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EFFECT OF THE IMMUNOMODULATORY DRUG EAPB0503 ON MUTANT NUCLEOPHOSMIN-1 FUNCTION IN ACUTE MYELOID LEUKEMIA

by

BATOUL HUSSEIN JISHI

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AN ABSTRACT OF THE THESIS OF

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Major: Physiology

Title: Effect of the Immunomodulatory Drug EAP0503 on Mutant Nucleophosmin-1 Function in Acute Myeloid Leukemia

Nucleophosmin-1 mutation (*NPM1c*) represents one of the most frequent mutations in Acute Myeloid Leukemia (AML). In *NPM1c* AML, NPM-1 is aberrantly exported to the cytoplasm, contributing to leukemogenesis. Ribosomal biogenesis depends on NPM1 binding with the SUMO-specific protease SENP3 or the $p14^{Arf}$ protein. While SENP3 catalyses desumoylation of NPM1/SUMO2 leading to 28S rRNA maturation; NPM1/$p14^{Arf}$ binding counteracts this activity. $p14^{Arf}$ binds and activates p53, through antagonizing its ubiquitin ligase MDM2. In this study, we explored the effect of NPM1c on the complex interaction with SENP3, p53, MDM2 and investigated the molecular basis of the immunomodulatory drug EAPB0503-induced NPM1c degradation on this interaction. We used two AML cell lines, OCI-AML2 and OCI-AML3 expressing NPM1 and NPM1c respectively. *In vitro*, the effect of EAPB0503 on cell growth, cell cycle, expression levels and localization of NPM1c, SENP3, p53, P-p53, MDM2 and p21 were assessed. EAPB0503 inhibited OCI-AML3 proliferation in a time-dependent manner and led to NPM1c degradation through p53 pathway activation. This was accompanied by restored NPM1 nucleolar localization in *NPM1c* AML cells. Importantly, *NPM1c* AML cells exhibited low basal levels of p53, and high basal levels of SENP3 and MDM2. EAPB0503 selectively degraded SENP3 in *NPM1c* AML cells, resulting in SUMOylation of NPM1c. *In vivo*, immunocompromised mice were injected intravenously with either cell lines. EAPB0503 was administered intraperitoneally, every other day, for 3 weeks. EAPB0503 selectively prolonged survival of OCI-AML3 xenograft mice, preserved the normal liver architecture, and reduced the number of bone marrow blasts along with NPM1c degradation, and $p14^{ARF}$ upregulation. This study provides a rationale for the therapeutic use of EAPB0503 in *NPM1c* AML.

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LIST OF ABBREVIATIONS

AML	Acute Myeloid Leukemia
RA	All Trans Retinoic Acid
allo-SCT	Allogeneic Stem Cell Transplant
ATO	Arsenic TriOxide
BER	Base Excision Repair
BRCA1-BARD	BRCA1-associated RING domain protein
CK2	Casein kinase 2
CEBPA	CCAAT/Enhancer Binding Protein
CR	Complete Remission
CBFβ	Core-binding factor, subunit beta
Crm1	Chromosome Region Maintenance 1
DNMT3A	DNA MethylTansferase 3A
DUB	DeUBiquitinating enzymes
ELN	European LeukemiaNet
EVI1	Ectopic Viral Integration site1
FDA	Federal Drug Administration
FLT3	Fms-Like Tyrosine kinase 3
FAB	French American British
HSCs	Hematopoietic Stem Cells
HSCT	Hematopoietic stem cell transplant
MRD	Minimal Residual Disease
MDS	MyeloDysplastic Syndrome
MDM2	Mouse double minute 2
MLLT3	Mixed lineage leukemia gene T3
MLL	Mixed lineage leukemia
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NOD-SCID	Nonobese diabetic/severe combined immunodeficiency
NoLS	Nucleolar Localization Signal
NPM1	Nucleophosmin
ORR	Objective Response Rate
OS	Overall Survival
PTM	Post-Translational Modification
PML	Promyelocytic Leukemia
PI3K	PhosphoInositide 3-Kinase
RUNX1-RUNX1T1	Runt-related transcription factor 1
SENP	SENtrin-specific Protease
SUMO	Small Ubiquitin-like Modifiers
TRIM	TRIpartite Motif-containing protein
TLS	TransLesion Synthesis
Ub	Ubiquitin
WHO	World Health Organization

LITERATURE REVIEW

CHAPTER 1: ACUTE MYELOID LEUKEMIA

A. Overview and epidemiology

Acute Myeloid Leukemia (AML) is a genetically heterogeneous and complex blood malignancy. AML is characterized by a clonal expansion of myeloid precursors with an increased proliferation rate and a reduced capacity to differentiate, resulting in the decreased production of normal mature blood cells and the accumulation of myeloblasts in the bone marrow (1). AML is one of the most frequent hematological malignancies in adults; it accounts for around 80% of acute leukemias (2). AML can occur at any age group with a median age of 68 in adults and a sharp increase of incidence with age (3). Patients older than 60 have a poor prognosis, with an overall survival (OS) of less than 10% at 2-years (4).

B. Classifications of AML

AML is a highly polyclonal disease that evolves over time. It can present at diagnosis with multiple clones and multiple mutations. The first classification of AML was conducted by the French-American British (FAB) system in 1976 and divided AML into eight subtypes (M0 to M7) based on morphological appearance of the blasts and their immuno-histochemical features (Table 1) (5).

Table 1. French-American-British (FAB) of AML (5)

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

This FAB classification was useful for decades. However, the discovery of genetic alterations in AML subtypes was better to predict clinical classification. Hence, in 2001, a new classification of AML was established by World Health Organization (WHO) in collaboration with the Society for Hemato-pathology and the European Association of

Hemato-pathology (6). This classification was revised by WHO in 2008 and included other prognostic factors (7) (Table 2).

Table 2. WHO classification of acute myeloid leukemia 2008

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

*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

Advances in sequencing technologies led to the discovery of new genetic mutations including *Fms-Like Tyrosine kinase 3 (FLT3),* and *Nucleophosmin-1 (NPM1).* Thus, AML classification was further revised. In 2010, the European LeukemiaNet (ELN) classification attempted to standardize the risk stratification in adult AML patients, by incorporating recurrent somatic mutations (8). They proposed a novel classification correlating cytogenetics and selected molecular alterations with clinical findings and treatment outcomes. Four groups of ELN classification were distinguished: favorable, intermediate-I, intermediate-II, and adverse. The latest AML classification was further simplified and divided patients according to their baseline cytogenetics into three major risk sub-categories: favorable, intermediate and adverse (9) (Table 3).

Table 3. 2017 ELN risk stratification of AML (9)

Risk Category	Genetic Abnormality
Favorable	t (8;21) (q22;q22.1); RUNX1-RUNX1T1
	inv(16) (p13.1q22) or t (16;16) (p13.1;q22); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD or with FLT3-ITD ^{low*}
	Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITD ^{high}
	Wild type NPM1 without FLT3-ITD or with FLT3-ITD $^{\mathbf{low*}}$ (w/o adverse risk genetic lesions)

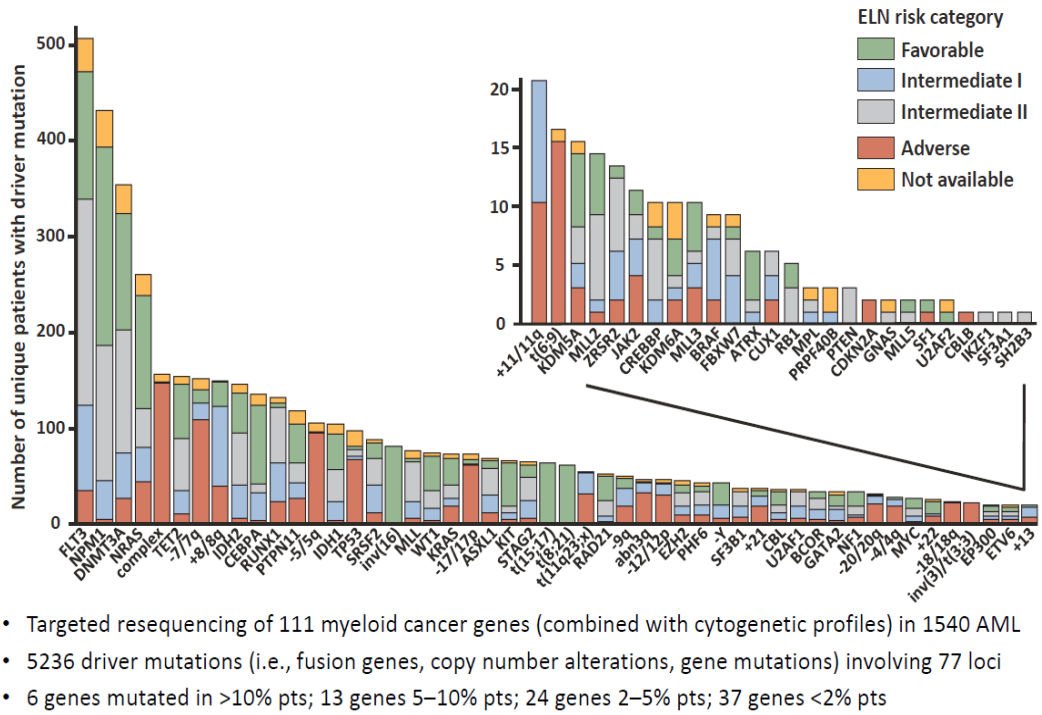
	t(9;11) (p21.3;q23.3); MLLT3-KMT2Ad
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t (6;9) (p23;q34.1); DEK-NUP214
	t (v;11q23.3); KMT2A rearranged
	t (9;22) (q34.1;q11.2); BCR-ABL1
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1)
	-5 or del(5q); -7; -17/abn(17p)
	Complex karyotype, monosomal karyotype
	Wild type NPM1 and FLT3-ITD ^{high*}
	Mutated RUNX1 †
	Mutated ASXL1 †
	Mutated TP53h

*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.

C. Genetic alterations in AML

While 50% of AML cases have chromosomal deletions or translocations (10), the remaining 50% are cytogenetically normal (CN-AML), with gene mutations (11). Exome sequencing in AML patients led to the identification of more than 20 driver recurrent mutations (12). The most significant mutations are: *Nucleophosmin 1* (*NPM1*) mutations, *DNA Methyltransferase 3A* (*DNMT3A*) mutations, *Fms-Like Tyrosine Kinase 3* (*FLT3*) mutations, *Isocitrate Dehydrogenase* (*IDH*) mutations, *Ten–Eleven Translocation 2* (*TET2*) mutations, *Runt-Related Transcription Factor* (*RUNX1*) mutations, *CCAAT Enhancer Binding Protein α* (*CEBPA*) mutations, *Additional Sex Comb-Like 1* (*ASXL1*) mutations, *Mixed Lineage Leukemia* (*MLL*) mutations, *Tumor Protein p53* (*TP53*) mutations, *c-Kit*

mutations, *Splicing Factor Gene* mutations and Cohesion Complex Members mutations (Figure 1) (13).



- Targeted resequencing of 111 myeloid cancer genes (combined with cytogenetic profiles) in 1540 AML
- 5236 driver mutations (i.e., fusion genes, copy number alterations, gene mutations) involving 77 loci
- 6 genes mutated in >10% pts; 13 genes 5–10% pts; 24 genes 2–5% pts; 37 genes <2% pts

Figure 1. Driver mutations in Acute Myeloid Leukemia (AML) (13)

Driver events in 1540 patients with AML. Each bar represents a distinct driver lesion; the lesions include gene mutations, chromosomal aneuploidies, fusion genes, and complex karyotypes. The colors in each bar indicate the molecular risk according to the European LeukemiaNet (ELN) classification. (13)

D. Clinical manifestations of AML

AML symptoms result mainly from a shortage of normal blood cells. Non-specific symptoms in AML patients include fatigue, loss of appetite, thrombocytopenia, anemia and/or neutropenia (14) . AML patients may experience others symptoms like bruising, weakness, anxiety, dizziness, depression, bleeding, shortness of breath (15). In addition to infections of variable severity, splenomegaly and hepatomegaly are seen in approximately one third of

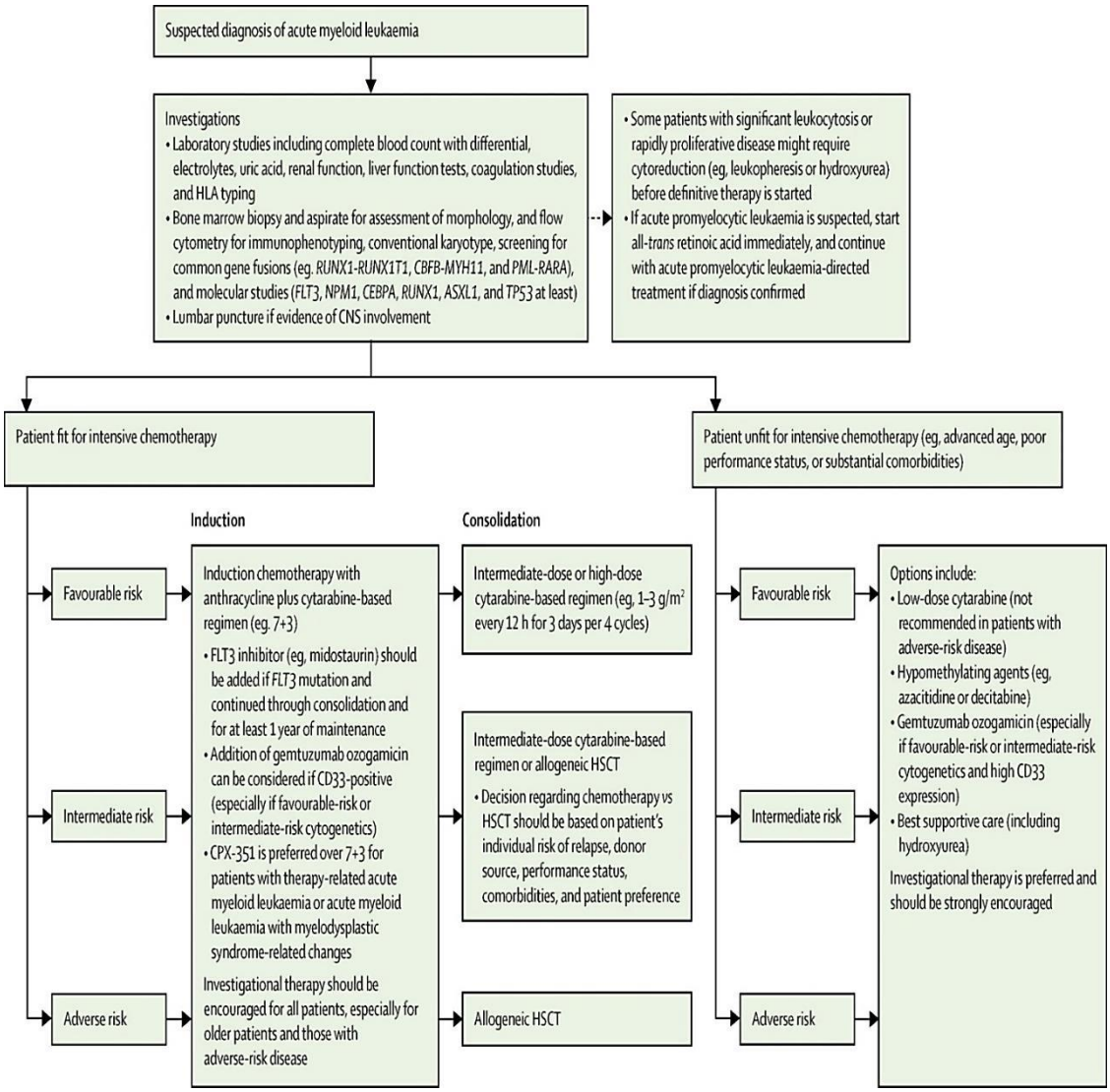
patients, especially in those with a monocytic or monoblastic morphologic subtype. Hemorrhagic manifestations including gingival bleeding, ecchymoses, epistaxis, or menorrhagia can also occur (16). In some patients, a serious bleeding diathesis can occur, particularly in the early phase of treatment, 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 (17). Moreover, chloroma, also called granulocytic sarcoma or myeloid sarcoma, is an extra-medullary manifestation of AML which was also reported as a rare manifestation of AML with an incidence of 2.5–9% (18).

