATO/RA combination rapidly triggered decrease of human cells in the bone marrow of xenografts. Thus, ATO/RA has clear *in vivo* activity in *NPM1c* AMLs.

These effects were originally believed to result from the degradation of *NPM1c*. Focusing of the mechanisms involved, we demonstrated that ATO initiates a PML/SUMO/RNF4-dependent *NPM1c* degradation. Both *NPM1c*-SUMOylation and ubiquitination were induced following 24h

of treatment with RA. ATO/RA-induced NPM1c degradation was followed by p14<sup>ARF</sup> restoration and p53 activation. One expects that the numerous downstream effects of NPM1c (Myc, Hox genes, metabolism...) will be reversed as well. Assessing the transcriptional effects of RA and ATO treatment in OCI-AML3 and control OCI-AML2 cells and found p53 activation following RA treatment in OCI-AML3 cells. Critically, ATO and/or RA-induced NPM1c degradation, p53 stabilization and cell death all require the presence on PML. Conversely, in AML3<sup>P53-/-</sup> cells, ATO/RA failed to induce cell death, although NPM1c was efficiently degraded. Thus, ATO/RA drive a PML-dependent degradation of NPM1c resulting in a selective p53 activation in NPM1c-AML cells and cell death.

Unexpectedly, the kinetic of p53 activation shows that ATO/RA-induced p53 stabilization was observed prior to any detectable NPM1c degradation, in OCI-AML3 cells and also in *ex-vivo* treated patients, implying the existence of other pathways. We found that PML expression was decreased in NPM1c expressing cell lines or primary patients' blasts. This results in fewer and smaller PML nuclear bodies and likely impedes, at least in part, the growth suppressive effects of PML. Mechanistically, NPM1c may interact with PML and sequester it in the cytoplasm, promoting its turnover. The exact pathway through which *NPM1c* AMLs exhibit very low basal levels of PML and disrupted NBs requires further investigation, but likely constitute a novel oncogenic mechanism downstream of NPM1c. NPM1c-driven p53 silencing may also be amplified by the loss of PML, in addition to ARF depletion. This PML down-regulation and disruption of PML NBs may contribute to ATO and RA responses. Indeed, ATO rapidly reforms PML nuclear bodies, as well explored in the APL model (Zhu, Koken et al. 1997, Lallemand-Breitenbach, Zhu et al. 2001, Jeanne, Lallemand-Breitenbach et al. 2010). RA was recently shown to inhibit the Pin-1 enzyme, a pathway that regulates PML stability (Reineke, Lam et al. 2008, Yuan, Lee et al. 2011, Wei, Kozono et al. 2015). Indeed, we found that RA and a Pin-1 inhibitor (AG17724) rapidly induce NB formation and stabilize PML or P53 levels in NPM1c expressing cells, resulting in a growth suppressive effect. Conversely, extinction of Pin-1 blocked RA-induced PML and p53 activation in OCI-AML3 and RA-induced loss of clonogenic activity. However, Pin-1 inhibitors failed to alter NPM1c expression in OCI-AML3 cells, consistent with our kinetic analysis. Finally, RA and AG17724 upregulated PML in AML3<sup>P53-/-</sup> cells, while no activation of p53 was observed in OCI-

AML3<sup>pml<sup>-/-</sup></sup>, demonstrating that PML (rather than P53) is the primary target of RA and Pin-1 inhibitor. Altogether, we have demonstrated that ATO and RA synergize to rapidly stabilize PML and p53 *via* Pin-1 inhibition, explaining the early response to the RA.

Our findings shed a novel light on the basis of the ATO/RA synergy, complementary with their roles in NPM1c degradation. Therapeutically, these two mechanisms (NPM1c degradation with restoration of p14<sup>Arf</sup> and P53 signaling and direct Pin-1 inhibition driving PML/P53 activation) may both play a role at different levels of AML clinical response to RA/ATO (senescence, apoptosis, growth arrest, loss of clonogenic activity). Contrary to APL, the combination of RA/ATO is not curative in *NPM1c* AML patients. Indeed, ATO/RA can transiently clear AML cells in *NPM1c* AML patients. This is likely due to the presence of other oncogenes, as *NPM1c* is usually a second hit and cannot initiate leukemia on its own. Hence, it will be important to combine RA and/or ATO to other drugs in the treatment of *NPM1c* AML. In that respect, we explored a possible synergy of RA with ActD, a drug that induces complete remissions in *NPM1c*-positive AMLs (Falini, Brunetti et al. 2015). RA and ActD were synergistic in inhibiting the clonogenic activity in OCI-AML3 cells, while they failed to induce any effect in OCI-AML3<sup>pml<sup>-/-</sup></sup> or AML3<sup>p53<sup>-/-</sup></sup> cells, again demonstrating the importance of the PML/P53 effector pathway. One of the most surprising observations from our studies is the similarity of this model with APL. They point to common mechanisms involving PML in terms of cell biology/oncogenesis (abnormal PML NB and blunting of P53 signaling) and in molecular mechanisms of response to RA/ATO combination (oncoprotein degradation via the PML/SUMO/RNF4/proteasome pathway and re-activation of the normal PML/P53 axis).

Our findings are also important with respect to the RA/Pin1 interplay. Previous studies described an ATO/RA synergy and attributed it to the ability of ATO to inhibit Pin-1 function (Kozono, Lin et al. 2018). By involving PML downstream of Pin1 inhibition, our studies suggest that a dual action to increase PML levels (RA) and target it onto PML bodies (ATO) contribute to the efficacy of this combination with respect to P53 activation. However, the fact that PML, rather than P53, is the first step downstream of RA-initiated Pin-1 inhibition does not exclude that p53 exerts a feed-forward loop on PML stabilization. Moreover, in *NPM1c* AMLs, RA-induced PML stabilization should facilitate ATO-induced *NPM1c* degradation and P53 activation will promote

PML expression and NB formation. The synergistic targeting of Pin-1 by ATO and RA was also proposed as an approach to target many types of cancer (Kozono, Lin et al. 2018). This should prompts investigations of the involvement of RA/Pin-1/PML/p53 axis in other malignancies, notably those where PML expression is low. In that respect, a long-term control of refractory follicular lymphoma was reported in one patient after treatment of secondary APL with ATO and RA. Unexpectedly, the patient achieved a remission of both FL and APL following ATO and RA treatment (Jain, Konoplev et al. 2018).

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# **ANNEX**

# **ANNEX 1**

## MYELOID NEOPLASIA

## Retinoic acid and arsenic trioxide trigger degradation of mutated NPM1, resulting in apoptosis of AML cells

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## Key Points

- RA/arsenic induces proteasomal degradation of mutant NPM1, yielding AML growth arrest and apoptosis.
- RA/arsenic treatment restored nucleolar localization of NPM1 and significantly reduced bone marrow blasts in NPM1 mutant AML patients.

Nucleophosmin-1 (NPM1) is the most frequently mutated gene in acute myeloid leukemia (AML). Addition of retinoic acid (RA) to chemotherapy was proposed to improve survival of some of these patients. Here, we found that RA or arsenic trioxide synergistically induce proteasomal degradation of mutant NPM1 in AML cell lines or primary samples, leading to differentiation and apoptosis. NPM1 mutation not only delocalizes NPM1 from the nucleolus, but it also disorganizes promyelocytic leukemia (PML) nuclear bodies. Combined RA/arsenic treatment significantly reduced bone marrow blasts in 3 patients and restored the subnuclear localization of both NPM1 and PML. These findings could explain the proposed benefit of adding RA to chemotherapy in NPM1 mutant AMLs, and warrant a broader clinical evaluation of regimen comprising a RA/arsenic combination. (Blood. 2015;125(22):3447-3454)

## Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous disease with a highly variable prognosis and an overall high mortality rate. The 5-year overall survival of adult AML patients is less than 50%, and only 20% of elderly patients survive over 2 years.<sup>1</sup> Cytogenetic alterations classify AML into 3 risk-based categories: favorable, intermediate, and unfavorable.<sup>2</sup> Patients with normal karyotype belong to the intermediate risk category and their prognosis is determined by specific genetic alterations, particularly Nucleophosmin-1 (NPM1) mutation and FMS-like tyrosine kinase 3 (FLT3) internal tandem duplication (ITD).<sup>3</sup>

NPM1 is an essential gene<sup>4</sup> encoding a nucleolar shuttling protein<sup>5</sup> with multiple functions, including stabilization of the p14<sup>Arf</sup> tumor suppressor protein, regulation of ribosome biogenesis, control of centrosome duplication, and p53 activation.<sup>4,5</sup> In mutant NPM1 proteins, critical tryptophan residues in the C-terminus are lost and a de novo nuclear export signal is created. This leads to accumulation of mutant NPM1, together with normal NPM1, in the cytoplasm of leukemic cells rather than in their nucleolus. NPM1 mutations drive leukemogenesis, as hematopoietic disorders were observed in

transgenics or knock-in mice.<sup>6-11</sup> Some studies suggested that addition of retinoic acid (RA) to conventional chemotherapy improves survival, selectively in AML patients harboring the NPM1 mutation in the absence of FLT3-ITD.<sup>12</sup> Other clinical studies reported negative results.<sup>13,14</sup> Overall, there is no consensus yet as to whether the addition of RA to chemotherapy improves the outcome of patients with NPM1 mutant AML. Moreover, the basis for the proposed clinical response to RA remains obscure.

Arsenic trioxide (arsenic) and RA are very effective treatments for acute promyelocytic leukemia (APL), a distinct AML subtype characterized by the expression of the promyelocytic leukemia (PML) RA receptor  $\alpha$  (RARA) fusion protein.<sup>15,16</sup> PML/RARA delocalizes PML, a protein implicated in control of p53-driven senescence. PML nuclear bodies (NB) are implicated in both APL pathogenesis and therapy response.<sup>17</sup> Both RA and arsenic induce degradation of PML/RARA through distinct pathways. Their combination cures APL in mice<sup>17-19</sup> and patients.<sup>20-22</sup>

Here, we demonstrate that arsenic and RA synergistically induce proteasome-mediated degradation of mutant NPM1, resulting in

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differentiation, growth arrest, and apoptosis, selectively in AML cells harboring a NPM1 mutation. Expression of NPM1 mutant protein was associated with altered formation of PML NBs. The RA/arsenic combination significantly reduced leukemic blasts in the bone marrow (BM) of 3 NPM1-mutant AML patients and restored nucleolar localization of NPM1 and PML NBs both *ex vivo* and *in vivo*. These findings raise an unexpected parallelism between APL and NPM1 mutant AMLs, provide a basis for its proposed RA sensitivity and warrant a broader evaluation of therapeutic regimen comprising a RA/arsenic combination, particularly, in elderly patients.

## Methods

### Patients, cells, and treatments

KG1a (ATCC CCL246.1), ML-2 (DSM ACC15), THP-1 (DSM ATCC 16), MOLM-13 (DSM ACC 554), and HEL (DSM ACC 11) AML cell lines (gift from F. Mazurier) with wild-type (WT) NPM1, were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and antibiotics. OCI-AML3 (DSM ACC582) AML cells (gift from D. Bouscary) and IMS-M2 (gift from B. Falini) harboring the NPM1 mutation without FLT3-ITD were grown in minimum essential medium—a containing 20% FBS and antibiotics. Cells were seeded at a density of  $2 \times 10^5$ /mL. Primary AML cells from the BM of 3 patients (patients 1-3) were extracted following Ficoll separation and cultured in minimum essential medium—a supplemented with 20% FBS and antibiotics. These samples were collected after approval by the Institutional Review Board and after patients provided informed consent in accordance with the Declaration of Helsinki. Arsenic was used at 0.1 or 1 mM, RA at 0.3 or 1 mM, and the proteasome inhibitor PS-341 at 10 nM. Cell growth was assessed using the CellTiter 96 cell proliferation assay kit (Promega Corp., Madison, WI) or by trypan blue dye assay. Five elderly AML patients (patients 4-8) with NPM1 mutation, who were judged unfit for chemotherapy, received compassionate RA (Vesanoid Roche) (45 mg/m<sup>2</sup> per day by mouth) and arsenic trioxide (Trisenox, Teva) intravenously (0.1 mg/kg per day).

### Fluorescence-activated cell sorter analysis

**Annexin V staining.** Phosphatidyl-serine exposure in treated AML cells was assessed using Annexin V-fluorescein isothiocyanate (Sigma-Aldrich). For terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay, fluorescein-conjugated dUTP incorporated in nucleotide polymers was detected and quantified using flow cytometry. For both annexin and TUNEL assays, approximately 10 000 cells per sample were acquired and analyzed using CellQuest software. For CD11b staining, mouse anti-human CD11b-APC/Cy7 antibody (BD Pharmingen) was used to detect and quantify CD11b cell surface differentiation marker. There were 10 000 events per sample acquired and BD fluorescence-activated cell sorter Diva software was used for analysis.

### Immunoblot analysis

Cells were solubilized at 4°C in lysis buffer. There were 50 mg of proteins loaded onto a 12% sodium dodecyl sulfate–polyacrylamide gel, subjected to electrophoresis and transferred onto nitrocellulose membranes. Blots were incubated with specific antibodies and washed, and proteins were visualized using the enhanced chemiluminescence system (Santa Cruz, Germany). The following antibodies were used: monoclonal anti-NPM1 recognizing both WT and mutated NPM1 (WT 1 c) (Abnova, Abcam), monoclonal anti-p53 (Santa Cruz), monoclonal anti-phospho-p53 (P-p53) (Cell Signaling), monoclonal anti-actin (Santa Cruz), monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (Abnova), polyclonal anti-mutated NPM1 (NPM1c) (Abnova), polyclonal anti-Fibrillarlin (Abcam), polyclonal anti-p21 (Santa Cruz), and polyclonal anti-poly-ADP-ribose polymerase (PARP) (Santa Cruz).

### Immunofluorescence and confocal microscopy

OCI-AML3 and THP-1 cells were cytospun onto glass slides (5 minutes, 800 rpm) and fixed with methanol at 220°C. Immunofluorescence assays were performed using primary antibodies against NPM1, the nucleolar marker Fibrillarlin, SUMO-1, or PML. Images were acquired by confocal microscopy using a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany) with a Plan Apochromat 63/1.4 numeric aperture oil-immersion objective, using Zen 2009 (Carl Zeiss). We have used a blinded method of counting NPM1, PML, and SUMO-1 bodies. Z-sections derived from 100 cells per condition were coded by one investigator and counted by another investigator for the presence of regular or faint PML bodies.

### Synergy studies and statistical analysis

Proliferation experiments on cell lines were repeated at least 3 times. Data are reported as the mean  $\pm$  standard error. Computerized combination index (CI) was generated automatically using CompuSyn software based on the CI-isobol method of Chou et al.<sup>23</sup> The CI was used to assess synergistic effects (CI < 1), additive effects (CI = 1), or antagonistic effects (CI > 1). Two statistical tests were performed to validate significance: the test and the one-way analysis of variance test.

## Results

### RA or arsenic induce differentiation, growth arrest, and apoptosis in NPM1 mutated AMLs only

AML cell lines with mutated NPM1 (OCI-AML3, IMS-M2) or WT NPM1 (KG1a, ML-2, THP-1, MOLM-13, HEL) were treated with RA and/or arsenic up to 48 hours. OCI-AML3 cells were much more sensitive to RA than IMS-M2, KG1a, ML-2 THP-1, MOLM-13, and HEL cells for cell cycle arrest and/or cell death (Figure 1A; supplemental Figure 1A, available on the Blood Web site). OCI-AML3, and IMS-M2 cells were also considerably more sensitive to arsenic than control cells (Figure 1A; supplemental Figure 1A). Synergy studies, analyzed using a computerized CI<sup>23</sup> revealed a strong synergistic effect of arsenic and RA for growth arrest in OCI-AML3 cells at 24 hours (CI 5 0.46 and 0.035, for low- or high-arsenic concentration, respectively) (Figure 1A). Increases in annexin-V, TUNEL positivity, and PARP cleavage were only observed in OCI-AML3 cells treated with arsenic or RA (Figure 1B-C; supplemental Figure 2). Thus, this combination synergized for induction of apoptosis, reaching 75% after 48 hours of treatment, exclusively in OCI-AML3 cells (Figure 1B-C; supplemental Figure 2). Furthermore, we observed a major induction of CD11b expression upon RA/arsenic treatment of OCI AML-3 and, to a lesser extent in IMS-M2, pointing to partial differentiation (Figure 1D; supplementary Figure 1A). Importantly, in OCI-AML3 cells, RA, or the RA/arsenic combination selectively upregulated p53, its active phosphorylated form and its downstream effector p21 (Figure 1E), likely explaining RA-induced cell cycle arrest and apoptosis (supplemental Figure 2).

Primary leukemic cells derived from BM of 3 AML patients (patients 1-3), presenting initially with 52%, 70%, and 74% of BM blasts, respectively, were treated *in vitro* with RA and/or arsenic, as indicated above. Patient 1 had APL with PML/RARA rearrangement; patient 2 harbored an NPM1 mutation without FLT3-ITD; and patient 3 had AML-M6 with WT NPM1. Cells from patients 1 and 2 were much more sensitive to RA and arsenic treatment than those derived from patient 3 (Figure 2A). Again, a strong synergy between RA and arsenic was observed, exclusively in patients 1 and 2 at any time point and dose (Figure 2A and data not shown). Collectively, RA and arsenic exert selective apoptosis on NPM1 mutant AMLs *ex vivo*.

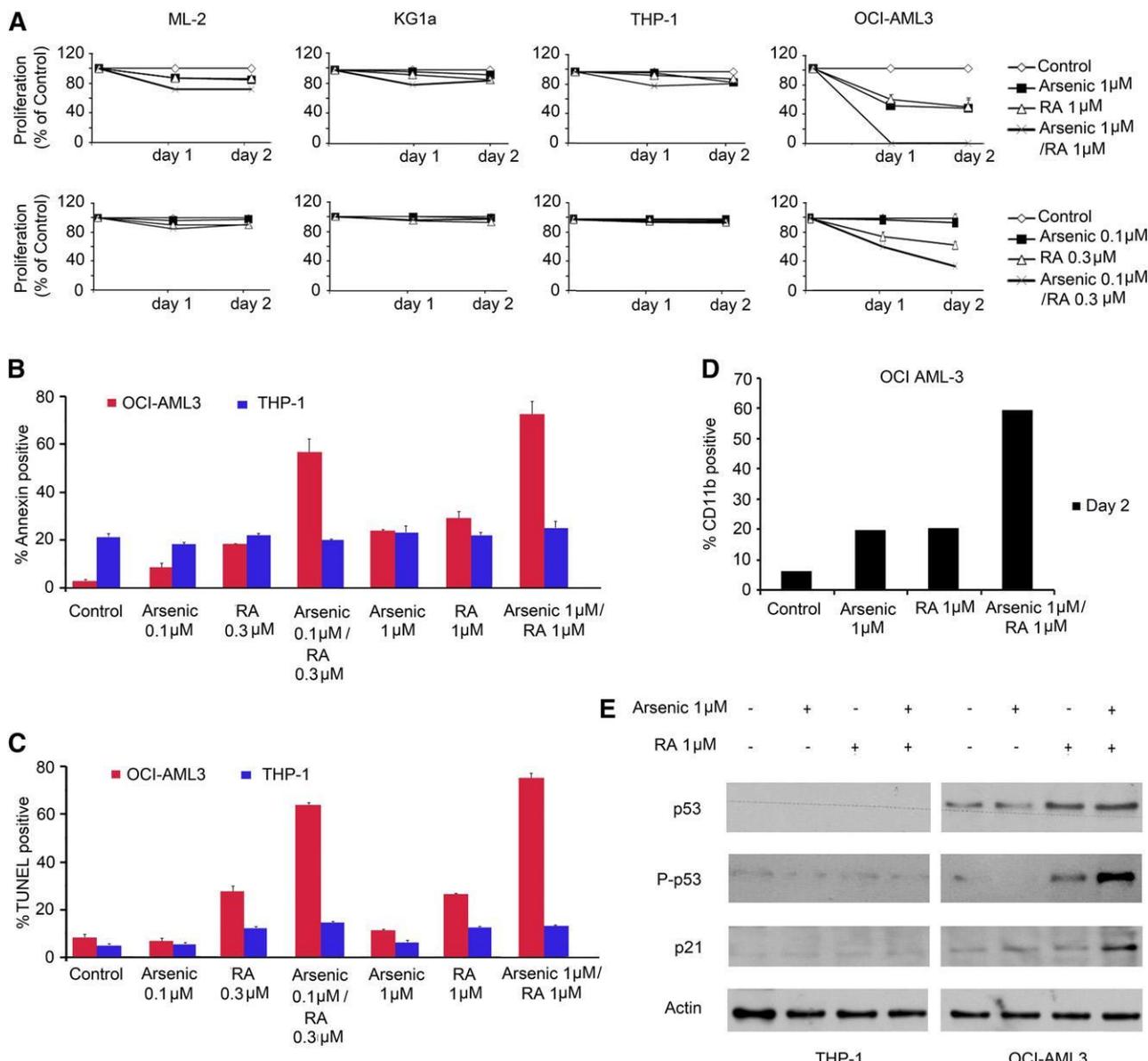


Figure 1. RA and arsenic induce growth inhibition and apoptosis in NPM1 mutated AML cell line. (A) AML cell lines with normal NPM1 (ML-2, KG1a, and THP-1) or mutated NPM1 (OCI-AML3 and IMS-M2) were treated with arsenic (1 mM or 0.1 mM), RA (1 mM or 0.3 mM) or a combination of both. Cell growth (percent of control) was assayed in triplicate wells. The results represent the average of at least 3 independent experiments. (B) Annexin V staining of THP-1 or OCI-AML3 cells treated for 48 hours as described. (C) TUNEL assay of THP-1 or OCI-AML3 cells treated for 48 hours as described. The results are the average of 3 independent experiments. (D) Flow cytometry analysis using CD11b differentiation marker on OCI-AML3 treated for 48 hours. (E) Western blot analysis for p53, P-p53, p21, or actin in THP-1 and OCI-AML3 cells treated for 48 hours as described.

**RA/Arsenic reduce marrow blasts in NPM1 mutant AML patients**

Compassionate use of RA and arsenic was initiated in 5 previously untreated or relapsed elderly AML patients (patients 4-8) with normal karyotype and mutated NPM1 that were judged unfit for chemotherapy. As expected from APL patients, this treatment was very well tolerated.<sup>24</sup> BM blasts significantly decreased in 3 patients (patients 4-6) on day 15 posttreatment (Figure 2B) as detailed below. In patient 4, analysis of BM aspirates revealed that blasts increased initially from day 1 to day 15 of RA treatment (day 8 of RA/arsenic) (15% to 38%, respectively), and subsequently normalized at day 23 of RA treatment (day 16 of RA/arsenic) (5%) in a normocellular marrow. Furthermore, peripheral blood (PB) blasts gradually decreased to disappear at day 21 of RA/arsenic. Throughout the treatment period, the patient became transfusion independent. In patient 5, BM and PB blasts decreased from

27% at day 1 to 2% to 3% at day 15 of RA/arsenic treatment. In patient 6, BM blasts decreased from day 1 to day 15 of RA/arsenic (from 55% to 14%) with a clearance of PB blasts at day 15. In these 3 patients, blast counts reincreased with discontinuation of treatment. Two additional patients<sup>7,8</sup> were treated; patient 7 died of invasive Aspergillosis at day 21 from RA/arsenic treatment with no evidence of response and patient 8 rapidly died of bilateral interstitial pneumonia at day 10 from RA/arsenic treatment (data not shown). Thus, RA and arsenic exerted a transient in vivo antileukemic effect in this subset of AML patients.

**Degradation of mutant NPM1 drives RA and/or arsenic-induced growth inhibition**

Because of the similarities between RA/arsenic effects on NPM1 mutant AML cells and APL cells (growth arrest, apoptosis and

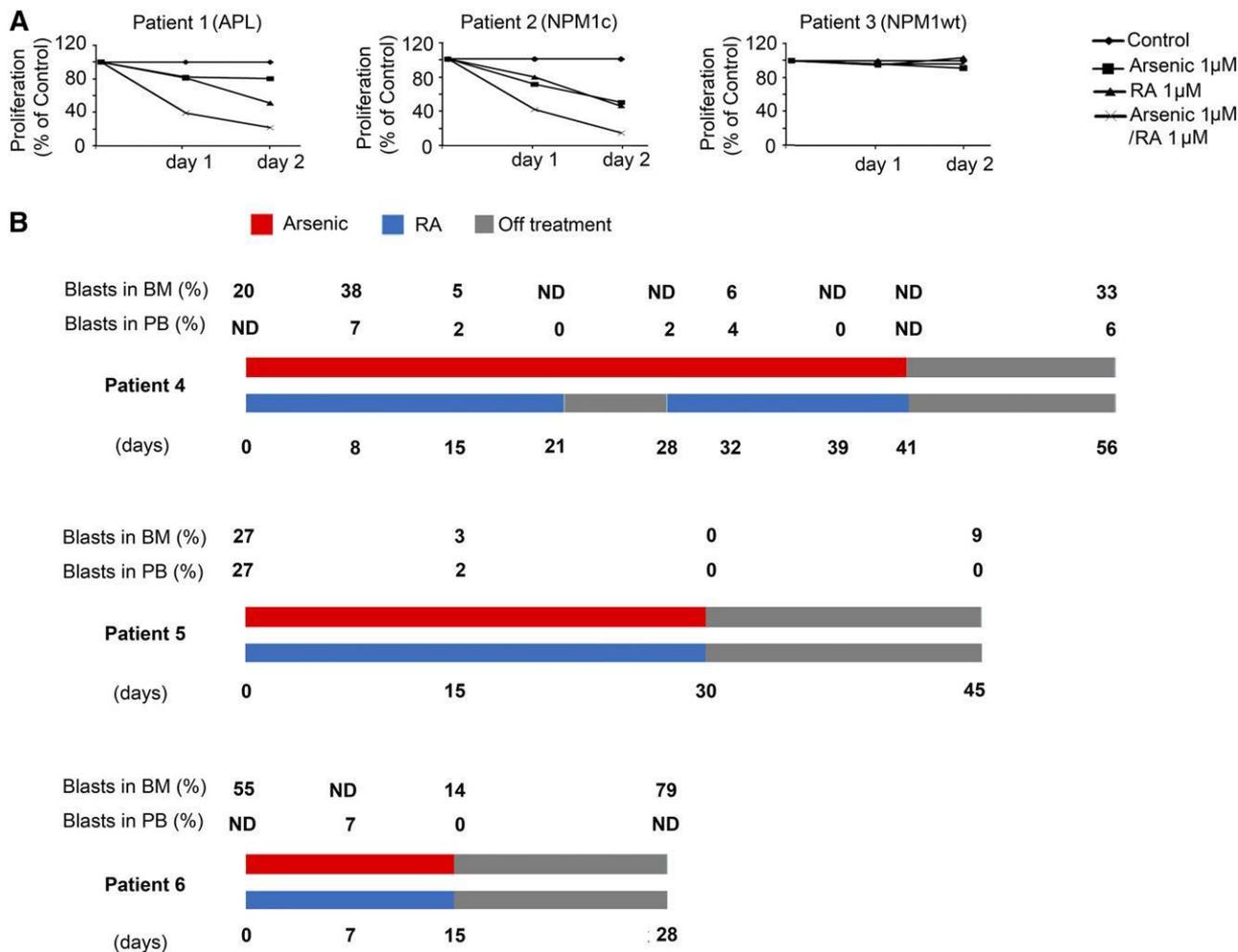


Figure 2. RA and arsenic induce growth inhibition in NPM1 mutated AML cells ex vivo and in vivo. (A) Primary AML cells from 3 different AML patients were treated with arsenic (1 mM), RA (1 mM) or a combination of both for 48 hours. Cell growth (percent of control) was assayed in duplicate wells. (B) Percent of PB and BM blasts and treatment schedule in 3 NPM1-mutated AML patients treated with RA and arsenic as indicated. ND, not done.

differentiation in vitro, in vivo anti-leukemic activity), we investigated the ability of these drugs to induce degradation of mutant NPM1 oncoproteins. In contrast, RA and, to a lesser extent, arsenic decreased NPM1 protein levels in OCI-AML3 cells, as assessed with an antibody detecting both WT and mutant proteins (Figure 3A). Using an antibody selective for NPM1 mutant protein, the amplitude of NPM1 downregulation was even higher. Similar findings were observed in primary patient cells (Figure 3B). In the IMS-M2 cells, degradation of the NPM1 mutant protein was observed with arsenic, not RA (data not shown). No effect of RA or arsenic on NPM1 expression was observed in THP-1, ML-2, HEL, or MOLM-13 cells (Figure 3A; supplemental Figure 1B). Critically, both NPM1 downregulation and growth arrest were reversed with the addition of the proteasome inhibitor PS-341 (Figures 3C-D and data not shown) (CI 5 4.58 at 24 hours). These results suggest that RA/arsenic-induced growth arrest of AML cells with mutant NPM1 is caused by proteasomal degradation of the mutated oncoprotein.

**RA and arsenic restore the normal localization of both NPM1 and PML**

In NPM1 mutant AMLs, NPM1 becomes delocalized to the cytoplasm (Figure 3E; supplemental Figure 3).<sup>25</sup> Importantly, treatment with

combined RA/arsenic, but not single agents alone, resulted in complete degradation of mutant NPM1 and accordingly restored the nucleolar localization of the remaining WT NPM1 protein in OCI-AML3 cells (Figure 3E; supplemental Figure 3). In vivo RA/arsenic treatment resulted in complete NPM1 nucleolar relocalization in the blasts of one patient (patient 7, Figure 4A), although mutant NPM1 was not fully degraded (data not shown). Thus, therapy corrects the defects in nucleolar organization (and presumably function) imposed by NPM1 mutation.