E. Current treatments of AML

The genetic complexity of AML renders the outcome of standard treatments very variable among patients. For more than three decades, the standard treatment of AML remained unchanged. 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 are adopted in newly diagnosed or relapsing/refractory patients and others are object of clinical investigation (19).

In newly diagnosed patients with AML, treatment with intensive chemotherapy is mainly dependent on the patient's fitness that relies mostly on age, performance status, and comorbidities to achieve complete remission (CR) (20). Patients who achieve CR from the induction therapy, should receive convenient post-remission consolidation therapy (21). Patients with increased risk of relapse receive hematopoietic stem cell transplantation (HSCT)

in the first remission (Figure 2). Various transplant models were designed to optimize decision-making about these patient candidates. Patients with favorable AML related features receive post-remission consolidation therapy whereas those of adverse risk receive HSCT in the first remission (22). In intermediate risk patients, decision regarding chemotherapy versus HSCT is based on patient's individual risk of relapse, donor source, performance status, comorbidities, and patient preference (Figure 2).



HLA=human leukocyte antigen. HSCT=hemopoietic stem cell transplant

Figure 2. Treatment plan of AML in adults (23).

1. Targeted therapy of AML

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 with resistance to chemotherapy. AML treatment is witnessing more personalized approaches with specific targeting of driving mutations. Targeted therapies are designed according to driver mutations and molecular alteration in each AML subtype. In 2017, several AML innovative drugs were approved by food and drug administration (Table 4) (24, 25). These include hypo-methylating agents, FLT-3 ITD inhibitors, Isocitrate-dehydrogenase (IDH) Inhibitors and monoclonal antibodies.

Among the FLT3 inhibitors, Sorafenib belongs to the first generation inhibitors and proved potent activity against AML (26, 27). Sorafenib was either added to standard chemotherapy in the first-line induction therapy in AML, or used as maintenance therapy after allo-HCT (28-30). Giltertinib is among the second generation of FLT3 inhibitors, that was FDA approved for the treatment of adult relapsing patients or in refractory AML with a *FLT3* mutation (31). Among the FLT3 inhibitors, Midostaurin is an oral multi-targeted kinase inhibitor. A large randomized international study suggested that adding Midostaurin to induction and consolidation therapy, and its single-agent use during maintenance therapy, improved overall survival (OS) and event-free survival (EFS), in patients 18–60 years of age with *de novo* FLT3-positive AML (32).

In adult AML patients having complex karyotype with no *NPM1* mutation, induction therapy is not effective alone and required the use of hypomethylating agents such as decitabine and azacitidine (33-35). 5-azacytidine and decitabine improve survival in AML patients. A phase III trial in elderly patients showed that azacytidine was associated with improved OS compared to patients who received common AML treatments (36).

A meta-analysis of randomized studies demonstrated that Gemtuzumab ozogamicin (GO), a humanized monoclonal antibody anti-CD33 drug conjugate carrying calicheamicin, a potent DNA damaging toxin, can be safely added to conventional induction therapy. This combination reduced the risk of relapse and led to a significant survival benefit for patients with intermediate, and particularly favorable, cytogenetics (37, 38).

Table 4. FDA approved targeted therapies for AML in 2017

	Drug class	Approved indication	Clinical outcomes
Midostaurin	Multi-targeted kinase inhibitor (FLT3, VEGFR2, PDGFR, and KIT)	Newly diagnosed FLT3-mutated acute myeloid leukemia in adults (given in combination with chemotherapy)	Chemotherapy and Midostaurin vs chemotherapy alone: median overall survival 74·7 months vs 25·6 months (HR 0·78; p=0·009) (32)
Enasidenib	IDH2 inhibitor	Relapsed or refractory IDH2-mutated acute myeloid leukemia	Overall response rate 40·3%, complete remission 19·3%, and median overall survival 9·3 months (39)
Gemtuzumab ozogamicin	Anti-CD33 antibody-drug conjugate	Newly diagnosed CD33-positive acute myeloid leukemia in adults and relapsed or refractory CD33-positive acute myeloid leukemia in adults or pediatric patients aged 2	For newly diagnosed, chemotherapy plus Gemtuzumab ozogamicin vs chemotherapy alone: median event free survival 15·6 months vs

		years and older	9·7 months (HR 0·58; p=0·0003) (40) and Gemtuzumab ozogamicin vs best supportive care: median overall survival 4·9 months vs 3·6 months (HR 0·69; p=0·005) (38) For relapsed or refractory, overall response rate 33·3%, complete remission 26·3%, and median overall survival 8·4 months(25)

Other targeted therapies were also designed against AML. In Acute Promyelocytic Leukemia (APL) patients, an AML subtype, a combination of Arsenic trioxide (ATO) and All-Trans Retinoic Acid (ATRA) became a standard treatment and led to high cure rates (41, 42).

Activation of the PI3K/AKT/mTOR signaling pathway is very common in AML (43, 44). Pan-PI3K, PI3Kδ, dual PI3K–mTOR and AKT inhibitors show anti-leukemic activity *in vitro* (45, 46) and some are currently in Phase I/ II trials (47, 48). In addition, a Phase I/II study of the mTOR inhibitor, everolimus, in combination with chemotherapy or azacitidine demonstrated good tolerability and high response rates in patients with relapsed AML (49) (50).

Finally, venetoclax, a bcl-2 inhibitor is used in combination with demethylating agents such as decitabine or azacitidine (51) or low dose Cytarabine (52) and showed tolerable safety and favorable Objective Response Rate (ORR) in elderly AML patients.

CHAPTER 2: NUCLEOPHOSMIN-1(NPM1)

A. NPM family

Nucleophosmin/nucleoplasmin (NPM) is a family of three histone chaperones (NPM1, NPM2, and NPM3), with NPM1 being the prevalent form in all tissues (53). 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 (54) (Figure 3).

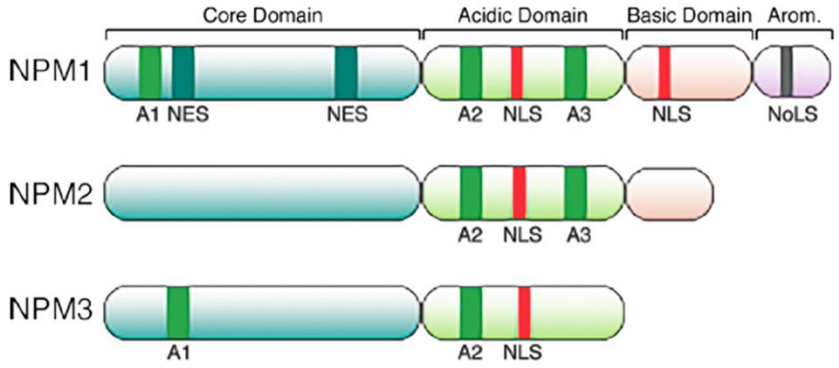


Figure 3. Domain representation of human NPM1, NPM2, and NPM3 proteins (54)

Schematic structure of human NPM proteins 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, NPM1 harbors 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 function exist between the three members of NPM family (55). The main functions of the three members are summarized in Table 5.

NPM1 (also known as NO38, numatrin or B23) is a ubiquitously expressed nucleolar phosphoprotein that constantly shuttles between the nucleus and the cytoplasm (56). NPM1 is directly implicated in human tumorigenesis (57). It is overexpressed in various tumors such as colon (58), ovarian (59) and prostate (60) carcinomas.

Table 5. NPM family members

NPM Family Member	Known As	Major function
NPM1	B23 (61, 62) or numatrin (63) in mammals and NO38 in amphibians (64)	• Ribosome Biogenesis (65) • Centrosome duplication (66) • Regulation of apoptosis through regulation of p53 and $p14^{ARF}$ (67) • DNA duplication (68) • Transcriptional regulation (69) • Histone chaperoning (70)
NPM2	Nucleoplasmin in amphibians	• Binds to histones and mediates the assembly of nucleosomes from DNA and histone proteins (71), • De-condensation and remodeling of paternal chromatin after fertilization (72)
NPM3	NO29 in amphibians (73)	• Ribosomal RNA biogenesis (74) • Paternal chromatin decondensation in mammals (75)

NPM1 is also known as B23 (61, 62) or numatrin (63) in mammals and NO38 in amphibians (64). NPM1 is mainly localized in the nucleolus (61) and has wide tissue distribution (76). It is involved in multiple cellular processes including ribosomal biogenesis (65). It is the most studied of all members of the Nucleophosmin family because of its frequent overexpression, mutations, rearrangement, and deletion in human cancers.

NPM2 was isolated from the eggs and the oocytes of *Xenopus laevis*; it is the most abundant nuclear protein (77, 78). NPM2 binds to histones and mediates the assembly of nucleosomes from DNA and histone proteins (71), and it also facilitate the de-condensation and remodeling of paternal chromatin after fertilization (72).

NPM3 is the most recent discovered NPM member (79), known as NO29 in amphibians (73). It is involved in ribosomal biogenesis by regulating NPM1 (74) and paternal chromatin de-condensation in mammals (75).

B. NPM1 gene and the structure of its encoded protein

NPM1 gene maps to chromosome 5q35 in humans and it contains 12 exons (53). It encodes three alternatively spliced isoforms: B23.1, B23.2, and B23.3. B23.1 is the dominant isoform (80); it is 37 kDa and consists of 294 amino acids (81). B23.1 is the only protein from the NPM family that has a unique RNA- binding domain at its C-terminus.

NPM1 has distinct domains allowing it to play multiple functions (Figure 4). The N-terminus contains a hydrophobic domain (82) involved in self-oligomerization (1-110 aa) and chaperone activity of NPM1 towards proteins, nucleic acids, and histones. It contains two leucine rich-nuclear export signals (NES) responsible for nucleo-cytoplasmic shuttling. The

first NES (42-49 aa) associates with ribosomal biogenesis (65), while the second NES sequence (94-102 aa) is required for centrosome localization (83).

In the middle, there are two highly acidic regions that are required for the binding of basic histone and ribosomal proteins, and facilitate nucleosome assembly and chromatin remodeling (68, 84). Between the two acidic regions, a ribonuclease activity motif is essential for ribosome biogenesis, and one nuclear localization signal (NLS 190-197 aa) are present (85, 86). The basic domain lies between amino acids 189-243 and is considered essential for nucleic acid binding.

The C-terminus of NPM1 (244-294 aa) has a nucleolar localization signal (NoLs) with two tryptophan residues at positions 288 and 290, which are critical for retaining NPM1 in the nucleolus. The shuttling property of NPM1 between the nucleolus, nucleus, and the cytoplasm is highly dependent on its NES, NoLs, and NLS motifs (87)(Figure 4).

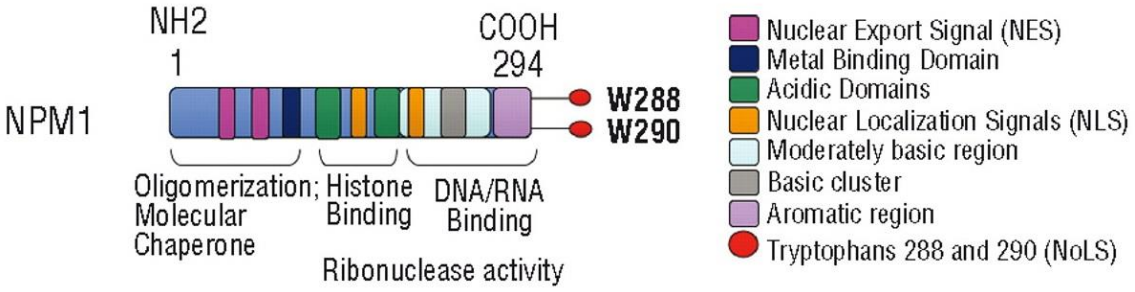


Figure 4. Structural and functional domains of wild-type NPM1 (88)

NPM1 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 (88).

The Shuttling properties of NPM1 depend on its functional domains. NoLs motif allows NPM1 to migrate from the cytoplasm to the nucleoplasm. The interaction of the two NES with the Crm1/exportin 1 ensures its export back to the cytoplasm (83) (Figure 5).

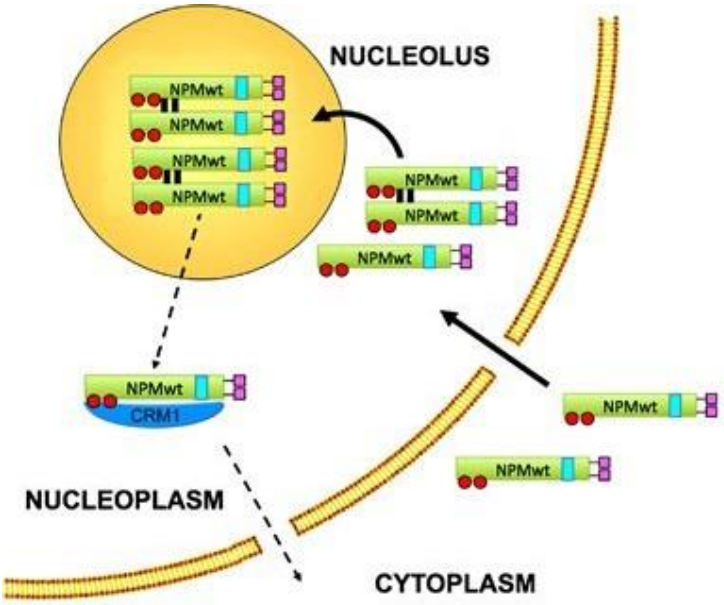


Figure 5. Mechanism of nucleo-cytoplasmic shuttling of NPM1 (89).

The nuclear import of the protein (arrow) greatly predominates over the nuclear export (dotted arrow). Thus, NPM1wt mainly resides in the nucleolus. (76)

C. NPM1 functions

While the major NPM1 function is ribosomal biogenesis, it is still involved in multiple cellular processes including genome stability, centrosome duplication, DNA repair, inhibition of apoptosis, histone chaperoning, cell cycle regulation, and regulation of $p14^{ARF}$-p53 pathway (90-95) (Table 5).

1. Ribosomal biogenesis

NPM1 is a key player in ribosomal biogenesis, contributing to cell growth and proliferation. NPM1 export signals and chaperoning capabilities aids in the transportation of ribosomal components from the nucleus to the cytoplasm (Figure 6). It aids in the processing and the assembly of ribosomes through its nucleocytoplasmic shuttling property. In addition, it has an intrinsic RNAase activity (65), it binds to nucleic acids (96), it is involved in processing of pre-RNA molecule (97) and chaperone activity (98) to prevent protein aggregation during ribosome assembly (99). A significant role of NPM1 is to mediate through a Crm1-dependant mechanism, the nuclear export of ribosomal protein L5/5S rRNA subunit complex (94). It interacts directly with several ribosomal proteins including RPL5 (94), RPS9 (100), and RPL23 (101). In addition, blocking nucleocytoplasmic shuttling inhibits ribosome subunit export (102). Ultimately, NPM1 aids in several distinct stages of ribosomal biogenesis.

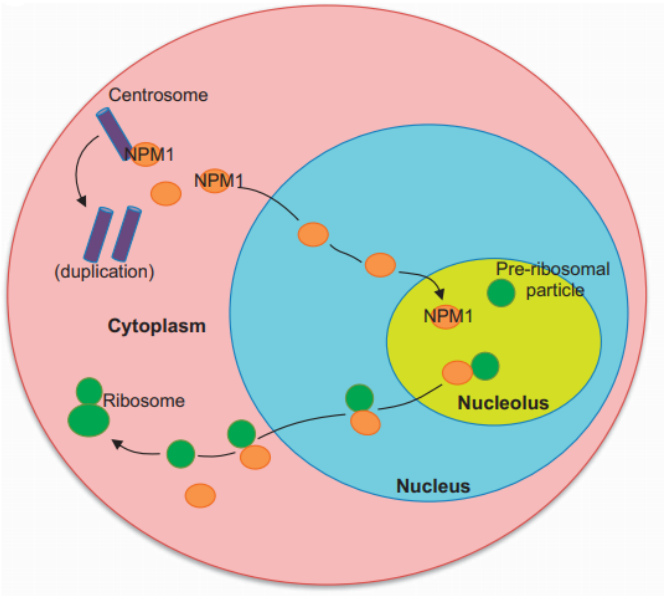


Figure 6. NPM1 role in ribosome biogenesis (103)

NPM1 binds unduplicated centrosomes in the cytoplasm to prevent duplication, and dissociates to allow duplication. NPM1 also mediates nuclear export of pre-ribosomal proteins for ribosomal assembly.

2. Maintenance of genome stability

NPM1 maintains genomic stability. It is involved in both DNA repair mechanisms (104, 105) and centrosome duplication (90). Depletion of NPM1 led to genomic instability with dramatic changes in nuclear morphology as well as distortion of nucleolar structure (106). In resting state, NPM1 associates with unduplicated centrosomes preventing their duplication (107) (Figure 7). At late G1, it dissociates from the centrosomes (107), enabling proper chromosome duplication and during mitosis, it re-associates with the centrosomes at the mitotic spindle.

NPM1 has a direct role in the repair of DNA lesions. It is involved in homologous recombination of DNA double-strand breaks (DSB) (104), and it is also involved in base excision repair pathway (108).

3. Stress response and apoptosis regulation

NPM1 interacts with the oncosupressors p53 and $p14^{ARF}$ and their mediator (MDM2) to regulate cell proliferation and apoptosis (Figure 7 and 8).

a. <u>NPM1 and p53</u>

p53 is a tumor suppressor gene described as the “guardian of the genome”. It is activated upon cell stress inducers, such as DNA damage, hypoxia, or oncogene activation (109). Once activated, it induces cell cycle arrest, promotes senescence, or induces apoptosis (109, 110). There is an important functional link between nucleolar integrity, NPM1, and p53

stability (111). Any disturbance in the nucleolar or ribosomal function leads to NPM1 delocalization from the nucleolus to the nucleoplasm to activate p53 (92). In fact, NPM1 maintains p53 stability by interacting and inhibiting Hdm2/MDM2, a p53 E3- ubiquitin ligase leading to its degradation (112) (Figure 7).

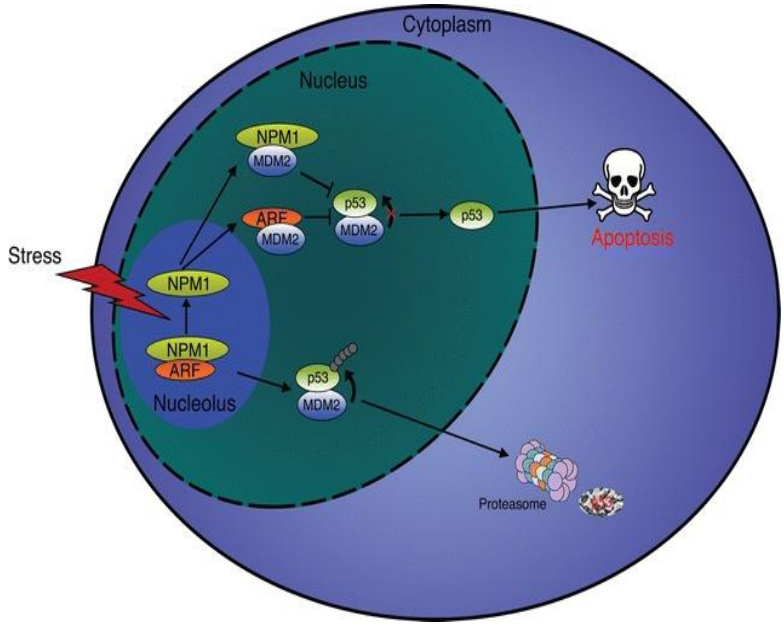


Figure 7. Regulation of apoptosis by NPM1 (54)

In normal cells, $p14^{ARF}$ and NPM1 form a dimer in the nucleoli, allowing MDM2 to target p53 for proteosomal degradation. Following a stress signal (DNA damage …), $p14^{ARF}$ and NPM1 dissociate and relocate to the nucleus were 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.

b. NPM1 and $p14^{ARF}$

$p14^{ARF}$ is a nucleolar tumor suppressor protein that induces cell cycle arrest, or apoptosis due to oncogenic stress. NPM1 and $p14^{ARF}$ control each other's stability and activity (Figure 8). NPM1 and $p14^{ARF}$ associate to form a high molecular weight complex within the nucleolus (91). This stabilizes $p14^{ARF}$ and delays its turnover (113) (Figure 8 a, c). An NPM1-interacting motif in the C-terminal region of $p14^{ARF}$, which corresponds to its predicted nucleolar localization signal was recently identified (114). Interestingly, mutant $p14^{ARF}$ doesn't associate with NPM1 and their complex is unstable and functionally impaired. Additionally, inhibition of the proteasome machinery only partially restored the stability of $p14^{ARF}$ mutants; hence, NPM1 protects $p14^{ARF}$ from proteasome degradation (113). $p14^{ARF}$ inhibits MDM2, by delocalizing it from the cytoplasm to the nucleolus (Figure 8b) (115), which leads to p53 stabilization (116). Cells lacking p53 and NPM1 have high proliferation rate, and their $p14^{ARF}$ protein is unstable and excluded from the nucleolus (67). Therefore, NPM1 maintains $p14^{ARF}$ stability and its nucleolar localization.

On the other hand, $p14^{ARF}$ inhibits cell proliferation upon stress independently from MDM2-p53 axis (Figure 8d). It interferes with ribosomal biogenesis by inhibiting the production of rRNA (117). More importantly, $p14^{ARF}$ promotes degradation of NPM1 by ubiquitylation and accelerated turnover (118) (Figure 8e). Several reports showed that $p14^{ARF}$ induces SUMOylation of many nucleolar proteins including NPM1 (160-163).

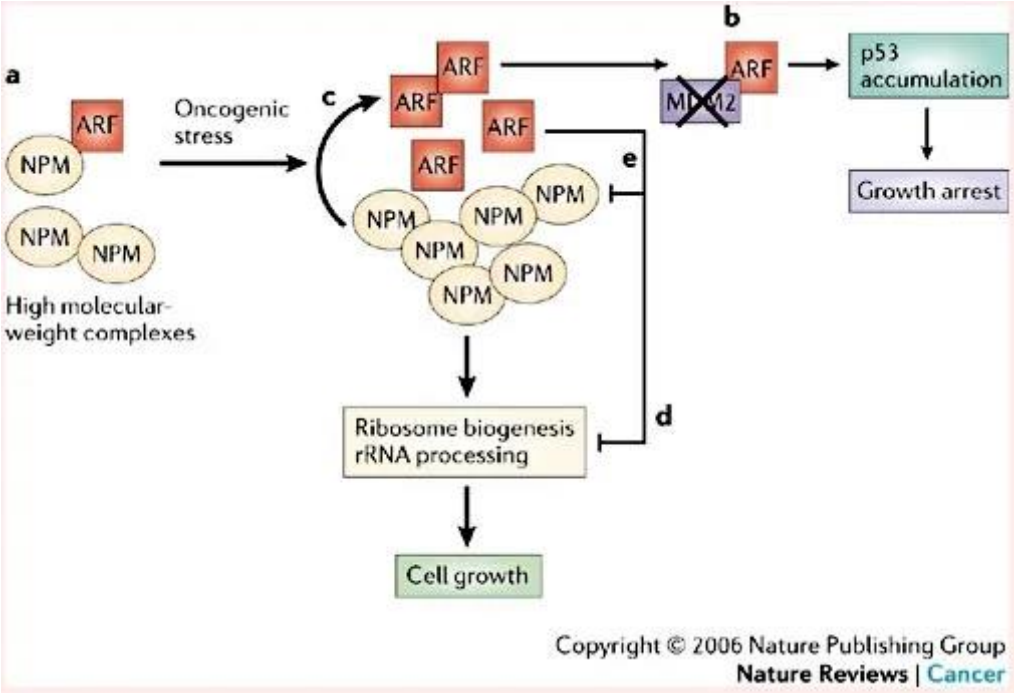


Figure 8. NPM and $p14^{ARF}$ control each other's stability and/or activity (57)**.**

a.Nucleophosmin (NPM) associates with $p14^{ARF}$ in the nucleolus. Increased expression of both $p14^{ARF}$ and NPM occurs in response to oncogenic stimulation. $p14^{ARF}$ activates and promotes both p53-dependent (b) and p53-independent growth-arrest pathways (c–e). b. $p14^{ARF}$ inhibits MDM2, which leads to p53 activation and the suppression of cell proliferation. c-e. Higher levels of NPM facilitate the accumulation of $p14^{ARF}$ by stabilizing it, whereas $p14^{ARF}$ negatively regulates ribosomal RNA processing (d), and even opposes NPM nucleo–cytoplasmic shuttling activity (e).

In addition to its tumor suppressor role, $p14^{ARF}$ is involved in the survival of cancer cells suggesting its pro-oncogenic function (119, 120)

D. Post-translational modifications of NPM1

Post-translational modifications (PTM) refer to covalent addition of functional groups on one or several amino acids. These include SUMOylation, ubiquitination and phosphorylation that aids in regulating the activity and the fate of many proteins including NPM1.

1. NPM1 SUMOylation

The ubiquitin-related SUMO (SUMOylation) system is conserved in all eukaryotes (121) and three SUMO isoforms were identified in mammals (SUMO-1, SUMO-2, and SUMO-3) (122, 123). SUMOylation is a reversible reaction. The de-modification process is called de-SUMOylation and involves specific proteases, referred to sentrin specific Isopeptidase (SENP). Six SENP (SENP-1, 2, 3, 5, 6, 7) were identified in humans (124). SENP1 and SENP2 dissociate SUMO-1 and SUMO-2/3 proteins, while SENP3, 5, 6 and 7 dissociate only SUMO-2/3 protein (125) (126).

a. SUMOylation/de-SUMOylation and Ribosomal biogenesis

The nucleolus is the main site for SUMOylation and de-SUMOylation. Among the six SENP proteins, SENP3 and SENP5 are heavily concentrated in the nucleolus (127, 128). Importantly, the tumor suppressor protein $p14^{ARF}$, which is also localized in the nucleolus, is involved in the SUMOylation of several nucleolar proteins including NPM1 (129-133). $p14^{ARF}$ inhibits the maturation of the 28S rRNA by interfering with NPM1 function (117, 118). Conversely, restoration of functional NPM1 is achieved by SENP3, which is involved in the processing of 32S rRNA to 28S rRNA then to 5.8S rRNA (134). Consequently, SENP3 and $p14^{ARF}$ balance each other's functions in modulating the SUMO status of NPM1, thus regulating ribosomal biogenesis (Figure 9).

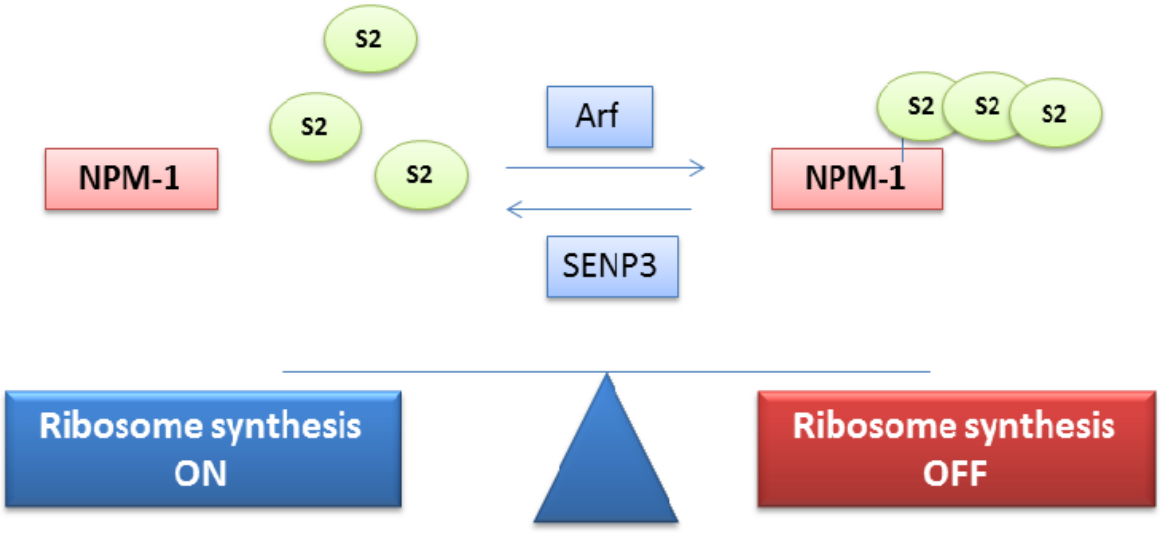


Figure 9. p14^{ARF}, SENP3, NPM1, and ribosomal biogenesis

SUMOylation –DeSUMOylation of NPM-1 is a crucial part of the regulatory network controlling ribosome synthesis and cell proliferation

2. NPM1 ubiquitination

NPM1 is known to be mono-ubiquitinated by E3 ubiquitin ligase BRCA1-BARD1 (BRCA1-associated RING domain protein) complex in a process not linked in protein degradation (135). In addition, it was reported that p14^{ARF} induces polyubiquitination and degradation of overexpressed NPM1 (67) while the ubiquitin specific peptidase 36 (USP36) deubiquitinates NPM1 leading to its stabilization (136).

CHAPTER 3: *NPM1* MUTATIONS IN AML

A. Types of NPM1 mutations

NPM1 mutations are one of the most frequent mutations in AML accounting for 30% of the cases; and the most common genetic alterations (50-60%) in AML patients with normal cytogenetic karyotype (CN-AML) (137). Most *NPM1* mutations are restricted to exon-12, are heterozygous and result in a dominant negative mutant for NPM1 altering its function. Immunohistochemistry staining of blasts from NPM1c-AML patients revealed that the wild type NPM1 (wtNPM1) dimerizes with NPM1c via a conserved N-terminal dimerization domain (138, 139). All these mutations lead to an ectopic translocation of the mutant protein (NPM1c) to the cytoplasm (140).

NPM1 mutations are named in the alphabetical order according to their discovery time (types A, B, C & D and others) (141) (Figure 10). Mutation A is the most common mutation accounting for 80% of the cases (142). It involves the duplication of TCTG (nucleotides 956–959), creating an insertion at position 960 (143).