PML NBs constitute platforms for posttranslational modifications, notably sumoylation, that have been repeatedly implicated in transformation.<sup>26-28</sup> Yet, alterations in PML NBs were not previously described in NPM1 mutant AMLs. Unexpectedly, in OCI-AML3 cells, PML NBs were significantly smaller than in THP-1 cells (Figure 4B). Accordingly, we could not detect nuclear bodies using SUMO-1 antibodies (Figure 4C). In primary NPM1 mutated AML cells, we again observed abnormal and heterogenous PML NBs, together with a significant overlap between nuclear, but extranucleolar, NPM1, and PML (Figure 4A). Treatment with RA/arsenic restored PML NB organization in both OCI-AML3 and THP-1 cells, however this effect was more pronounced in NPM1 mutated cells and was accompanied by enhanced SUMO-1 NB formation (Figure 4B-C). These results are highly suggestive for mutant

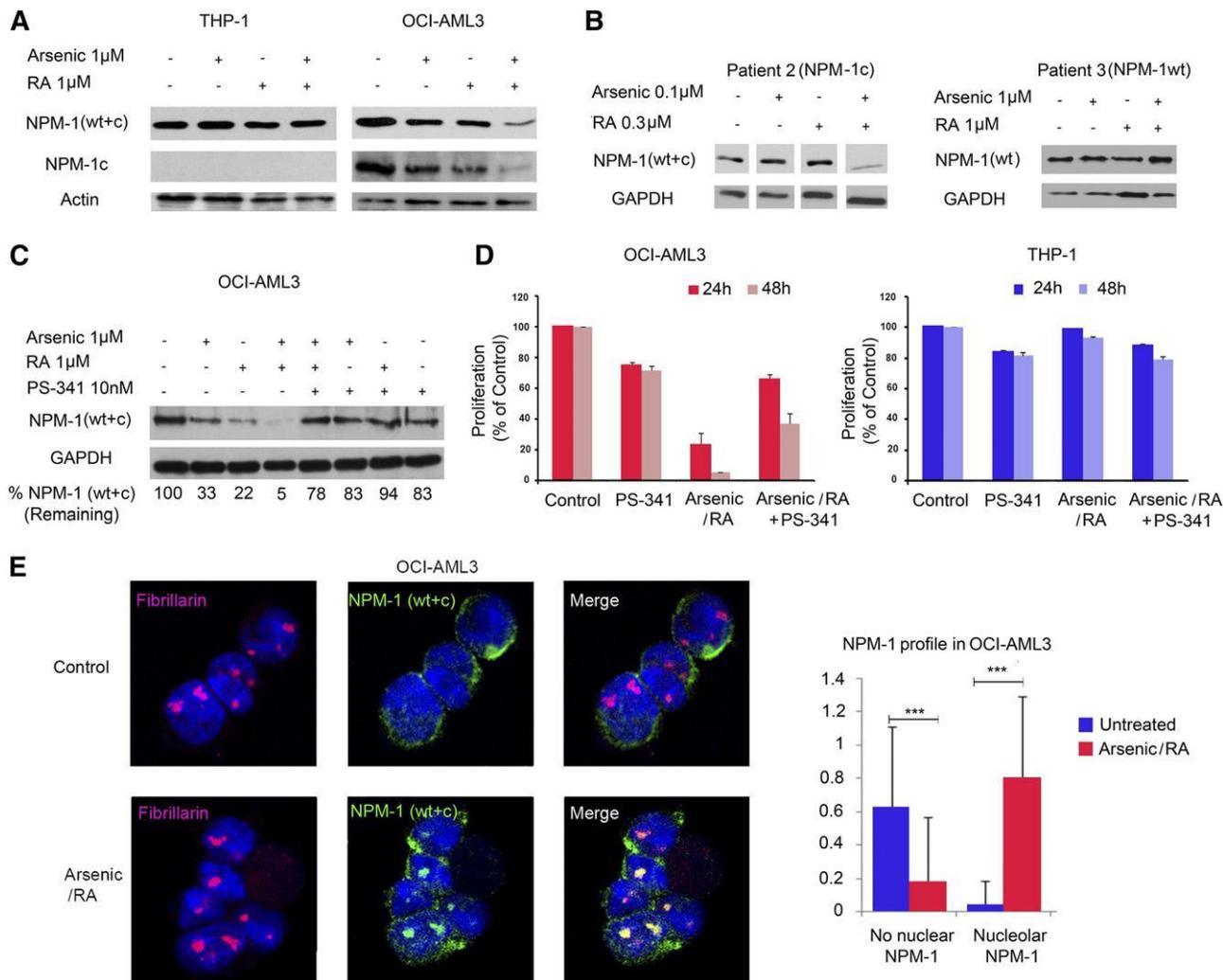


Figure 3. RA and arsenic induce proteasomal degradation of mutant NPM1 and restore NPM1 nucleolar localization. (A) Western blot analysis for NPM1 recognizing both WT and mutated NPM1 (WT 1 c), mutated NPM1 (NPM1c), actin in THP-1, and OCI-AML3 cells treated with arsenic (1 mM), RA (1 mM), or a combination of both for 48 hours as indicated. A representative of 3 independent experiments is shown. (B) Western blot analysis for NPM1 (WT 1 c) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in primary leukemia cells derived from AML patients treated with arsenic (0.1 or 1 mM), RA (0.3 or 1 mM), or a combination of both for 48 hours as indicated. (C) Western blot analysis for NPM1 (WT 1 c) and GAPDH in OCI-AML3 cells treated with arsenic (1 mM), RA (1 mM), PS-341 (10 nM), either alone, or in combination for 48 hours as indicated. Percentages indicate the amount of remaining NPM1 (WT 1 c) after normalization to GAPDH. (D) Cell count with trypan blue staining (percent of control) of OCI-AML3 and THP-1 cells treated with arsenic (1 mM), RA (1 mM), PS-341 (10 nM), either alone, or in combination for up to 48 hours. Cell growth (percent of control) was assayed in triplicate wells. The results depict one representative experiment among 3 independent ones. (E) Confocal microscopy analysis of nucleolar NPM1 localization in OCI-AML3 or THP-1 cells after treatment with RA/arsenic for 48 hours. NPM1 was stained with an antibody recognizing NPM1 (WT 1 c) (green), nucleoli were stained with anti-Fibrillarin (red), and nuclei were stained with 4,6 diamidino-2-phenylindole (blue). Images represent Z sections. Graphs show quantification of nucleolar NPM1 as averages of one Z section/cell from 30 different cells of 3 independent experiments. Significant P values are indicated by asterisks.

NPM1-mediated disorganization of PML/SUMO-1 NBs. As in APL, NB restoration could contribute to the therapeutic efficacy of the RA/arsenic combination.<sup>17,29</sup>

## Discussion

In APL, both ourselves and others, have demonstrated that PML/RARA degradation by arsenic or RA restores PML nuclear bodies and activates a p53 senescence checkpoint that is required for APL eradication.<sup>15,30,31</sup> The results obtained here with NPM1 mutant AML unexpectedly bear some similarities with APL. First, mutant NPM1 is degraded with RA or arsenic exposure, and p53 signaling is activated. Second, mutant NPM1 disorganizes PML bodies and RA/arsenic restores nuclear organization (nucleolar NPM1 with normal PML/

SUMO bodies) ex vivo and in vivo. Finally, BM blasts are reduced in some treated patients. Mechanistically, these AMLs are addicted to continuous expression of the mutant protein<sup>32</sup> so that degradation of mutant NPM1 most likely triggers cell cycle arrest, apoptosis, and differentiation. Reorganization of PML bodies could also contribute to P53 activation and therapy response.<sup>17,33</sup>

Cell cycle arrest and apoptosis induced by RA most likely reflect P53 activation with degradation of mutant NPM1. RA very efficiently degrades mutant NPM1 in OCI-AML3 cells, but not IMS-M2 cells. It remains obscure how RA selectively targets mutant NPM1 protein and this should be the focus of future studies. A variety of mutations were described in NPM1. Some of these may only confer sensitivity to RA-initiated and/or arsenic-initiated degradation. PML NBs are SUMO-dependent degradation factories activated by interferons and oxidative stress.<sup>27</sup> Arsenic enhances formation of PML NBs and promotes the degradation of some PML-associated proteins. That pretreatment with

Blasts from *in vivo* treated patient

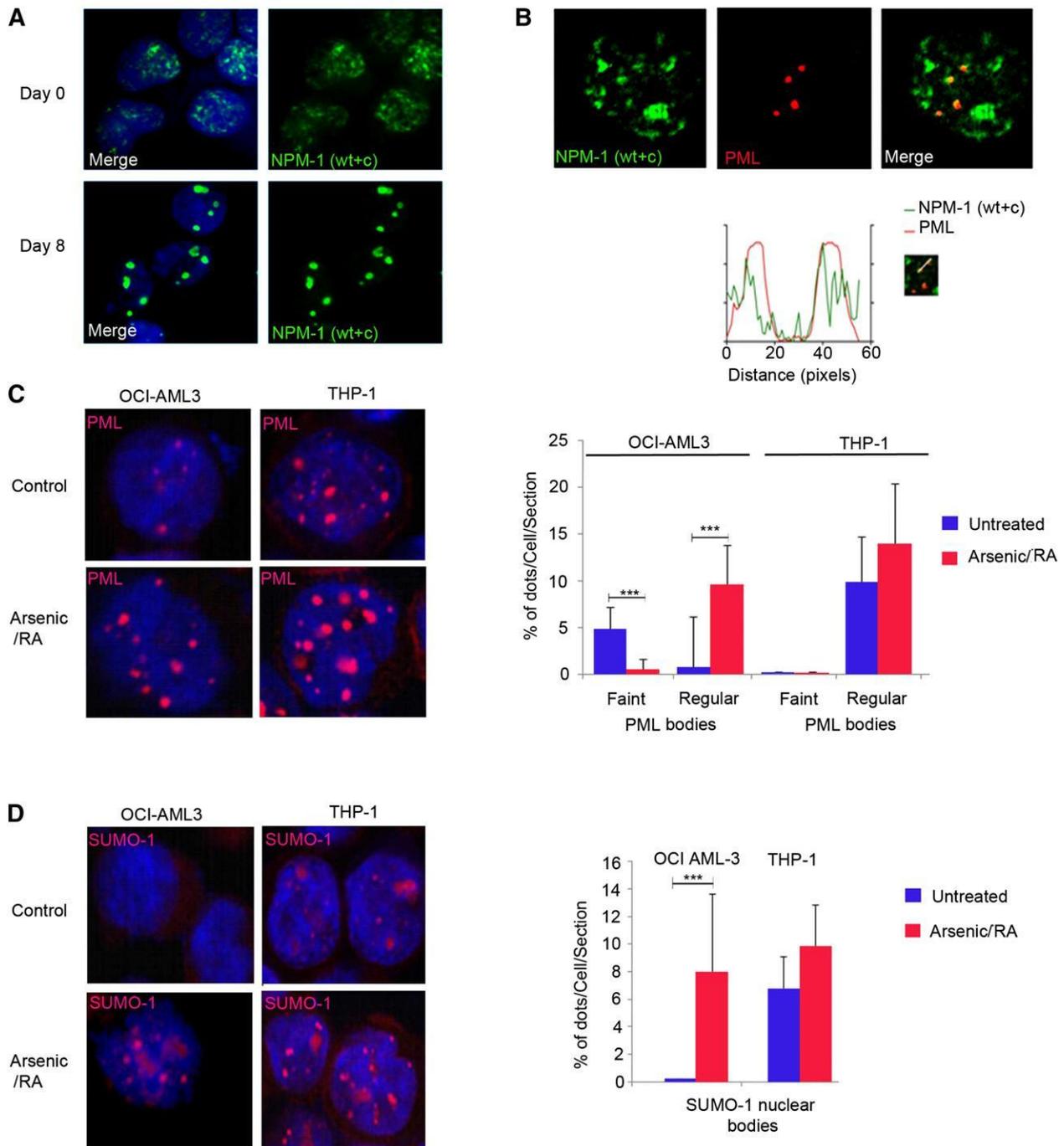


Figure 4. Combination of RA and arsenic restores PML and SUMO-1 nuclear body formation. (A-B) Primary leukemic blasts from one NPM1 mutated AML patient on days 0 and 8 after *in vivo* treatment with RA/arsenic were analyzed by confocal microscopy. NPM1 was stained with anti-NPM1 (WT + c) antibody (green); PML was stained with anti-PML antibody (red). (C-D) OCI-AML3 cells (left panels) and THP-1 cells (right panels) treated with RA/arsenic for up to 48 hours and analyzed by confocal microscopy. (C) Treatment of OCI-AML3 cells with RA/arsenic leads to PML nuclear body reorganization. (D) Treatment of OCI-AML3 cells with RA/arsenic leads to SUMO-1 nuclear body formation. The results (C-D) depict one representative of 3 independent experiments. Graphs show quantification of PML and SUMO-1 NBs, as averages of one Z-section/cell from 30 different cells. Significant P values are indicated by asterisks.

interferon- $\alpha$  significantly accelerated arsenic-induced degradation of mutant NPM1 (data not shown), which could suggest a role of PML and/or SUMOs in the arsenic-triggered degradation process. In that respect, altered PML NBs biogenesis in NPM1 mutant AMLs could reflect a physical interaction between PML and mutant NPM1, a protein which is massively sumoylated.<sup>34</sup> Other similarities between

PML and NPM1 exist, such as link to p53 control, interferon signaling, or oxidative stress.<sup>35</sup>

Because elderly NPM1-mutated AML (.80 years old and/or with severe comorbidities) are unlikely to be eligible for treatment with chemotherapy, some patients were treated on a compassionate basis with the RA/arsenic combination used in APL. Although we did not

observe complete remissions, the leukemia clearly regressed in several patients. This combination is unlikely to be curative alone, but may be part of a broader therapeutic strategy. We could demonstrate the relocalization of NPM1 to the nucleolus in primary AML blasts, pointing to therapy-induced restoration of nuclear organization. Yet, this combination was insufficient to obtain complete degradation of mutant NPM1 in AML patients *in vivo*. In APL, clinical response mirrors the extent of PML/RARA degradation and only full oncoprotein catabolism yields remissions.<sup>17,19</sup> Some *in vivo/ex vivo* differences may be responsible for this blunted *in vivo* degradation by the RA/arsenic combination. For example, the arsenic-induced oxidative stress required for full NPM1 degradation may not be reached *in vivo*. Preclinical optimization, for example using xenografted mouse models,<sup>36</sup> could address this point. Although five AML cell lines with WT NPM1 were not affected by RA/arsenic therapy *ex vivo*, we cannot exclude that other AML genotypes may be sensitive to RA/arsenic and/or that this combination may not elicit degradation of other oncoproteins.

These unexpected findings provide an intriguing parallel to the RA/arsenic-mediated degradation of PML/RARA in APL. They warrant further biochemical studies to elucidate the basis for the selective catabolism of NPM1 mutants. Our observations could explain the survival benefit of adding RA to chemotherapy in this subset of patients and warrants clinical evaluation of new therapeutic combinations incorporating frontline RA/arsenic in elderly ones.

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## Authorship

Contribution: H.E.H., H.d.T., and A.B. designed the study and wrote the manuscript; Z.D., C.B., R.H., N.T., A.S., and U.S. performed experiments; E.R., L.A., O.L., M.M., H.D., and P.F. treated AML patients; N.D. and K.Z. participated in study design and approved the final version of the manuscript.

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## **Retinoic acid and arsenic trioxide trigger degradation of mutated NPM1, resulting in apoptosis of AML cells**

Hiba El Hajj, Zeina Dassouki, Caroline Berthier, Emmanuel Raffoux, Lionel Ades, Olivier Legrand, Rita Hleihel, Umut Sahin, Nadim Tawil, Ala Salameh, Kazem Zibara, Nadine Darwiche, Mohamad Mohty, Hervé Dombret, Pierre Fenaux, Hugues de Thé and Ali Bazarbachi

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# **ANNEX 2**

# Imidazoquinoxaline Derivative EAPB0503: A Promising Drug Targeting Mutant Nucleophosmin 1 in Acute Myeloid Leukemia

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**BACKGROUND:** Nucleophosmin 1 (NPM1) is a nucleocytoplasmic shuttling protein mainly localized in the nucleolus. NPM1 is frequently mutated in acute myeloid leukemia (AML). NPM1c oligomerizes with wild-type nucleophosmin 1 (wt-NPM1), and this leads to its continuous cytoplasmic delocalization and contributes to leukemogenesis. Recent studies have shown that Cytoplasmic NPM1 (NPM1c) degradation leads to growth arrest and apoptosis of NPM1c AML cells and corrects wt-NPM1 normal nucleolar localization. **METHODS:** AML cells expressing wt-NPM1 or NPM1c or transfected with wt-NPM1 or NPM1c as well as wt-NPM1 and NPM1c AML xenograft mice were used. Cell growth was assessed with trypan blue or a CellTiter 96 proliferation kit. The cell cycle was studied with a propidium iodide (PI) assay. Caspase-mediated intrinsic apoptosis was assessed with annexin V/PI, the mitochondrial membrane potential, and poly(adenosine diphosphate ribose) polymerase cleavage. The expression of NPM1, p53, phosphorylated p53, and p21 was analyzed via immunoblotting. Localization was performed with confocal microscopy. The leukemia burden was evaluated by flow cytometry with an anti-human CD45 antibody. **RESULTS:** The imidazoquinoxaline 1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine (EAPB0503) induced selective proteasome-mediated degradation of NPM1c, restored wt-NPM1 nucleolar localization in NPM1c AML cells, and thus yielded selective growth arrest and apoptosis. Introducing NPM1c to cells normally harboring wt-NPM1 sensitized them to EAPB0503 and led to their growth arrest. Moreover, EAPB0503 selectively reduced the leukemia burden in NPM1c AML xenograft mice. **CONCLUSIONS:** These findings further reinforce the idea of targeting the NPM1c oncoprotein to eradicate leukemic cells and warrant a broader preclinical evaluation and then a clinical evaluation of this promising drug. *Cancer* 2017;123:1662-73. © 2017 American Cancer Society.

**KEYWORDS:** acute myeloid leukemia, apoptosis, 1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine (EAPB0503), nucleophosmin 1, xenograft mice.

## INTRODUCTION

Acute myeloid leukemia (AML) is a complex, heterogeneous blood malignancy in which a failure to differentiate and an overproliferation of undifferentiated myeloid precursors result in impaired hematopoiesis and bone marrow (BM) failure. AML is associated with a highly variable prognosis and a high mortality rate, with overall survival exceeding 2 years for only 20% of elderly patients and 5 years for less than 50% of adult patients.<sup>1</sup>

The prognosis of AML is mostly dependent on somatic genetic alterations used to classify the risk as favorable, intermediate, or unfavorable.<sup>2</sup> In AML patients with a normal karyotype, the most important genetic mutations influencing both the prognosis and the treatment strategies are mutations in nucleophosmin 1 (NPM1) and FMS-like tyrosine kinase 3 (FLT-3) internal tandem duplication.<sup>3</sup> Recently, more heterogeneous genomic categories for AML have been reported.<sup>4</sup>

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The first 2 authors contributed equally to this article.

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Additional supporting information may be found in the online version of this article.

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NPM1 is an essential gene<sup>5</sup> encoding a phosphoprotein<sup>6</sup> continuously shuttling between the nucleus, nucleolus, and cytoplasm but mainly residing in the nucleolus.<sup>7,8</sup> NPM1 has many functions, including p14<sup>Arf</sup> stabilization, ribosomal biogenesis regulation, centrosomal duplication control, and p53 activation in response to stress stimuli.<sup>5,6,9</sup> In AML, NPM1 mutations account for approximately one-third of patients, and this makes it one of the most frequently mutated genes.<sup>6,10</sup> These mutations lead to the creation of a de novo nuclear export signal,<sup>6,10,11</sup> which results in cytoplasmic accumulation of NPM1c, along with wild-type nucleophosmin 1 (wt-NPM1) and thus leukemogenesis in these AML patients.<sup>10</sup>

Despite all the advances in genetic and epigenetic changes in AML, there is still little progress in the treatment of the disease. Although complete remission is reached by almost 70% of patients with standard induction chemotherapy, refractory disease is common, and relapse represents the major cause of treatment failure.<sup>12</sup> Stem cell transplantation remains the best chance for long-term survival but is associated with several complications.<sup>13</sup> Therefore, new therapeutic approaches, specifically ones directly targeting the products of AML genetic alterations, are needed.

In NPM1c AML, degradation of the NPM1c oncoprotein leads to leukemic cell growth arrest and apoptosis.<sup>14-16</sup> We and others have recently shown that arsenic trioxide and retinoic acid selectively induce NPM1c proteasomal degradation and thus lead to apoptosis in NPM1c AML cells.<sup>15,16</sup> This combined treatment restores NPM1 nucleolar localization ex vivo and in vivo. However, although the clearance of AML blasts was observed in a few treated patients, no cure was achieved, likely because of the complexity and status of the disease burden. This underlies the need for novel therapies to improve treatment outcomes.

Imiquimod is a toll-like receptor 7 immunomodulator<sup>17,18</sup> used to treat certain skin cancers<sup>19</sup> and genital warts.<sup>20</sup> Imiquimod analogues, called imidazoquinolines, have been synthesized<sup>21</sup>; among them, 1-(2-phenyl-ethyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine (EAPB0203) and 1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine (EAPB0503) have been reported with promising antitumor activity.<sup>22,23</sup> Indeed, EAPB0203 displayed pronouncedly higher in vitro potency against melanoma and adult T-cell leukemia cells in comparison with imiquimod.<sup>23,24</sup> Later, EAPB0503 showed 10-fold higher cytotoxicity than EAPB0203 against melanoma cells.<sup>25</sup> More recently, EAPB0503

showed a potent apoptotic effect in chronic myeloid leukemia cells through BCR-ABL degradation.<sup>26</sup>

Here we demonstrate that EAPB0503 induces NPM1c proteasomal degradation selectively in NPM1c AML cells and leads to their apoptosis. Importantly, introducing NPM1c to wt-NPM1-harboring cells sensitizes them to EAPB0503. Moreover, EAPB0503 treatment restores wt-NPM1 nucleolar localization in vitro and also in ex vivo treated blasts and selectively reduces the leukemia burden in NPM1c AML xenograft mice. These findings expand the antileukemic use of EAPB0503, reinforce the idea of targeting oncoprotein degradation to kill leukemia cells, and warrant a broader preclinical evaluation and then a clinical evaluation of this promising drug.

## MATERIALS AND METHODS

### Cell Lines

KG-1a, ML-2, and THP-1 cell lines (from F. Mazurier) and IMS-M2 (from H. de The) were grown in Roswell Park Memorial Institute 1640 medium. OCI-AML3 cells (from D. Bouscary) were grown in minimum essential medium a. Cells were seeded at a concentration of  $2 \times 10^5$ /mL. EAPB0203 or EAPB0503 was used at 0.1 to 5  $\mu$ M, the caspase inhibitor Z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (zVAD) (Bachem Bioscience) was used at 50 mM, and the proteasome inhibitor PS-341 was used at 10 nM.<sup>15</sup> Cell growth was assessed with trypan blue or a CellTiter 96 proliferation kit (Promega).

Primary AML cells from patients' BM were extracted as described by El Hajj et al<sup>15</sup> after approval by the institutional review board at the American University of Beirut and after the patients had consented according to the Declaration of Helsinki.

### Drugs

The synthesis of EAPB0203 and EAPB0503 was performed as described by Deleuze-Masquefa et al.<sup>21,22</sup> Further optimization of EAPB0503 synthesis was achieved with microwave-assisted chemistry.<sup>27</sup>

### Generation of Cells Expressing wt-NPM1 or NPM1c

Green fluorescent protein (GFP) wt-NPM1 or NPM1c inserts were amplified and ligated into a pByte lentiviral vector by the EcoRI site. Stable OCI-AML2 expressing wt-NPM1 or NPM1c was generated by lentiviral transduction followed by blasticidin selection. GFP-positive cells were sorted with the FACSAria Special Order Research Product (Becton Dickinson) and grown in minimum essential medium a before the cell growth assessment.

HeLa cells were transfected with pcDNA hemagglutinin (HA) expressing wt-NPM1 or NPM1c (from G. Tell)<sup>28</sup> with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations and were grown in Dulbecco's modified Eagle's medium.

#### Xenograft Animal Studies

NOD/Shi-scid IL2r<sup>2/2</sup> (NSG) mice were obtained from Jackson Laboratories (United States). Mouse protocols were approved by the institutional animal care and utilization committee of the American University of Beirut. OCI-AML3 or THP-1 cells ( $1 \times 10^6$ ) were injected into the tail vein of 8-week-old females (5 mice per group). On day 5 after the AML injection, the mice were treated with EAPB0503 (15 mg/kg) for 5 days a week over a period of 2 weeks. EAPB0503 was dissolved in dimethyl sulfoxide and diluted in an equal volume of lipofundin (vehicle) before its intraperitoneal administration to the mice.<sup>24,29</sup>

#### Flow Cytometry

##### Cell cycle analysis

Propidium iodide (PI) staining was used to assess the cell cycle as described by El Hajj et al.<sup>15</sup>

##### Annexin V staining

An annexin V–fluorescein isothiocyanate kit (BD Pharmingen) was used to assess phosphatidylserine exposure. Cells were treated with 1  $\mu$ M EAPB0503 for 24 hours before annexin V/PI labeling and flow cytometry analysis.

##### Mitochondrial membrane potential (MMP)

The MMP was assessed by a cell's ability to retain rhodamine 123 (Sigma-Aldrich), as described by Saliba et al.<sup>26</sup>

A Becton Dickinson FACS instrument was used; 10,000 events per condition were acquired, and FlowJo software (FlowJo LLC) was used for the analysis of the results.

##### Human CD45 staining

BM from the femurs and tibias of euthanized animals was flushed at the end of week 3 after AML inoculation. Cell surface staining was performed on 100  $\mu$ L of a sample with 20  $\mu$ L of an anti-human CD45 Peridinin Chlorophyll Protein (PerCP) conjugated antibody (345809; Becton Dickinson). After incubation for 15 minutes in the dark, erythrocytes were lysed with 1 mL of an FACS lysis solution (Becton Dickinson). Labeled samples were washed twice and analyzed on a Guava flow cytometer.

#### Immunoblot Analysis

After 48 hours of treatment with EAPB0203 or EAPB0503, proteins were probed with poly(adenosine di-phosphate ribose) polymerase (PARP), p53, p21, HA (Santa Cruz), phosphorylated p53 (Biolabs), or NPM1 (Abcam) before incubation with the monoclonal horse-radish peroxidase–conjugated secondary antibodies. The loading control was performed via probing with the mouse horseradish peroxidase–conjugated glyceraldehyde 3-phosphate dehydrogenase antibody (Abnova) or b-actin (Abcam). Immunoblots were detected with a luminol detection kit (Santa Cruz), and images were captured with the X-OMAT or BioRad ChemiDoc MP system.

#### Immunofluorescence Microscopy

AML cells or patients' blasts were spun down onto glass slides, fixed, and permeabilized with ice-cold methanol for 30 minutes. Immunostaining was performed with a monoclonal antibody against anti-B23 NPM1 (Santa Cruz) and a polyclonal antibody against the nucleolar marker fibrillarin (Abcam). Primary antibodies were revealed by Alexa Fluor 488– or Fluor 594–labeled secondary antibodies (Santa Cruz). Images were acquired with a Zeiss LSM 710 laser scanning microscope operated with Zen 2009 software (Carl Zeiss).

#### Statistical Analysis

Data are reported as averages and standard deviations. Statistical analyses were performed with the Student t test; a P value less than .05 was considered significant.

## RESULTS

### EAPB0203 and EAPB0503 Induce Growth Arrest in NPM1c AML Cells

We used 3 wt-NPM1 cell lines (THP-1, KG-1a, and MOLM-13) and the 2 available NPM1c AML cell lines (OCI-AML3 and IMS-M2) to test for EAPB0203 and EAPB0503 effects on cell growth and viability. We tested a range of drug concentrations (0.1–5 mM) and assessed cell growth for 5 days after treatment. Both treatments resulted in pronounced time-dependent growth inhibition of OCI-AML3 cells (Fig. 1A,B). EAPB0203 at 5 mM resulted in significant OCI-AML3 growth inhibition ( $P < .05$ ), which started 72 hours after treatment. Strikingly, EAPB0503 was more potent and at 0.1 mM resulted in significant growth inhibition, which started 96 hours after treatment ( $P < .001$ ). Similarly significant results were obtained for both OCI-AML3 and IMS-M2: a concentration of 0.5 mM induced growth inhibition starting 72 hours after treatment ( $P < .001$ ), and concentrations

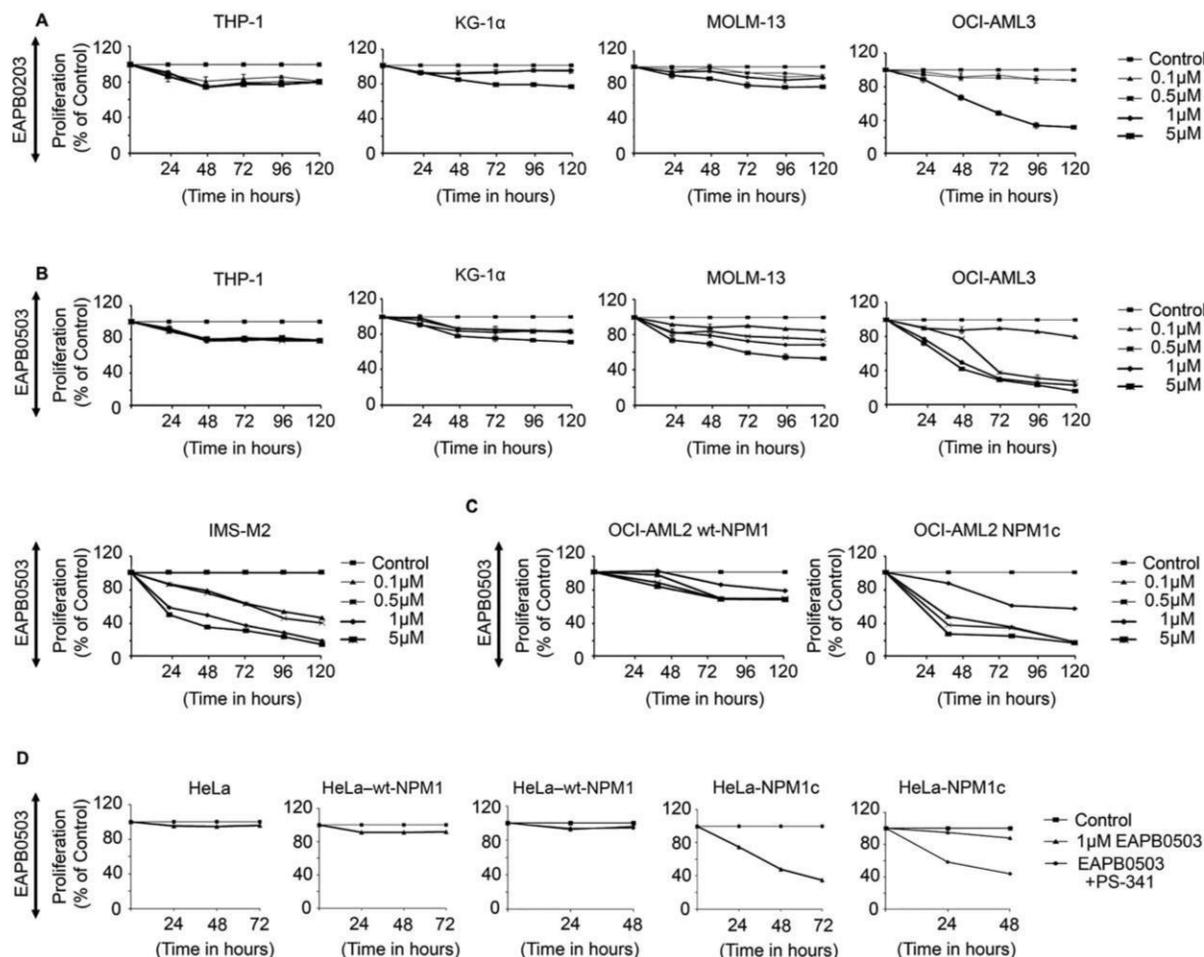


Figure 1. EAPB0503 induces selective growth inhibition in NPM1c AML cells. AML cell lines with normal NPM1 (THP-1, KG-1a, and MOLM-13) and NPM1c (OCI-AML3 and IMS-M2) were treated with increasing concentrations (0.1–5 mM) of (A) EAPB0203 and (B) EAPB0503 for 24, 48, 72, 96, and 120 hours. (C) Stably transfected OCI-AML2 with green fluorescent protein wt-NPM1 or NPM1c was treated with increasing concentrations (0.1–5 mM) of EAPB0503 for 24, 48, 72, 96, and 120 hours. (D) HeLa cells transfected with hemagglutinin-tagged wt-NPM1 or NPM1c were treated with 1 mM EAPB0503 alone or in combination with 10 nM PS-341 for 24, 48, and 72 hours as indicated. Cell growth (percentage of the control) was assayed in triplicate. The results represent the average of at least 3 independent experiments. AML indicates acute myeloid leukemia; EAPB0203, 1-(2-phenylethyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; EAPB0503, 1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; NPM1, nucleophosmin 1; wt-NPM1, wild-type nucleophosmin 1; Cytoplasmic NPM1 (NPM1c) Hemagglutinin (HA)-tagged confirmed green fluorescent protein (GFP)-tagged wt-NPM1 or NPM1c.

of 1 and 5 mM induced the same inhibitory effect 24 hours after treatment ( $P < .05$  and  $P < .001$ , respectively; Fig. 1B). Importantly, a median inhibitory concentration of 1 mM in OCI-AML3 and IMS-M2 cells was achieved 2 days after treatment with EAPB0503 ( $P < .05$  and  $P < .001$ , respectively), whereas a concentration of 5 mM was achieved after treatment with EAPB0203 in OCI-AML3 (Fig. 1A,B). This more potent effect of EAPB0503 versus EAPB0203 is in line with previously reported results.<sup>26</sup> THP-1 and KG-1a cells were minimally sensitive to the compounds, with only approximately 20% growth inhibition even 5 days after treatment (Fig. 1A,B). MOLM-13 cells were also minimally sensitive to

EAPB0203 but displayed approximately 50% growth inhibition 72 hours after treatment with EAPB0503 (Fig. 1A,B). This percentage did not become more pronounced even 5 days after treatment, and the only significant result was obtained with concentrations of 1 and 5 mM, 120 and 72 hours after treatment, respectively ( $P < .05$ ; Fig. 1B).