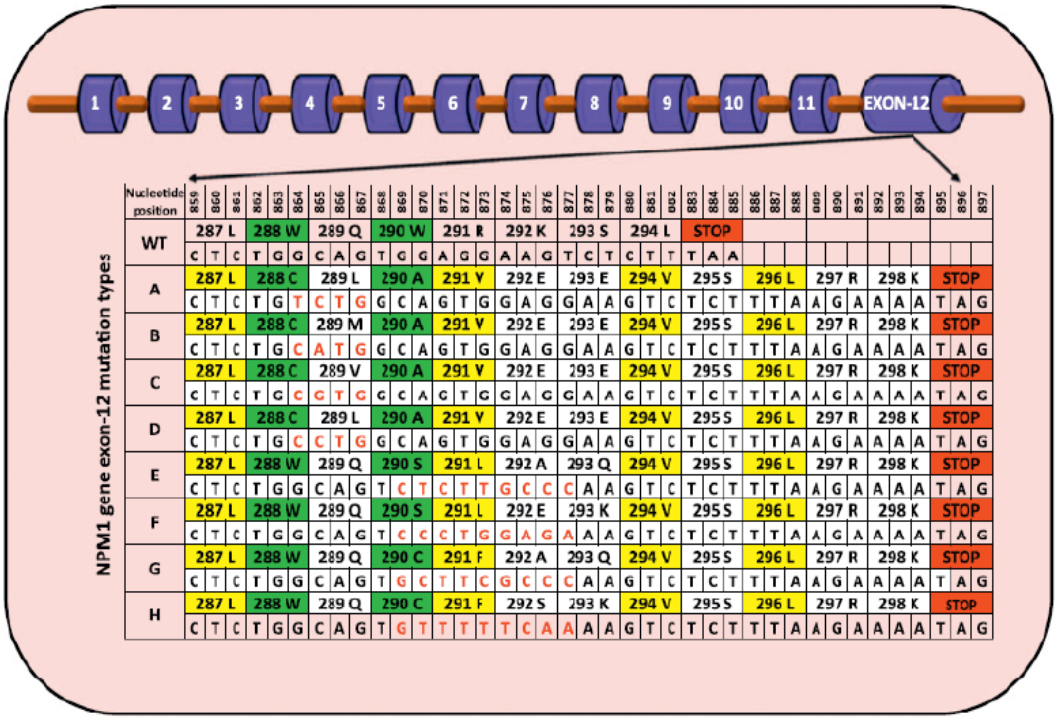


Figure 10. Types of NPM1 exon-12 mutations in AML (144)

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.

B. Consequences of NPM1 mutations

1. Abnormal trafficking of NPM1c

All mutations of NPM1 result in a frameshift in their C-terminal domain. Hence, the last seven amino acids (WQWRKSL) are replaced with 11 different residues (137). This mostly affects tryptophan residues 288 and/or 290 leading to the loss of the NoLs and creates an additional third NES (139). This additional NES motif enhances the binding of NPM1c protein to the nuclear export receptor exportin 1, and translocates it to the cytoplasm (Figure 11).

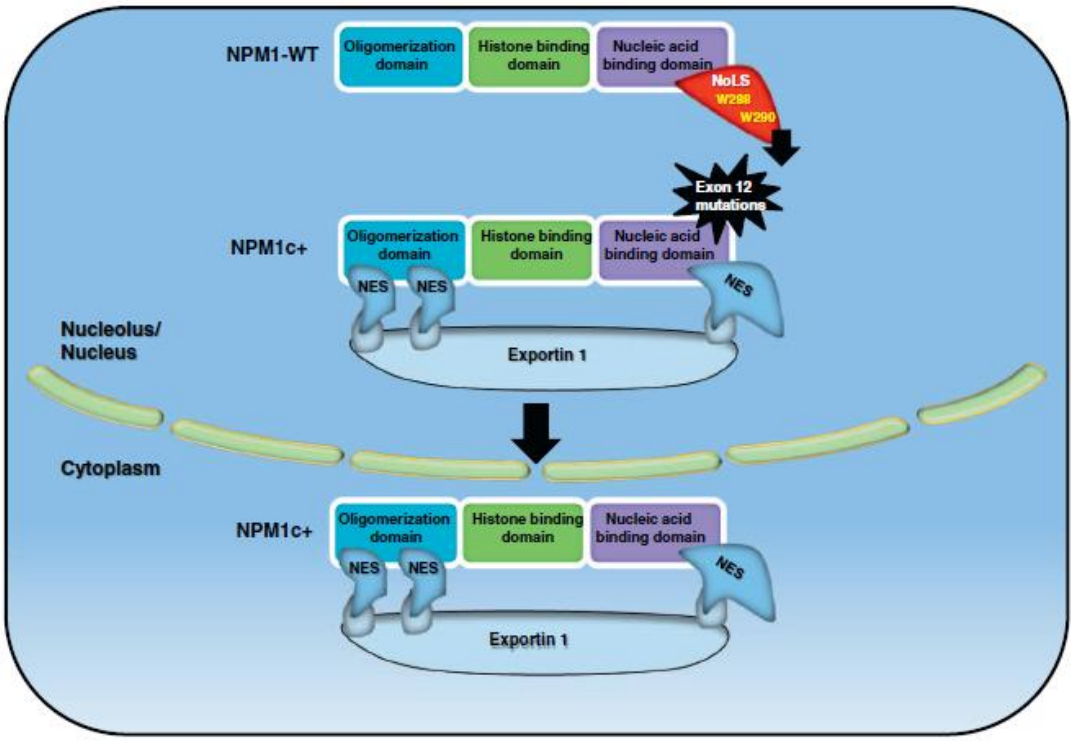


Figure 11. Abnormal trafficking of NPM1c (144)

Exon 12 mutations result in the loss of the C-terminal NoLs and a new NES is created. Exportin 1 binds to NES bearing NPM1c and exports it into the cytoplasm.

2. NPM1c effect on interacting proteins

Given its pleiotropic functions, *NPM1* mutations drive leukemia through a combination of loss of functions and gain of functions in distinct cellular processes. Since $p14^{ARF}$ is stabilized upon its interaction with NPM1, NPM1c is unable to stabilize $p14^{ARF}$ in the cytoplasm, leading to its degradation and the reduction of its half-life (138, 145) (Figure 12a). As a result, MDM2 will remain bound to p53, favoring its inactivation and its proteosomal degradation. Thus, $p14^{ARF}$ delocalization inhibits its ability to induce cell cycle arrest and this may lead to leukemogenesis. In contrast, in $p14^{ARF}$ null cells, NPM1c shows

normal anti-proliferative activity (138). Consequently, the perturbation in $p14^{ARF}$ function is not sufficient to explain the leukemogenic transformation in NPM1 mutation.

It was reported that wt-NPM1 regulates the turnover of the c-Myc oncoprotein, by stabilizing F-box protein Fbw7γ, an E3 ligase complex, and maintaining its nucleolar localization (146). NPM1c interacts with Fbw7γ, inducing delocalization to the cytoplasm and thus its degradation. As a result, Myc oncoprotein is overexpressed in cells expressing NPM1c (146) (Figure12b). Although high Myc levels induces $p14^{ARF}$ and p53 dependent cell cycle arrest, cells expressing NPM1c have this pathway turned off due to $p14^{ARF}$ cytoplasmic delocalization (146). These changes could lead to leukemogenesis.

Transgenic mouse model expressing NPM1c showed that NPM1c alone is not sufficient to induce AML (147-150). Similarly, knock-in mouse models revealed the same outcome of myeloproliferation and features of NPM1c AML, but it couldn't initiate leukemia (150). Thus the exact mechanism of NPM1c mutation driving AML is not yet defined.

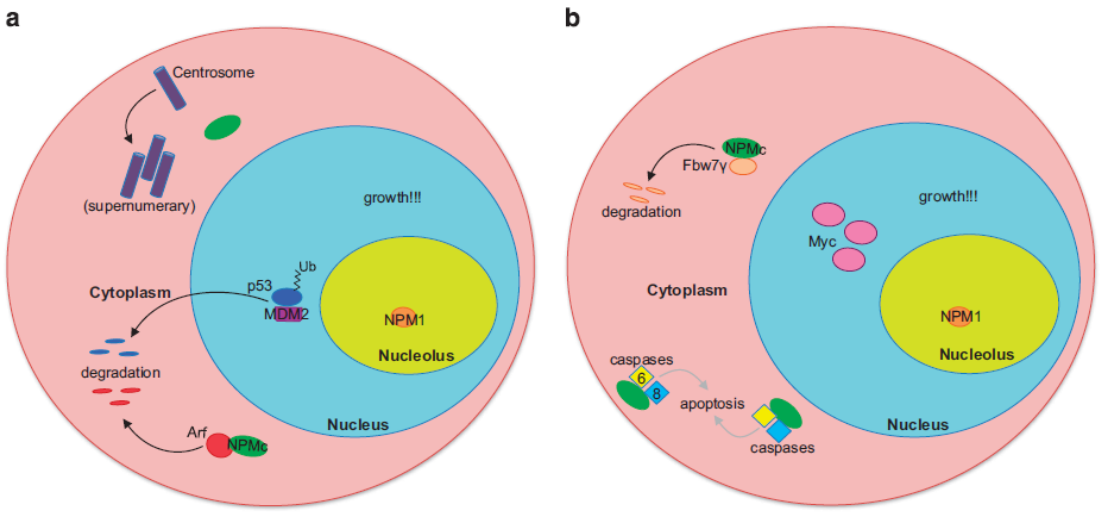


Figure 12. NPM1c and leukemogenesis (103).

(a) NPM1c interacts with and sequesters $p14^{ARF}$ to the cytoplasm, reducing its stability and causing its degradation; p53 ubiquitination by MDM2 causes p53 degradation, antagonizing growth inhibition; NPM1 haplo-insufficiency leads to supernumerary centrosomes. (b) NPM1c binds and delocalizes Fbw7γ to the cytoplasm, decreasing stability and causing degradation; without Fbw7γ, Myc oncogene levels increase, promoting growth and proliferation; NPM1c interacts with and inhibits caspase-6/-8, indirectly promoting growth.

C. Targeted therapies in NPM1c AML

Since important tumor suppressors are deregulated by NPM1c ($p14^{ARF}$, p53, PTEN,…), NPM1c can be an excellent therapeutic target. Several drugs were designed to target multiple deregulated aspects in NPM1c. These include:

1. NSC348884

NPM1 exists in dimers and oligomers through its N- terminus oligomerization domain (57). NPM1c heterodimerizes with wtNPM1 and translocates it to the cytoplasm. NSC348884 (N, N, N′, N′-tetrakis [(5- methyl-1H-benzimidazol2-yl) methyl] ethane-1, 2-diamine) was identified as an NPM1 inhibitor that disrupts its ability to oligomerize. NSC348884 induces apoptosis (151) and activates p53 and its downstream effector p21 in AML cell lines (152). Moreover, a synergistic effect was observed between NSC348884 and ATRA selectively in cells expressing NPM1c (153).

2. Dactinomycin

Dactinomycin (also known as actinomycin D), is considered one of the first antibiotics isolated from soil bacteria, and displaying an anticancer activity (154). It has a role in inhibiting ribosomal biogenesis by intercalating GC pairs, repressing the action of RNA polymerase I (155). Falini et al. reported the therapeutic advantage of dactinomycin in *NPM1c* AML patients in the absence of FLT3 ITD. Dactinomycin led to complete morphological and

immune-histochemical remission after two cycles of therapy and complete molecular remission after the fourth cycle (156).

3. Selinexor

Selinexor ((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 Exportin 1 (XPO1) inhibitor. Exportin 1 mediates the export of leucine rich nuclear export signal- dependent protein from the nucleus to the cytoplasm. NPM1c acquires a third NES which is a key player in leukemogenesis (89) (see Figure 11). Selinexor induces anti-leukemic effects in cultured and primary AML cells by inhibiting the translocation of NPM1c. It is also implicated in upregulating p53 and CEBPA and thus induces myeloid differentiation (157). The limitation of XPO1 inhibitors is a lack of specificity, whereby it targets, in addition to NPM1c, all shuttling proteins and molecules from the nucleus to the cytoplasm.

4. Oridonin

Oridonin is a bitter tetracycline chemotherapeutic agent used in Chinese medicine. Its use was reported in colon cancer, pancreatic cancer, and hepatocellular cancer as well as with hematological malignancies (158). Li et al. showed that oridonin induces apoptosis in *NPM1c* AML cell lines, restores nucleolar translocation of NPM1 and upregulates p53 and $p14^{ARF}$ (158).

5. Avrainvillamide

Avrainvillamide is a natural alkaloid product with an antiproliferative role on different cancer cell lines. It binds to several proteins, including NPM1c by S-alkylation of its cysteine residues (159). Mukherjee et al reported that avrainvillamide restores the nucleolar

localization of NPM1c and proves that the interaction between avrainvillamide and Cys275 of NPM1c mediates this relocalization (160).

6. All-trans retinoic acid and arsenic trioxide

All-trans retinoic acid (ATRA) alone or in combination with arsenic trioxide (ATO) is used for the treatment of APL (161). In AML, some studies suggested that addition of ATRA to conventional chemotherapy improves survival, selectively in *NPM1c* AML patients (162). We and others showed that ATRA and ATO synergistically induce proteosomal degradation of NPM1c; leading to differentiation and apoptosis in AML cell lines or primary blasts derived from *NPM1c* AML (163). Treatment of a small number of NPM1c elderly AML patients with ATRA and ATO reduced bone marrow and peripheral blood blasts. However, blast counts re-increased upon discontinuation of treatment (163) suggesting that ATRA and ATO exert transient anti-leukemic activities.

7. Epigallocatechin-3-Gallate (EGCG)

Hoang et al showed that EGCG down regulates the expression of NPM1, inhibits cell proliferation, and induces apoptosis in cells expressing NPM1c by but not in cells which have wtNPM1 (164).

CHAPTER 4: EAPB0503

A. Imiquimod

Imiquimod (Figure 13), (S-26308, R-837) (1-(2-methylpropyl)-1H-imidazo [4, 5-c] quinolin-4-amine), is the first member of the imidazoquinolone family, and belongs to the class of medications called immune response modifiers. This nucleoside analogue of the imidazoquinoline family was the first immune response modifier used for the treatment of infectious skin conditions and shown great anti-viral and anti-tumor activities *in vivo* (165). This agent was FDA approved in 1997 for the topical treatment of external peri-anal warts by increasing the activity of the body's immune system. This drug is also efficacious as a topical therapy for certain types of skin cancers: basal cell carcinoma, Bowen's disease, superficial squamous cell carcinoma, some superficial malignant melanomas, cutaneous B-cell lymphomas and actinic keratosis (166).

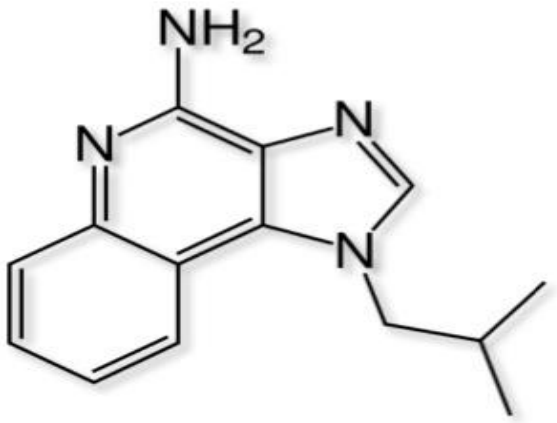


Figure 13. Chemical structure of imiquimod

The exact mechanism of action by which imiquimod activates the immune system is not yet known. Nevertheless, it is known that imiquimod activates immune cells by ligating the

Toll-like receptor 7 (TLR-7), commonly involved in pathogen recognition, on the cell surface (167). There is evidence that imiquimod, when applied to skin, can lead to the activation of Langerhans cells, which subsequently migrate to local lymph nodes to activate the adaptive immune system (167). Other cell types activated by imiquimod include natural killer cells, macrophages, and B lymphocytes.

B. EAPB0503

The initials EAPB stand for Equipe d'Accueil Pharmacochimie et Biomolécules (the laboratory where the compounds are being synthesized) and the numbers "0" for imidazo [1, 2-a] quinoxaline series, "5" for methoxyphenyle, and "03" for methylamine substituents.

1. Chemical structure

Imiquimod (1-(2-methylpropyl)-1*H*-imidazo [4, 5-*c*] quinolin-4-amine) is made up of a quinoline component, a 1*H*-imidazole ring and a methylpropyle group. In EAPB0503 (1-(3-methoxyphenyl)-*N*-methylimidazo [1, 2-*a*] quinoxalin-4-amine, the quinoline part is modified into quinoxaline, maintaining the three intracyclic nitrogen in the imidazoquinoxaline rings. Imidazoquinoxaline differ from Imiquimod by the presence of bridgehead nitrogen between the quinoxaline and imidazole rings, which both constitute the tricyclic heterocyclic core of the compounds. The amine (-NH2) group of the Imiquimod molecule is replaced by methylamine (-NHCH3) in EAPB0503. The methylamine group seems to be essential for increased potency of this compound (168). The methylpropyle group of the Imiquimod was substituted with 3-methoxyphenyle in EAPB0503, probably increasing polarity and binding properties of this compound (Figure 14).

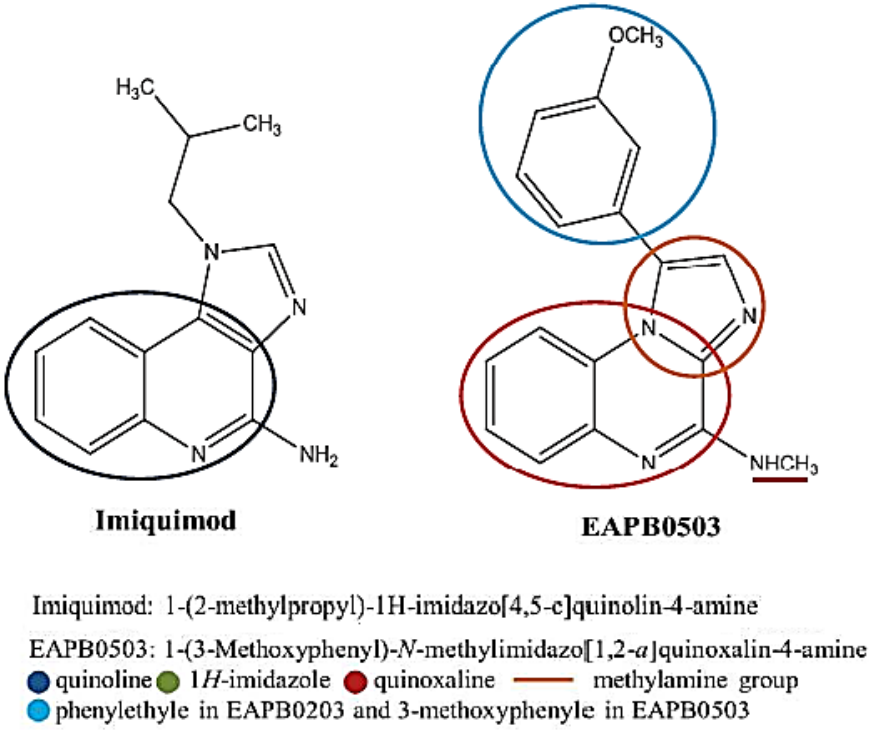


Figure 14. Chemical structural differences between Imiquimod and EAPB0503

2. Imidazoquinoxaline and cancer treatment

EAPB0503 displays higher cytotoxicity than Imiquimod, against melanoma cells (169). EAPB0503 induced cell cycle arrest and apoptosis in chronic myeloid leukemia cells by degrading the fusion BCR-ABL oncoprotein (170). Importantly, our group demontsrated that EAPB0503 displays a potent and selective activity in *NPM1c* AML. Indeed, Nabbouh et al. showed that EAPB0503 induces growth inhibition and apoptosis in *NPM1c* AML cell lines, in a time and dose dependent manner. Apoptosis was accompanied by the dissipation of MMP and PARP cleavage. EAPB0503 selectively targets NPM1c proteosomal degradation and restores wt-NPM1 to the nucleolus. *In vivo*, EAPB0503 selectively reduces leukemia bone marrow burden in NPM1c AML xenograft mice (171).

AIM OF THIS STUDY

NPM1 is one of the most frequently mutated proteins in AML. Our group previously demonstrated that EAPB0503 exerts a selective and potent activity against *NPM1c* AML. In this study, we investigated the molecular basis of EAPB0503 potency. We explored its effect on the p53/MDM2 axis and SENP3/NPM1/$p14^{ARF}$ interplay. We also examined the long-term effect of EAPB0503 on the survival, organ infiltration and NPM1/$p14^{ARF}$ in *NPM1c* AML xenograft mice.

MATERIALS AND METHODS

A. Cell lines

OCI-AML2 (from Dr. H. de Thé) and OCI AML3 cells (from Dr. D Bouscary) (Table 6) were used in this study. Cells were grown in minimum essential medium alpha (MEM-α) supplemented with 10 and 20% fetal bovine serum for AML2 and AML3 respectively. Primary AML cells from patients' BM were extracted as described by (El Hajj et al. 2015) after approval by the Institutional Review Board at the American University of Beirut and after consented agreement of patients according to Helsinki's Declaration.

Table 6. Characteristics of the used AML cell lines

AML cell line	**Description**	**Origin and source**	**Morphology**	**FLT-3/ p21/p53 status**	**NPM-1 status**
OCI-AML3	Acute myeloid leukemia	Peripheral blood Male, 57 years	Single, round to oval cells	Wild-type	**Mutant**
OCI-AML2	Acute myeloid leukemia	Peripheral blood Male, 65 years	Single, round to oval cells	Wild-type	**Wild-type**

B. EAPB0503

EAPB0503 was synthesized as described by Deleuze-Masquefa *et al* (169, 172). Synthesis was further optimized by microwave-assisted chemistry (173). EAPB0503 powder was dissolved in dimethylsulfoxide (DMSO) (Amresco, OH, USA) at a stock solution of at 0.1 M, aliquoted, and stored at -20°C.

C. Generation of cells expressing NPM1 or NPM1c

Hela cells were transfected with PE-GFP expressing wt-NPM1 or NPM1c (from Clontech) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations and were grown in Dulbecco's modified Eagle's medium.

D. Cell viability

OCI-AML2 and OCI-AML3 cells were seeded at a concentration of $2x10^5$/ml. Cell growth was assessed using the trypan blue exclusion dye assay. EAPB0503 was tested at the concentration of 1 μM as described (171). Cell viability and molecular studies were analyzed at three different time points of treatment (6, 24 and 48 hours).

E. Immunoblotting

After 6, 24 or 48h of treatment with EAPB0503, total protein was extracted in 50 μl of Laemmli buffer (Bio-Rad laboratories, Hercules, California, USA) added to the harvested pellets. Proteins were quantified using Nanodrop (Thermo Scientific, ND-1000, Massachusetts, USA). Equal amount of proteins were loaded on 12% SDS-PAGE gel and transferred onto nitrocellulose membranes, which were blocked and probed with the following antibodies (Table 7).

Table 7. List of antibodies used this study

Antibody	Company	Catalog Number	Mouse/rabbit	Dilution
NPM1c	Thermo Fisher SCIENTIFIC	PA1-46356	Rabbit	1:1000

SENP3 (D20A10) XP	Cell Signaling	5591	Rabbit	1:1000
MDM2 [2A10]	Abcam	ab16895	mouse	1:250
p21 Waf1/Cip1 (12D1)	Cell Signaling	2947	Rabbit	1:1000
Phospho-p53 (Ser15)	Cell Signaling	9284	Rabbit	1:1000
$p14^{ARF}$	Thermo Fisher SCIENTIFIC	MA5-14260	mouse	1:200
p53 (DO-1) sc-126	Santa Cruz Biotechnology	D1717	mouse	1:200
c-Myc (9E10)	Thermo Fisher SCIENTIFIC	13-2500	Mouse	1:200
b-actin (8H10D10)	Cell Signaling	3700	Mouse	1:1000
GAPDH	Abnova	MAB5476	Conjugated	1:20000

F. Immunofluorescence and confocal microscopy

OCI-AML2 and OCI-AML3 were fixed with ice-cold methanol at -20°C for 20 minutes and cytospun onto glass slides. Immunostaining was performed with a rabbit polyclonal antibody against NPM1c (Invitrogen), and a monoclonal anti-NPM1 recognizing both wt. and mutated NPM1c (Abcam mouse AB10530). Primary antibodies were revealed by Alexa Fluor 488– or Fluor 594–labeled secondary antibodies (Abcam). Staining of nuclei was performed with 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen). Images were acquired by confocal microscopy using a Zeiss LSM710 confocal microscope (Zeiss, Oberkochen, Germany), and images were processed using Zen 2009 (Carl Zeiss).

G. Proximity ligation assay (PLA) and confocal microscopy

Hela cells were fixed with paraformaldehyde onto glass coverslips. Protein-protein interactions were visualized using the Duolink *in situ* proximity ligation assay (PLA) system (Olink Bioscience) following the manufacturer's instructions. Anti-SUMO2/3 (Santa Cruz sc-32873), anti-GFP (Santa Cruz (B-2): sc-9996) antibodies were used. Staining of nuclei was performed with DAPI (Invitrogen). Images were acquired by confocal microscopy using a Zeiss LSM710 confocal microscope (Zeiss, Oberkochen, Germany), and images were processed using Zen 2009 (Carl Zeiss).

H. Xenograft Animal Studies

NOD/Shi-scid IL2rγ-/- (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-AML2 cells were injected into the tail vein of eight-week-old mice (12 mice per group). 7 days post AML cells' injection, mice were treated intraperitoneally with EAPB0503 (2.5mg/kg, 50μg/mouse) every other day over a period of three weeks. EAPB0503 was dissolved in DMSO and diluted in equal volume of lipofundin (vehicle) before intraperitoneal administration to mice. Six mice per condition were sacrificed for bone marrow (BM) flushing, CD45 stain and organ infiltration while the remaining six mice were kept to monitor survival.

I. Human CD45 staining

Three weeks post-treatment with EAPB0503, BM cells were flushed from the femurs and tibias of euthanized animals. Cell surface staining was performed on 100µl of the sample using 20µl of anti-human CD45 PerC-P antibody (BD#345809). Labeled samples were analyzed on a Guava flow cytometer. BM cells were also used to assess proteins $p14^{ARF}$ and NPM1c levels by western blot.

J. Histopathology

Livers from either treated or untreated mice were fixed in neutral buffer formalin (Sigma-Aldrich), embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy.

K. Statistical analysis

All *in vitro* experiments described in this study were run in at least three independent experiments. Data were reported as the average ± standard deviations. Statistical analysis was done using Student's t test p-value of less than 0.05 was considered as significant.

RESULTS

A. EAPB0503 prolongs the survival of OCI-AML3 xenograft mice, reduces BM leukemia burden

To assess the effect of EAPB0503 on *NPM-1c* AML xenograft mice, eight-week old NSG mice were intravenously injected with OCI-AML2 and OCI-AML3 cells. Seven days post-injection, xenograft mice were treated intraperitoneally with EAPB0503 every other day over a period of 3 weeks, according to timeline described in Figure 15A. By the end of week four after AML cells inoculation, a group of six mice was assessed for survival. While untreated control mice, or OCI AML2 treated mice succumbed at day 40, EAPB0503 selectively prolonged the survival in OCI AML3 xenograft mice for up to 100 days (Figure 15B).

To understand the molecular basis of this prolonged survival, a group of six mice were sacrificed at the end of treatment. BM cells of femurs and tibias were flushed, and stained for hCD45, a prototypic receptor-like protein tyrosine phosphatase expressed on all nucleated hematopoietic cells (174, 175). Flow cytometry analysis revealed that OCI-AML3 burden in the BM of xenograft mice was significantly reduced from 47% to 25% upon EAPB0503-treatment ($p<0.05$) (Figure 15C), as compared to OCI-AML2 burden (24% in untreated versus 34% in EAPB0503 treated mice) (Figure 15C).

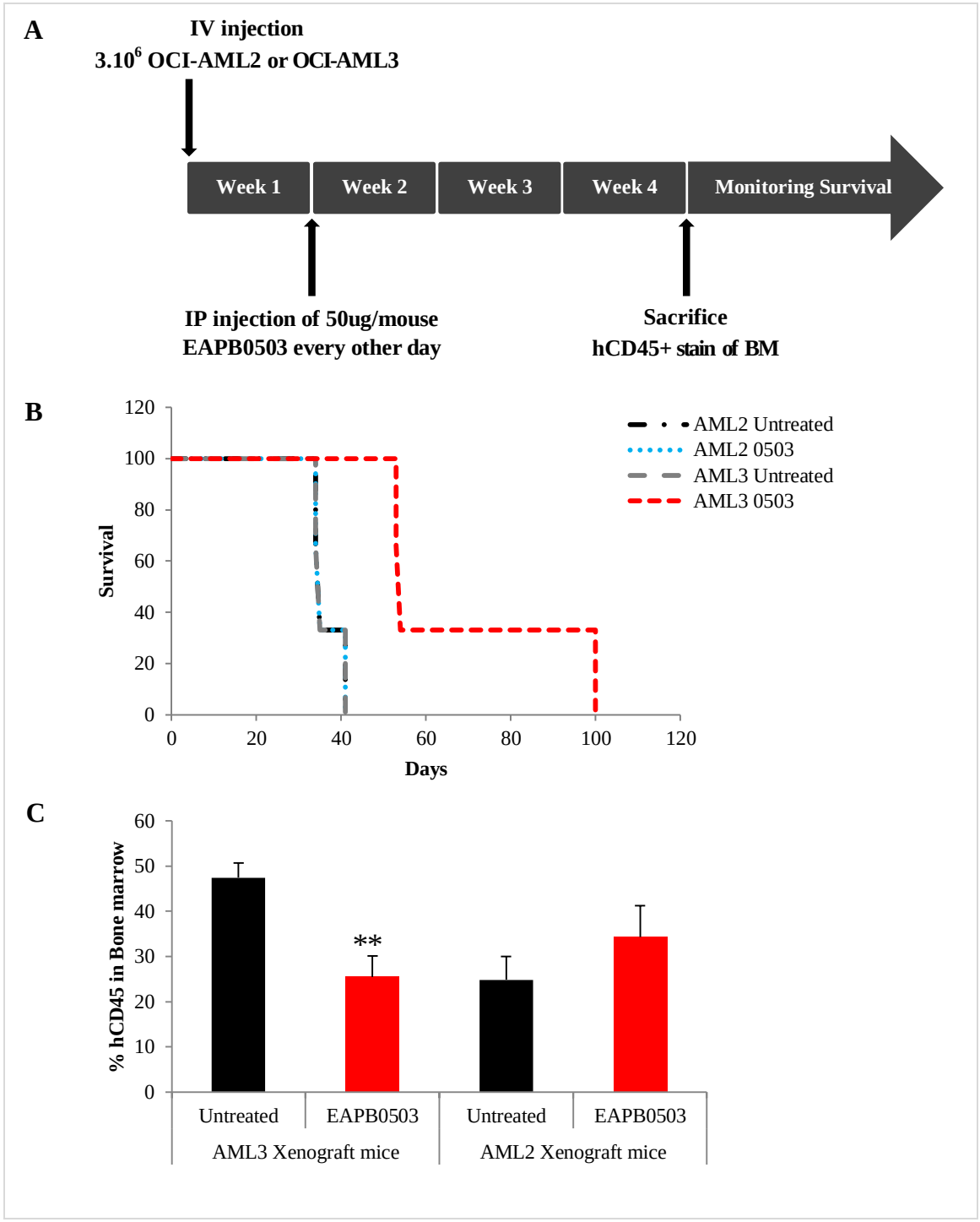


Figure 15. EAPB0503 prolongs the survival and reduces the leukemia bone marrow burden in OCI-AML3 xenograft NSG mice.