#### Introduction of NPM1c Into wt-NPM1-Expressing Cells Sensitizes Them to EAPB0503

To examine whether the growth inhibition solely observed in NPM1c cell lines was due to NPM1 mutations, we introduced NPM1c to wt-NPM1-expressing cells and checked for their sensitivity to EAPB0503. We used the

wt-NPM1-expressing AML cell line (OCI-AML2) and generated by lentiviral transduction and then blasticidin selection cells stably expressing either GFP-tagged wt-NPM1 or NPM1c. GFP-positive cells were sorted, and a range of EAPB0503 concentrations (0.1-5 mM) were tested to assess cell growth more than 5 days after treatment. Interestingly, stable expression of NPM1c in OCI-AML2 resulted in significantly pronounced growth inhibition at 0.1 mM that started 72 hours after treatment and at 0.5, 1, and 5 mM that started 48 hours after treatment ( $P < .05$ ; Fig. 1C). A minimal effect was observed in wt-NPM1 OCI-AML2: maximum growth inhibition of 30% (nonsignificant) was obtained 48 hours after treatment with concentrations of 0.5, 1, and 5 mM (Fig. 1C). Similar results were obtained with HeLa cells: a concentration of 1 mM induced growth arrest starting 48 hours after treatment in HA NPM1c-transfected cells ( $P < .001$ ) but not wt-NPM1-transfected cells (Fig. 1D). This growth inhibition was reversed upon the addition of PS-341 only in NPM1c-expressing cells both 24 and 48 hours after treatment ( $P < .05$ ; Fig. 1D). Our results strongly suggest that introducing NPM1c into cells harboring wt-NPM1 sensitizes them to EAPB0503. Because of its potency, especially in NPM1c AML cells, only EAPB0503 was adopted at its median inhibitory concentration of 1 mM for the remainder of the study.

#### EAPB0503 Induces Massive Apoptosis in NPM1c AML Cells

To examine the mechanisms dictating growth inhibition and cell death, a cell cycle analysis was performed 48 hours after treatment with 1 mM EAPB0503. A sharp increase in the pre-G<sub>0</sub> cell percentage, which reached more than 80%, was obtained upon the treatment of OCI-AML3 with EAPB0503. Minimal effect was observed in the wt-NPM1 cells (THP-1, KG-1a, and MOLM-13; Fig. 2A and Supporting Fig. 1A [see online supporting information]). The cell cycle distribution showed no major variation in all the tested AML cells untreated or treated with EAPB0503 (Fig. 2B and Supporting Fig. 1A [see online supporting information]), and this shows that the drug is mostly inducing pre-G<sub>0</sub> accumulation in NPM1c AML without affecting the other cell cycle phases.

To confirm the apoptosis, annexin V/PI labeling was performed, and a significant increase of 40% in annexin V positivity was observed only in OCI-AML3 cells treated with 1 mM EAPB0503 for 24 hours ( $P < .005$ ; Fig. 2C and Supporting Fig. 1B [see online supporting information]). In contrast, all wt-NPM1 cells remained virtually annexin V-negative upon treatment with the drug (Fig. 2C and Supporting Fig. 1B [see online supporting information]).

#### EAPB0503-Induced Apoptosis in NPM1c AML Cells Involves the Dissipation of MMP and Caspase Activation

The intrinsic apoptotic cascade is characterized by many steps, the earliest of which is the disruption of the MMP.<sup>30</sup> Because EAPB0503 induces apoptosis in NPM1c AML cells, we measured MMP in untreated cells or 2 days after treatment with EAPB0503. Treated OCI-AML3 cells failed to retain the rhodamine 123 dye inside their mitochondria (Fig. 2D and Supporting Fig. 1C [see online supporting information]). Conversely, all wt-NPM1 AML cells showed no loss of MMP up to 48 hours after treatment (Fig. 2D and Supporting Fig. 1C [see on-line supporting information]).

To study the effect of MMP dissipation in EAPB0503-treated AML cells on the caspase cascade, we examined PARP cleavage. The treatment of OCI-AML3 for 48 hours with EAPB0503 but not with EAPB0203 led to PARP cleavage into its death-associated fragment (Fig. 2E); this occurred to a much lesser extent in the wt-NPM1 AML cells treated with either drug (Fig. 2E). Interestingly, the cotreatment of cells with the general caspase inhibitor zVAD and EAPB0503 reversed EAPB0503 growth-induced inhibition in OCI-AML3 (Fig. 2F), whereas no effect was observed in wt-NPM1 cells (THP-1 and MOLM-13; Fig. 2F). Altogether, our results indicate that the selective growth arrest obtained in NPM1c AML with EAP0503 involves caspase activation.

#### EAPB0503 Treatment Activates p53 Signaling in NPM1c AML Cells

To determine whether the EAPB0503-associated growth inhibition and apoptosis were p53-mediated, p53 protein levels were monitored 48 hours after treatment with 1 mM EAPB0203 or EAPB0503, and the results were compared with untreated controls. EAPB0503 induced substantial upregulation of total p53 protein levels and the p53 phosphorylated form exclusively in the NPM1c OCI-AML3 cell line (Fig. 2G), whereas no effect was observed upon the treatment of these cells with EAPB0203 (Fig. 2G). Accordingly, p21 protein levels were upregulated only in EAPB0503-treated OCI-AML3 (Fig. 2G). Because p53 is mutated in both THP-1 and KG-1a cell lines,<sup>31</sup> we tested p53 only in the wt-NPM1 MOLM-13 cell line and found that p53, phosphorylated p53, and p21 protein levels remained unchanged upon treatment with either drug (Fig. 2G). Altogether, these results show that EAPB0503 is a potent inducer of apoptosis exclusively in NPM1c AML cells.

### EAPB0503 Induces NPM1c Proteasomal Degradation and Restores wt-NPM1 Nucleolar Localization in NPM1c AML Cells

Given the selective activity of EAPB0503 in NPM1c AML cells, we examined its effect on NPM1c oncoprotein

degradation. Although no effect of EAPB0203 or EAPB0503 on NPM1 expression was obtained in THP-1, MOLM-13, or KG-1a cells (Fig. 3A), EAPB0503 but not EAPB0203 triggered NPM1 downregulation in OCI-AML3 cells (Fig. 3B), and this suggests that NPM1c is the

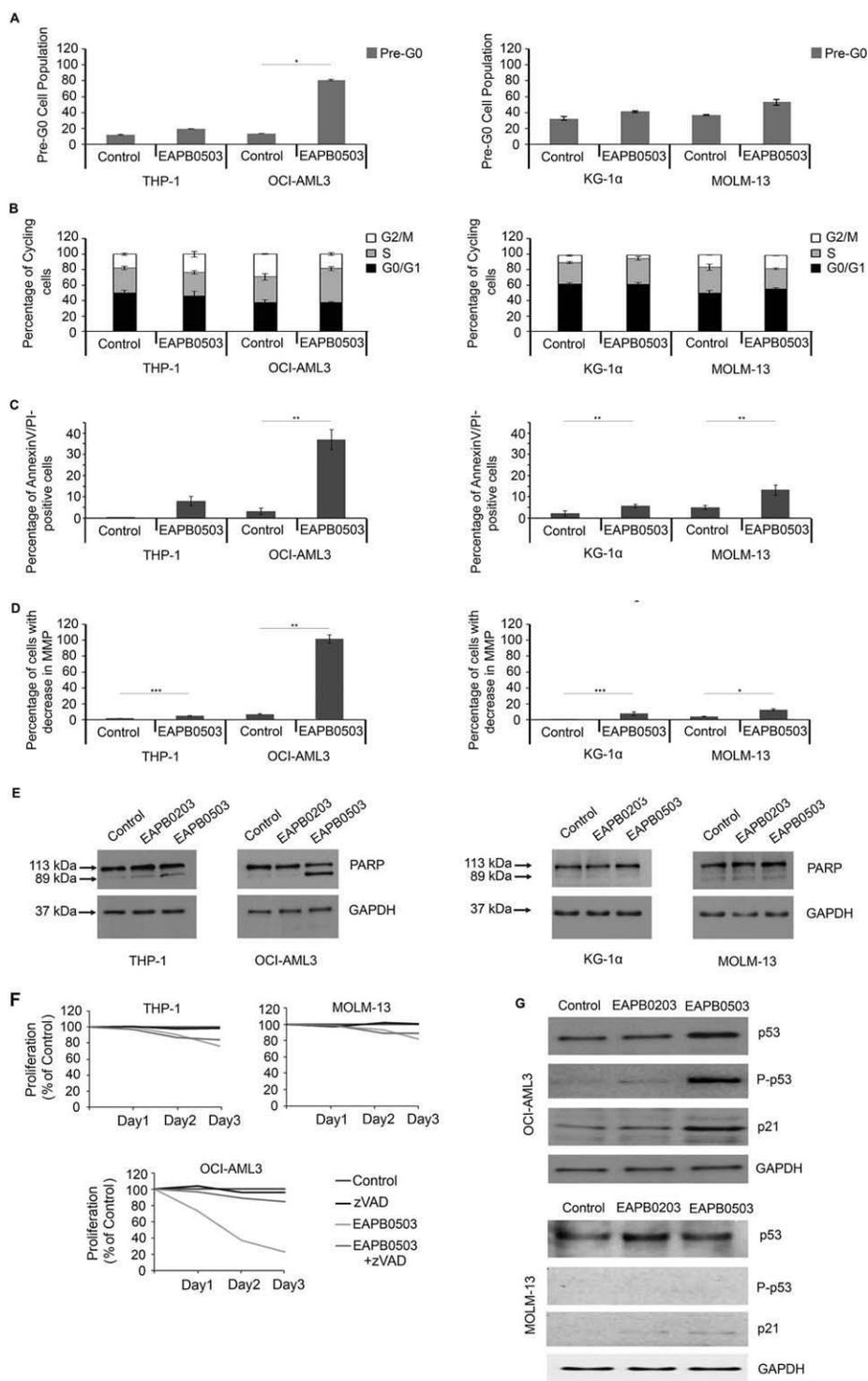


Figure 2.

primary target of EAPB0503. Critically, adding the proteasome inhibitor PS-341 reversed both NPM1 downregulation and growth arrest (Fig. 3C) specifically in OCI-AML3 (Supporting Fig. 2 [see online supporting information]). To eliminate any potential off-target effect of the treatment, we treated HA-tagged, wt-NPM1- or NPM1c-transfected HeLa cells with EAP0503 alone or in combination with PS-341. With an anti-HA antibody, our results showed that EAPB0503 proteasome-mediated degradation was selective for NPM1c and was reversed upon the addition of PS-341 (Fig. 3D). Using primers specific for either wt-NPM1 or NPM1c messenger RNA, we found that neither transcript level was affected in EAPB0503-treated cells (Supporting Fig. 3 [see online supporting information]), and this shows that NPM1 downregulation occurs at the protein level. Collectively, these results strongly suggest that EAPB0503-treated NPM1c AML cells are secondary to oncoprotein degradation.

In NPM1c AML, wt-NPM1 oligomerized with NPM1c and was delocalized to the cytoplasm (Fig. 3E),<sup>6,10,11</sup> whereas the treatment of THP-1 cells with EAPB0503 did not affect NPM1 nucleolar localization (Fig. 3E), EAPB0503 treatment of OCI-AML3 restored the nucleolar localization of the remaining NPM1 protein (Fig. 3E). This suggests that EAPB0503-triggered degradation of NPM1c releases wt-NPM1 and thus corrects the nucleolar organization defect.

#### EAPB0503 Selectively Inhibits Proliferation, Induces NPM1c Degradation, and Restores wt-NPM1 Nucleolar Localization in Ex Vivo Treated NPM1c AML Blasts

Primary blasts derived from the BM of 6 AML patients were treated with EAPB0503. Patient 1 had acute promyelocytic leukemia with PML/RARA rearrangement, patients

2 and 6 were AML patients with wt-NPM1, and patients 3 to 5 harbored an NPM1 mutation without FLT-3 internal tandem duplication. Although leukemic cells derived from patients 1, 2, and 6 were not sensitive to EAPB0503 treatment, those derived from patients 3 to 5 were highly sensitive, and almost all died within the first 48 hours after treatment (Fig. 4A). Moreover, EAPB0503 induced NPM1c selective degradation in patients 3 to 5 (Fig. 4B) and restored the wt-NPM1 nucleolar localization only in those patients (Fig. 4C). Collectively, EAPB0503 exerts its growth-inhibition effect, induces NPM1c degradation, and corrects the wt-NPM1 nucleolar localization selectively in treated NPM1c AML blasts *ex vivo*.

#### EAPB0503 Selectively Reduces the Leukemia BM Burden in OCI-AML3 Xenograft Mice

Several xenograft mouse models have been generated.<sup>32,33</sup> Furthermore, OCI-AML3 and THP-1 cells are known to express the hCD45 marker.<sup>33,34</sup> To assess the *in vivo* efficacy of EAPB0503, we injected NSG mice with OCI-AML3 or THP-1 cells. Five days after the AML cell injection, xenograft mice were treated intraperitoneally with EAPB0503 or its respective vehicle (dimethyl sulfoxide/lipofundin) once daily for 5 consecutive days a week over a period of 2 weeks. At the end of week 3 after the AML cell inoculation, BM was flushed from the femurs and tibias of untreated mice and vehicle- or EAPB0503-treated mice. Human AML xeno-graft cells were stained with the human-specific hCD45<sup>1</sup> antibody and analyzed with flow cytometry. Our results show that the OCI-AML3 BM burden was markedly reduced from 34% to 10% upon EAPB0503 treatment ( $P < .05$ ; Fig. 5A,B), whereas the THP-1 burden was not affected (22% for untreated mice vs 23% for EAPB0503-treated mice; Fig. 5B,C). These results indicate that EAPB0503 is a promising drug that selectively

Figure 2. EAPB0503 induces caspase-mediated apoptosis in NPM1c AML cells. (A) Pre-Go cell population after PI staining upon the treatment of AML cell lines with the median inhibitory concentration dose (1 mM) of EAPB0503 for 48 hours. (B) Percentage of cycling cell populations after PI staining upon the treatment of AML cells for 48 hours as described previously. Histograms represent the relative distributions of nonapoptotic cells between the G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases. (C) Annexin V staining of AML cells treated for 48 hours as described previously. (D) MMP assay. After the treatment with AML cells as described previously and rho-damine 123 staining, rhodamine 123 was excited at 488 nm, and the fluorescence emission at 525 nm was assessed with flow cytometry. (E) Western blot analysis for PARP upon the 48-hour treatment of AML cells with EAPB0203 and EAPB0503. (F) Pro-liferation assay after the treatment of AML cells (THP-1, MOLM-13, and OCI-AML3) with 1 mM EAPB0503 alone or in combination with 50 mM zVAD (general caspase inhibitor) for 24, 48, and 72 hours. Cell growth is represented as the percentage of the control as indicated. (G) Western blot analysis for p53, P-p53, p21, and GAPDH in OCI-AML3 and MOLM-13 cells treated for 48 hours as described. In all flow cytometry assays, histograms represent 1 of 3 independent experiments. P values less than .05 were considered significant (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ). AML indicates acute myeloid leukemia; EAPB0203, 1-(2-phenylethyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; EAPB0503, 1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, mitochondrial membrane potential; PARP, poly(adenosine diphosphate ribose) polymerase; PI, propidium iodide; P-p53, phosphorylated p53; zVAD: z-Val-Ala-DL-Asp(Ome)-fluoromethylketone Rhodamine 123 phosphorylated-p53.

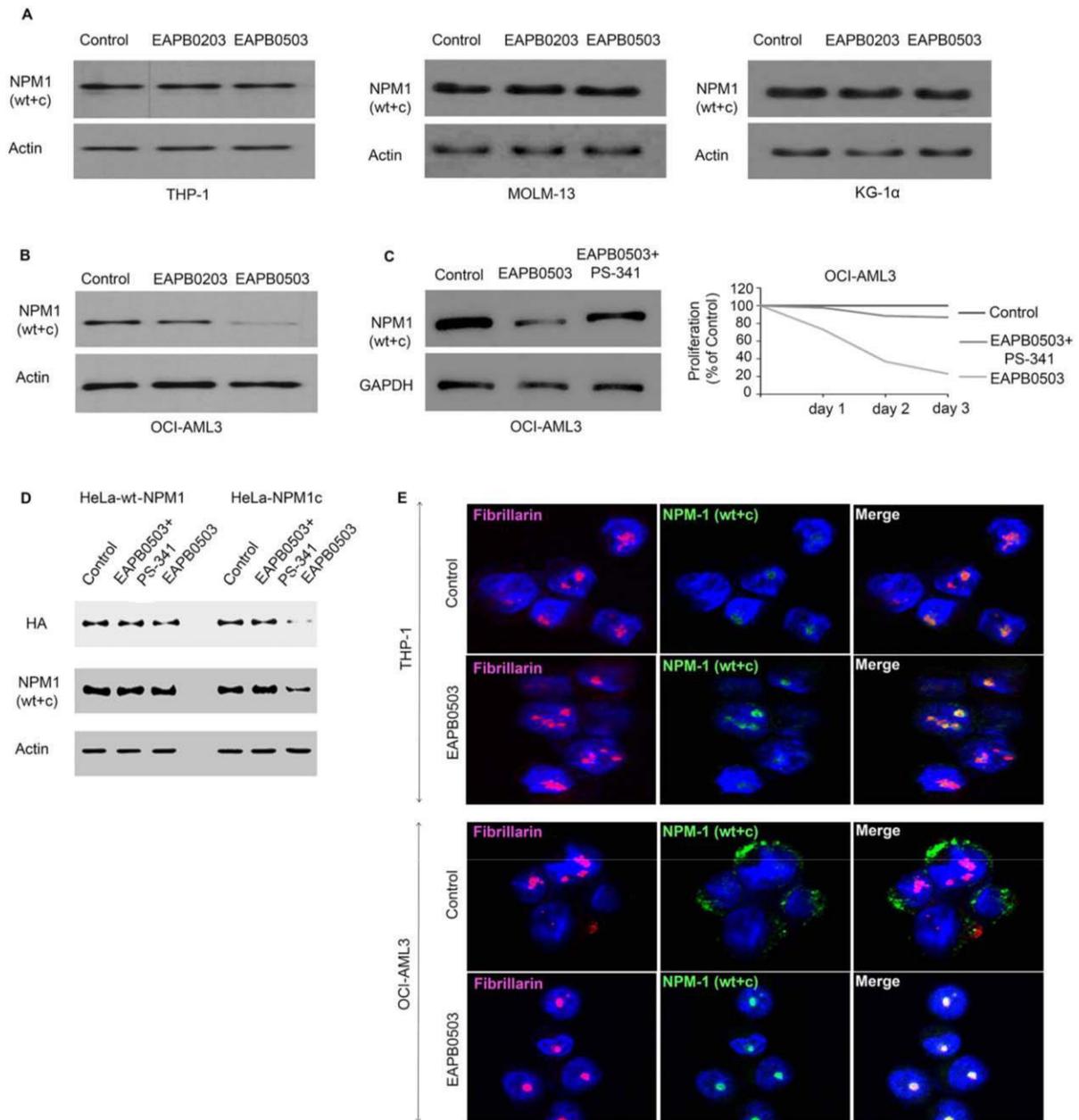


Figure 3. EAPB0503 induces proteasomal degradation of the NPM1c protein and restores the correct wt-NPM1 nucleolar localization in the NPM1c OCI-AML3 cell line. Western blot analysis of NPM1 recognizing both NPM1 (wt+c) and actin in (A) AML cell lines with wt-NPM1 (THP-1, MOLM-13, and KG-1 $\alpha$ ) and (B) NPM1c OCI-AML3 cell lines treated with 1 mM EAPB0203 or EAPB0503 for 48 hours as indicated. (C) NPM1 (wt+c) and GAPDH in OCI-AML3 treated with 1 mM EAPB0503 alone or in combination with 10 nM PS-341 (proteasome inhibitor) for 48 hours as indicated and proliferation assay after the treatment of OCI-AML3 with 1 mM EAPB0503 alone or in combination with 10 nM PS-341 for 24, 48, and 72 hours. Cell growth is presented as the percentage of the control as indicated. (D) Western blot analysis for HA, NPM1 (wt+c), and actin in HeLa cells transfected with HA-tagged wt-NPM1 or NPM1c and treated with 1 mM EAPB0503 alone or in combination with 10 nM PS-341 for 48 hours as indicated. (E) Confocal microscopy analysis of NPM1 localization in THP-1 or OCI-AML3 cells after treatment with EAPB0503 for 48 hours. NPM1 was stained with an antibody recognizing NPM1 (wt+c) (green), nucleoli were stained with anti-fibrillarlin (red), and nuclei were stained with 4,6-diamidino-2-phenylindole (blue). Images represent z-sections. AML indicates acute myeloid leukemia; EAPB0203, 1-(2-phenylethyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; EAPB0503, 1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HA, hemagglutinin; NPM1, nucleophosmin 1; wt-NPM1, wild-type nucleophosmin 1; NPM1c, cytoplasmic NPM1; NPM-1 (wt+c): wild type and cytoplasmic NPM1.

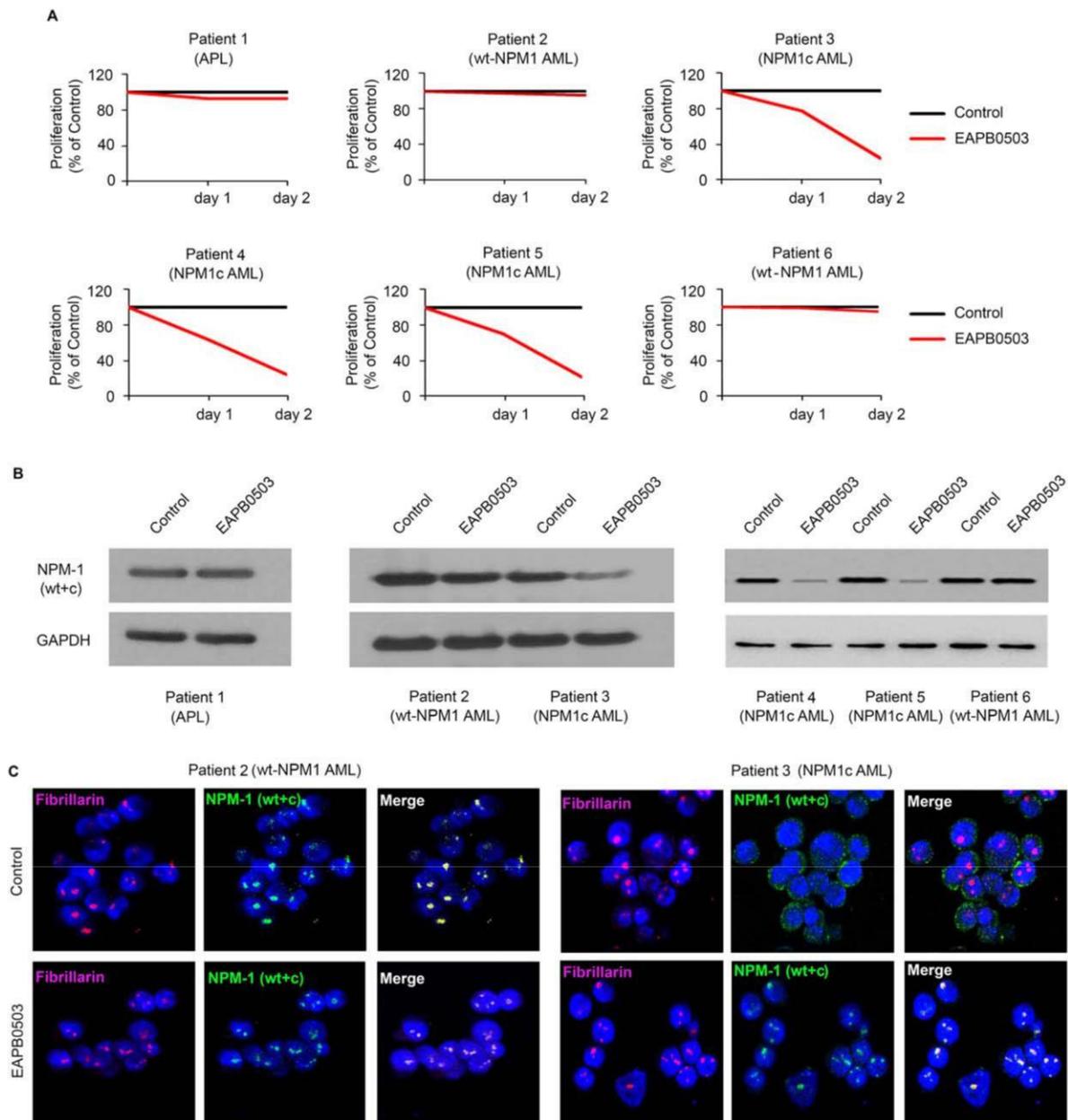


Figure 4. EAPB0503 inhibits proliferation, induces the degradation of NPM1c, and restores the nucleolar localization of wt-NPM1 selectively in ex vivo treated blasts derived from NPM1c AML patients. Primary leukemic blasts were harvested from 3 patients and treated with 1 mM EAPB0503. Patient 1 had APL with PML/RARA rearrangement, patients 2 and 6 were AML patients with wt-NPM1, and patients 3 to 5 were AML patients harboring an NPM1 mutation without FLT-3 internal tandem duplication. (A) Pro-liferation of AML blasts after treatment for 24, 48, and 72 hours. Cell growth is represented as the percentage of the control. (B) Western blot analysis for NPM1 (wt+c) and GAPDH in treated AML blasts as indicated previously. (C) Confocal microscopy of de-ri-ved blasts from patients 2 and 3. NPM1 (wt+c) was stained with an anti-NPM1 (wt+c) antibody (green), nucleoli were stained with anti-fibrillarin (red), and nuclei were stained with 4,6-diamidino-2-phenylindole (blue). Images represent z-sections. AML indicates acute myeloid leukemia; APL, acute promyelocytic leukemia; EAPB0503, 1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; FLT-3, FMS-like tyrosine kinase 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NPM1, nucleophos-min 1; wt-NPM1, wild-type nucleophosmin 1; NPM1c, cytoplasmic NPM1; NPM1 (wt+c), wild-type and cytoplasmic NPM1.

reduces the NPM1c AML BM burden in xenograft animals and warrants more preclinical investigation and then a clinical investigation.