(A) Eight-week-old female NSG mice were injected with OCI-AML2 or OCI-AML3 cells intravenously (12 mice per cell line per condition). EAPB0503 was intraperitoneally administered every other day for 3 weeks. By the end of week 3 of treatment, one group of six mice per condition was monitored for survival. The bone marrow of the remaining six mice per condition was harvested from femurs and tibias, and then stained with the anti-hCD45 antibody. (B) Kaplan–Meier overall survival of untreated NSG mice injected with OCI-AML2 or OCI-AML3 (n=6, Black line and Gray line respectively) or treated with EAPB0503 (n=6, blue line and red line respectively). (C) Histograms showing the hCD45 PerCP percentage in xenograft animals (n=6 per condition). Black histograms show hCD45 stained BM from untreated OCI-AML3 and OCI-AML2 xenograft mice. Red histograms show stained BM-derived OCI AML3 and OCI-AML2 cells with the hCD45 antibody from EAPB0503-treated animals. The t-test was performed to validate significance. *, ** and *** indicate p values $\leq$ 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

B. EAPB0503 decreases the leukemic infiltration into the liver of OCI-AML3 NSG mice:

Liver failure due to blast infiltration was reported in AML patients (176). Upon sacrifice, gross pathology of the liver revealed pale color and white nodules in untreated xenograft mice. EAPB0503 treatment showed a normal gross macroscopy of livers (Figure 16A). Consistent with these results, H&E stain showed a clear infiltration of the liver with OCI AML3 cells in untreated xenograft mice. EAPB0503 treatment preserved the normal architecture of the liver with a very low number of infiltrating leukemic cells (Figure 16B). These results illustrate the potency of EAPB0503 against leukemic blast infiltration into the liver of *NPM1c* AML xenografted animals.

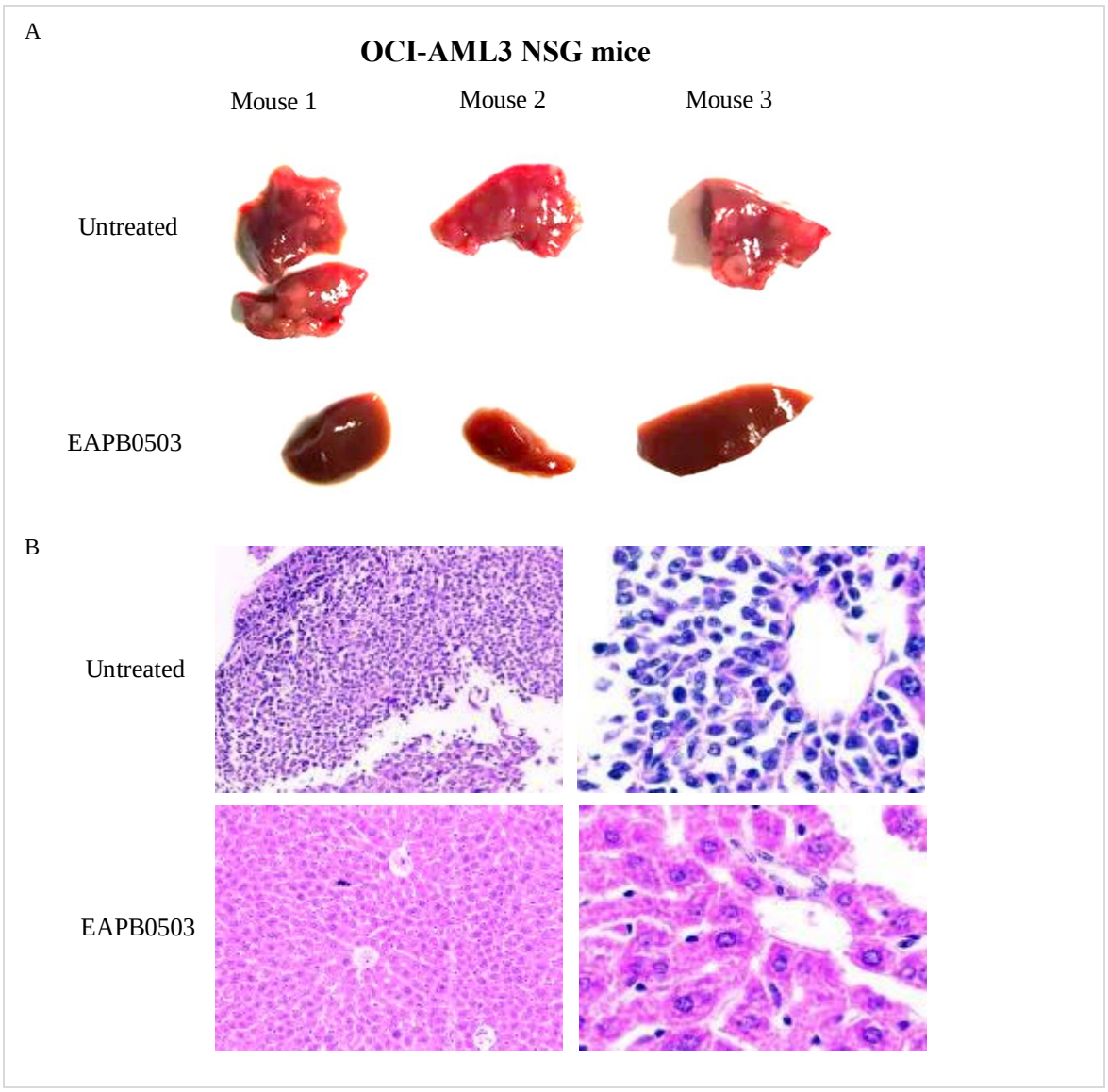


Figure 16. EAPB0503 reduced leukemic infiltration of OCI-AML3 blasts into the liver of xenograft mice.

(A) Gross pathology of livers of three representative untreated (upper panel) or EAPB0503-treated (lower panel) OCI AML3 xenograft mice. (B) Histological analysis (H&E stain) of the liver of a representative untreated or EAPB0503 treated OCI-AML3 xenograft mice (left panel, magnification 10x, right panel, magnification 40x).

C. EAPB0503 induces NPM1c degradation and $p14^{ARF}$ upregulation in the BM of OCI-AML3 NSG mice

Given that EAPB0503 prolonged the survival and reduced the BM leukemic burden exclusively in OCI-AML3 xenograft mice, we tested the expression level of NPM1c in the BM of treated versus untreated mice. Consistent with the beneficial selective effect of EAPB0503 against *NPM1c* AML xenograft mice, EAPB0503 induces NPM1c degradation and $p14^{ARF}$ upregulation in the BM blasts of OCI-AML3 xenograft mice (Figure 17).

Altogether, these results show the promising therapeutic potency of EAPB0503 *in vivo* and expand our previously published data to demonstrate prolonged survival and selective reduced leukemia burden in BM of *NPM1c* AML xenograft mice, following NPM1c degradation.

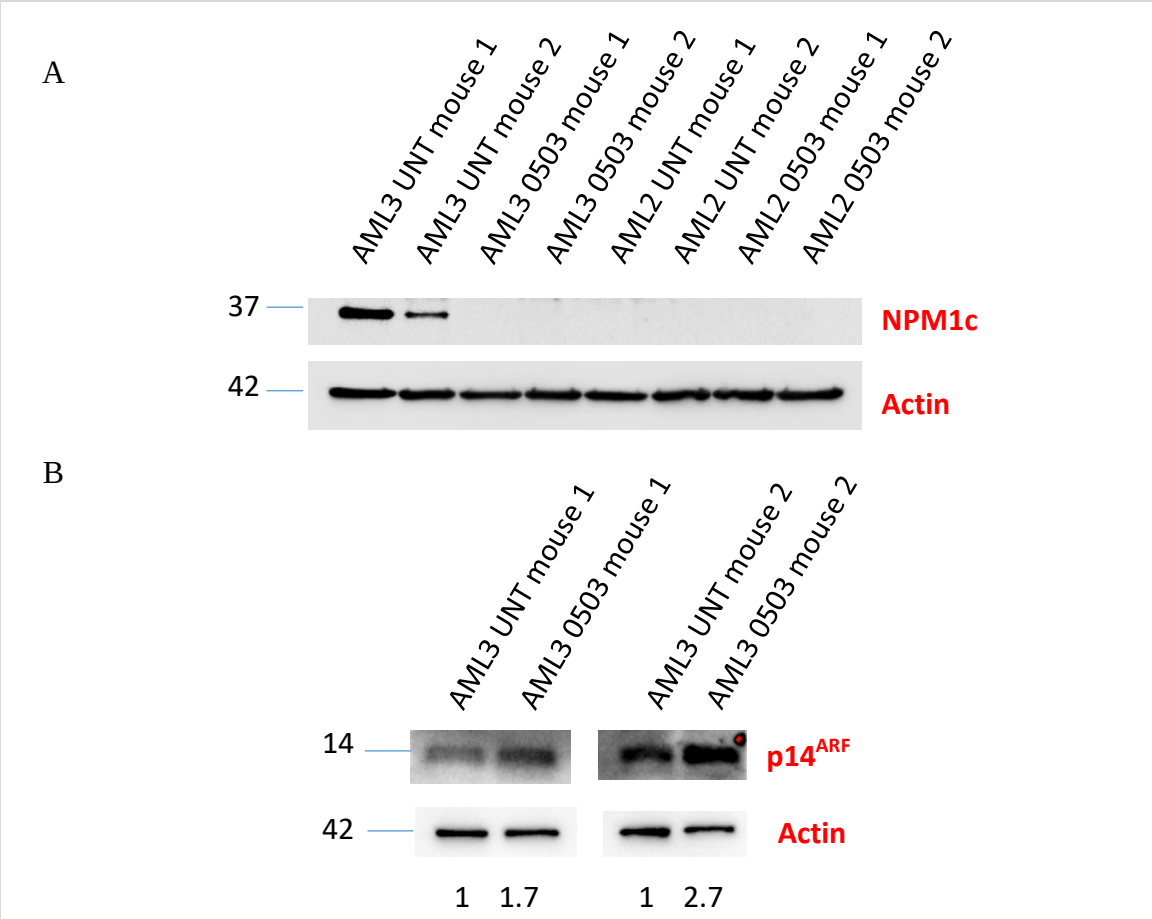


Figure 17. EAPB0503 induces NPM1c degradation in the BM of OCI-AML3 NSG mice

A. Western blot of NPM1c in BM cells from NSG mice xenografted with OCI-AML3 or OCI-AML2 cells (2 representative mice out of six are shown), after in vivo treatment with EAPB0503. B. Western blot of $p14^{ARF}$ in BM cells from NSG mice xenografted with OCI-AML3 (2 representative mice out of six are shown). Eight-week-old NSG mice were injected with 3 million OCI-AML3 or OCI-AML2 cells intravenously. At day 7 post-leukemic cells injection, EAPB0503 was administered intraperitoneally every other day, over a period of 3 weeks.

D. EAPB0503 induces growth inhibition in OCI-AML3 cell line

To further explore the molecular mechanisms of EAPB0503 potency, and due to the small amount of blasts obtained from BM of xenograft mice, we investigated the effect of this drug *in vitro*. We started by reproducing the results obtained by Nabbouh et al, and tested earlier

time points. For that purpose, we assessed the effect of EAPB0503 on the cell growth and viability of the two cell lines, OCI-AML2 and OCI-AML3, harboring wt-NPM1 and NPM1c respectively. We chose the optimal drug concentration of 1 μM representing the inhibitory concentration IC50 of the drug (171). Consistent with the published results, EAPB0503 resulted in a pronounced and selective time-dependent growth inhibition of OCI-AML3 cells, at 24-, and 48-hours post-treatment ($p<0.001$, Figure 18). This effect wasn't observed in OCI-AML2 cells with similar growth after 24-, and 48-hours treatment compared to untreated controls. These results reproduce the selectivity of EAPB0503 against *NPM1c* AML cells.

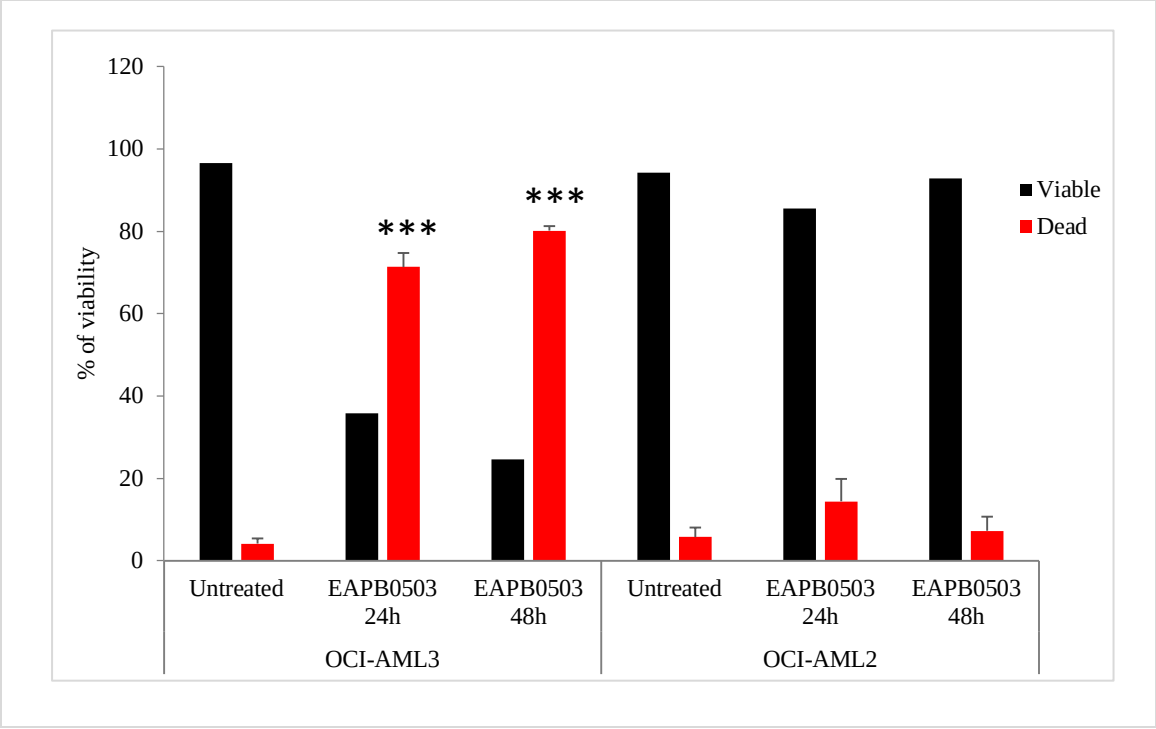


Figure 18. EAPB0503 induces selective growth inhibition in OCI-AML3 cells.

Trypan blue assay of OCI-AML2 and OCI-AML3 cells at 24-, and 48-hours post-treatment with EAPB0503. Black histograms represent the percentage of viable cells. Red histograms represent the percentage of dead cells. The results represent the average of at least 3 independent experiments. The t-test was performed to validate significance. *, ** and ***

indicate p values ≤ 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

E. OCI-AML3 express high SENP3 and MDM2 and low p53 protein levels

NPM1 interacts with p53 regulatory molecules MDM2 activating the MDM2-p53 pathway (112). In addition, NPM1 is involved in ribosomal biogenesis through a balance between SENP3 and $p14^{ARF}$ (134). We first screened the effect of NPM1c on the basal levels of SENP3 and demonstrated that, OCI-AML3 cells expressing NPM1c (Figure 19A), exhibit high levels of SENP3 as compared to OCI AML2 (Figure 19B). High SENP3 expression may indicate a sustained ribosomal biogenesis, presumably playing a role in protein synthesis supporting the leukemic properties of these cells.

We then investigated the p53/MDM2 pathway and demonstrated that OCI AML3 express high MDM2 basal protein levels (Figure 19C). Consistent with this data, p53 basal levels were low and p53 was inactive in OCI AML3 (Figure 19D). Our results demonstrate that NPM1c inhibits p-p53 by upregulating its ubiquitin ligase MDM2, presumably to inhibit apoptosis and confer survival properties to these cells.

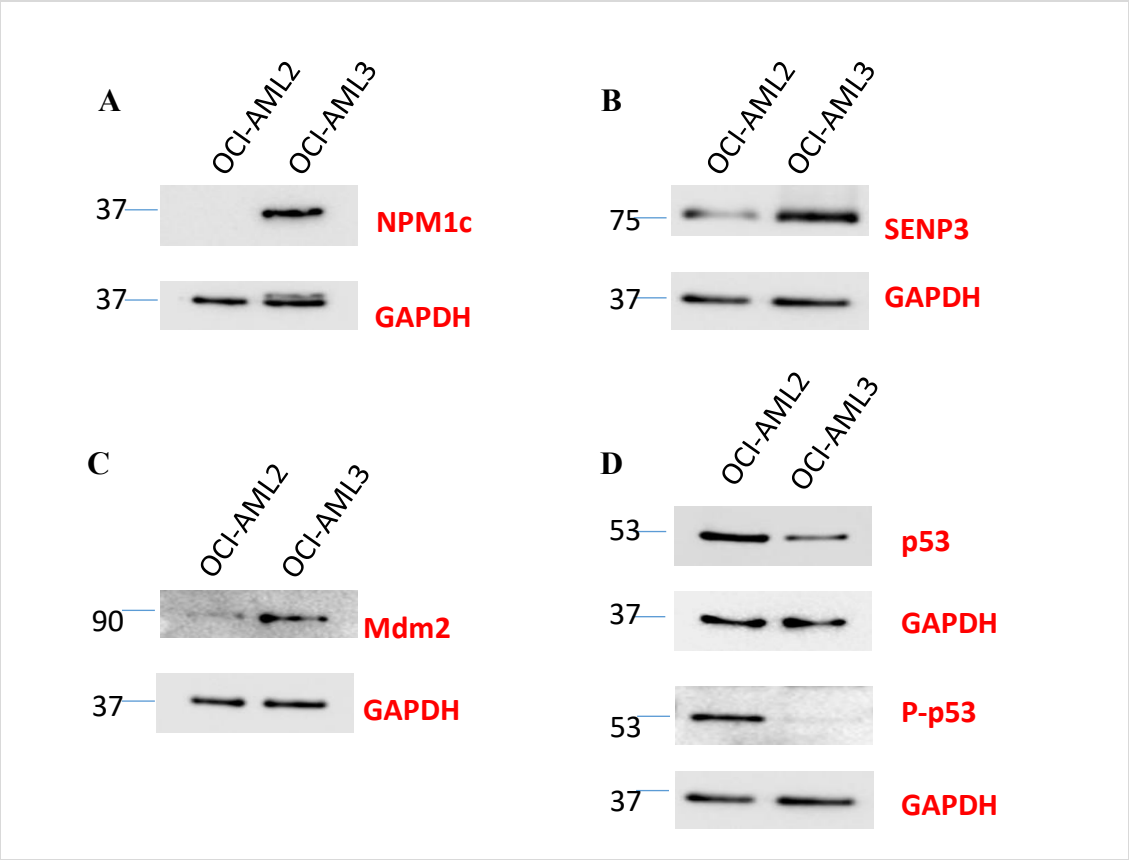


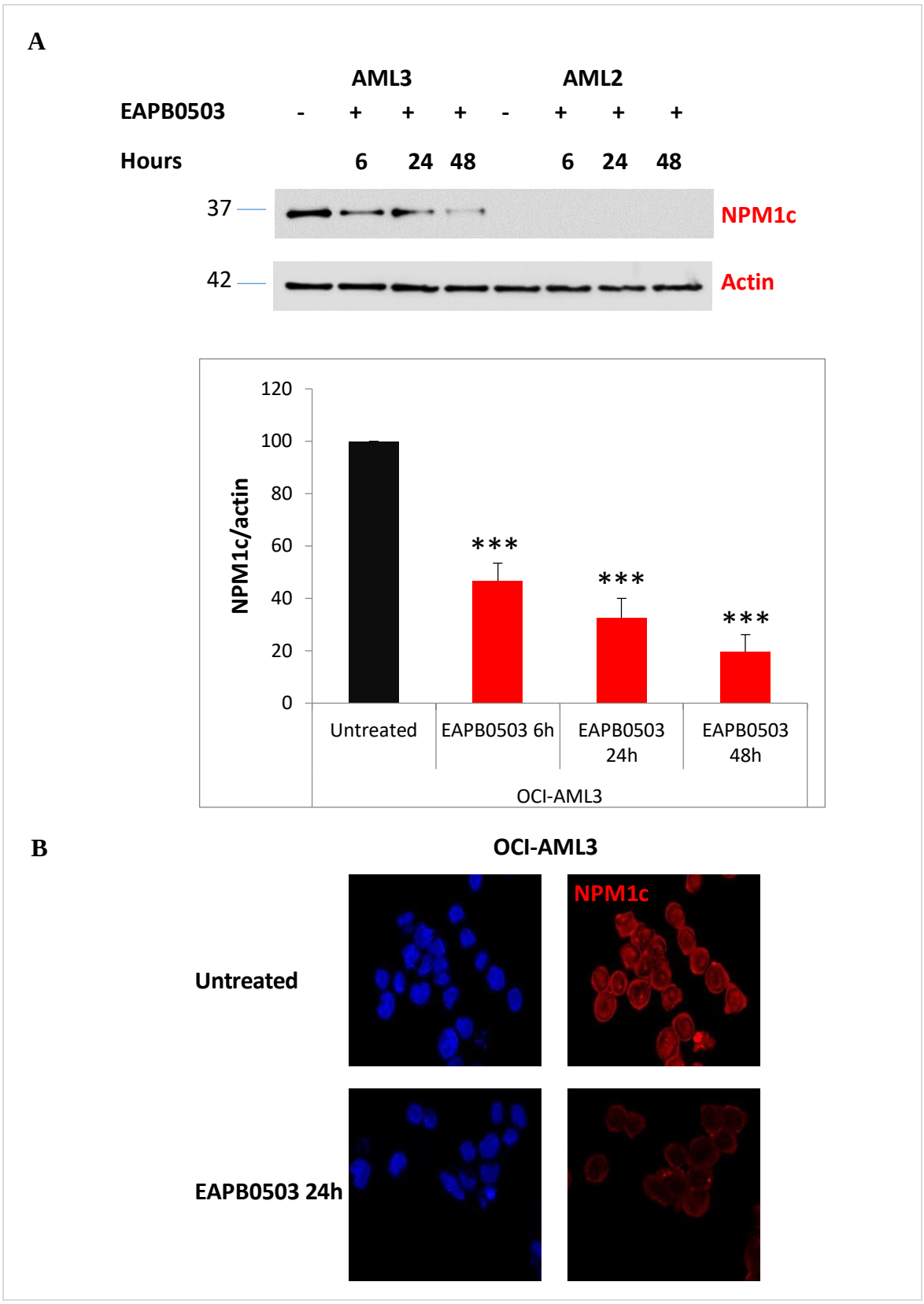
Figure 19. Basal level protein expression of cells expressing NPM1c and wt-NPM1.

Western blot analysis of NPM1c (A), SENP3 (B), MDM2 (C), p53 and P-p53 (D) in OCI-AML2 and OCI-AML3 cells depicted from one representative experiment out of at least three independent experiments.

F. EAPB0503 induces NPM1c degradation as early as 6h and restores the wild type NPM1 nucleolar localization in OCI-AML3 at 24h post-treatment

We tested the expression and localization of NPM1c after EAPB0503 treatment at earlier time points (6h) (171). Upon treatment, EAPB0503 triggered NPM1c downregulation in a time dependent manner. Indeed, NPM1c degradation was initiated at 6 hours post-treatment in OCI-AML3 cells (Figure 20A, $P<0.001$). Results were confirmed using Immunofluorescence assays (Figure 20B).

In *NPM1c* AML, NPM1c oligomerizes with wt-NPM1 and translocates it to the cytoplasm (140). Consistent with published reports, we observed the cytoplasmic localization of total NPM1 in untreated OCI-AML3 cells after probing with anti-NPM1 (wt+c) antibody. Upon treatment, EAPB0503 restored the nucleolar localization of the remaining NPM1 protein in OCI-AML3 cells (Figure 20C, upper panel), whereas nucleolar localization of NPM1 was not affected in OCI-AML2 treated cells (Figure 20C, lower panel). While NPM1c degradation started at 6h, restoration of nucleolar localization of wt-NPM1 was not observed until 24h post-treatment with EAPB0503. Hence, our results demonstrate that NPM1c degradation occurs earlier than the re-localization of wt-NPM1 into the nucleolus.


A
AML3
AML2
EAPB0503
- + + + - + + +
Hours
6 24 48 6 24 48
37
NPM1c
42
Actin
120
100
80
60
40
20
0
NPM1c/actin
***
***
***
Untreated
EAPB0503 6h
EAPB0503 24h
EAPB0503 48h
OCI-AML3
B
OCI-AML3
NPM1c
Untreated
EAPB0503 24h

C

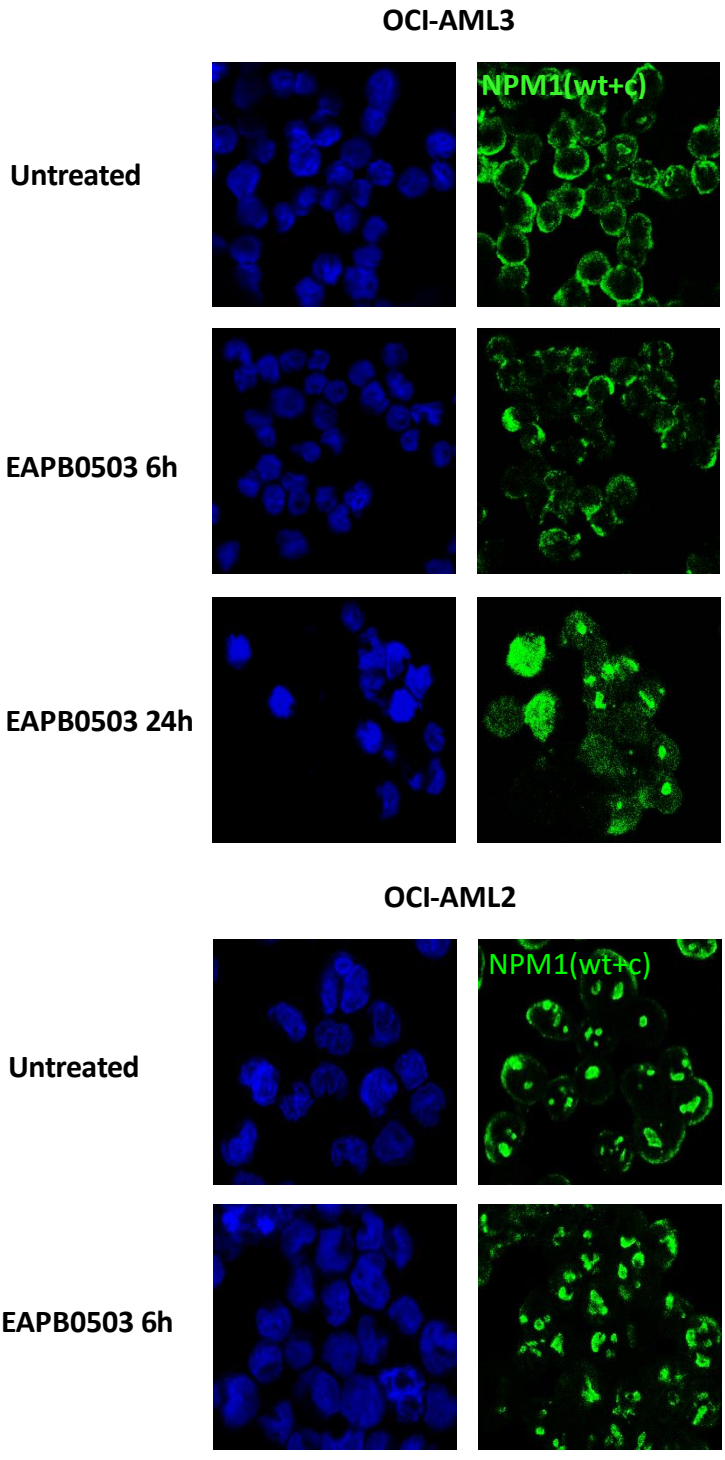
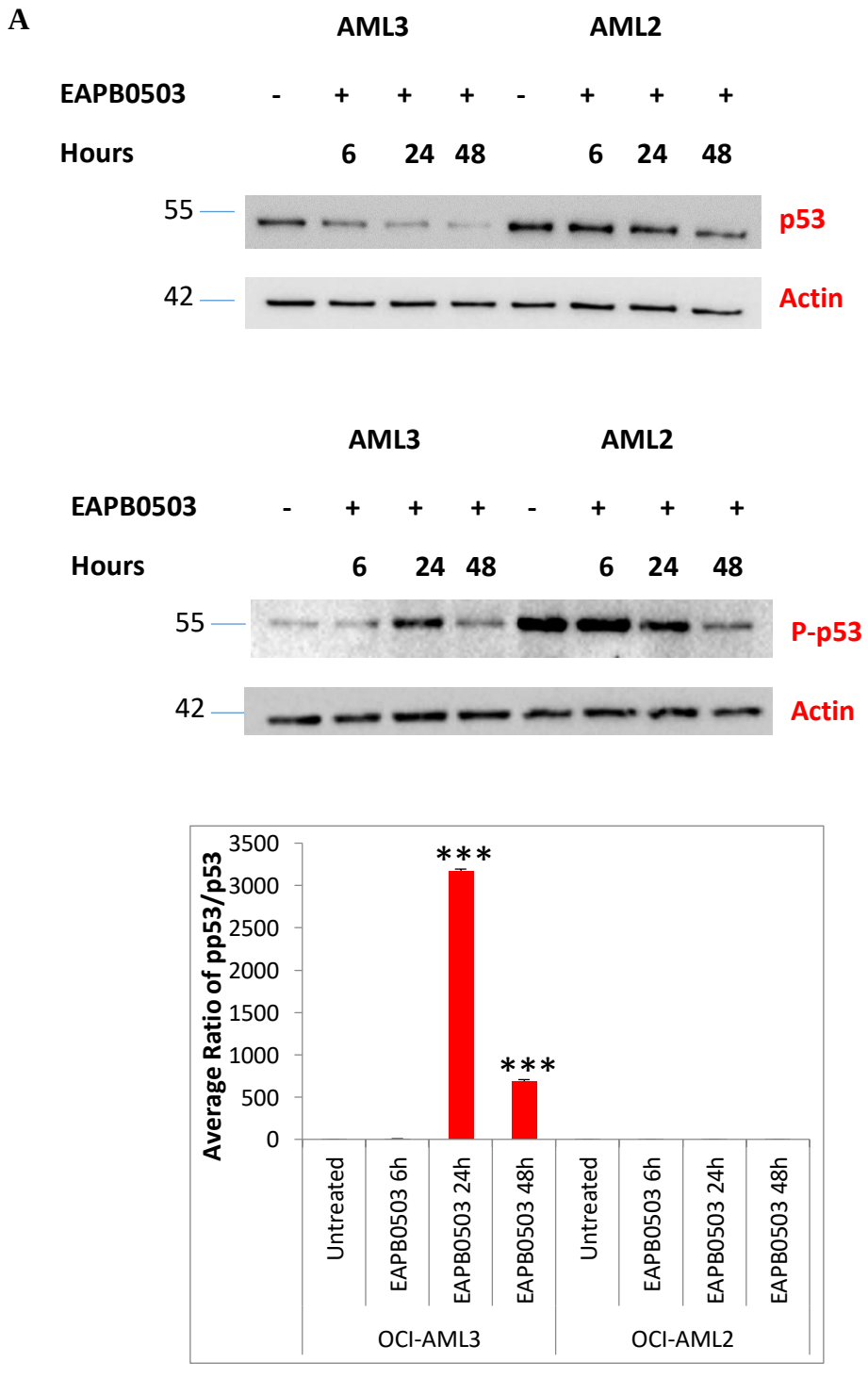

OCI-AML3
NPM1(wt+c)
Untreated
EAPB0503 6h
EAPB0503 24h
OCI-AML2
NPM1(wt+c)
Untreated
EAPB0503 6h

Figure 20. EAPB0503 induces degradation of the NPM1c protein as early as 6h and restores the correct wt-NPM1 nucleolar localization after 24h of treatment of the OCI-AML3 cell line.