## DISCUSSION

In this report, we examine the effects of EAPB0503 and EAPB0203, 2 imidazoquinoxaline agents, on AML cell

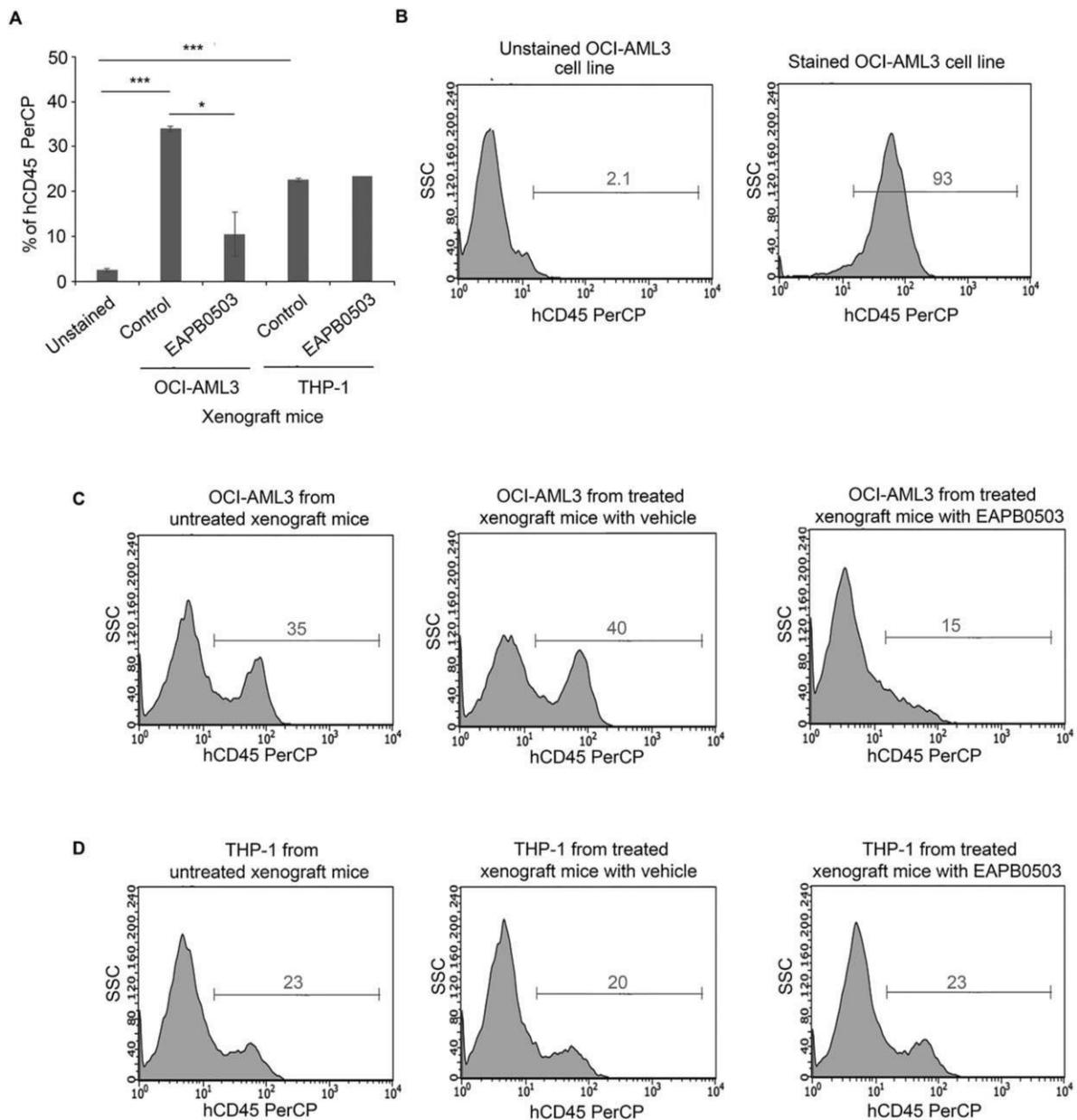


Figure 5. EAPB0503 selectively reduces the leukemia bone marrow burden in OCI-AML3 xenograft NSG mice. Eight-week-old fe-male NSG mice were injected with  $1 \times 10^6$  OCI-AML3 or THP-1 cells intravenously. EAPB0503 or its vehicle was administered for 5 days per week over a period of 2 weeks intraperitoneally. At the end of week 3, bone marrow was harvested from femurs and tibias of xenograft mice and then stained with the anti-hCD45 antibody. (A) Histograms showing the hCD45 PerCP percentage in xenograft animals. (B) Unstained and stained OCI-AML3 cell lines with the hCD45 antibody. (C) Representative histograms of stained and untreated OCI-AML3 xenograft mice, OCI-AML3 xenograft mice treated with the vehicle, and OCI-AML3 xenograft mice treated with EAPB0503. (D) Representative histograms of stained and untreated THP-1 xenograft mice, THP-1 xenograft mice treated with the vehicle, and THP-1 xenograft mice treated with EAPB0503. EAPB0503 indicates 1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine; NSG, NOD/Shi-scid IL2rg<sup>2/2</sup>; PerCP: peridinin chlorophyll protein (\*P <.05, \*\*P <.01, \*\*\*P <.001); SSC, side scatter.

lines. Imidazoquinoxalines have arisen as promising anti-cancer drugs on the basis of their in vitro activity in T-cell leukemia and chronic myeloid leukemia and their in vivo activity in melanoma.<sup>22,23,26</sup> We show that EAPB0503

has a specific growth-inhibition effect on NPM1c OCI-AML3 and IMS-M2 cells in a dose- and time-dependent manner. EAPB0503 activity in OCI-AML3 cells is considerably more pronounced than EAPB0203

activity, and this in line with its higher antitumor potency in other cancer types.<sup>24,25</sup> Introducing NPM1c into cells harboring wt-NPM1 sensitizes them to EAPB0503. The phenyl group is directly linked to the core imidazoquinoline heterocycle in EAPB0503, whereas an ethyl link exists in EAPB0203 between the 2 parts. This ethyl linker in EAPB0203 appears to abolish the antileukemic activity in most of the tested leukemia models in comparison with the direct linkage in the EAPB0503 compound.<sup>26</sup> Indeed, this change in the EAPB0503 structure enhanced its *in vitro* activity and led to better bioavailability in rats.<sup>29</sup>

We have shown that EAPB0503 induces growth arrest and apoptosis in NPM1c AML cells. Apoptosis is accompanied by the dissipation of MMP and PARP cleavage, and this strongly suggests the involvement of the intrinsic apoptotic pathway. Our results are consistent with previous studies showing antitumor activity of EAPB0503 in melanoma and chronic myeloid leukemia with a mode of action similar to the mode of this compound.<sup>23,26</sup>

NPM1c characterizes one-third of AML patients,<sup>6,10</sup> and when it alone is present in the case of a normal karyotype, it confers a better prognosis.<sup>35</sup> NPM1 mutations mediate malignancies as observed in transgenic and knock-in mice.<sup>36</sup> Mutated NPM1 is the key hallmark of OCI-AML3 and IMS-M2 cells for maintaining their malignant proliferation. In NPM1c AML, emerging studies have shown that therapies targeting NPM1c oncoprotein degradation lead to inhibition of proliferation and the cell death of leukemic cells.<sup>14-16</sup> In line with these findings, we have demonstrated that EAPB0503 degrades the NPM1c oncoprotein in a proteasome-dependent manner. This results in correcting the wt-NPM1 nucleolar localization in both NPM1c AML cells and *ex vivo* treated blasts derived from NPM1c AML patients. Furthermore, in *in vivo* NPM1c AML xenograft animals, EAPB0503 showed a selective reduction of the BM leukemia burden.

Recently, EAPB0503 was shown to exert potent inhibition of tubulin polymerization that correlated with its antiproliferative activity.<sup>27</sup> Therefore, the corrective effect of wt-NPM1 nucleolar localization after NPM1c degradation warrants testing the disruption of the microtubule network in NPM1c AML cells to further explain the mechanism of cell death.

Nowadays, most AML patients are still dying, especially because the basic therapies have remained unchanged or have only slightly changed over the last 2 decades. Nonetheless, before novel clinical therapies are introduced, a deep understanding of the therapeutic approach is required. The evolutionary changes emerging in

AML classification based on the morphology and cytogenetic/genetic changes reflect the importance of identifying the subtype-specific biology to determine the appropriate targeted therapy triggering degradation of the byproducts of these genetic modifications.<sup>13</sup> Our results suggest that EAPB0503 holds promise for the treatment of NPM1c AML, especially in those patients with mutation A,<sup>37</sup> which represents 80% of NPM1 mutations in AML<sup>38</sup> and is the hallmark mutation present in OCI-AML3 and IMS-M2.<sup>39</sup> These promising results were translated *in vivo*: among treated mice, EAPB0503 decreased the BM leukemia burden only in NPM1c xenograft mice. Further *in vivo* studies (survival and organ infiltration) and *ex vivo* studies (treated blasts) are required for us to have a complete idea of EAPB0503's mechanism of action.

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## CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

## AUTHOR CONTRIBUTIONS

Ali I. Nabbouh: Performance of experiments and reporting to Hiba A. El Hajj. Rita S. Hleihel: Performance of experiments and reporting to Hiba A. El Hajj. Jessica L. Saliba: Performance of experiments and reporting to Hiba A. El Hajj. Martin M. Karam: Performance of experiments and reporting to Hiba A. El Hajj. Maguy H. Hamie: Performance of experiments and reporting to Hiba A. El Hajj. Hsin-Chieh J.M. Wu: Performance of experiments and reporting to Hiba A. El Hajj. Caroline P. Berthier: Performance of experiments and reporting to Hiba A. El Hajj. Nadim M. Tawil: Performance of experiments and reporting to Hiba A. El Hajj. Pierre-Antoine A. Bonnet: Planning of study. Carine Deleuze-Masquefa: Planning of study. Hiba A. El Hajj: Planning of study and writing of manuscript.

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# **ANNEX 3**

# Antitumor Effect of the Atypical Retinoid ST1926 in Acute Myeloid Leukemia and Nanoparticle Formulation Prolongs Lifespan and Reduces Tumor Burden of Xenograft Mice



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## Abstract

Acute myeloid leukemia (AML) is one of the most frequent types of blood malignancies. It is a complex disorder of undifferentiated hematopoietic progenitor cells. The majority of patients generally respond to intensive therapy. Nevertheless, relapse is the major cause of death in AML, warranting the need for novel treatment strategies. Retinoids have demonstrated potent differentiation and growth regulatory effects in normal, transformed, and hematopoietic progenitor cells. All-trans retinoic acid (ATRA) is the paradigm of treatment in acute promyelocytic leukemia, an AML subtype. The majority of AML subtypes are, however, resistant to ATRA. Multiple synthetic retinoids such as ST1926 recently emerged as potent anticancer agents to overcome such resistance. Despite its lack of toxicity, ST1926 clinical development was restricted due to its limited bioavailability and rapid excretion. Here, we investigate the preclinical efficacy of

ST1926 and polymer-stabilized ST1926 nanoparticles (ST1926-NP) in AML models. We show that sub-mmol/L concentrations of ST1926 potently and selectively inhibited the growth of ATRA-resistant AML cell lines and primary blasts. ST1926 induced-growth arrest was due to early DNA damage and massive apoptosis in AML cells. To enhance the drug's bio-availability, ST1926-NP were developed using Flash NanoPre-cipitation, and displayed comparable anti-growth activities to the naked drug in AML cells. In a murine AML xenograft model, ST1926 and ST1926-NP significantly prolonged survival and reduced tumor burden. Strikingly, *in vivo* ST1926-NP antitumor effects were achieved at four fold lower concentrations than the naked drug. These results highlight the promising use of ST1926 in AML therapy and encourage its further development.

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## Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous disorder characterized by clonal expansion of myeloid blast progenitor cells in the bone marrow and peripheral blood of patients (1). AML is associated with a highly variable prognosis dictating a high mortality rate with an overall survival exceeding 2 years only in 20% of elderly patients and 5 years in less than 50% of adult patients (2). Further-

more, standard chemotherapy with or without hematopoietic stem cell transplantation leads to 40% cure rates in approximately 40% of adult patients but only in 10% of elderly patients (3). Thus, developing effective and safer therapies remains urgently needed.

Retinoids are natural vitamin A derivatives or synthetic molecules with vitamin A activities that regulate a wide range of biological processes, including development, differentiation, proliferation, and cell death, particularly in hematopoietic cells (4). Differentiation therapy using the natural retinoid all-trans retinoic acid (ATRA) became the paradigm in management of an AML subtype, the acute promyelocytic leukemia (APL; ref. 5). However, in non-APL AML patients, ATRA is only effective on those presenting with nucleophosmin-1 (NPM1) mutations without fms-like tyrosine kinase 3 internal tandem duplication (FLT-3 ITD) mutations, and is often linked with acquired resistance and disease relapse (4, 6). Arsenic trioxide combined with ATRA displayed a potent and selective efficacy in this category of AML patients both *ex vivo* and *in vivo* (7, 8). Although clearance of AML blasts was observed in few treated patients, no cure was achieved. In a randomized clinical trial, the combination of decitabine and ATRA revealed beneficial response rates (9–11). In many trials, retinoid related molecules were developed to overcome ATRA limitations by increasing their specificity and decreasing their toxicity (12, 13). So far, one of the most potent and less toxic retinoid-related molecules

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is the synthetic adamantyl retinoid ST1926; a derivative of CD437 (14, 15). ST1926 has shown a potent antitumor activity in several *in vitro* and *in vivo* cancer models of ovarian carcinoma (16, 17), lung carcinoma (18), neuroblastoma (19, 20), rhabdomyosarcoma (21), teratocarcinoma (22), *in vitro* two-dimensional and three-dimensional breast cancer models (23), and several leukemia animal models (13, 22, 24). This anti-tumor activity is independent of retinoic acid receptors (RAR) and p53 signaling pathways. ST1926 displayed a favorable pharmacokinetic profile when compared to CD437 (14) offering promise in cancer therapeutics. ST1926 exhibits its growth inhibitory effects by inducing early DNA damage in various types of tumor cells (18, 21–25). ST1926-induced resistance resulted in delayed and reduced DNA damage, highlighting the critical role of DNA damage pathways in ST1926-mediated cell death (18, 26). ST1926 is orally bioavailable (13), while rapidly achieving effective micromolar (mmol/L) concentrations in mouse plasma (21). Consequently, ST1926 reached phase I clinical trials for patients with advanced ovarian carcinoma (27, 28). However, its development was halted due to rapid glucuroconjugation resulting in sharp decrease of plasma concentrations to sub-mmol/L levels (27).

Nanomedicine has recently gained widespread attention, as it enables more efficient drug delivery, increased stability, and bioavailability, and reduced drug toxicity (29). The promise of nanomedicine in therapeutics is expanding rapidly. It has been applied to several cancer applications where the FDA (USA) has approved some formulations that are currently undergoing clinical trials (30). For instance, Doxil (doxorubicin hydrochloride liposome injection, Orthobiotech) was the first FDA approved nanodrug used to treat metastatic ovarian cancer and Kaposi's sarcoma, where it improved the balance between treatment, efficacy, and toxicity (31). The albumin bound nanoparticle (NP) formulation of paclitaxel, Abraxane (Bioscience, Astra), is currently approved in the clinic against metastatic breast, pancreatic, and non-small cell lung cancer (32, 33). Interestingly, ATRA loaded in polymer poly(lactide-coglycolide) NPs reversed AML cell growth and induced cell differentiation and apoptosis (34). This latter formulation was also shown to increase ATRA's anti-cancer activity and bioavailability relative to its free form in liver carcinoma models (35).

In this study, we investigated the preclinical efficacy of ST1926 and polymer-stabilized ST1926 NPs (ST1926-NP) in both AML *in vitro* and *in vivo* models. We showed that ST1926 at low sub-mmol/L concentrations potently inhibited the growth of several tested human ATRA-resistant AML cell lines, and primary AML patients-derived blasts, while sparing resting and activated normal leukocytes as well as hematopoietic and mesenchymal stem cells (MSCs). ST1926 induced early DNA damage and massive apoptosis in all tested AML cell lines. In a murine AML xenograft mouse model, ST1926 and ST1926-NP significantly reduced tumor burden and prolonged survival. Strikingly, ST1926-NP prolonged survival in AML xenografted animals at four-fold lower concentrations than the naked drug with no detectable toxicity on normal animals. More importantly, ST1926-NP significantly reduced bone marrow leukemia burden in THP-1 xenograft mice and this effect was similar to that observed at four-fold higher concentrations of the naked ST1926. These results highlight the promise of ST1926 in AML therapy and warrant further clinical development of this drug.

## Materials and Methods

### Cell culture and reagents

THP-1 (DSM ATCC 16), KG1-a (ATCC CCL246.1), MOLM-13 (DSM ACC 554), ML-2 (DSM ACC15), and HEL (DSM ACC 11) human AML cell lines (kindly provided by Dr. Fred Mazurier in 2014) were cultured in RPMI1640 (Lonza) medium supplemented with 10% FBS (Sigma) and 50 U/mL penicillin–streptomycin antibiotics (Lonza). Cell lines were authenticated in December 2016 by Laragen Cell Line Authentication and tested in 2016 for mycoplasma contamination by Hoechst staining. Cells were passaged on average 10 between thawing and use. Primary AML cells from the bone marrow of two patients were collected as previously described (10). Peripheral blood mononuclear cells (PBMC) were obtained from four healthy donors following Ficoll-Hypaque (Lymphoprep) separation. Activated PBMCs were cultured as previously described (22). Human samples were collected after approval by the Institutional Review Board at the American University of Beirut and after consented agreement of patients according to Helsinki's Declaration.

ST1926 (E-3-(4<sup>0</sup>-hydroxy-3<sup>0</sup>-adamantylbiphenyl-4-yl) acrylic acid) was kindly provided by Biogem, dissolved in DMSO at a concentration of 1 10<sup>2</sup> mol/L, and stored in amber tubes at 80 C. The final DMSO concentration never exceeded 0.1% which showed no effect on the growth of all tested cells. Caffeine (Sigma Chemical Co.) was dissolved in water to a final concentration of 100 mmol/L, and diluted to a 2 mmol/L final concentration in cell culture media.

### Cell growth and cell-cycle analysis

Cell growth was assessed using thiazolyl blue tetrazolium bromide (MTT) dye (Sigma). Optical density (OD) was measured by the microplate ELISA reader (Multiscan EX) at 595 nm. Cell viability was determined by trypan blue dye exclusion assay. Cell-cycle analysis was performed using propidium iodide (50 mg/mL; Sigma) staining and a FACSAria SORP flow cytometer (Becton Dickinson), and cell-cycle distribution was analyzed using FACSDiva software (Becton Dickinson), as previously described (36).

**Mitochondrial membrane potential measurement** Mitochondrial membrane potential was quantified using Rhodamine (R123) retention (Sigma) as previously described (24).

### Immunoblot analysis

Cellular protein lysates [0.25 mmol/L Tris-HCL (pH 7.4), 20% β-mercaptoethanol, and 5% SDS] were prepared, quantified, and separated by SDS-PAGE, and were then transferred onto nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in TBS (50 mmol/L Tris-HCL and 150 mmol/L NaCl), and were incubated overnight with specific primary antibodies at 4 C. Secondary antibodies were added after mild washing for 2 hours at room temperature while shaking. Proteins were visualized by enhanced chemiluminescence (ECL) using the ECL system. The following antibodies were used: p53 (sc-126) and PARP (sc-7150; Santa Cruz Biotechnology), GAPDH (MAB5476; Abnova), and γ-H2AX (2577; Cell Signaling).

### Nanoparticle formulation

ST1926-NP formulations were prepared by Flash NanoPrecipitation (FNP; ref. 37). ST1926 and polystyrene-*b*-poly(ethylene

oxide) diblock copolymer (PS-PEO; Polymer Source, Dorval, Canada) at a mass ratio of ST1926/PS-PEO of 1/5 were dissolved in 3 mL of tetrahydrofuran (THF). The PS-PEO used has a polystyrene block size of 1,500 g/mole, and a poly(ethylene oxide) block size of 2,400 g/mole. To form NPs, the THF solution of ST1926 and PS-PEO was mixed in a confined volume with water using a multi-inlet vortex mixer (MIVM) at a volumetric ratio of 1/9 THF/H<sub>2</sub>O. Flow rates of the THF and water streams were set to 12 and 108 mL/min, respectively, using Harvard apparatus PHD2000 syringe pumps. The resulting NP suspension was collected at the mixer outlet for further processing and analysis.

NP formulations used in mice experiments were concentrated using 50-kDa MicroKros hollow fiber filter (Spectrum, D04-E050-05-S) with 235 cm<sup>2</sup> surface area and maximum pressure of 30 psig (2 bar) as previously described (37). The concentration of ST1926 in the NP formulation was determined by UV analysis. A sample of ST1926 NP was dissolved in THF to obtain a molecularly dissolved solution. The A360 UV absorbance was determined using a DeNovix DS-11 spectrophotometer (DeNovix), and correlated to the ST1926 concentration using a calibration curve obtained for ST1926 in solution.

#### Dynamic light scattering

The NP size was determined using dynamic light scattering (DLS; Brookhaven Instruments, BI-200SM) following NP formation (38). The hydrodynamic particle size distribution was determined by measuring light scattering at 90°. NP formulations were freshly prepared each week weekly for *in vivo* treatments, and the particle size monitored by DLS.

#### Animal studies

NOD SCID and NOD-scid IL2rg<sup>null</sup> (NSG) mice were obtained from Jackson Laboratories. Mice protocols were approved by the Institutional Animal Care and Use Committee of the American University of Beirut. The comparative toxicity of the naked drug ST1926 to its NP formulation was first assessed in healthy NOD SCID mice. ST1926 at 7.5 and 30 mg/kg in its naked form or encapsulated in NP at the same corresponding concentrations, as well as the solvent and NP controls were tested in groups of five healthy adult mice. Animals were treated every other day for a period of 4 weeks. Mice were monitored on a daily basis for any change (fur, movement, . . .) and weighed on a weekly basis to check for any signs of toxicity.

For survival experiments, NOD SCID male and female mice (6 weeks old; average weight of 20 g) were intraperitoneally injected with 3 × 10<sup>6</sup> THP-1 AML cells. Animals were housed in a specific pathogen-free facility. Mice were divided randomly into five groups: control only injected with THP-1 cells (n = 2), ST1926 control (n = 5), ST1926 treatment (n = 7), NP control (n = 7), and ST1926-NP treatment (n = 7). Health status of the mice and nodule formation around the abdominal region were monitored every other day, over the period of the treatment. Mice were humanely euthanized when moribund as defined by: weight loss >15% to 20%, lethargy, ruffled fur, slow motion, after deep anesthesia by isoflurane followed by cervical dislocation.

For ST1926 treatment, 0.6 mg of ST1926 was dissolved in 10 mL DMSO and subsequently diluted in 90 mL of 1:10 ethanol/cremophor: 1 PBS solution. ST1926-treated animals received 100 mL of ST1926 intraperitoneally (equivalent to 30 mg/kg body weight) as this dosage was reported to cause survival

advantage in AML mice with no signs of toxicity (13). ST1926-NP-treated animals were intraperitoneally injected with 200 mL of the suspension, which provides an ST1926 dose of 7.5 mg/kg body weight. This concentration was selected based on optimal NP encapsulation efficacy and formulation stability and was determined by UV analysis. Treatment protocols started 3 days after malignant cell injection, and were delivered three times a week for up to 5 weeks. Mice survival was recorded. Peritoneal volumes were measured and recorded once a week by a digital caliper (Model DC150-S) applying the general formula:  $V = \frac{4}{3} \pi r^3$ .

To assess bone marrow tumor burden, 3 × 10<sup>6</sup> THP-1 cells were injected into the tail vein of 8-week-old male and female NSG mice (n = 3 per group). On day 3 post THP-1 injection, mice were treated intraperitoneally with 7.5 or 30 mg/kg of naked ST1926, NP charged with 7.5 mg ST1926, and corresponding control for 3 days a week, every other day over a period of 3 weeks.

#### Human CD45 staining

Bone marrow from the femurs and tibias of euthanized animals were flushed at the end of week 3 post-THP-1 cells inoculation. Cell surface staining was performed on 100 mL of sample using 20 mL of the anti-human CD45 PerC-P antibody (Becton Dickinson, cat. no. 345809) as described (39). After incubation for 15 minutes in the dark, erythrocytes were lysed using 1 mL FACS Lyse (Becton Dickinson, cat. no. 345809). Labeled samples were washed twice and analyzed by Guava flow cytometer.

#### Statistical analysis

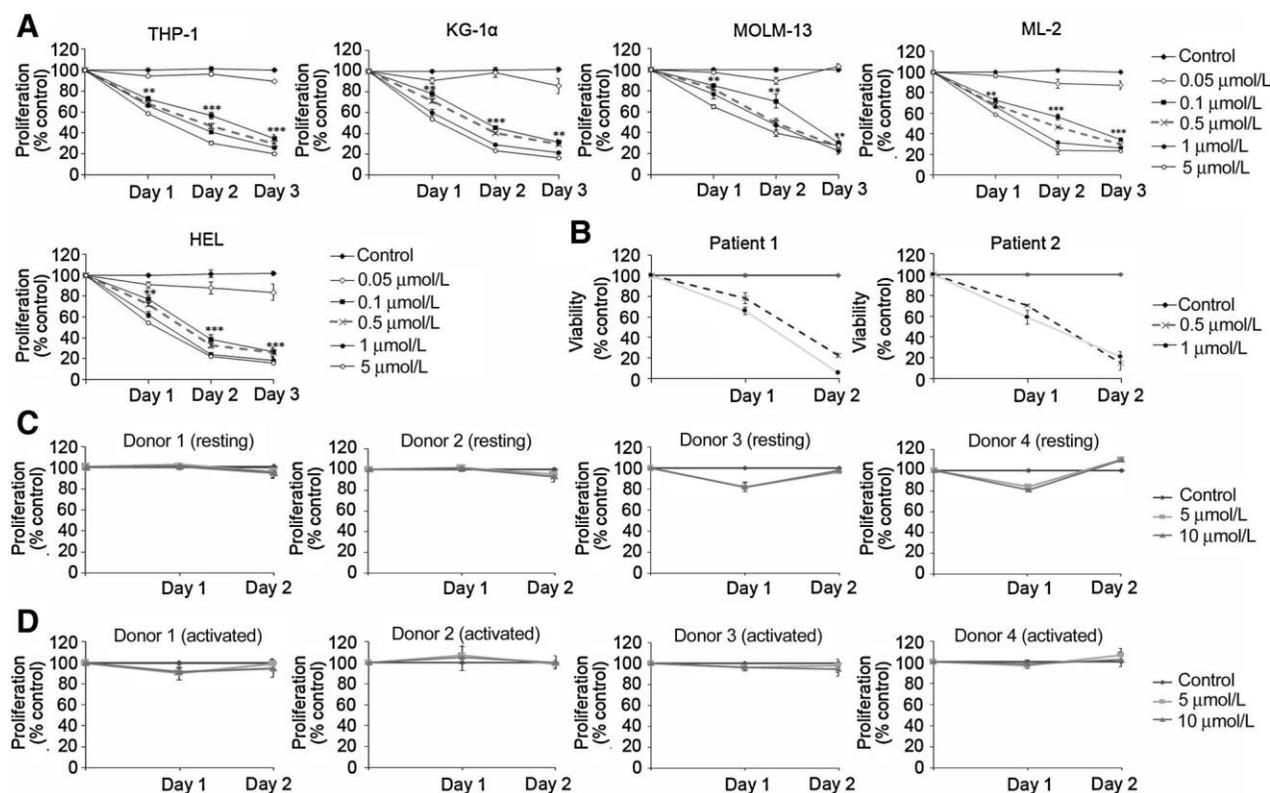
Statistical comparisons were done using Microsoft Excel 2010. Nonpaired and paired t test was used for comparison of two groups, whereas one-way ANOVA was used for three or more groups of treatments. , , and indicate P values 0.05, 0.01, and 0.001, respectively. Kaplan–Meier method with statistical significance was used for survival analysis. Log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests were used for statistical analysis between the different groups; differences were considered significant only when P values were less than 0.05.

## Results

ST1926 inhibits growth of AML cells at sub-mmol/L concentrations while sparing normal leukocytes at 100-fold higher levels

To test the effect of ST1926 on cell growth and viability, we used five human non-APL AML cell lines (THP-1, KG-1a, MOLM-13, ML-2, and HEL) harboring different genetic mutations. These AML cells were shown to be resistant to ATRA (7). Using the MTT cell proliferation assay, we observed that pharmacologically achievable sub-mmol/L concentrations of ST1926 as low as 0.1 mmol/L, with long plasma retention times (21, 27) significantly inhibited the proliferation of all tested AML cells, irrespective of their mutational signatures, in a time-dependent manner (Fig. 1A). ST1926 at 0.1 mmol/L concentrations resulted in approximately 70% growth inhibition at 72 hours in tested cells. Similar trends were observed when cell viability was assessed using trypan blue exclusion assay (Supplementary Fig. S1). Despite the growth inhibitory effect of ST1926 in AML cells, no effect was noticed on their differentiation status, as assessed by the similar CD11b expression in untreated or 0.5 mmol/L ST1926-treated cells up to 72 hours.

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**Figure 1.** ST1926 inhibits AML cell growth. **A**, Effects of ST1926 treatment on the growth of AML cell lines. THP-1, KG1-a, MOLM-13, ML-2, and HEL cells were treated with 0.1% DMSO or the indicated concentrations of ST1926 up to 3 days. Cell growth was assayed in triplicate wells with the MTT cell proliferation assay. Results are expressed as a percentage of control (0.1% DMSO) and represent the average of three independent experiments SE ( $P < 0.01$ ;  $P < 0.001$ ). **B**, Primary AML cells are sensitive to ST1926. Primary AML cells from two patients were treated with the indicated concentrations of ST1926 up to 2 days. Cell viability was determined in triplicate wells by the trypan blue exclusion assay. Results are expressed as percentage of control (0.1% DMSO) and represent the average of triplicate wells SD. Leukocytes are resistant to suprapharmacological concentrations of ST1926. **C**, Resting and **D**, phytohemagglutinin-activated PBMCs from four healthy donors were treated with 0.1% DMSO or 5 to 10 mmol/L ST1926 up to 2 days. Cell growth was assayed in triplicate wells with the CellTiter 96 nonradioactive MTT Cell Proliferation Kit. Results are expressed as percentage of control (0.1% DMSO) SD.

Furthermore, we have tested ST1926 effect on primary leu-kemic blasts derived from the bone marrow of two AML patients [patient 1: AML-M1/NPM1/FLT3-ITD positive; patient 2: APL/t(15;17) PML-RAR $\alpha$  positive], and have demonstrated that 0.5 mmol/L ST1926 treatment for 48 hours reduced their viability by approximately 80% (Fig. 1B). Importantly, high concentrations of ST1926 up to 10 mmol/L had no effect on resting (Fig. 1C) and phytohemagglutinin-activated normal PBMCs from four healthy donors (Fig. 1D) as previously reported (22). Furthermore, minimal effect was observed on the growth of normal human CD34<sup>b</sup> fraction of mononuclear cells and MSCs at the working concentrations of 0.5 and 1 mmol/L (Supplementary Fig. S2). In summary, 0.5 mmol/L ST1926 concentrations were used in subsequent experiments as they drastically reduced cell growth and viability of all tested AML cells.

ST1926-induced growth inhibition of AML cells is irreversible and causes a massive accumulation of treated cells in the presumably apoptotic sub-G<sub>1</sub> region

To assess whether ST1926-growth inhibition was sustained after drug removal, THP-1 and MOLM-13 cells, harboring p53

and FLT-3 mutations (40), respectively, were pretreated with 0.5 mmol/L ST1926 for 24 hours, then cells were resuspended in drug-free media for up to 3 days. Using MTT assay, our results indicate that ST1926 growth suppression was irreversible where THP-1 and MOLM-13 growth was significantly reduced by 70% and 60%, respectively, 2 days postdrug removal (Fig. 2A). To study the mechanisms involved in ST1926-induced growth inhibition, cell-cycle analysis was performed in THP-1, KG1-a, MOLM-13, and ML-2 cells treated with 0.5 mmol/L ST1926 for up to 48 hours. No major variation in cell-cycle distribution was observed between control AML cell lines (Fig. 2B and Supplementary Fig. S3). ST1926-treated THP-1, MOLM-13, and ML-2 cells induced a massive accumulation of cells in the presumably apoptotic sub-G<sub>1</sub> region of the cell cycle as early as 24 hours except for KG1-a, where this accumulation was pronounced after 48 hours of treatment (Fig. 2B). In fact, approximately 80%, 40%, 70%, and 80% of THP-1, KG1-a, MOLM-13, and ML-2 treated cells for 48 hours, respectively, accumulated in the sub-G<sub>1</sub> region (Fig. 2B). We observed a minor G<sub>0</sub>-G<sub>1</sub> cell-cycle arrest in KG1-a, MOLM-13, and ML-2 treated cells for 24 hours, whereas S-phase arrest was detected in THP-1-treated cells (Fig. 2B).

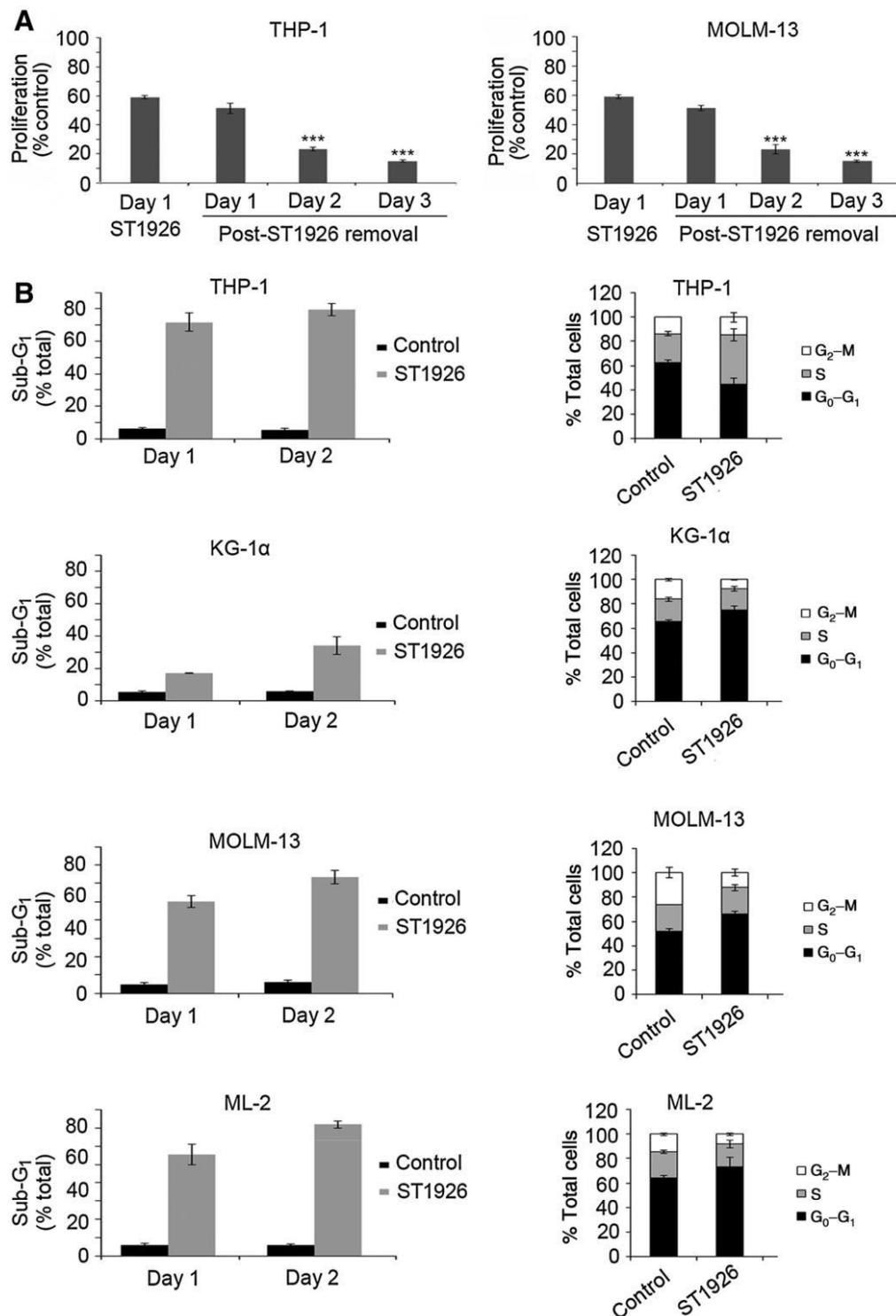


Figure 2.

Effects of ST1926 on the cell-cycle distribution of AML cells. A, Irreversible effect of ST1926 on AML cell growth. THP-1 and MOLM-13 cells were treated with 0.5 mmol/L ST1926 for 24 hours. One day posttreatment, cells were resuspended in drug-free media up to 3 days. Cell growth was assayed in triplicate wells with the CellTiter 96 nonradioactive MTT Cell Proliferation Kit. Results are expressed as percentage of control (0.1% DMSO) and represent the average of three independent experiments (SE;  $P < 0.001$ ). B, Cell-cycle distribution of ST1926-treated AML cells. THP-1, KG-1 $\alpha$ , MOLM-13, and ML-2 cells were treated with 0.5 mmol/L ST1926 up to 2 days. Cell-cycle analysis was performed as described. Left panel shows the effect of ST1926 on sub-G<sub>1</sub> region cell accumulation, whereas right panel shows the percentage of nonapoptotic cells after 24 hours of ST1926 treatment. Results are expressed as percentage of control (0.1% DMSO) and represent the average of three independent experiments (SE).

ST1926 induces apoptosis, dissipation of mitochondrial membrane potential, and DNA damage in AML cells

Apoptosis induction upon ST1926 treatment was confirmed in AML cells. THP-1, KG1-a, MOLM-13, and ML-2 cells treated with 0.5 mmol/L ST1926 revealed PARP (113 kDa) cleavage into its death associated fragments (89 and 24 kDa) as early as 24 hours (Fig. 3A). ST1926-induced apoptosis in AML cells was associated with dissipation of the mitochondrial membrane potential as early as 24 hours in 0.5 mmol/L ST1926-treated THP-1 and MOLM-13 cells (Fig. 3B). The intracellular fluorescence of Rho-damine-123 dye decreased significantly by approximately 60% in THP-1 and MOLM-13 treated cells for two days (Fig. 3B). To further understand ST1926-mediated growth inhibition and cell death, p53 protein levels were monitored in THP-1 and MOLM-13 cell lines harboring a mutated and wild type p53 profile (40), respectively. MOLM-13-treated cells with 0.5 mmol/L ST1926 displayed a remarkable increase in total p53 protein levels where-

as as expected no changes were detected in THP-1-treated cells with mutated p53 (Fig. 3C).

ST1926 has been previously characterized as a genotoxic drug that causes early DNA damage in various types of tumor cells (15, 22–26). Here, we show a substantial increase in the phosphorylation of the DNA damage marker, H2AX to  $\gamma$ -H2AX, as early as 30 minutes in THP-1 and MOLM-13 cells treated with 0.5 mmol/L ST1926 (Fig. 3D). To further investigate the role of DNA damage in ST1926 effects, Caffeine was used in combination with ST1926 in AML cells. Caffeine is known to inhibit the DNA damage response pathway (41) and to mitigate ST1926-induced S-phase arrest in rhabdomyosarcoma cells (21). In our studies, the effect of ST1926 on THP-1 and MOLM-13 cell growth inhibition was significantly reversed upon cotreatment with Caffeine (Fig. 3E). Altogether, these results show that ST1926 is a potent inducer of apoptosis and DNA damage in AML cells at sub-mmol/L concentrations independently of p53 status.

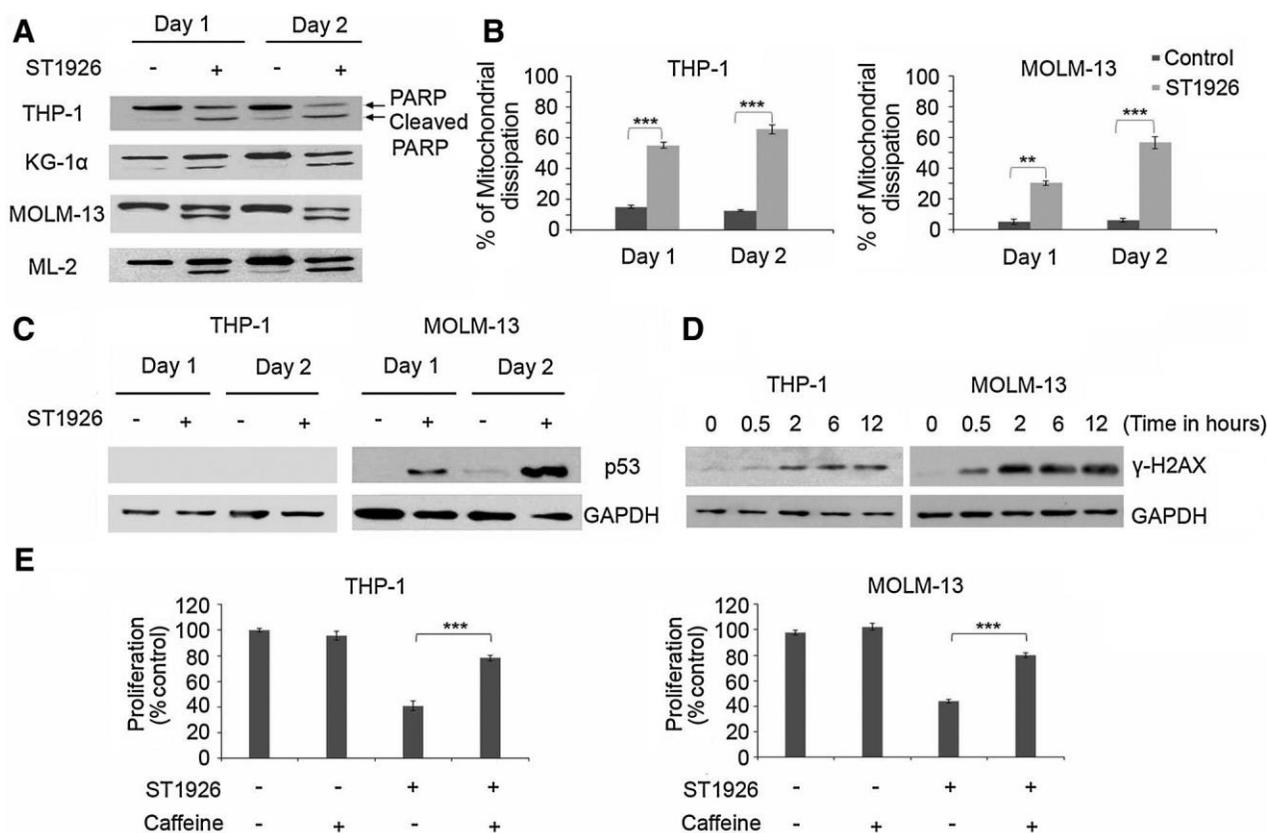


Figure 3.

ST1926 induces apoptosis and DNA damage-mediated mechanism of action in AML cells. A, Induction of PARP cleavage in ST1926-treated AML cells. THP-1, KG1-a, MOLM-13, and ML-2 cells were treated with 0.1% DMSO or 0.5 mmol/L ST1926. Protein lysates (50 mg/lane) were immunoblotted against PARP antibody. Results are representative of three independent experiments. B, ST1926 treatment results in loss of mitochondrial membrane potential. THP-1 and MOLM-13 cells were treated as in A. Cells were then exposed to Rhodamine-123 and change in mitochondrial Rhodamine dye intracellular accumulation was measured through FACSscan flow cytometer. C, THP-1 and MOLM-13 cells were treated as in A, and protein lysates (50 mg/lane) were immunoblotted against total p53 antibodies. Blots were re-probed with GAPDH antibody to ensure equal protein loading. D, Induction of DNA damage. THP-1 and MOLM-13 cells were treated with 0.5 mmol/L of ST1926, and protein lysates (100 mg/lane) were immunoblotted against  $\gamma$ -H2AX and re-probed with GAPDH antibody. Results shown are representative of three independent experiments. E, ST1926-induced growth inhibition in AML cells is reversed upon the abrogation of DNA damage. THP-1 and MOLM-13 cells were pretreated with 2 mmol/L caffeine for 24 hours then washed and exposed to 0.1% DMSO, 2 mmol/L caffeine alone, 0.5 mmol/L of ST1926 alone, or both ST1926 and caffeine for up to 48 hours. Cell growth was measured in triplicate wells with the MTT cell proliferation assay. Results are expressed as percentage of control (0.1% DMSO) and represent the average of two independent experiments (SE;  $P < 0.01$ ;  $P < 0.001$ ).

ST1926 naked drug and nanoparticle formulation show a similar and stable growth-inhibitory effect in AML cells

Solubility and bioavailability problems are major reasons for which many drug candidates fail in clinical development. ST1926 is a highly hydrophobic drug (Fig. 4A) that was found to undergo major glucuroconjugation leading to its poor bio-availability and rapid excretion by the liver (27). To enhance ST1926 bioavailability, we generated polymer-coated ST1926-NP using Flash NanoPrecipitation technique (37, 42). ST1926 was formulated into NP with a drug to polymer mass ratio of 1:5 using 5 mg of ST1926 and 25 mg of polystyrene-b-poly (ethylene oxide) block copolymer (PS1.5-PEO2.4) with a poly-styrene hydrophobic block. The resulting solution was mixed at an optimized rate using the MIVM mixer (Fig. 4B). ST1926-NP's size was determined using the DLS apparatus and was found to be on average 190 nm and to remain stable at room temperature even after 24 hours (Fig. 4C).

To test whether formulating ST1926 into NPs attenuates its efficacy, THP-1 and MOLM-13 cells were treated with the same concentrations of naked drug and formulated NPs, and their cell growth was determined using the MTT proliferation assay. Interestingly, both ST1926 and ST1926-NP exhibited similar growth-inhibitory effects with an approximate  $IC_{50}$  values of 0.5  $\mu\text{mol/L}$  at 48 hours in THP-1 and MOLM-13 cells (Fig. 4D). This suggests that drug-carrier interactions are not rate-limiting for drug diffusion out of the NP, which could otherwise reduce its efficacy.

Formulating ST1926 into nanoparticles prolongs the survival and reduces the peritoneal volume of AML xenografted mice at low concentrations

To assess the *in vivo* efficacy of ST1926 and its formulated NPs, we injected 3 million THP-1 cells intraperitoneally into NOD SCID mice. Three days post-AML cell injection, THP-1 xenografted

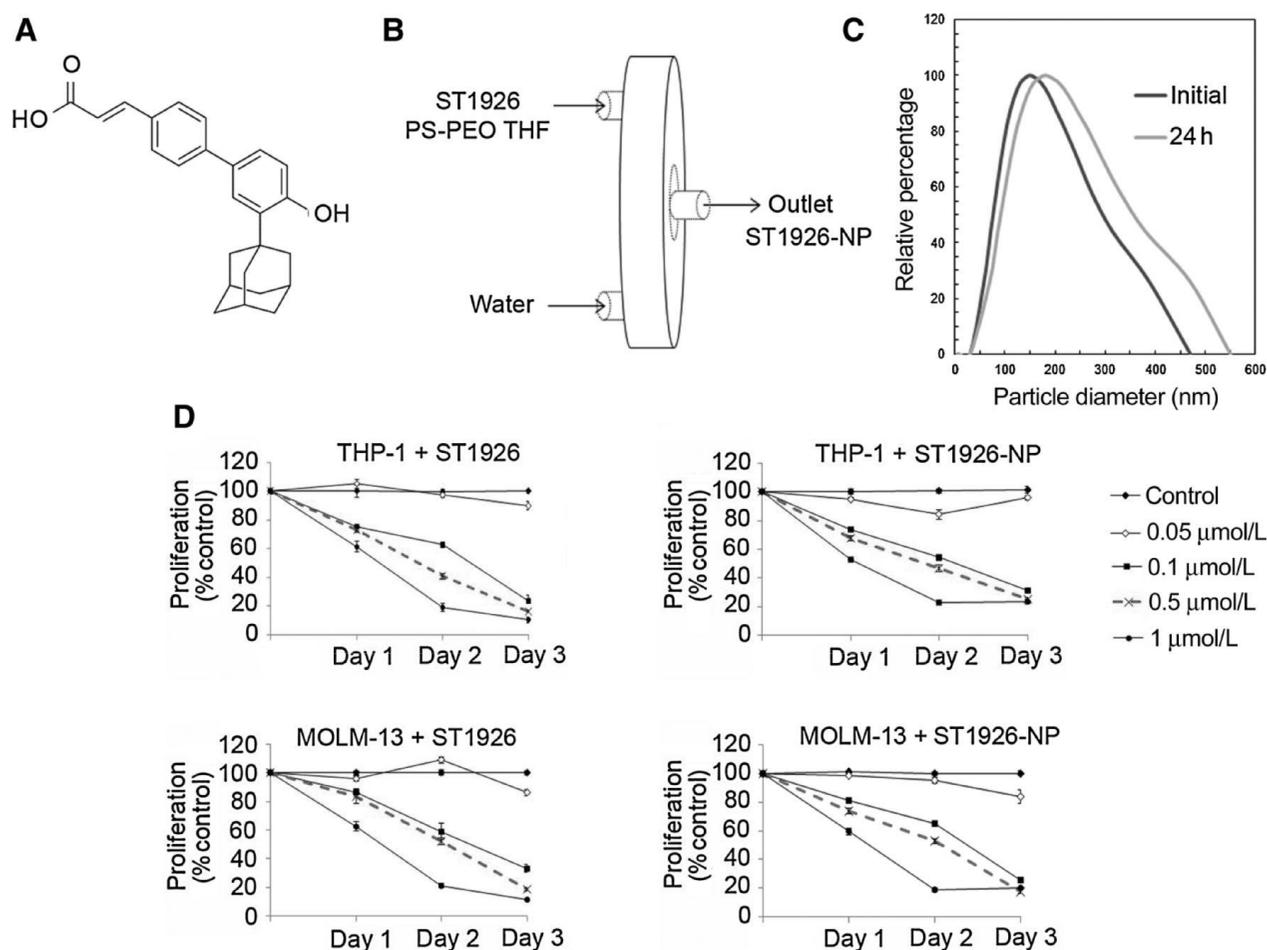


Figure 4.

Nanoparticle formulation of ST1926 and effects on AML cell growth. A, Chemical structure of ST1926. B, Schematic representation of the multiple inlet vortex mixer (MIVM) design used to generate ST1926 nanoparticles (ST1926-NP) by Flash NanoPrecipitation. ST1926 was formulated into nanoparticles with a drug to polymer mass ratio of 1:5. 5 mg of ST1926 and 25 mg of polystyrene-b-poly(ethylene oxide) block copolymer (PS1.5-PEO2.4) with a polystyrene hydrophobic block. The resulting solution was mixed at an optimized rate using the MIVM mixer in which streams of solution were mixed at high velocity in the small chamber at a volume ratio of 1:9 THF:H<sub>2</sub>O. C, Intensity-based particle size distribution of ST1926-NP. The size of the polymer-coated ST1926 was determined by dynamic light scattering initially and after 24 hours. Results represent the average of three independent experiments. D, ST1926 and nanoparticle formulation show similar AML cell growth inhibition. THP-1 and MOLM-13 cells were treated with 0.1% DMSO or indicated concentrations of ST1926 or ST1926-NP for up to 3 days. Cell growth was measured in triplicate wells with the MTT cell proliferation assay. Results are expressed as percentage of control (0.1% DMSO) and represent an average of three independent experiments (SE).

mice were intraperitoneally treated with either 30 mg/kg ST1926, 7.5 mg/kg ST1926-NP, or their respective vehicles once daily, 5 days a week, for up to 5 weeks. ST1926-NP concentration was selected at optimal formulation stability to reduce aggregation and other instabilities (43).

We observed a marked survival advantage in both ST1926 and ST1926-NP-treated animals with a median survival of 63 and 62 days, respectively, when compared to their respective controls with a median survival of 42 and 46 days (Fig. 5A). Strikingly, even at four-fold lower concentrations, ST1926-NP exhibited similar survival profile as ST1926 (Fig. 5A). Importantly, after 24 days of treatment, we noted a sharp increase in the peritoneal volume of control mice reaching more than

100 cm<sup>3</sup> versus less than 50 cm<sup>3</sup> in ST1926 and ST1926-NP treated animals (Fig. 5B and Supplementary Table S1). One out of seven mice survived for 242 days due to ST1926 treatment at 30 mg/kg and for 280 days, in ST1926-NP treated animals at four-fold lower concentrations (Fig. 5A). These results indicate that ST1926 treatment prolongs the survival and reduces the peritoneal volume of AML xeno-grafted animals and its NP formulation results in similar effects at significantly lower concentrations than the naked drug.

Formulating ST1926 into nanoparticles diminishes the leukemia bone marrow burden in an AML orthotopic mouse model at four fold lower ST1926 concentrations than the naked drug

We have used a well-established AML orthotopic mouse model where THP-1 cells were injected intravenously in NSG mice (39, 44). Three days postleukemic cells injection, animals were treated with 7.5 or 30 mg/kg of naked ST1926, or 7.5 mg ST1926-NP, and compared to untreated controls. All mice were sacrificed at the end of the third week of treatment. Since THP-1 cells express the human CD45 (hCD45<sup>b</sup>) marker (44, 45), bone marrow cells were harvested from untreated or treated animals and stained for hCD45<sup>b</sup> to evaluate leukemia burden. ST1926-NP containing 7.5 mg of drug significantly reduced bone marrow leukemia burden in AML mice ( $P < 0.01$ ; Fig. 5C). Importantly, the effect of 7.5 mg/kg ST1926-NP was similar to that of the naked drug at four-fold higher concentrations. In contrast, naked ST1926 at the same concentrations of 7.5 mg/kg did not show any reduction in tumor burden underscoring the superiority of the new ST1926-NP formulation (Fig. 5C).

These results highlight the promising impact of this new formulation in reducing AML tumor burden at lower drug concentrations.

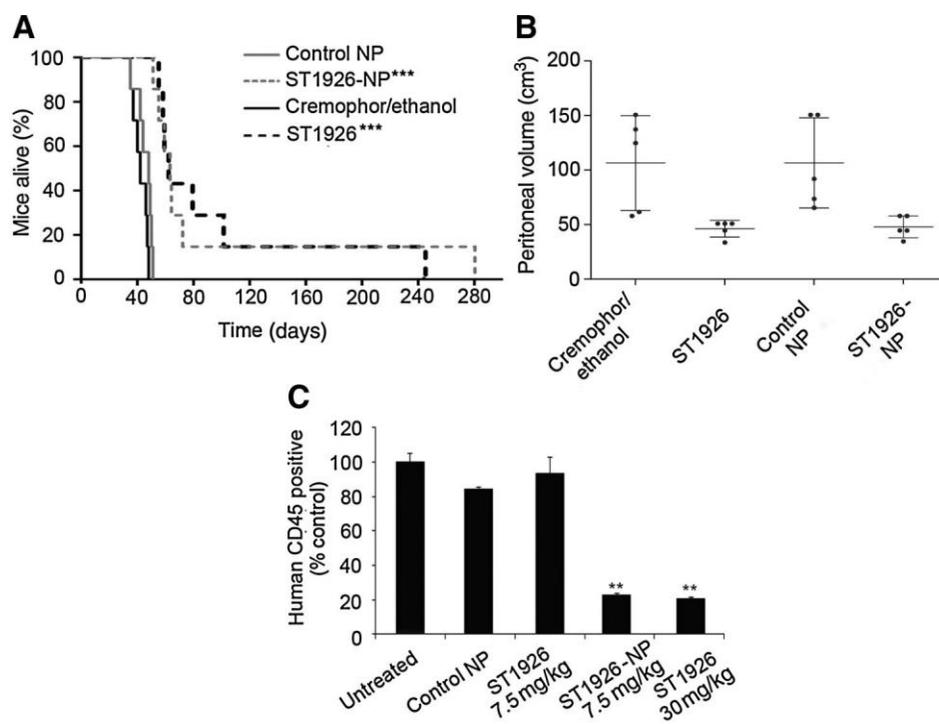


Figure 5.

ST1926 and nanoparticle formulation prolong survival and reduce tumor burden in AML xenograft mouse model. A, In vivo efficacy of ST1926 and its formulated nanoparticles (ST1926-NP). AML xenografted mice: ST1926 control (cremophor/ethanol;  $n = 5$ ), ST1926 treatment ( $n = 7$ ), NP control ( $n = 7$ ), and ST1926-NP treatment ( $n = 7$ ) were treated intraperitoneally with either 30 mg/kg ST1926, 7.5 mg/kg ST1926-NP, or their respective vehicles once daily for 5 days a week up to 5 weeks. Kaplan–Meier plots of overall survival are displayed ( ,  $P < 0.001$ ). B, Peritoneal volumes of treated ( $n = 5$  for each group) determined at 24 days posttreatment. C, 8-week-old NSG mice were intravenously injected with  $3 \times 10^6$  THP-1 cells. On day 3 post THP-1 injection, mice were treated intraperitoneally with 7.5 or 30 mg/kg of naked ST1926, NP charged with 7.5 mg/kg ST1926, and untreated control for 3 days a week, every other day over a period of 3 weeks. At the end of treatment period, bone marrow was harvested from tibias and femurs of xenografted mice and then stained with anti-hCD45 antibody. Labeled samples were washed twice and analyzed on a Guava flow cytometer as 10,000 cells/group. Results are expressed as percentage of control ( SD; ,  $P < 0.01$ ).

## Discussion

In this study, we showed that ST1926 potently inhibited the growth and induced massive apoptosis of ATRA-resistant AML cells at low sub-mmol/L concentrations through the activation of DNA damage and dissipation of the mitochondrial membrane potential. This ST1926 mechanism of action is similar to previously reported results in other tested hematological malignancies (13, 22, 24–26) and solid tumors (16, 19, 21, 23, 46). Importantly, sub-mmol/L ST1926 concentrations exerted the same inhibitory effect in primary AML patients' derived blasts, while sparing normal leukocytes and normal human hematopoietic and MSCs. We also report that ST1926 cell death effect is independent of p53 status as previously published (14, 21–23) and of several commonly encountered mutations in AML.

ST1926 has been previously characterized as a genotoxic drug that causes early DNA damage in various types of tumor cells (18, 21–26). Here we show that ST1926-treated AML cells at sub-mmol/L concentrations resulted in early DNA damage. Co-treatment with DNA damage inhibitors reversed the drug's effects highlighting the crucial role of ST1926 in DNA damage. In fact, ST1926 resistance was demonstrated to be due to a defective DNA damage response in the NB4 APL (26), H460 lung carcinoma (18), and colon carcinoma HCT116 cells (unpublished results). Previous work has established that ST1926 inhibits the growth of the p53 mutated AML NB4 cells, and of the p53 null HL-60 AML cells, underlying a p53-independent mechanism of action (13). Similarly to its CD437 parental compound, ST1926 was shown to work independently of the RAR signaling pathway (15, 23, and our unpublished data). Recently, CD437 was demonstrated to directly inhibit DNA polymerase  $\alpha$ , the enzyme responsible for initiating DNA synthesis during the S-phase of cell cycle, which is encoded by POLA1 (47). It would be interesting to check on the status of POLA1 mutations and/or expression in AML cells that develop resistance to ST1926 and to investigate whether this mutation confers resistance to CD437 as well.

ST1926 clinical testing was halted in phase I for ovarian cancer, as it was found to undergo glucuroconjugation on its phenolic hydroxyl group leading to its poor bioavailability and rapid excretion by the liver (27). In fact, short-lived ST1926 mmol/L plasma concentrations were observed in humans (27) and mice (21), which abruptly dropped to more sustained sub-mmol/L levels. Given ST1926 potent activities and lack of toxicity in humans and in various tumor models, multiple efforts were taken to synthesize analogs with more favorable properties (48). Another approach was to enhance ST1926 bioavailability through the use of NP. Furthermore, NPs have great potential as cellular drug delivery vehicles. They are shown to promote targeted efficacy by enhancing the drugs' stability, protecting them from degradation, reducing side effects as well as bioavailability, and retention at the target site of action (49, 50). Moreover, most approved NP-based therapies are nowadays administered to patients by intravenous injection underscoring their promising clinical use (51). Here, we report the generation of polymer-stabilized ST1926-NP formulations using Flash NanoPrecipitation technique (37, 42).

Both ST1926 and NP formulations significantly prolonged survival, and reduced peritoneal volume and bone marrow tumor burden in AML xenografted mice. Furthermore, both treatments were well-tolerated in animals and no signs of behavioral abnormalities, undesirable side effects, or toxicities were noted. Interestingly, ST1926 antitumor properties in AML xenografted mice

were observed at four-fold lower concentrations when formulated in NPs versus the naked drug. Indeed, the higher potency of ST1926-NP obtained solely *in vivo* when compared to the naked drug, may be due to the complexity of the tumor microenvironment (52) that might be providing enhanced accessibility of ST1926-NP than the naked drug after circulation in the plasma. Further *in vivo* studies are required to study the mechanism of action of ST1926 and ST1926-NP in AML models. PEG used as hydrophilic block has been demonstrated to prolong the NPs circulation time *in vivo* and to provide a steric barrier to the particle and a reduction in its opsonization (53). We have preliminary data showing that the NP formulation did not enhance the plasma ST1926 concentrations than the naked drug. It remains to be determined whether ST1926-NP results in lower blood absorption but greater extravascular tissue and bone marrow distribution than the free drug. Future assays with high sensitivity should be optimized to detect ST1926 and/or its glucuroconjugated form in plasma, bone marrow, and urine. These pharmacokinetic studies may explain our observed beneficial effect of using ST1926-NP in AML xenograft mouse models. Interestingly, we have previously observed a significant extravascular distribution of the naked ST1926 in mice which may explain the observed antitumor effect of ST1926 in animal models (21).

The observed potent antitumor properties of ST1926 in several types of cancers that are resistant to conventional therapies and independently of p53 signaling as well as its low drug toxicity underline the need for its further clinical development. In summary, we highlight the promise of ST1926 NP formulation in AML therapy and call for its drug development through formulation strategies or synthesis of analogs with more favorable pharmacological properties (48).

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Molecular Cancer Therapeutics

## Antitumor Effect of the Atypical Retinoid ST1926 in Acute Myeloid Leukemia and Nanoparticle Formulation Prolongs Lifespan and Reduces Tumor Burden of Xenograft Mice

Leeanna El-Houjeiri, Walid Saad, Berthe Hayar, et al.

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# **ANNEX 4**

SHORT REPORT

Open Access



# The HTLV-1 oncoprotein Tax is modified by the ubiquitin related modifier 1 (Urm1)

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## Abstract

**Background:** Adult T-cell leukemia/lymphoma (ATL) is an aggressive malignancy secondary to chronic human T-cell lymphotropic virus 1 infection, triggered by the virally encoded oncoprotein Tax. The transforming activity and subcellular localization of Tax is strongly influenced by posttranslational modifications, among which ubiquitylation and SUMOylation have been identified as key regulators of the nuclear/cytoplasmic shuttling of Tax, as well as its ability to activate NF- $\kappa$ B signaling.

**Results:** Adding to the complex posttranslational modification landscape of Tax, we here demonstrate that Tax also interacts with the ubiquitin-related modifier 1 (Urm1). Conjugation of Urm1 to Tax results in a redistribution of Tax to the cytoplasm and major increase in the transcription of the NF- $\kappa$ B targets *Rantes* and *interleukin-6*. Utilizing a tax-transgenic *Drosophila* model, we show that the Urm1-dependent subcellular targeting of Tax is evolutionary conserved, and that the presence of Urm1 is strongly correlated with the transcriptional output of Dipterin, an antimicrobial peptide and established downstream target of NF- $\kappa$ B in flies.

**Conclusions:** These data put forward Urm1 as a novel Tax modifier that modulates its oncogenic activity and hence represents a potential novel target for developing new strategies for treating ATL.

**Keywords:** ATL, HTLV-1, Tax, Urm1, NF- $\kappa$ B, Oncogenesis

## Background

The Human T-cell Leukemia Virus type 1 (HTLV-1) transactivator Tax initiates adult T-cell leukemia/lymphoma (ATL), an aggressive T-cell lymphoproliferative malignancy with poor prognosis [1, 2]. Multiple cellular effects such as activation of NF- $\kappa$ B signalling, inhibition of apoptosis and interfering with DNA repair, Tax triggers an oncogenic phenotype and often confers resistance to chemotherapy [3–6]. By inducing proteasomal degradation of Tax, the combination of arsenic

trioxide (arsenic) and interferon-alpha (IFN) selectively kills ATL cells and has the potential to cure ATL in mice and patients [7–13].

Tax is post-translationally modified by phosphorylation, acetylation and O-GlcNAcylation, as well as the attachment of ubiquitin-like molecules (UBLs), such as ubiquitin and the small ubiquitin modifiers (SUMOs) [14–19]. Ubiquitylation and SUMOylation have been reported to regulate the activity, turnover, subcellular localization, and protein-protein interactions of Tax [8, 20–23].

Ubiquitin-related modifier 1 (Urm1) is a dual function UBL [24, 25] with an established role as a sulfur transferase important for tRNA modification [26–29], as well as a posttranslational modifier that conjugates to target proteins during oxidative stress [30, 31]. An evolutionary conserved role of Urm1 for survival and oxidative stress responses has recently been reinforced by our recent report on the Urm1/Uba4 conjugation machinery

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in *Drosophila melanogaster* [32]. In this study we have unravelled Tax as a novel target of urmylation in both humans and flies and demonstrated a patho-physiologically relevant role of Urm1 as a regulator of the subcellular localization, as well as signaling activity of Tax.

## Results

### The HTLV-1 oncoprotein Tax is urmylated and subsequently localized to the cytoplasm

We have previously showed that the combination of arsenic and IFN induces G1 arrest and apoptosis in ATL leukemic cells, associated with induction of oxidative stress and Tax degradation in the proteasome [7, 8, 10, 12]. Importantly, degradation of Tax degradation is mediated by PML-dependent SUMOylation, followed by RNF4 dependent ubiquitylation [8]. Given that Tax is heavily modified by ubiquitin and SUMO (SUMO-1, 2 and 3), we were interested to analyze whether it is also modified by another ubiquitin-like molecule, Urm1, which is known to rapidly conjugate to multiple target proteins in response to oxidative stress. Indeed, arsenic is known to induce oxidative stress [33] and the combination of arsenic and IFN results in Tax recruitment to PML nuclear bodies, a well-known stress sensor [7–12].

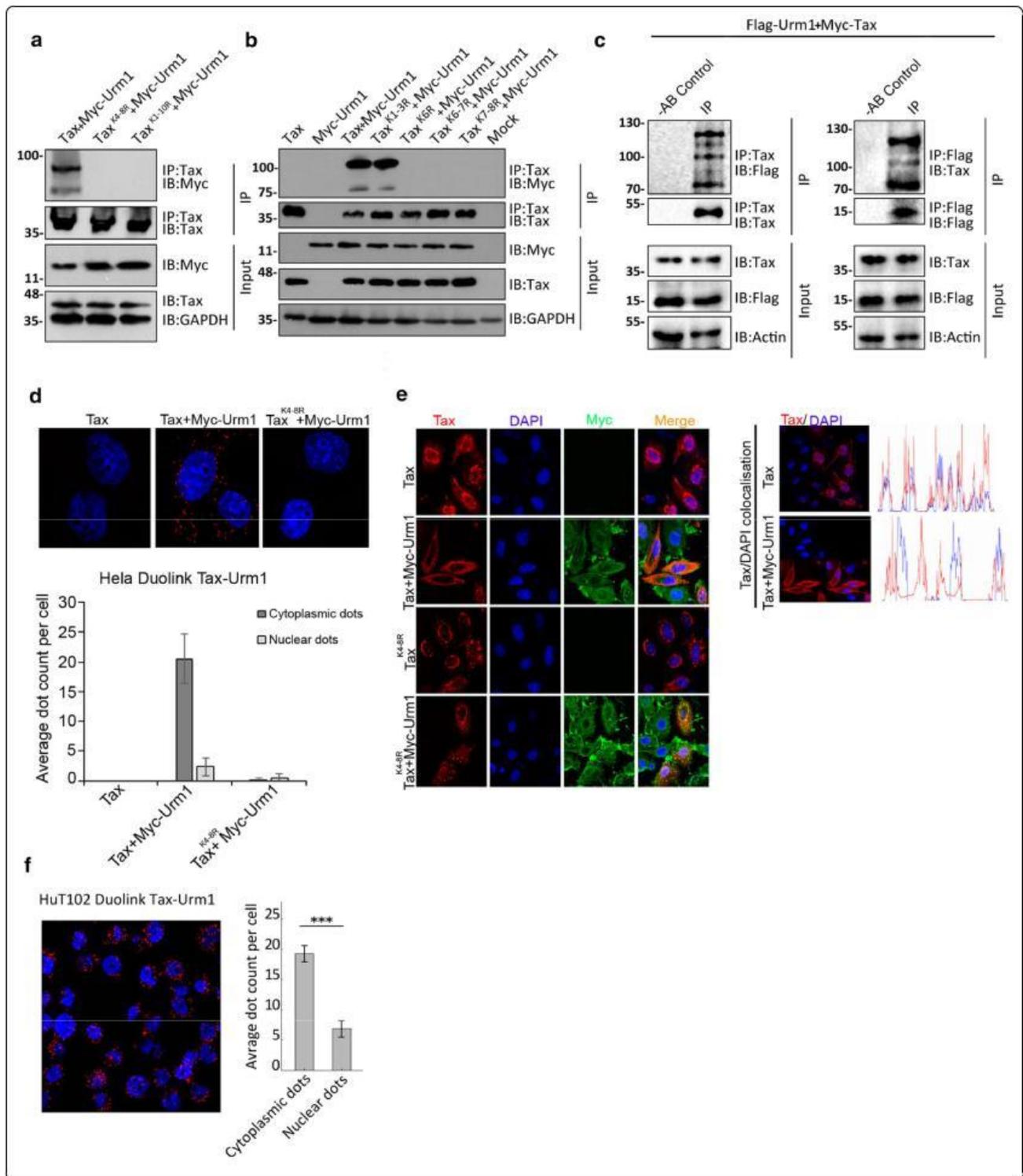
Initially, we used *tax*-transfected HeLa cells to confirm that Tax is modified by urmylation, and found Urm1 to conjugate to wild type Tax, but not to a Tax mutant where all lysine residues were replaced by arginine, TaxK1-10, or a Tax variant in which only lysines 4-8 were mutated, TaxK4-8R (Fig. 1a). In an attempt to further map the lysine targeted by Urm1, we concluded that most likely multiple Urm1 moieties attach to Tax via lysine residues 4-8, since neither of the tested mutants in this area could be urmylated in our assay (Fig. 1a, b). It is interesting in light of the reported targeting of lysine 4-8 by both ubiquitin and SUMO [16, 17]. In addition,

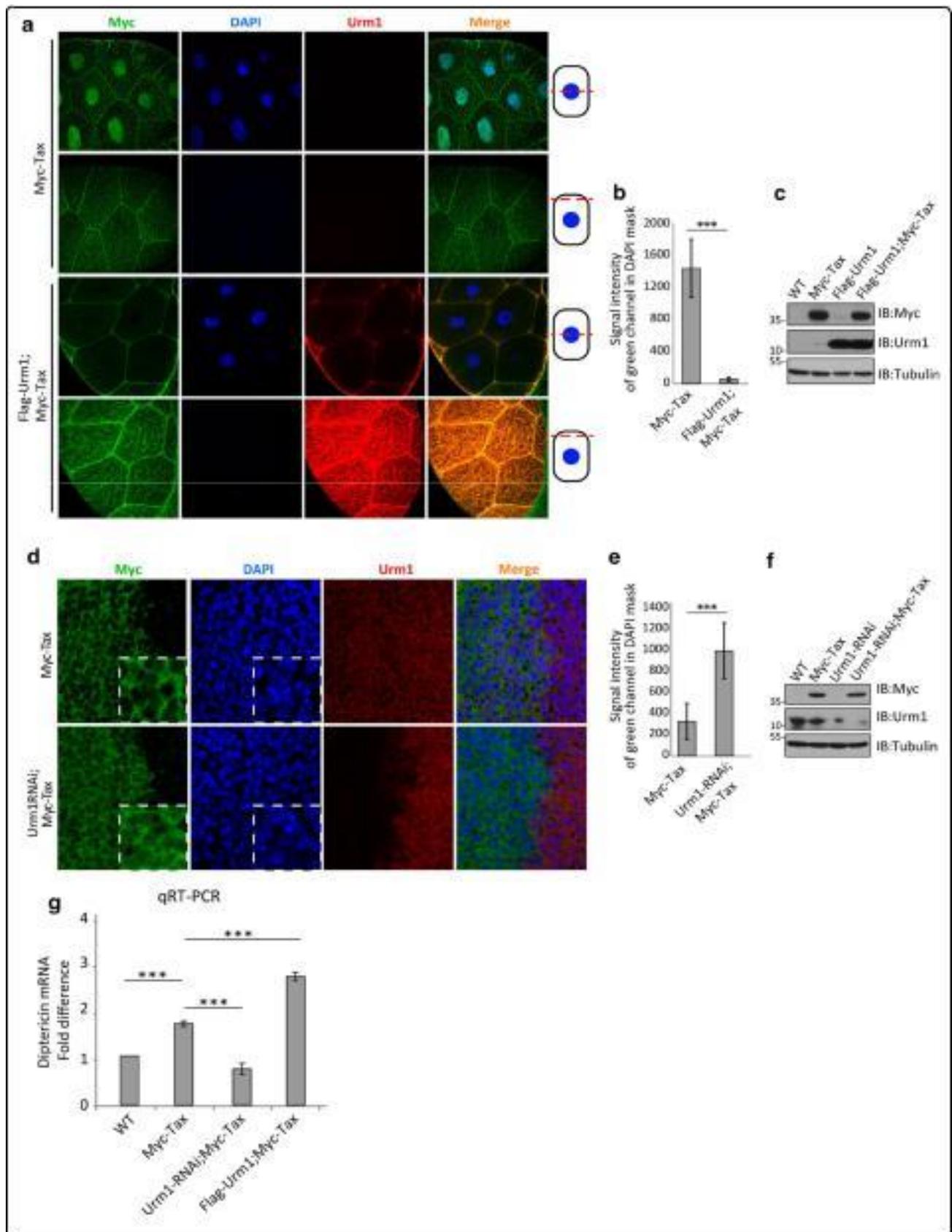
utilizing an established transgenic *Drosophila* model for Tax-driven transformation [32], in which Myc-tagged Tax is expressed under control of the UAS/GAL4 system, we further confirmed that Tax is urmylated also when expressed in fly tissues, and that similar to HeLa cells, the conjugation of Urm1 to Tax results in a size shift of Tax also in fly lysates (Fig. 1c). To decipher the intracellular localization of Tax/Urm-1 interaction, we utilized the proximity ligation assay (Duolink) methodology. We found that Urm1 interacts with Tax in the cytoplasm (Fig. 1d). Since modification by other UBLs strongly influences the subcellular localization of Tax, we tested the impact of Urm1 overexpression on the subcellular localization of Tax (Fig. 1e). Immunofluorescence analysis demonstrates that whereas transfected Tax has a pre-dominant nuclear distribution, a significant shift to the cytoplasm is observed upon addition of URM-1 (Fig. 1f). In agreement with a targeting of lysines 4-8 by Urm1, TaxK4-8R mutant failed to interact with Urm1 in these cells (Fig. 1d) and showed a similar subcellular localization both in the presence and absence of Urm1 (Fig. 1e), implying that the localization of TaxK4-8R mutant is not affected by Urm1. Importantly, in ATL-derived HuT-102 cells, cytoplasmic interaction of endogenous Tax and endogenous Urm1 was also evident (Fig. 1f).

Next, we investigated whether the Urm1-mediated shuttling of Tax is conserved also in *Drosophila*. In third instar larval salivary glands, which commonly are used to visualize nuclear-cytoplasmic shuttling of molecules, co-expression of Urm1 and Tax by the UAS/GAL4 system caused a marked export of Tax out from the nucleus (Fig. 2a–c). In agreement with an Urm1-dependent cytoplasmic targeting of Tax, RNAi-mediated reduction of Urm1 protein levels reciprocally resulted in increased levels of nuclear Tax, as compared to expressing Tax alone (Fig. 2d–f).

(See figure on next page)

**Fig. 1** Urmylation of the HTLV-1 oncoprotein Tax strongly influences the subcellular localization of Tax. **a** Tax is targeted by urmylation upon co-expression of wild-type Tax and Myc-tagged Urm1 in HeLa cells. Mutagenesis of Tax, replacing all lysine residues (Tax<sup>K1-10R</sup>) or lysine 4-8 (Tax<sup>K4-8R</sup>) for arginines, completely abolished Urm1 conjugation to Tax. **b** Mutational analysis of the indicated lysine residues in Tax, expressed in HeLa cells, indicates that Urm1 targets the same lysine residues as ubiquitin and SUMO for conjugation, lysine residues 4-8. **c** Tax is urmylated in protein lysates derived from *Drosophila melanogaster* adult eyes expressing Myc-Tax and Flag-Urm1 under control of the GAL4/UAS system, using GMR-GAL4 as driver. – AB control represents a negative control, in which no antibody was added to the immunoprecipitation. **d** Duolink in situ proximity ligation assay performed in HeLa cells expressing Tax and Myc-tagged Urm1, depicting that Tax-Urm1 protein complexes are primarily localized in the cytoplasmic compartment  $n = 25$ ,  $P < 0.0001$ . **e** Co-expression of Myc-Urm1 (green) and Tax (red) in HeLa cells causes a shift in the subcellular localization of Tax, with a clear increase of cytoplasmic Tax, as compared to cells expressing Tax alone (two upper panels). The Urm1-dependent nuclear exclusion of Tax is abrogated in cells expressing the -Tax<sup>K4-8R</sup> mutant, indicating that lysine 4-8 is required for Urm1-mediated regulation of Tax (two lower panels). **f** Also in ATL-derived HuT102 cells, interaction between endogenous Tax and endogenous Urm1 proteins is primarily encountered in the cytoplasm,  $n = 42$ ,  $P < 0.0001$ . Representative images for the -Duolink experiments and immunohistochemical analysis in HeLa cells were acquired by confocal microscopy using either a Zeiss LSM 510 META confocal laser microscope or a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany) with a Plan Apochromat 63/1.4 numeric aperture oil-immersion objective using Zen 2009 (Carl Zeiss). High-resolution images were obtained with a deconvolution program (Autodeblur; Image Quant), using blind iterative algorithms





(See figure on previous page)

**Fig. 2** Posttranslational modification of Tax by urmylation regulates the nuclear-cytoplasmic shuttling of Tax, and in extension its ability to activate NF- $\kappa$ B signaling. **a** Co-expression of Flag-Urm1 (red) and Myc-Tax (green) in 3rd instar larval *Drosophila* salivary glands (employing Sgs-GAL4 as driver) results in a complete blockage of the nuclear Myc-Tax accumulation, which is observed upon expression of Myc-Tax alone. Quantification is shown in **(b)**,  $n = 12$ ,  $P < 0.0001$ . **c** Verification of the expression levels of Myc-Tax and Flag-Urm1, induced by the UAS/GAL4 system in *Drosophila*, by Western Blot. **d** RNAi-mediated knockdown of Urm1 promotes a significant increase in the amount of nuclear Tax, analyzed in the 3rd instar larval wing disc of flies with the indicated genotypes. Engrailed-GAL4 was used to drive expression of Myc-Tax (green) alone (top) and together with Urm1-RNAi (red) (bottom) specifically in the posterior half of the wing disc (left side of each image), while preserving wild-type tissue in the anterior part (right side of each image). The images are taken at the border between the anterior and posterior side, thus displaying the control wild-type tissue as well as the genetically modified area expressing Myc-Tax and/or Urm1-RNAi in the same view. Quantification of Tax/DAPI colocalization is shown in **(e)**,  $n = 14$ ,  $P < 0.0001$ . **f** Verification of the GAL4/UAS-mediated induction of Myc-Tax expression and the efficiency of RNAi-mediated knockdown of Urm1 in *Drosophila*, visualized by Western Blot. **g** The ability of Tax to induce activation of the NF- $\kappa$ B pathway is strongly correlated with the expression levels of Urm1, as indicated by qRT-PCR analysis of Dipterecin, an established transcriptional target of NF- $\kappa$ B in the adult *Drosophila* fat body. Expression of Myc-Tax, Urm1-RNAi and Flag-Urm1 was induced by the UAS/GAL4 system, utilizing FB-GAL4 as driver, and the qRT-PCR was performed in triplicates on two biological replicates ( $P < 0.001$ ). Images depicting *Drosophila* salivary glands in **(a)** and wing imaginal discs in **(d)** were acquired using a Nikon C1 confocal microscope, magnifications,  $\times 60$  Plan Apo VC NA 1,40 oil and  $\times 100$  Plan Apo VC NA 1,40 oil and EZ-C1 software. The -Duolink images were acquired as described in Fig. 1

### Nuclear- cytoplasmic shuttling of Tax is modulated upon urmylation *in vivo* and *in vitro*

Tax-related oncogenesis has been linked to the activation of several pro-survival and proliferative signaling pathways, among which NF- $\kappa$ B is most prominent. To investigate whether urmylation affects the signaling activity of Tax, we monitored the mRNA levels of the antimicrobial peptide Dipterecin, an established downstream transcriptional target of NF- $\kappa$ B in *Drosophila*, as readout for NF- $\kappa$ B activity. Since the primary source of Dipterecin in *Drosophila* is the fat body, a fat-body specific GAL4 driver (FB-GAL4) was used to assess the signaling capacity of Tax in the presence and absence of Urm1. As expected, we found a clear correlation between Urm1-dependent nuclear/cytoplasmic shuttling of Tax and Dipterecin transcription, as indicated by a threefold induction of Dipterecin in flies co-expressing Tax and Urm1, and a concomitant reduction in flies with reduced levels of Urm1 (Fig. 2g).

### Urm1 strongly influence Tax- induced transcription of NF- $\kappa$ B target genes

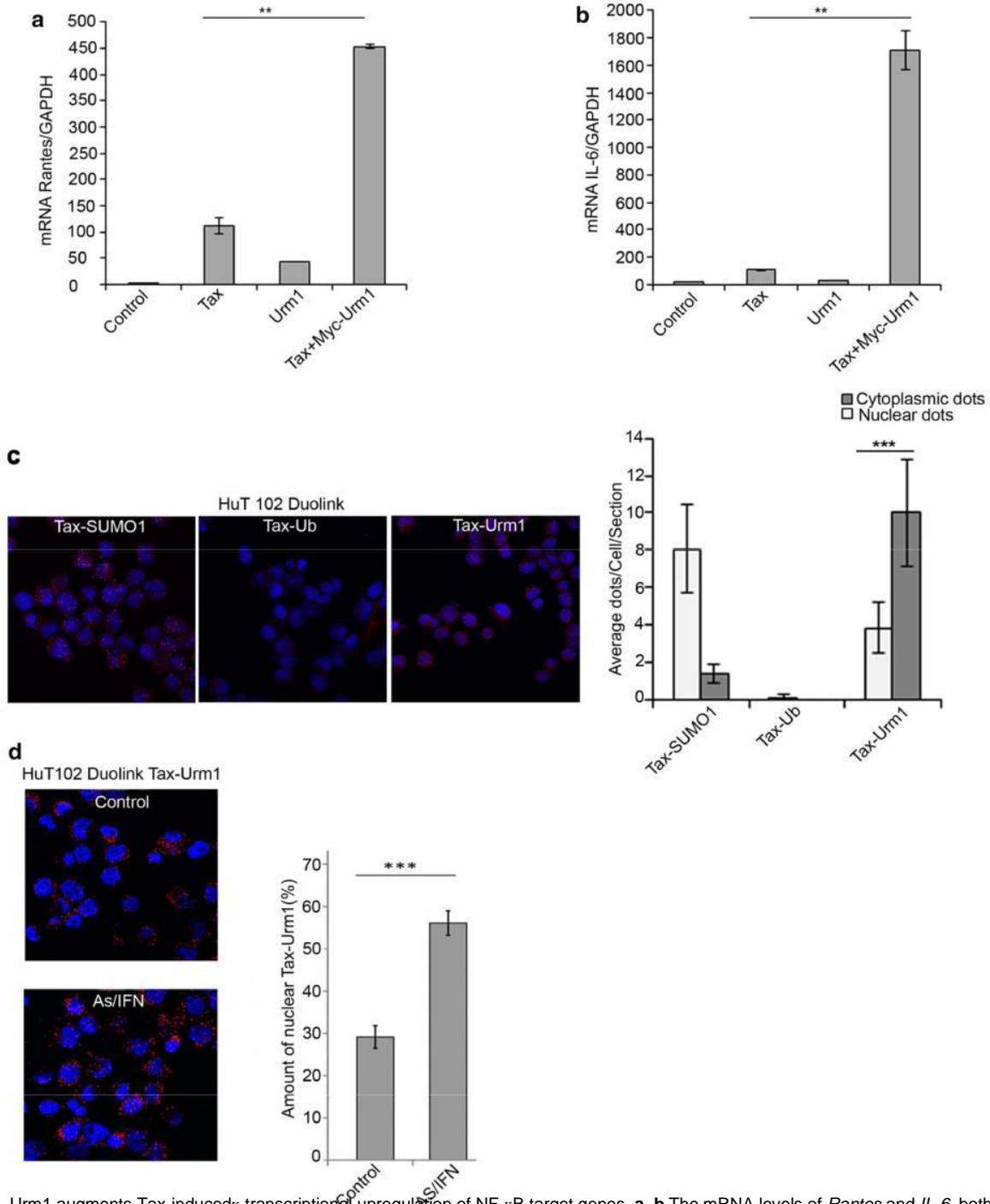
To investigate the functional impact of Tax urmylation on the ability of Tax to activate the NF- $\kappa$ B pathway, we assessed the effect of Urm1 addition on Tax-induced upregulation of transcription of NF- $\kappa$ B targets such as Rantes and interleukin-6 (IL6). Consistent with the increase of Tax-induced Dipterecin transcription in *Drosophila*, we observed a significant elevation of Tax-induced transcripts for both Rantes (Fig. 3a) and IL-6 (Fig. 3b) in HeLa cells upon addition of Urm-1, supporting an important role for Urm1 in Tax-mediated cellular signaling.

Finally, we examined the interaction between endogenous Tax and endogenous Urm1 by proximity ligation assay in HuT-102 cells and compared it to other UBLs,

which are known to modify Tax. Interestingly, whereas Tax - Urm1 complexes were predominantly localized in the cytoplasm, Tax - SUMO1 interaction was most frequently observed in the nucleus and no basal Tax-ubiquitin interaction was noted (Fig. 3c). We finally assessed how Tax-Urm1 complex formation was affected by arsenic/IFN treatment of HuT-102 cells, and discovered an increased nuclear targeting of urmylated Tax in response to treatment (Fig. 3d).

### Conclusions

We have uncovered a novel role of the UBL-molecule Urm1 as a modifier of the viral oncoprotein Tax, involved in the subcellular targeting and signaling activity of Tax. Specifically, we provide evidence that Urm1 is covalently conjugated to Tax in transfected HeLa cells and in HTLV-I transformed HuT-102 cells, as well as in *Drosophila* tissues. In all cases, Tax urmylation is associated with a size shift of Tax and clearly affects its subcellular localization by promoting a cytoplasmic accumulation of Tax. Most likely multiple Urm1 moieties attach to Tax via lysine residues 4-8, since neither of the tested mutants in this region were urmylated in our assay. Furthermore, Urm1-dependent cytoplasmic localization of Tax is correlated with NF- $\kappa$ B signaling, since the mRNA levels of *Rantes* and *IL-6*, both known as transcriptional targets of NF- $\kappa$ B, are dramatically increased in HeLa cells expressing Tax together with Urm1, as compared with cells expressing Tax alone. Interestingly, treatment of HuT-102 cells with arsenic/IFN increased the nuclear targeting of urmylated Tax. Our data put forward Tax as the first oncoprotein to be identified as a target of the UBL Urm1, and paves the way for further studies aimed at elucidating the outcome of Tax urmylation, such as pathogenicity and degradation mechanisms. In order to fully understand the functional implication of Tax urmylation, it will in addition be



**Fig. 3** Urm1 augments Tax-induced transcriptional upregulation of NF- $\kappa$ B target genes. **a, b** The mRNA levels of *Rantes* and *IL-6*, both known as transcriptional targets of NF- $\kappa$ B, are dramatically increased in HeLa cells expressing Tax together with Urm1, as compared with cells expressing Tax alone. Quantitative RT-PCR, where the levels of *Rantes* (**a**) and *IL-6* (**b**) mRNA are normalized against the housekeeping gene GAPDH,  $P < 0.001$ . **c** Comparison of the subcellular localization of interaction events between Urm1 and Tax, in relation to complex formation involving Tax and ubiquitin, versus Tax and SUMO-1, visualized using proximity ligation assay in HuT102 cells. **d** Treatment of HuT102 cells with arsenic/IFN counteracts the Urm1-dependent cytoplasmic shuttling of Tax, resulting in an increased accumulation of Tax1-Urm1 protein complexes in the nucleus,  $n = 42$ ,  $P < 0.001$

essential to investigate the crosstalk between the different UBLs that conjugate to Tax and experimentally address whether Tax ubiquitylation, SUMOylation and urmylation occur independently, sequentially, or simultaneously, in order to regulate Tax activation.

## Methods

### Fly stocks

Wild type *white*<sup>1118</sup>, *GMR-GAL4*, *Sgs3-GAL4* and *FB-GAL4* was from Bloomington *Drosophila* Stock Center, Indiana, USA. e RNAi line for Urm1 *P{GD15862}v48364* was from Vienna *Drosophila* Resource Center, Vienna, Austria [33]. *UAS:Urm1*<sup>WT</sup> [32] and *UAS:Tax*<sup>WT</sup> [34] have been described previously.

### Cells and Plasmids

HeLa cells were cultured and transfected with Lipofectamine 2000™ (Gibco, Invitrogen) as previously described [22]. PSG5 M-Tax lysine to arginine mutants *TaxK4-8R*, *TaxK1-3R*, *TaxK6R*, *TaxK6-7*, *TaxK7-8R* and *Tax-K1-10R* have been previously described [18]. pcDNA4:Urm1 (DC01337) was from Abgent.

### Immunoprecipitation and Western Blot

HeLa cells or adult flies were lysed and sonicated in 2% SDS and 50 mM Tris-HCl, pH 8. Immunoprecipitation were performed in 50 mM Tris-HCl, pH 8, 200 mM NaCl, 0.1 mM EDTA, 0.5% NP-40, 10% glycerol, and protease inhibitors, with Tax antibodies (#168-A51 from the National Institutes of Health AIDS Research and Reference Reagent Program) and protein A-agarose (Sigma). Following washing and elution in sample buffer, immunoprecipitations and lysate controls were analyzed by Western blot, as previously described [8], using anti-myc(9E10) at 1:250 (Santa Cruz), anti-Tax at 1:250, anti-GAPDH at 1:20000 (Abnova), *Drosophila* anti-Urm1 at 1:500 [32] and anti-Tubulin at 1:5000 (Sigma). *Drosophila* immunoprecipitations were performed using lysates from fly heads expressing UAS:Myc-Tax and/or UAS:Flag-Urm1 under control of GMR-GAL4 (UAS/GAL4 system).

### In situ proximity ligation assays (Duolink) and confocal microscopy

HeLa or HuT-102 cells were fixed in methanol onto glass coverslips or by cytospin, respectively. Protein-protein interactions were visualized using the Duolink in situ proximity ligation assay (PLA) system (Olink Bioscience), employing anti-Tax, anti-Urm1 [32], anti-SUMO-1 C9H1 (Cell Signaling Technology), anti-ubiquitin FL-76 (Santa Cruz) and anti-Myc ab9106 (Abcam).

### Immunofluorescence

Immunofluorescence staining of *Drosophila* tissues was performed as previously described [32], employing the antibodies anti-Urm1 at 1:500 [32], anti-Myc (9E10) at 1:500 (Sigma), and DAPI for nuclear visualization. HeLa cells were stained with anti-Tax (as above) at 1:50, and anti-Myc ab9106 (Abcam).

### Quantitative PCR

Total RNA, extracted with TRIzol<sup>®</sup> (Thermo Fischer Scientific), was template for cDNA synthesis with Random Hexamers and SuperScript<sup>®</sup> II Reverse Transcriptase (Thermo Fischer Scientific). Purification of DNA<sup>™</sup> was performed using Clean and Concentrator kit (Zymo Research), followed by qPCR employing the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems). Primer sequences for Urm1 were 5'-GGGCGGAGTTACTATTTGGT-3' and 5'-TCATAACCGATTTCACTCAAGTTT-3' and for Diptericin 5'-GTTTACCATTGCCGTCGCCCTTAC-3' and 5'-CCCAAGTGCTGTCCATATCCTCC-3'.

IL-6 levels were assessed using the primers 5'-GGA GACTTGCTGGTGAA-3' and 5'-GCATTTGTGGTTG GGTC-3', whereas Rantes was monitored using 5'-AC CACACCCTGCTGCTTTGC-3' and 5'-CCGAACCC ATTTCTTCTCTGG-3' primers.

### Abbreviations

ATL: adult T-cell leukemia/lymphoma; HTLV-1: human T-cell lymphotropic virus 1; HuT-102: human T-cell lymphoma cell line 102; IFN: interferon-alpha; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; SUMO: small ubiquitin modifier; UBL: ubiquitin like molecule; Urm1: ubiquitin-related modifier 1.

### Authors' contributions

MS, CG and AB designed the research. RH, BK, ID, HO, CM, and ZD performed the experiments. CG, MS, BK, RH, MES, AB analyzed the data and provided technical skills. CG, MS and AB wrote the paper. All authors contributed with discussions regarding the results and commented on the manuscript. All authors read and approved the final manuscript.

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### Competing interests

The authors declare that they have no competing interests.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

**Consent for publication**

This manuscript does not contain personal data from any individuals.

**Ethics approval and consent to participate**

This manuscript does not contain personal data from any individuals.

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# **ANNEX 5**



# Mouse Models That Enhanced Our Understanding of Adult T Cell Leukemia

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Adult T cell Leukemia (ATL) is an aggressive lymphoproliferative malignancy secondary to infection by the human T-cell leukemia virus type I (HTLV-I) and is associated with a dismal prognosis. ATL leukemogenesis remains enigmatic. In the era of precision medicine in oncology, mouse models offer one of the most efficient *in vivo* tools for the understanding of the disease biology and developing novel targeted therapies. This review provides an up-to-date and comprehensive account of mouse models developed in the context of ATL and HTLV-I infection. Murine ATL models include transgenic animals for the viral proteins Tax and HBZ, knock-outs for key cellular regulators, xenografts and humanized immune-deficient mice. The first two groups provide a key understanding of the role of viral and host genes in the development of ATL, as well as their relationship with the immunopathogenic processes. The third group represents a valuable platform to test new targeted therapies against ATL.

**Keywords:** adult T cell leukemia, HTLV-I, mouse models, Tax, HBZ

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## INTRODUCTION

### Human T Cell Leukemia Virus

Human T-cell leukemia virus type I (HTLV-I) retrovirus belongs to the deltaretroviridae family of viruses (reviewed in Matsuoka and Jeang, 2007). It is the first described oncogenic retrovirus and is responsible for a spectrum of diseases, the most aggressive of which is Adult T Cell Leukemia (ATL) (Poiesz et al., 1980; Hinuma et al., 1981; Yoshida et al., 1982, reviewed in Watanabe, 2017; Zhang et al., 2017). Approximately 5–20 million people are infected with HTLV-I worldwide (reviewed in Gessain and Cassar, 2012). However, the highest prevalence is reported in endemic areas that include Japan, the Caribbean, South America, inter-tropical Africa, Pacific islands, some areas in the Middle East, and Romania (Nosaka et al., 2017, reviewed in Edlich et al., 2003; Gessain and Cassar, 2012). The genome of this virus encodes for classical structural proteins required for retroviral replication and a series of accessory and regulatory proteins including the viral transcriptional activator Tax (Lee et al., 1984, reviewed in Azran et al., 2004) and the HTLV-I bZIP factor gene (HBZ), a viral protein encoded from the 3<sup>0</sup> long terminal repeat (LTR) in the complementary strand of the proviral genome (Gaudray et al., 2002, reviewed in Matsuoka and Jeang, 2011; Giam and Semmes, 2016; Ma et al., 2016). Both Tax and HBZ are linked to HTLV-I pathogenesis (reviewed in Boxus and Willems, 2009; Kannian and Green, 2010; Giam and Semmes, 2016).

## Adult T Cell Leukemia

Adult T cell leukemia is an aggressive hematological malignancy with very poor prognosis, high relapse rate, resistance to therapy, and a limited survival rate (Takatsuki et al., 1977; reviewed in Hermine et al., 1998; Bazarbachi et al., 2004, 2010; Goncalves et al., 2010; Marçais et al., 2013; Nasr et al., 2017; Watanabe, 2017). ATL develops in around 5% of infected carriers, following a long latency period exceeding 20 years (reviewed in Ishitsuka and Tamura, 2014; Bangham and Ratner, 2015).

Adult T cell leukemia is characterized by the presence of leukemic cells with atypical morphology and lobulated nucleus (Shimoyama et al., 1983). The majority of these cells are mature CD3<sup>C</sup> CD4<sup>C</sup> CD25<sup>C</sup> CD7 cells exhibiting an increased expression of the alpha chain of interleukin 2 receptor (IL-2R) but also an overexpression of Foxp3, a marker of T regulatory (T<sub>Reg</sub>) cells (Waldmann et al., 1984; Shimoyama, 1991; Okayama et al., 1997; Karube et al., 2004).

## Tax as a Viral Oncoprotein

Tax is a 40 kDa viral transactivator protein that promotes viral transcription via the 5'<sup>0</sup>-LTR. Tax functions as an oncogene resulting in leukemia (Grassmann et al., 1989; Tanaka et al., 1990). In rat fibroblasts, the expression of Tax is sufficient for induction of transformation and development of tumors (Grassmann et al., 1989, 1992; Tanaka et al., 1990). Tax trans-activates transcription by activating promoters implicated in cell proliferation, activation, and survival leading to accumulation of diverse genetic and epigenetic mutations, genetic instability, cell cycle checkpoint disruption, and damage of DNA repair mechanisms (reviewed in Marriott and Semmes, 2005; Kfoury et al., 2012; Bazarbachi, 2016; Watanabe, 2017). Tax binds critical transcription factors including the cAMP response element binding protein (Zhao and Giam, 1992; Brauweiler et al., 1995; Giebler et al., 1997), AP-1 (Fujii et al., 2000; Iwai et al., 2001), and serum response element (SRF) (Fujii et al., 1991, 1992). In addition, Tax inactivates tumor suppressor genes including p53 (Pise-Masison et al., 1998; Portis et al., 2001) and p16 (Suzuki et al., 1996), represses the expression of cyclin A, and antagonizes apoptosis through inhibiting apoptotic genes expression such as Bax and promoting anti-apoptotic ones including Bcl-xL and BFI-1 (Brauweiler et al., 1997; Nicot et al., 2000; Marriott and Semmes, 2005; Macaire et al., 2012).

Importantly, Tax activates the NF- $\kappa$ B pathway (Sun et al., 1994; Good and Sun, 1996; Mori et al., 1999; Hironaka et al., 2004), after binding the regulatory subunit of the I $\kappa$ B kinase (IKK) complex called nemo or IKK-g (Harhaj and Sun, 1999; Kfoury et al., 2008; Wang et al., 2016) resulting in the activation of downstream effector genes (reviewed in Kfoury et al., 2005). Furthermore, Tax induced NF- $\kappa$ B activation is highly dependent on its post translational modifications namely ubiquitylation and sumoylation (reviewed in Kfoury et al., 2012). Tax also affects the expression of various micro RNAs (mi-RNA) including mi-RNA31 known to inhibit the expression of the NF- $\kappa$ B non-canonical pathway components (Yamagishi et al., 2012). Tax-induced mi-RNA31 downregulation occurs via a deregulation of polycomb proteins, leading to a consequent activation of NF- $\kappa$ B,

and inhibition of apoptosis in ATL cells (Yamagishi et al., 2012). Moreover, Tax modulates the microenvironment and increases ATL cells' invasion and extravasation through affecting gap junctions between endothelial cells and infected cells (El-Sabban et al., 2002; Bazarbachi et al., 2004).

Due to genetic/epigenetic alterations in the HTLV-I genome, including mutations and promoter methylation, most ATL cells lack detectable Tax expression (Takeda et al., 2004). Despite its undetectable levels in ATL patients (Matsuoka and Jeang, 2007), Tax is essential for ATL cells survival as its silencing results in cell death (Dassouki et al., 2015; Bazarbachi, 2016). Recent data revealed transient Tax expression in the form of Tax bursts (Tax expression switching on/off) occurring in a small fraction of ATL-derived or HTLV-I transformed cells (Mahgoub et al., 2018, reviewed in Bangham and Matsuoka, 2017). In a similar context, ATL epigenome was analyzed and an ATL-specific "epigenetic code" paramount for cell identity was deciphered (Fujikawa et al., 2016). In more details, Tax was shown to induce an epigenetic-dependent global alteration including increased polycomb complex 2 (PRC2) trimethylation at histone 3 (H3k27me3) resulting in cellular transformation, immortalization, and epigenome reprogramming, similar to that observed in ATL patients (Fujikawa et al., 2016). All these properties make Tax an ideal oncoprotein for *in vivo* investigation.

## HBZ Biology in ATL

HBZ is a nuclear protein encoded by the complementary strand of HTLV-I RNA genome (Larocca et al., 1989; Gaudray et al., 2002). Unlike Tax that is often undetected in ATL cells, *Hbz* gene undergoes no abortive mutations and the protein is expressed in all ATL patients and HTLV-I infected carriers (Fan et al., 2010; Kataoka et al., 2015; reviewed in Satou et al., 2006; Matsuoka and Jeang, 2011). HBZ was found to be a negative regulator of Tax-mediated viral transcription (Gaudray et al., 2002). This opposite expression pattern of the two proteins may indicate a possible differential role in HTLV-I pathogenesis and suggests HBZ as a candidate for a possible HTLV-I vaccine (Mahieux, 2015; Sugata et al., 2015). The mRNA of HBZ positively correlates with the proviral load of HTLV-I in carriers, and ATL patients (Saito et al., 2009). *In vitro*, HBZ promotes the proliferation of ATL cells but its suppression by short hairpin RNA (shRNA) results in modest inhibition of ATL cells proliferation (Satou et al., 2006; Arnold et al., 2008). HBZ affects several cellular pathways implicated in cellular proliferation such as NF- $\kappa$ B (Zhao et al., 2009; Panfil et al., 2016), AP-1 (Matsumoto et al., 2005), JunD (Thebault et al., 2004; Kuhlmann et al., 2007), c-Jun, JunB (Basbous et al., 2003), and CREB (Lemasson et al., 2007). In contrast to Tax which constitutively activates both canonical and non-canonical NF- $\kappa$ B pathways, HBZ was shown to inhibit the canonical pathway of NF- $\kappa$ B via proteasomal degradation of p65 while the non-canonical pathway was not affected (Zhao et al., 2009; Panfil et al., 2016).

## Animal Models in ATL

Due to the complexity of HTLV-I associated diseases and the enigmatic mechanisms dictating their occurrence, in particular

in ATL, animal models have been instrumental in providing a platform for answering pivotal questions related to HTLV-I infection, disease progression, and importantly developing new effective therapeutic approaches (reviewed in El Hajj et al., 2012; Niewiesk, 2016). Among these models, rabbits, monkeys but also rats were useful to understand early HTLV-I viral infection and transmission as well as the induced host immune response against the virus (reviewed in El Hajj et al., 2012). More recently, transgenic *Drosophila* models expressing Tax in the compound eye and plasmacytes were generated (Shirinian et al., 2015). However, mice remain by far one of the most efficient tools helping in understanding the biology of this affliction. Murine ATL models include transgenic animals for the viral proteins Tax and HBZ, xenografts inoculated with ATL cells (either cell lines or patient-derived cells) and humanized mouse models (reviewed in Panfil et al., 2013; Niewiesk, 2016). In this review, we attempt to provide an updated summary of these various mouse models, the key advances they offered in the understanding of HTLV-I infection, as well as their contribution to ATL research and drug development.

## MOUSE MODELS OF ATL

### Immunocompromised Mouse Models

Mice are relevant tools to study the molecular mechanisms of carcinogenesis and to develop new antitumor therapies. However, in immunocompetent mice, transplantation is often hindered by the functional host immune response resulting in low or no tumor engraftment. This problem was overcome after the discovery of the immunocompromised CB17 *scid/scid* (SCID) mouse model making a revolution in the cancer field. These mice harbor a spontaneous non-sense mutation in the *scid* gene, encoding for the protein kinase DNA activated catalytic polypeptide (Pkrdc), indispensable for efficient B and T lymphocytes recombination (Bosma et al., 1983). The loss of *Pkrdc* results in impaired adaptive immunity whereby B and T cells are both non-functional. Despite the lack of adaptive immunity, SCID mice retain a normal innate immunity in which macrophages, antigen-presenting cells, and natural killer (NK) cells carry normal functions (Bosma et al., 1983).

To further improve tumor engraftment, a non-obese diabetic (NOD/SCID) model exhibiting additional mutations resulting in further impairment of NK activity was generated (Shultz et al., 1995). This model was further immunosuppressed to generate the NOD/SCID b2-microglobulin<sup>null</sup> mice in which the *b2-microglobulin* gene was deleted resulting in a complete abolishment of the NK cell activity (Koller and Smithies, 1989). Importantly, a NOD/SCID IL2-Rg<sup>−</sup> or NSG model was generated by deletion or truncation of the gamma chain of IL-2R (Ito et al., 2002), reviewed in (Ito et al., 2008). Therefore, in addition to all the abnormalities of their predecessors, NSG mice possess a defective production of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 as well as a severe impairment of the dendritic cell (DC) and their capacity to produce interferon  $\gamma$  (IFN- $\gamma$ ) upon stimulation (Ito et al., 2002; Ishikawa et al., 2005). For further immunosuppression, the Rag2<sup>−</sup> gc<sup>−</sup> model was

established. These mice have a deletion of the Recombination Activating Genes (*RAG2*), impairing the production of both T and B cells and NK cell-mediated immunity in murine hosts. Moreover, because the rag proteins are not involved in DNA repair, *RAG2*-deleted mice do not show the leakiness or radio-sensitivity observed in SCID mice (Traggiai et al., 2004; reviewed in Chicha et al., 2005). Because of these properties, these mice allowed to study human hematopoiesis (Hiramatsu et al., 2003; Ishikawa et al., 2005; reviewed in Pearson et al., 2008).

### ATL Development in Xenograft Mouse Models

The use of xenograft mice has provided invaluable information pertaining to the tumorigenic and proliferative potential of ATL (Ohsugi et al., 1994). Under this section, we will provide an overview of most tested immunocompromised animals injected with HTLV-I-transformed or ATL-derived cell lines or those injected with patient-derived ATL cells. Initial xenograft studies investigating ATL development and progression were performed in the SCID mouse model (Ishihara et al., 1992; Feuer et al., 1993; Kondo et al., 1993). Later studies reported the use of NOD/SCID and SCID/Beige mice for injection of transformed or immortalized cell lines (Liu et al., 2002). More recently, an HBZ xenograft mouse model was generated by transplantation of retrovirally transduced T cells with Bcl-xL, AKT, and HBZ (Kasugai et al., 2016). Only mice groups transplanted with T cells triply transduced with plasmids encoding for the three proteins generated tumors, highlighting the need of key components from different cellular pathways along with the viral HBZ for cellular transformation (Kasugai et al., 2016) (Table 1).

### Xenograft Mouse Models as Platforms for ATL Targeted Drug Development

Given its resistance to therapy and its high relapse rate, ATL remains an aggressive disease with an unfavorable prognosis (reviewed in Bazarbachi et al., 2011; Nasr et al., 2017). Despite remarkable progress in ATL therapies with the use of antiviral therapy and allogeneic stem cell transplantation (Bazarbachi et al., 2010, 2014), most patients relapse highlighting the necessity of novel therapeutic approaches (Table 2).

### Targeting the NF- $\kappa$ B pathway in ATL therapy

Adult T cell leukemia development entails the deregulation of multiple cellular pathways, including the constitutive activation of the NF- $\kappa$ B pathway (reviewed in Kfoury et al., 2005), making this pathway an attractive therapeutic target against ATL. Accordingly, a wide array of NF- $\kappa$ B inhibitors including specific (Bay 11-7082), and non-specific inhibitors (such as bortezomib and Dehydroxymethylepoxyquinomicin DHMEQ), were tested in ATL xenograft mouse models.

Bay 11-7082, specifically inhibiting the NF- $\kappa$ B DNA binding activity, prevented primary tumor growth and leukemic organ infiltration in NSG mice xenografted with HTLV-I infected cell lines (Dewan et al., 2003). Bortezomib prevented tumor growth in an ED, ATL-derived T cell line, xenografted SCID model (Satou et al., 2004). DHMEQ, known to inhibit the activation of NF- $\kappa$ B by preventing the nuclear translocation of its active subunit p65

**TABLE 1 |** Summary of the contribution of mouse models to ATL biology.

Model	Contribution to ATL biology	Reference
<b>Xenografts</b>		
Xenograft mouse models injected with HTLV-I transformed, ATL-derived cell lines or ATL patients derived cells	<p>Represented a platform for targeted drug development.</p> <p>Provided a potential tool to test therapeutic agents:</p> <ul style="list-style-type: none"> <li>– Targeting the constitutively active NF-<math>\kappa</math>B pathway.</li> <li>– Specifically targeting leukemic cells.</li> </ul> <p>Allowed a better understanding of the immune response against HTLV-I.</p> <p>Was instrumental for studying early stages of primary HTLV-I infection and subsequent clonal proliferation.</p> <p>Allowed the assessment of early steps of HTLV-I infection, proviral load, and clonal proliferation.</p>	<p>Zimmerman et al., 2011; Ishitsuka et al., 2012; Masaki et al., 2013; Saitoh et al., 2016 Phillips et al., 2000; Zhang et al., 2003, 2005; Maeda et al., 2010, 2015 Ishihara et al., 1992; Feuer et al., 1995; Stewart et al., 1996; Uchiyama, 1996; Liu et al., 2002 Miyazato et al., 2006</p> <p>Takajo et al., 2007</p>
<b>Humanized</b>		
HTLV-I infected human CD34 <sup>C</sup> in Rag2 <sup>-/-</sup> $\gamma$ c <sup>-/-</sup> mice models	<p>Allowed the understanding of early infection stages.</p> <p>Confirmed the in vivo correlation of Tax and NF-<math>\kappa</math>B activation upon expansion of CD4<sup>C</sup>CD25<sup>C</sup> malignant cells.</p>	Villaudy et al., 2011
HTLV-I infected human CD133 <sup>C</sup> in NSG	<p>Generated a human adaptive immune system in immunodeficient mice.</p> <p>Was the closest model to recapitulate the in vivo ATL development.</p> <p>Assessed the initiated immune system against the virus and clonal selection.</p>	Tezuka et al., 2014
<b>Tax Transgenics</b>		
LTR-Tax-Tg	<p>Established Tax as an oncoprotein and HTLV-I as transforming virus resulting in mesenchymal tumors and neurofibroma.</p> <p>Showed that Tax expression in oxidative fibers resulted in HTLV-I associated myopathies.</p> <p>Showed that Tax induced autoimmune like Sjogren like syndrome.</p> <p>Showed that Tax induced skeletal abnormalities and fragile bones similar to ATL patients.</p> <p>Showed that Tax is arthrogenic and induced ankylotic arthropathy.</p> <p>Showed that Immune system activation contributes to ATL pathogenesis in infected carriers.</p>	<p>Hinrichs et al., 1987; Nerenberg et al., 1987</p> <p>Nerenberg and Wiley, 1989</p> <p>Green et al., 1989</p> <p>Ruddle et al., 1993</p> <p>Habu et al., 1999</p> <p>Swaims et al., 2010</p>
Double transgenic -bgal-Tg model LTR-Tax HTLV-I LTR	<p>Unveiled tissues supporting tax-mediated transcriptional transactivation.</p> <p>Provided a model system to study the mechanism of gene regulation by Tax.</p>	Bieberich et al., 1993
huGMZBTax GMZ-Tax-Tg Granzyme promoter	<p>First model to generate leukemia (LGL), tumor infiltration, and splenomegaly partly resembling ATL.</p> <p>Showed that Tax functionally inactivates P53 contributing to late stage tumor progression.</p> <p>Showed that innate immune system, specifically IFN-<math>\gamma</math>, is crucial for ATL development.</p> <p>Revealed malignant hypercalcemia and osteolytic bone lesions resembling human ATL.</p> <p>Showed that Tax expression in vivo induced constitutive activation of HTLV-I.</p> <p>Revealed that Tax activation of lymphocytes recruits, activates, and transforms NK/T-cells.</p>	<p>Grossman et al., 1995</p> <p>Portis et al., 2001</p> <p>Mitra-Kaushik et al., 2004</p> <p>Gao et al., 2005</p> <p>Bernal-Mizrachi et al., 2006</p> <p>Rauch et al., 2009b</p>
CD3- Tax Tg CD3-epsilon promoter/enhancer	<p>Model failed to develop leukemia.</p> <p>Tax expression closely associated with apoptosis in vivo.</p>	Hall et al., 1998
tTA/Tax mice Bi-transgenic doxycycline inducible model, EmuSR alpha promoter-enhancer	<p>Revealed ATL-like cutaneous lesions and splenomegaly via HTLV-I activation.</p> <p>Showed that Tax or HTLV-I suppression resolves cutaneous symptoms.</p>	Kwon et al., 2005
lck-Tax-tg model Lck-proximal promoter	<p>Showed diffuse large cell lymphoma after prolonged latency.</p> <p>Model exhibits acute ATL like symptoms and HTLV-I activation.</p> <p>Provided a candidate ATL stem cells of CD38<sup>-</sup> /CD71<sup>-</sup> /CD117<sup>C</sup> phenotype and decreased expression of Tax, Notch, BMI1 were isolated.</p>	<p>Hasegawa et al., 2006</p> <p>Yamazaki et al., 2009</p>

(Continued)

TABLE 1 | Continued

Model	Contribution to ATL biology	Reference
Ick-Tax-tg model Lck-Distal promoter	Showed leukemia of mature CD4 <sup>C</sup> cells resembling mature CD4 <sup>C</sup> ATL cells	Ohsugi et al., 2007b
<b>HBZ transgenic</b> HBZ-Tg model CD4-specific promoter/enhancer/silencer	Showed systemic inflammation and later lymphoma in 30% of mice upon aging. Increased effector/memory CD4 <sup>C</sup> cells and functionally impaired CD4 <sup>C</sup> Foxp3 <sup>C</sup> T <sub>reg</sub> cells. Showed that HBZ promotes a pro-inflammatory phenotype via labile Foxp3 expression. Showed that HBZ suppresses Th1 cytokines and impairs cell-mediated immunity.	Satou et al., 2011 Yamamoto-Taguchi et al., 2013 Sugata et al., 2012 Kuribayashi et al., 2016
<b>Double Transgenic Tax/HBZ</b> HBZ/Tax model CD4 promoter/enhancer	HBZ-Tg mice exhibit ATL stem cells of c-kit <sup>C</sup> /CD4 <sup>C</sup> /CD8 phenotype. This model failed to generate ATL-like leukemia. Skin lesions, T-cell lymphoma, and splenomegaly with increased CD4 <sup>C</sup> memory and Foxp3 <sup>C</sup> T <sub>reg</sub> cells.	Zhao et al., 2014

TABLE 2 | ATL mouse models as platform for ATL targeted therapy.

Model	Drug	Drug target	ATL therapy	Reference
Xenograft model	Bay 11-7082	NF-kB	Prevented tumor growth and infiltration.	Dewan et al., 2003
	Bortezomib	NF-kB	Prevented tumor growth in an ED SCID.	Satou et al., 2004
	DHMEQ	NF-kB	Prolonged survival and Prevented tumor growth.	Ohsugi et al., 2007a
	9-aminoacridine and Campath-1H	NF-kB and CD25	Prolonged survival and induced P53-mediated apoptosis.	Ju et al., 2014
	Compound E, Bortezomib, and Romidepsin	g-secretase, NF-kB, and HDAC	Assessed interaction between Notch-1 and HTLV-I pathways, combination exhibited synergy supporting clinical trials.	Yu et al., 2015
	AR-42	HDAC	Prolonged survival.	Zimmerman et al., 2011
	Campath-1H and HAT or MEDI 507	CD2 and CD25	Targeting different CD25 epitopes exhibited synergy.	Zhang et al., 2006
	Flavopiridol and HAT	cyclin-dependent kinase and CD25	Synergy enhancing antitumor effect and survival.	Chen et al., 2009
	HAT, MAT, and 7G7B6	CD25	Inhibited tumor growth.	Maeda et al., 2010
	7G7/B6 and daclizumab	CD25	Presented osteoponin-integrin interaction as novel therapeutic target for ATL.	Maeda et al., 2015
	Daclizumab and Depsipeptide	CD25 and HDAC inhibition	Improved survival and attenuated tumor infiltration and viral production.	Ikebe et al., 2013
	A20 ShRNA	A20, ubiquitin-editing Enzyme	Decreased tumor growth and revealed a novel role for ubiquitin-editing enzymes in ATL development.	Saitoh et al., 2016
	ABT-737	Bcl-2 and Bcl-xL inhibition	Inhibited tumor growth.	Ishitsuka et al., 2012
Adoptive patient-autologous Tax-CTL	ATL cells	Decreased tumor infiltration and enhanced survival in vivo.	Masaki et al., 2013	
Humanized model	Tinofovir and Azidothymidine	Reverse Transcriptase inhibition	Prophylactic potential by blocking primary infection in vivo.	Miyazato et al., 2006
	TARC-PE38	CCR-4	CCR4 is a potential ATL target.	Hiyoshi et al., 2015
Transgenic model	Arsenic/IFN	NF-kB, Tax, LIC	Cured ATL via LIC elimination.	Ei Hajj et al., 2010
	ST1926	NF-kB, Tax, LIC	Highlights retinoids as promising therapies by enhancing survival and decreasing tumor infiltration.	Ei Hajj et al., 2014

DHMEQ, dehydroxymethyllepoxyquinomicin; HDAC, histone deacetylase; HAT, humanized anti-Tac; MAT, murin anti-Tac; arsenic, arsenic Trioxide; IFN, interferon-alpha; LIC, leukemia initiating cells.

(Ohsugi et al., 2006), showed a significantly prolonged survival and prevented tumor growth in an ATL NSG xenograft mouse model (Ohsugi et al., 2007a).

In a similar context, a small molecule, 9-aminoacridine (9AA) selectively induced *in vitro* ATL cell death through inhibition of

the NF-kB pathway and induction of p53 responsive genes (Ju et al., 2014). The efficacy of 9AA alone or in combination with Campath-1H (a monoclonal antibody directed against CD52) was assessed using a xenograft NOD/SCID model, inoculated with MET-1 cells. MET-1 are activated T cells that express CD2,

CD3, CD4, CD25, CD122, and CD52. An enhanced survival of tumor bearing mice was reported upon treatment with both compounds as compared to either compound alone. This involved the induction of p53, PARP cleavage, and apoptosis of splenic cells from leukemia-bearing mice (Ju et al., 2014).

Finally, and in order to assess the physical and functional interaction between Notch-1 signaling (increased in some ATL patients) and NF- $\kappa$ B activation, the therapeutic efficacy of the g-secretase inhibitor compound E along with bortezomib and romidepsin, a histone deacetylase inhibitor (HDACI), was explored in an NSG model injected with MT-1 cells (Yu et al., 2015). Each of the three reagents alone or in double combination inhibited tumor growth as monitored by tumor size, the level of tumor markers in the serum, and significantly prolonged the survival of tumor-bearing animals (Yu et al., 2015).

At the cellular and molecular levels, and to assess the *in vivo* implication of NF- $\kappa$ B in ATL pathogenesis, Nitta et al. (2008) investigated ATL development in a defective NF- $\kappa$ B setting. In this study, alymphoplasia (*aly/aly*) mice bearing an NF- $\kappa$ B inducing kinase (NIK) mutation were used. These mice harbored defects in lymphoid organs development and severe deficiencies in both humoral and cell-mediated immunity. In contrary to BALB/c and C57BL/6J control mice, *aly/aly* animals inoculated with HTLV-I producing MT-2 cells, did not maintain the provirus and antibodies against HTLV-I were not detected suggesting that NIK is required for the initial proliferation and maintenance of HTLV-I infected cells in mice (Nitta et al., 2008).

#### Targeted ATL drug development other than NF- $\kappa$ B inhibition

One of the suggested approaches in ATL treatment is virotherapy. Oncolytic virotherapy is a relatively new approach in cancer treatment, utilizing replication-competent viruses that selectively target cancer cells while sparing the normal healthy ones (Russell et al., 2012). MET-1 NOD/SCID model was used to evaluate the potential of measles-virus-virotherapy in treating ATL. Measles virus treatment of tumor cells lacking type I interferon (IFN- $\alpha$ ) secretion was shown to be more efficient both *in vitro* and *in vivo* as compared to other tumor cells (Parrula et al., 2011). Using the same animal model, Zimmerman et al. (2011) investigated the therapeutic effect of AR-42, an HDACI, in alleviating HTLV-I-associated lymphoid malignancies. A dietary formulation of AR-42 was shown to prolong survival of ATL engrafted mice as compared to controls.

Non-obese diabetic/SCID xenograft mice were also used to test ABT-737, a small molecule inhibitor of Bcl-2 and Bcl-xL, whereby ABT-737 resulted in a significant inhibition of the tumor growth *in vivo* (Ishitsuka et al., 2012).

In another study, Ikebe et al. (2013) investigated the efficacy of 17-DMAG, an HSP90 inhibitor, as a therapeutic agent against ATL. Oral administration of 17-DMAG dramatically attenuated the aggressive infiltration of multiple organs in an ATL xenograft mouse model. It also inhibited the *de novo* viral production and improved the overall survival of ATL mice (Ikebe et al., 2013).

More recently, an unrecognized role of ubiquitin-editing enzyme, A20, in the survival of HTLV-I-infected cells was unveiled in a SCID model. In brief, the ATL-derived HuT-102 cell line was first transduced with A20 shRNA and then inoculated

into SCID mice (Saitoh et al., 2016). Depletion of A20 induced apoptosis and affected the *in vivo* growth of HTLV-I infected cells, highlighting the importance of ubiquitin in ATL development.

#### Monoclonal antibodies as an ATL therapy in xenograft mouse models

The expression of specific cell surface markers on ATL cells implanted into mice, renders them an excellent model for testing the pre-clinical therapeutic potential of monoclonal antibodies. In this context, and given that ATL cells express CD2 and CD25 among other surface markers, the effect of monoclonal antibodies targeted against these two surface markers was performed in NOD/SCID mice intraperitoneally injected with MET-1 cells (Zhang et al., 2003). Furthermore, the effect of campath-1H (anti CD52), either alone or in combination with a humanized anti-Tac (HAT) or MEDI 507, a monoclonal antibody directed against CD2, was investigated. Campath-1H led to a striking prolongation of the survival of MET-1 ATL-bearing mice. This survival was significantly longer than that of the group receiving HAT. Moreover, the study revealed the anti-leukemic mechanism of action of Campath-1H which involves FcR-gamma-containing receptors (e.g., FcRgamma-III) present on polymorphonuclear leukocytes and macrophages, known to normally mediate antibody-dependent cellular cytotoxicity (ADCC) and/or trigger cross-linking induced apoptosis (Zhang et al., 2003). The same group explored the use of flavopiridol, a cyclin-dependent kinase inhibitor, alone or in combination with HAT. HAT/flavopiridol combination resulted in a prolonged survival and dramatic enhancement of the antitumor effect in MET-1 NOD/SCID mice as compared to the control group (Zhang et al., 2005).

A MET-1 NOD/SCID mouse model was also used to investigate the anti-leukemic effects of specific antibodies targeting IL-2R (Phillips et al., 2000) whereby HAT, murine anti-Tac (MAT), and 7G7/B6, all of which targeting IL-2R $\alpha$ , significantly delayed leukemia progression resulting in enhanced survival (Phillips et al., 2000). To decipher the mechanism of action of these antibodies, comparison between treated-NOD/SCID and NSG mice was carried out (Zhang et al., 2004). In contrast to what was seen in NOD/SCID mice, treatment of NSG mice did not affect leukemia growth nor improved mice survival highlighting that the immune system difference, specifically polymorphonuclear cells, plays a crucial role in leukemia elimination by anti-IL-2R antibodies (Zhang et al., 2004).

Another promising therapeutic target is CC chemoreceptor 4 (CCR4). CCR4 is a chemokine receptor expressed by tumor cells in about 90% of ATL patients (Ishida et al., 2003). Several studies have investigated the effect of monoclonal Anti-CCR4 antibodies *in vivo* as potential therapies in ATL (Yano et al., 2008; Ito et al., 2009; Ishii et al., 2010; Hiyoshi et al., 2015). Using an ATL SCID xenograft mouse model, Yano et al. tried to augment the ADCC effect induced by defucosylated chimeric Anti-CCR4 IgG1 monoclonal antibody KM2760 via addition of granulocyte colony stimulating factor (G-CSF). Addition of G-CSF resulted in a more robust antitumor effect as compared to KM2760 alone (Yano et al., 2008). Using the same model, another monoclonal

anti-CCR4 antibody KW-0761 was investigated (Ishii et al., 2010). KW-0761 also presented promising antitumor activity where tumor volume was significantly decreased (Ishii et al., 2010).

Anti-CD25 antibodies such as 7G7/B6 and daclizumab, directed against different epitopes of CD25 were also tested either alone or in combination (Zhang et al., 2006). Overall, 91% of the mice receiving the combination survived showing the promising synergistic effect of these two antibodies, especially when compared to the single antibody treatment (Zhang et al., 2006). In a similar study and using the same model, daclizumab was investigated in combination with an HDAl, depsipeptide, and showed an enhanced antitumor effect and survival of the leukemia-bearing mice, compared with those in the depsipeptide or daclizumab alone groups (Chen et al., 2009).

Given that some ATL cells express CD30 on their surface, the therapeutic effect of two anti-CD30 monoclonal antibodies was also investigated. SGN-30, a chimeric anti-CD30 mAb, and SGN-35, a monomethyl auristatin E-conjugated anti-CD30 mAb, were used. Maeda et al. (2010) treated NOD/SCID mice subcutaneously engrafted with HTLV-I-infected cells and reported a significant inhibition of the tumor growth upon treatment with either antibodies (Maeda et al., 2010).

In another context, the therapeutic efficacy of adoptive patient-autologous Tax-specific cytotoxic T cells (Tax-CTL) was assessed in NSG mice bearing primary ATL cells from three patients. Tax-CTL treatment resulted in a significant decrease of ATL cell infiltration into blood, spleen, and liver as well as a significant prolonged survival time in ATL NSG mice that received cells from two out of three patients (Masaki et al., 2013).

More recently, the NSG mice were used to investigate the physiological roles of osteopontin (OPN)-integrin interaction in ATL pathogenesis *in vivo*. ATL cell lines inoculated into NSG mice resulted in an increased OPN plasma levels. Treatment of these mice with anti-OPN mAbs inhibited not only tumor growth but also tumor invasion and metastasis suggesting a pivotal role of OPN in these processes (Maeda et al., 2015).

Altogether, the above studies highlight the importance of xenograft mice models in targeting and understanding ATL.

## Humanized Mouse Models of ATL

Despite the importance of ATL xenograft mouse models, these models present with the limitation of being injected with ATL-derived or HTLV-I-transformed cell lines that were maintained for years in culture. This entails the potential changes in their genetic drift and the attenuation in their *in vivo* potential. Moreover, ATL xenograft mouse models do not provide answers to how HTLV-I induces ATL at early steps and how it maintains leukemogenesis (reviewed in Niewiesk, 2016). In this context, humanized mouse models were generated (reviewed in Panfil et al., 2013; Duc Dodon, 2014; Niewiesk, 2016) (Table 1).

### Humanized Mouse Models: A Transformation in ATL Biology and Immune Responses

The lack of an adaptive and/or innate immune system is advantageous for HTLV-I replication and tumor engraftment.

Injecting CD34<sup>C</sup> hematopoietic stem cells into NSG mice

resulted in generation of human lymphocytes. The first humanized ATL model was developed by Miyazato et al. (2006) in which human peripheral blood mononuclear cell (PBMC) were first injected in NSG mice to establish a human-like setting followed by inoculation of HTLV-I virus producing MT-2 cells. MT-2 cells ensured cell-to-cell transmission required for HTLV-I infection. In this model, proviral load was increased in both CD4<sup>C</sup> and CD8<sup>C</sup> cells (Miyazato et al., 2006).

In a similar study, PBMC from HTLV-I infected carriers were injected in NSG mice to establish HTLV-I infection (Takajo et al., 2007). Despite the different methodology, both studies resulted in mice harboring human infected cells, which later undergo clonal proliferation (Miyazato et al., 2006; Takajo et al., 2007).

Another humanized model was developed by Villaudy et al. (2011) whereby CD34<sup>C</sup> human umbilical stem cells (HUSC) were intra-hepatically inoculated into newborn BALB/c/Rag22/2IL-2Rgc2/2 (also known as BRG mice) mice generating human lymphocytes. These lymphocytes were later infected by intraperitoneal injection of irradiated HTLV-producing MT-2 cell line. This study reported an alteration in T cell development alongside an increase in the proviral load and expansion of CD4<sup>C</sup>CD25<sup>C</sup> cells. Mice also developed ATL-like splenomegaly as well as lymphoma (Villaudy et al., 2011).

In a different study, intra-bone marrow injection (IBMI) of cord blood CD133<sup>C</sup> stem cells intratibially into sublethally irradiated NSG mice was performed (Tezuka et al., 2014). This study is based on the idea that CD133<sup>C</sup> cells are believed to be the ancestral of CD34<sup>C</sup> in hematopoiesis and carry the potential to differentiate to any hematopoietic cells including lymphocytes (Tezuka et al., 2014; reviewed in Duc Dodon, 2014). One month following CD133<sup>C</sup> inoculation, human CD45<sup>C</sup> leukocytes were found to completely reconstitute the murine bone marrow. Later, human B and T lymphocytes were detected and a balanced B/T lymphocyte ratio was attained and remained stable for up to 8 months (Tezuka et al., 2014). After establishing a human immune system in this model, HTLV-I infection involved the injection of sublethally irradiated MT-2 cells known to produce HTLV-I. Shortly after infection, an increase in CD4<sup>C</sup> cells was reported where CD4<sup>C</sup> CD25<sup>C</sup> clones gradually dominated indicating clonal selection. ATL like features including splenomegaly, hepatomegaly with ATL infiltration, and ATL-like “flower cells” were also reported (Tezuka et al., 2014). Screening of cytokines profiles demonstrated an initial elevation in the levels of IL-6, IL-8, IL-10, IL-12, IL-13, IFN- $\gamma$ , and TNF- $\alpha$ . In addition, granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokine (C-C motif) ligand 4 were also increased, clearly referring to an initiated immune response against the virus (Tezuka et al., 2014). One of the main findings of this study was the generation of a functional adaptive immune response in a humanized mouse model, summarized by detection of anti-HTLV-I antibodies as well as Tax-specific cytotoxic T cells (CTL). These CTL inversely correlated with proviral load of infected cells (Tezuka et al., 2014). Thus Tezuka’s model was by far the closest one in recapitulating the development of ATL *in vivo*. While most of the other humanized models developed lymphoma and/or thymoma, Tezuka’s model exclusively developed leukemia. This may be due to the fact that

infection was carried out after a humanized immune system may be fully developed.

Another humanized ATL model was generated by Nakamura et al. (2015) where PBMC from ATL patients were injected into NOD/SCID/Jak3-null mice (NOJ mice). In Brief, the model involved subcutaneous injection of the ATL S1T cell line into mice, followed by subcutaneous, intraperitoneal, or intravenous transplantation of primary ATL cells. ATL cells successfully infiltrated various organs and could transplant into secondary mice establishing NOJ mice as successful models for primary ATL xenotransplantation (Nakamura et al., 2015).

### Humanized Models as Platforms for ATL Therapy

After the revolution that humanized mouse models generated in the ATL field, they provided a strong platform for testing anti-ATL therapies (Table 2). Two antiviral reverse transcriptase inhibitors, Tenofovir and Azidothymidine, were tested in the first humanized ATL model developed by Miyazato et al. (2006). After the uncertain role of these agents in halting HTLV-I infection, their prophylactic potential was investigated. Both agents were reported to block primary infection in these mice (Miyazato et al., 2006).

Moreover, humanized models proved instrumental in investigating the anti-tumor potential of anti-CCR4 antibodies. NSG mice inoculated with primary ATL cells and autologous immune cells belonging to the same patient were used to assess the antitumor efficacy of KM2760 antibody (Ito et al., 2009). KM2760 decreased the number of ATL cells in blood, spleen, and liver, as well as ATL lesions and organ infiltration, and lowered IL-2R concentration in serum (Ito et al., 2009). Using a humanized mouse model similar to that developed by Villaudy et al. (2011), the efficacy of TARC-PE38 targeting CCR4 was investigated (Hiyoshi et al., 2015). TARC-PE38 is a complex of thymus and activation-regulated chemokine (TARC), CCR4 ligand, fused to a truncated *Pseudomonas aeruginosa* exotoxin A (PE38). TARC-PE38 efficiently killed HTLV-I-infected cell lines and shrank HTLV-I-associated solid tumors size. Moreover, TARC-PE38 markedly inhibited the proliferation of HTLV-I-infected human CD4<sup>C</sup>CD25<sup>C</sup> or CD4<sup>C</sup>CD25<sup>C</sup>CCR4<sup>C</sup> cells and reduced the proviral loads in PBMC obtained from both patients and asymptomatic carriers (Hiyoshi et al., 2015).

Using the NOJ model generated by Nakamura et al. (2015) the antitumor effect of pyrrolidine dithiocarbamate (PDTC), an antioxidant agent, was tested in ATL and showed that PDTC significantly enhanced the survival of these mice (Nakamura et al., 2015).

### Transgenic Mouse Models of HTLV-I

To decipher the oncogenic potential role of HTLV-I proteins *in vivo*, transgenic mice overexpressing the viral oncoproteins Tax or HBZ were generated. So far several *Tax* transgenic models expressing Tax under different promoters and two *HBZ* models were developed. Despite exhibiting many features of ATL, none of these models could exactly recapitulate HTLV-I-associated ATL. For instance, and as opposed to HTLV-I infection where HBZ- and Tax-specific CTLs and antibodies are generated, transgenics for both oncoproteins lack a host induced immune response

against these viral proteins. the importance of transgenic models in the ATL context lies in confirming the oncogenic functions of HTLV-I proteins, Tax and HBZ, *in vivo* and in disclosing various host pathways manipulated by these proteins ultimately leading to tumor generation (Table 1).

### Tax Transgenic Models Develop ATL-Like Leukemia/Lymphoma

#### Tax transgenic mice

The generation of *Tax* transgenic models relied on the earliest discoveries of the *in vitro* Tax oncogenic potential, from independent laboratories (Grassmann et al., 1989; Tanaka et al., 1990). To investigate the *in vivo* role of Tax in leukemogenesis, several mouse models have been generated (reviewed in Ohsugi, 2013; Niewiesk, 2016). Depending on the used promoter, Tax expression resulted in various tumors (Ohsugi, 2013). Except for models utilizing the lymphocyte-specific protein tyrosine kinase p56 (lck) or granzyme promoters, most of the models could not generate leukemia/lymphoma and rather resulted in less typical HTLV-I tumors and manifestations (Ohsugi, 2013; Panfil et al., 2013; Niewiesk, 2016). Nerenberg et al. (1987) established the first *Tax* transgenic mouse where *Tax* gene was under the control of the natural HTLV-I promoter, the LTR promoter [Tg (HIV-*tat*) 6-2Gja] (Nerenberg et al., 1987). Referred to initially as tat protein, Tax exhibited tissue-specific expression and LTR-*Tax* mice mainly developed mesenchymal tumors in the nose, ear, mouth, tail, as well as the foot. Despite not recapitulating human ATL, this research was novel in establishing Tax as an oncoprotein and HTLV-I as a transforming virus *in vivo* (Nerenberg et al., 1987). Beside mesenchymal tumors, LTR-*Tax* mice developed tumors at other multiple sites resembling neurofibroma making this model useful for studying neurofibromatosis (Hinrichs et al., 1987). Apart from tumors, mice were shown to develop myopathies similar to those associated with HTLV-I, which are due to the atrophy/degeneration of oxidative muscle fibers (Nerenberg and Wiley, 1989). Using the same model, Habu et al. (1999) reported high incidence of inflammatory polyarthropathy resembling arthritis. Profound skeletal alterations resulting in fragile bones with high turnover rate were also reported (Ruddle et al., 1993). Salivary and lacrimal glands showed exocrinopathy and lesions resembling Sjogren's syndrome due to the attack by immune cells (Green et al., 1989).

Afterwards, a bi-transgenic mouse was generated by crossing LTR-*Tax* mice with LTR-b *gal* mice (b-galactosidase) to better visualize the organ involvement in ATL (Bieberich et al., 1993). Tax acts on the LTR resulting in an increased b-gal expression; and this enzyme was detected in specific tissues including bones, muscles, exocrine glands, as well as mesenchymal tumors (Benvenisty et al., 1992; Bieberich et al., 1993). Using this same model, Swaims et al. (2010) assessed the interaction with the host immune system and demonstrated that the activation of infected CD4<sup>C</sup> T cells may induce Tax expression and thus may contribute to ATL pathogenesis in infected carriers. In addition, infected CD4<sup>C</sup> cells harboring Tax exhibited changes in the expression of surface markers and resulted in changes in CD4<sup>C</sup> subtype specifications (Swaims et al., 2010, reviewed in Kress et al., 2011). Despite not developing leukemia/lymphoma, the LTR-*Tax*

model provided a clear-cut evidence that *Tax* expression is solely sufficient for tumor induction establishing *Tax* as an oncoprotein *in vivo*.

In another attempt, a *Tax* transgenic model of C57BL/6TgN mice (huGMZB*Tax*) was developed where *Tax* expression was controlled by the granzyme B promoter (Grossman et al., 1995). This promoter restricted the expression of *Tax* to the T cell compartment specifically CD4<sup>C</sup> and CD8<sup>C</sup>, NK, and lymphokine activated killer cells. In this model, mice developed large granular lymphocytic leukemia (LGL), neutrophil dominated inflammation, as well as tumors on the ears, tail, and leg (Grossman et al., 1995). Mice also developed symptoms resembling human ATL such as high white blood cell count, splenomegaly, neutrophilia, and lymphadenopathy. LGL cells disseminated to distant organs such as lungs, bone marrow, and liver (Grossman et al., 1995). Despite not fully recapitulating the human disease, this model was instrumental in showing that *Tax* expression in the lymphocytes is sufficient to cause leukemia. Later, Gao et al. (2005) demonstrated that these mice also exhibited malignant hypercalcemia and symptoms associated with metastasis such as osteolytic bone lesions which again resemble the disease in ATL patients (Gao et al., 2005). Activated NK and T cells from this model demonstrated *Tax*-mediated constitutive activation of NF- $\kappa$ B in both its canonical and non-canonical pathways (Bernal-Mizrachi et al., 2006). Using the same model, the role of p53 inactivation in *Tax*-induced tumor development was assessed and showed that the p53 apoptotic pathway was functionally inactivated (Portis et al., 2001). P53 mutations in tumors were also detected but were associated with secondary organ infiltration. In the same context, mating of these *Tax*-transgenic mice with P53-deficient mice did not accelerate the initial tumor development (Portis et al., 2001). However, it significantly increased disease progression and mortality in p53 heterozygous mice. This suggested that *Tax* functionally inactivates p53 which contributes to late stage tumor progression rather than initial tumor formation (Portis et al., 2001).

In a different study, the role of the innate immune system and inflammation in ATL development was assessed. The *Tax*-granzyme model was mated with an *IFN-g* knock-out model. The resulting mice exhibited enhanced tumorigenesis with accelerated lesions development (Mitra-Kaushik et al., 2004). Using the same model, Rauch et al. generated *Tax*-LUC double transgenic mouse model whereby luciferase bioluminescent imaging techniques allowed to track tumor engraftment *in vivo* (Rauch et al., 2009a,b). The onset of peripheral subcutaneous tumors was preceded by the formation of microscopic intra-epithelial lesions. This suggests that *Tax* activates lymphocytes, which then recruit NK/T-cells to be activated and transformed (Rauch et al., 2009b). Another study from the same group suggested that in *Tax*-LUC model, lymphoma development is promoted by an inflammatory stimulus whereby T cell activation via T cell receptor (TCR) was shown to promote/exacerbate tumorigenesis (Rauch et al., 2009a). Afterwards, the same *Tax*-LUC model was utilized to investigate the role of IL-15 in spontaneous lymphoma development (Rauch et al., 2014). IL15<sup>+</sup> *Tax*-LUC mice were generated and resulted in an aggressive lymphoma development and accelerated mortality,

suggesting that IL-15 contributes to the antitumor immunity in ATL. Knocking out IL-15 also resulted in elevation of IL-1a and IL-1a driven cytokines (Rauch et al., 2014). Treatment with anti-IL-1a antibodies resulted in decreased tumor growth. This study suggested IL-15 and IL-1a as potential therapeutic options in ATL.

Hall et al. (1998) established another *Tax* transgenic model where *Tax* gene was under the control of *CD3-epsilon* promoter-enhancer sequences. These mice, of C57/CBA origins, developed a variety of tumors including salivary and mammary adenomas as well as mesenchymal tumors, specifically at wound sites, yet failed to develop leukemia (Hall et al., 1998).

In an attempt to target *Tax* expression to the leukocyte compartment, a bi-transgenic doxycycline inducible model [Tg (*EmuSR-tTa*) 83Bop] was generated (Kwon et al., 2005). This conditional “tet off” *Tax* transgenic model targeted both wild-type *Tax* and *Tax* mutants that selectively compromise NF- $\kappa$ B or CREB pathways to the leukocyte compartment. Wild type *Tax* transgenic mice developed a lethal cutaneous disease with skin lesions infiltrated by CD3<sup>C</sup>CD4<sup>C</sup>MHC-2<sup>C</sup> T cells, similar to those seen in ATL patients. Mice also developed systemic lymphadenopathy and splenomegaly. Moreover, inflammatory cytokines including TNF- $\alpha$ , IL-6, IL1  $\alpha/\beta$ , IFN- $\gamma$  were induced (Kwon et al., 2005). Of note, suppression of *Tax* by doxycycline administration resulted in disappearance of skin lesions directly linking *Tax* to this dermal pathogenesis (Kwon et al., 2005).

To further restrict *Tax* expression to the thymus compartment, being the site of maturation of T cells, the two *Lck* promoters, distal and proximal, were used. The proximal promoter drives gene expression in thymocytes while the distal one restricts gene expression specifically to mature T lymphocytes (Hasegawa et al., 2006; Ohsugi et al., 2007b; reviewed in Ohsugi, 2013). Hasegawa et al. (2006) generated a *Tax* transgenic model using the *Lck* proximal promoter [C57BL/6-Tg (Lck-HTLV-I *Tax*)]. As compared to previous transgenic models, Hasegawa's model was the closest to recapitulate ATL. In this model, mice developed CD4<sup>C</sup> CD8<sup>C</sup> CD44<sup>C</sup>CD25<sup>C</sup> diffuse large cell lymphoma and leukemia after a prolonged latency period of around 18 months resembling the latency period required in humans to generate tumors (Hasegawa et al., 2006). Interestingly, mice exhibited clinical and histological resemblance to acute ATL seen in patients, such as characteristic “flower cells” in blood smears, lymphadenopathy, splenomegaly with extensive infiltration by lymphomatous cells, as well as distant organ infiltration of bone marrow, liver, kidney, lung, skin, and meninges. Infiltrating T cells were of malignant phenotype with increased expression of CD25<sup>C</sup> cell surface marker (Hasegawa et al., 2006). In addition, mice exhibited further common features with ATL patients such as significant leukocytosis, hypercalcemia, elevated LDH, and constitutive NF- $\kappa$ B activation (Hasegawa et al., 2006). A major difference between Hasegawa's mice and human ATL is in the phenotype of leukemic cells; mice exhibited immature CD4<sup>C</sup> cells while human ATL involves leukemia generally of mature CD4<sup>C</sup> cells (reviewed in Ohsugi, 2013). Since the development of leukemia required a long time, splenocytes derived from this *Tax* transgenic model were intraperitoneally injected in SCID mice. This model recapitulated most of the ATL phenotypes, similar to

the transgenic model and to ATL patients, in less than 1 month (Hasegawa et al., 2006; El Hajj et al., 2010). Moreover, this model was the first to be used for the isolation of candidate ATL stem cells of CD38<sup>+</sup>/CD71<sup>+</sup>/CD117<sup>C</sup> phenotype (Hasegawa et al., 2006; Yamazaki et al., 2009). These stem cells exhibited decreased levels of Notch, Tax, and BMI-1 expression all of which indicate their early hematopoietic cell origin (Yamazaki et al., 2009).

The distal promoter of *Lck* was also used to develop Tax transgenic mice (Ohsugi et al., 2007b). The expression of Tax under the distal promoter resulted in leukemia of mature CD4<sup>C</sup> cells unlike the immature ones obtained in the Hasegawa et al. generated Tax transgenic model (Ohsugi et al., 2007b).

### Tax transgenic models for ATL-targeted therapy

Despite the improvements in prolonging survival of the leukemic subtypes of ATL upon using zidovudine and IFN- $\alpha$  (IFN) (Bazarbachi et al., 2010), most patients relapse underlying the urgent need for novel therapies and targets. Different drugs and drug combinations were investigated, of which a very promising treatment is the combination of arsenic trioxide (arsenic) and IFN- $\alpha$  (Table 2). *In vitro* studies involving ATL cell lines and primary patients' leukemic cells showed the efficacy of the arsenic/IFN combination in triggering Tax degradation by the proteasome resulting in cell cycle arrest and apoptosis (Bazarbachi et al., 1999; El-Sabban et al., 2000; Nasr et al., 2003). To assess the efficacy of this combination *in vivo*, El Hajj et al. used spleen cells derived from the murine Tax transgenic ATL model (Hasegawa et al., 2006) and injected them into SCID mice (El Hajj et al., 2010). Approximately 1 month after transplantation, mice recapitulated ATL manifestations (Hasegawa et al., 2006; El Hajj et al., 2010). Strikingly, arsenic/IFN cured ATL in these mice. Although this combination did not rapidly decrease the tumor bulk, as ATL cells only underwent modest cell cycle arrest and apoptosis, the curative efficacy of arsenic/IFN occurred through clearance of leukemia initiating cells (LIC). Briefly, using the serial transplantation method, ATL cells derived from primary mice treated with the arsenic/IFN combination resulted in lower leukemia transplantation ability in untreated secondary mice and no transplantation in untreated tertiary mice (El Hajj et al., 2010). Addition of the proteasome inhibitor bortezomib to arsenic/IFN treatment of primary mice reversed all the observed phenotypes and led to normal ATL development in serial transplantation experiments, demonstrating that Tax degradation is the critical step for LIC exhaustion which further highlighted the oncogenic addiction of ATL cells to Tax (El Hajj et al., 2010). Later, Kchour et al. (2009) have investigated the effect of the triple combination of zidovudine, arsenic, and IFN, and translated the promising pre-clinical results to patients. Interestingly, the triple drug combination of zidovudine/arsenic/IFN resulted in 70% complete remission rate and 100% overall response rate in chronic ATL patients, as well as an unprecedented prolonged survival in some patients, strongly suggesting a similar mechanism of LIC eradication in patients (Kchour et al., 2009).

Using the same *in vivo* model, the preclinical efficacy of a synthetic retinoid ST1926 was investigated (El Hajj et al.,

2014) (Table 2). Oral treatment of ST1926 induced massive apoptosis, prolonged survival and decreased tumor infiltration, leukocytosis, and splenomegaly as compared to untreated group of animals. This study highlights the potential of synthetic retinoids as a promising therapy for ATL (El Hajj et al., 2014). It remains to be determined whether ST1926 treatment alone targets LIC in ATL.

## HBZ Transgenic Models Generate T-Cell Lymphoma but Not Leukemia

### HBZ transgenic models

The first *in vivo* HBZ transgenic mouse model (HBZ-Tg mice) was generated by Satou et al. (2006), where the HBZ gene was expressed under the control of a murine CD4-specific promoter/enhancer/silencer (Satou et al., 2006). Restricted HBZ expression to CD4<sup>C</sup> T cells resulted in systemic inflammation and development of T cell lymphoma in only 30% of mice after a long latency period. HBZ-Tg spontaneously developed systemic dermatitis, alveolitis, and later lymphoma upon aging (Satou et al., 2011). At the cellular level, HBZ increased the generation and proliferation of Foxp3<sup>C</sup> T cells as well as the transcription of Foxp3 mRNA. HBZ-Tg mice exhibited an increase in the number of functionally impaired CD4<sup>C</sup> Foxp3<sup>C</sup> Treg cells as well as effector/memory CD4<sup>C</sup> T cells (Satou et al., 2011). Recently, this model was used to investigate whether the proliferation of CD4<sup>C</sup> T cells is increased *in vivo*. Allergic encephalomyelitis was experimentally induced by immunization with myelin oligodendrocyte glycoprotein (MOG)/complete Freund's adjuvant.

Disease severity was not increased but the number of CD4<sup>C</sup> T cells was increased only in the immunized HBZ-Tg mice suggesting that HBZ-expressing T cells have higher susceptibility to immune stimulation *in vivo* (Kinosada et al., 2017). Using the same model, Yamamoto-Taguchi et al. (2013) showed that HBZ promotes inflammation through labile Foxp3 expression; Treg cells induced by HBZ have unstable Foxp3 expression and tend to convert to Foxp3 T cells producing IFN- $\gamma$ . This HBZ-induced pro-inflammatory phenotype of CD4<sup>C</sup> T cells was suggested to be involved in the HTLV-I-associated pathogenesis and inflammation (Yamamoto-Taguchi et al., 2013). Later, Mitagami et al. (2015) closely investigated HBZ-induced inflammation and revealed that in HBZ-Tg mice, inflammation severity significantly correlate with lymphoma development. The study suggested a link between HBZ inflammation and oncogenesis in CD4<sup>C</sup> T cells (Mitagami et al., 2015).

In another context, HBZ expression was found to impair the cell-mediated immunity of HBZ-Tg via suppression of Th-1 cytokine production (Sugata et al., 2012). Despite being of low immunogenicity, anti-HBZ antibodies can be detected in patient's serum. Sugata et al. (2012) have utilized the HBZ-Tg mouse model to investigate the possibility of generation of HBZ-targeted HTLV-I vaccine. Accordingly, splenocytes from HBZ mice, called HT-48, were inoculated into immunodeficient mice generating an ATL model. C57BL/6 mice immunized by a recombinant vaccinia virus-based HBZ vaccine generated HBZ-specific CD4 and CD8 T-cell response. Afterwards, inoculation of anti-HBZ cytotoxic T cells into the generated HT-48 mouse model increased survival of these mice suggesting that HBZ

might be a candidate for vaccine production (Sugata et al., 2012). Recently, ATL stem cells were identified in an HBZ model (Kuribayashi et al., 2016). In this study, HT-48 cells from *HBZ-Tg* mice were injected intraperitoneally into C57BL/6 mice, then serial transplantation experiments were done to assess the presence of LIC where nine consecutive transplantations were done (Kuribayashi et al., 2016). Surprisingly, HT-48 cells were able to regenerate leukemia in all transplantations. In this model, ATL stem cells were identified as c-kit<sup>C</sup>/CD4<sup>C</sup>/CD8 cells. Compared to the T cell progenitors, reported ATL stem cells had a similar gene expression profile (Kuribayashi et al., 2016).

More recently, a transgenic *HBZ* mouse model using Granzyme B (*Gzmb-HBZ*) was generated (Esser et al., 2017). In addition to splenomegaly, abnormal white cell count, tumors developing after 18 months, in two thirds of the *Gzmb-HBZ* mice, as well as pathologic bone loss and hypercalcaemia were obtained (Kinosada et al., 2017) (Table 1).

### The Double Transgenic HBZ/Tax Mouse Model Fails to Recapitulate ATL

Zhao et al. (2014) established a double transgenic mouse model expressing both Tax and HBZ viral proteins. In this model, both proteins were exclusively expressed in CD4<sup>C</sup>T cells. The concomitant transgenic expression of both Tax and HBZ resulted in skin lesions, T-cell lymphoma, and splenomegaly resembling in part diseases observed in HTLV-I infected individuals (Zhao et al., 2014). In addition, HBZ/Tax double expression resulted in an increase in the number of CD4<sup>C</sup> memory T cells and Foxp3<sup>C</sup> T<sub>reg</sub> cells. Overall, they reported that little difference is seen between the phenotype produced by *HBZ-Tg* model and *HBZ/Tax* double transgenic model. However, in contrast to all published findings, this study reported that “Tax expression alone failed to generate major health problems” and did not result in tumor development (Zhao et al., 2014). In addition, this

model resulted in lymphoma and thus failed to generate ATL-like leukemia (Table 1).

## CONCLUSION

In an attempt to unveil the molecular mechanisms dictating ATL development and to advance novel therapeutic options, multiple animal models were utilized. The use of animal models to study HTLV-I infection and ATL development has been instrumental in providing valuable data concerning disease progression and potential therapy targets. Besides ATL patients, mice remain the most valuable and useful tools for *in vivo* investigation of ATL. Generation of ATL xenograft models strikingly advanced the search for targeted therapies for ATL. To assess ATL pathogenesis in a more human setting, humanized models were remarkably helpful. These models are critical for studying early steps of HTLV-I infection. Transgenic ATL models expressing HTLV-I proteins, Tax, HBZ, or both have disclosed the potential roles and contributions of either protein to ATL pathogenesis. In this context, *Tax* transgenic mice, specifically *Lck*-promoter-models, were shown to develop ATL-like leukemia with pathology and molecular changes resembling acute ATL including activation of NF-κB pathway. *Tax* transgenic mice also served as means of testing targeted drug therapies. On the other hand, *HBZ* transgenic mice showed manifestations of systemic inflammation establishing HBZ as a pro-inflammatory protein. Finally, despite the noted progress in disease understanding and its treatment strategies, an animal model that can fully recapitulate the human ATL disease has not been achieved yet.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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