(A) Western blot analysis of NPM1c in OCI-AML2 and OCI-AML3 cells depicted from one representative experiment. Black histograms and red histograms represent the densitometry of NPM1c/actin of untreated or EAPB0503-treated OCI-AML3 at 6-, 24-, or 48h in at least three independent experiments. *, ** and *** indicate p values $\leq$ 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant. (B) Confocal microscopy analysis of NPM1 localization in OCI-AML3 cells after treatment with EAPB0503 for 24 hours. NPM1c was stained with an antibody recognizing only NPM1c (red), and nuclei were stained with DAPI (blue). (C) Confocal microscopy analysis of NPM1 localization in OCI-AML2 or OCI-AML3 cells after treatment with EAPB0503 for 24 hours. NPM1 was stained with an antibody recognizing both NPM1 (wt+c) (green), and nuclei were stained with DAPI (blue).

G. EAPB0503 activates p53 signaling pathway in OCI-AML3

We previously demonstrated that EAPB0503 induces apoptosis of NPM1c cells following activation of the p53 pathway (171). We investigated the time point at which this pathway is activated by exploring p53, its phosphorylated form P-p53, its downstream effector p21 and its ubiquitin ligase MDM2. P-p53 is upregulated significantly starting 24h post-treatment exclusively in OCI-AML3 cell line ($p<0.001$), with no significant effect in OCI-AML2 cell line (Figure 21A). Surprisingly, p21 upregulation was concomitant with the early degradation of NPM1c at 6h post-treatment ($p<0.001$) (Figure 21B), to reach its maximum 24 and 48h post-treatment, likely indicating that the activation of p21 might be p53-independent. In addition, we observed a gradual degradation of MDM2 protein levels, that reached its maximum at 48h post treatment selectively in OCI-AML3 cell line ($p<0.001$) (figure 21C). These results support the notion that p53 activation through its phosphorylation and MDM2 degradation orchestrate the pro-apoptotic activity observed in *NPM1c* AML- EAPB0503 treated cells.


A
AML3
AML2
EAPB0503
- + + + - + + +
Hours
6 24 48 6 24 48
55
p53
42
Actin
AML3
AML2
EAPB0503
- + + + - + + +
Hours
6 24 48 6 24 48
55
P-p53
42
Actin
Average Ratio of pp53/p53
3500
3000
2500
2000
1500
1000
500
0
***
***
Untreated
EAPB0503 6h
EAPB0503 24h
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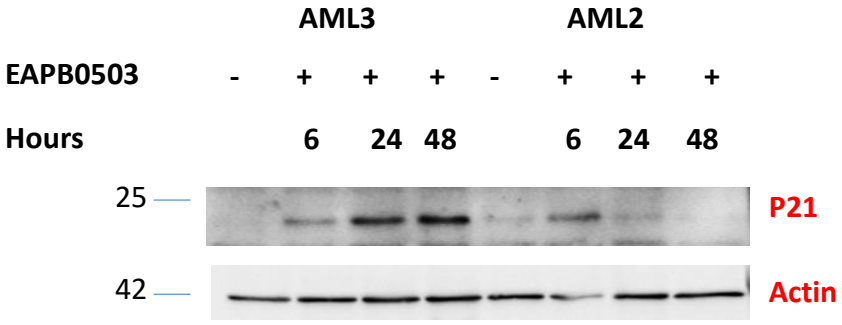
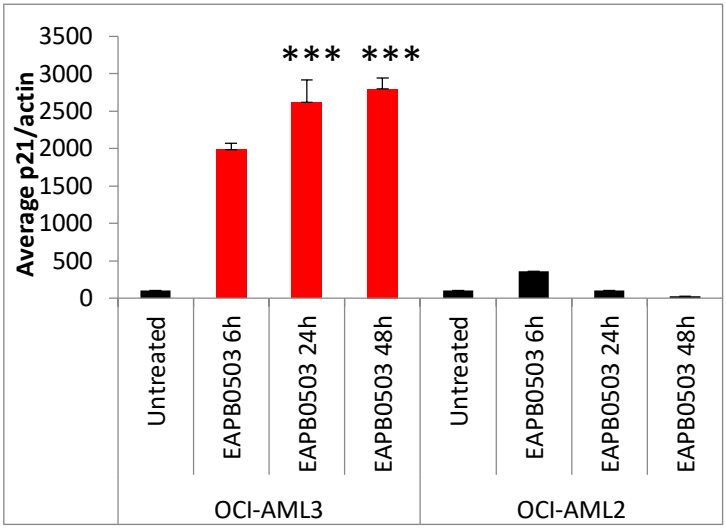
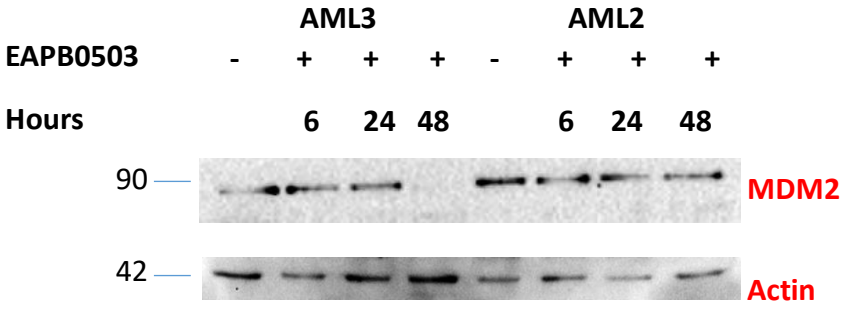
Untreated
EAPB0503 6h
EAPB0503 24h
EAPB0503 48h
OCI-AML3
OCI-AML2

B


AML3
AML2
EAPB0503 - + + + - + + +
Hours 6 24 48 6 24 48
25
P21
42
Actin


Average p21/actin
3500
3000
2500
2000
1500
1000
500
0
*** ***
Untreated
EAPB0503 6h
EAPB0503 24h
EAPB0503 48h
Untreated
EAPB0503 6h
EAPB0503 24h
EAPB0503 48h
OCI-AML3
OCI-AML2

C


AML3
AML2
EAPB0503 - + + + - + + +
Hours 6 24 48 6 24 48
90
MDM2
42
Actin

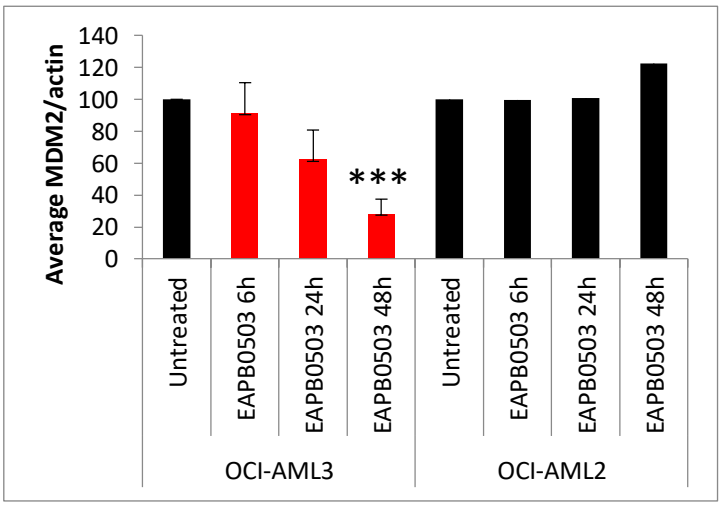


Figure 21. EAPB0503 activates p53 signaling pathway and degrades its ubiquitin ligase MDM2.

(A) Western blot analysis of p53, P-p53, and actin in OCI-AML3 and OCI-AML2 treated with EAPB0503 for 6-, 24-,, 48- hours as indicated, depicted from one representative experiment. Black histograms and red histograms represent the densitometry of the ratio P-p53/p53 of untreated or EAPB0503-treated OCI-AML3 at 6-, 24-, or 48h in three independent experiments. (B) Western blot analysis of p21 in OCI-AML3 and OCI-AML2 treated with EAPB0503 for 6, 24 48 hours as indicated, depicted from one representative experiment. Black histograms and red histograms represent the densitometry of p21/actin of untreated or EAPB0503-treated OCI-AML3 at 6-, 24-, or 48h in three independent experiments. (C) Western blot analysis of MDM2 in OCI-AML3 and OCI-AML2 treated with EAPB0503 for 6, 24 48 hours as indicated, depicted from one representative experiment. Black histograms and red histograms represent the densitometry of MDM2/actin of untreated or EAPB0503-treated OCI-AML3 at 6-, 24-, or 48h in three independent experiments. *, ** and *** indicate p values $\leq$ 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

H. EAPB0503 induces NPM1c degradation and activates p53 pathway in ex-vivo treated NPM1c AML blasts

Primary blasts derived from the BM of three AML patients were treated with EAPB0503. Patient 1 has both *NPM-1* and *FLT-3 ITD* mutations; patient 2 has only *NPM-1* mutation, whereas patient 3 has *wt-NPM-1*. Consistent with our obtained results *in vitro*, EAPB0503

selectively degrades NPM1c and activates p53 signaling pathway in *NPM1c* AML patients (Figure 22). These results validate the efficacy of EAPB0503 on patient-derived blasts.

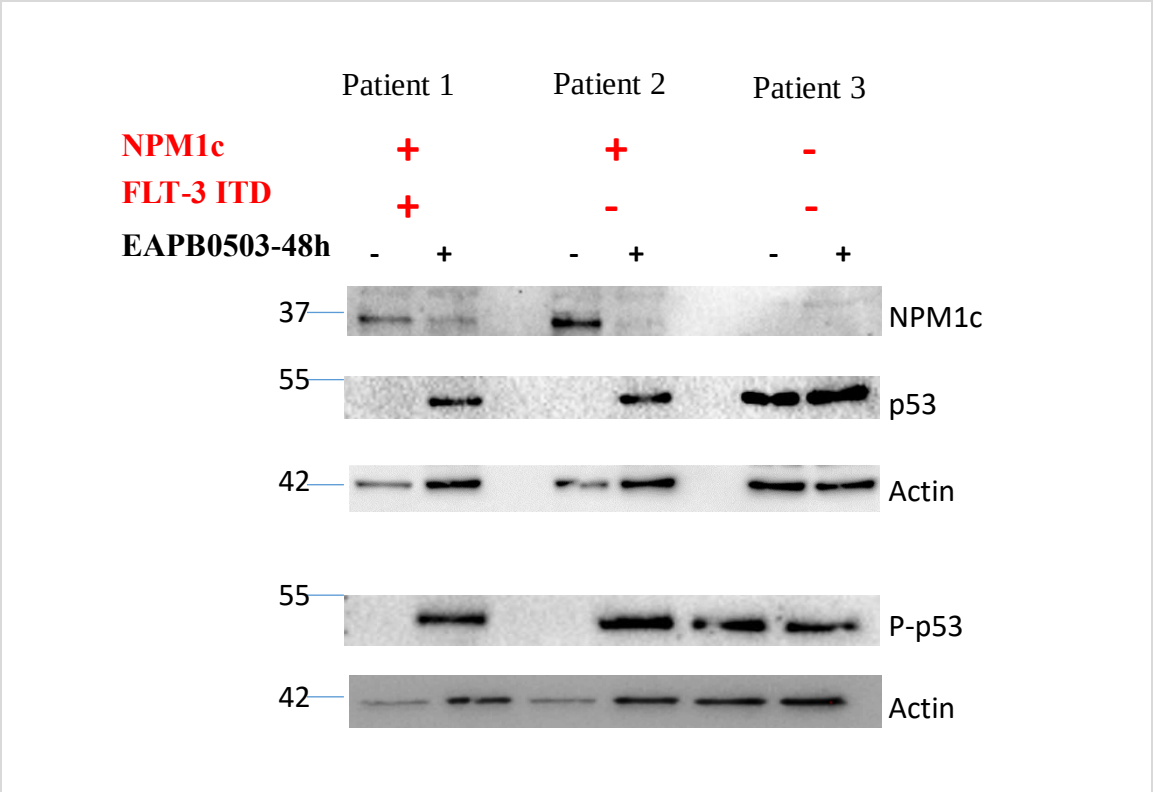


Figure 22. EAPB0503 induces NPM1c degradation and p53 upregulation in NPM1c AML patients.

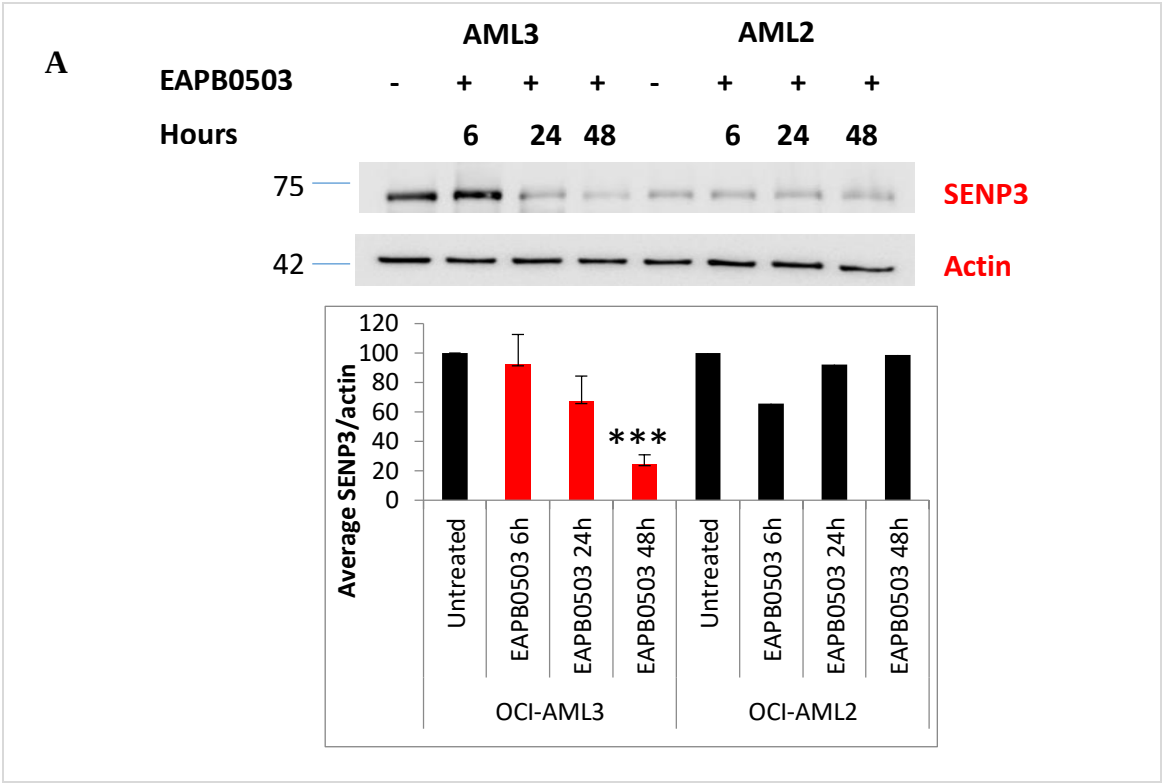
Primary leukemic blasts were harvested from 3 patients and treated with 1 μM EAPB0503 for 48h. Western blot analysis for NPM1c, p53, P-p53, and actin in treated AML blasts as indicated.

I. EAPB0503 induces SENP3 degradation in OCI-AML3 and enhances the SUMOylation of NPM1c by SUMO2/3

NPM1 is involved in ribosomal biogenesis through interplay between SENP3 and $p14^{ARF}$ via cycles of SUMOylation/deSUMOylation. It is well documented that $p14^{ARF}$ SUMOylates NPM1 prohibiting ribosomal biogenesis**.** Conversely, the nucleolar deSUMOylating enzyme SENP3 deSUMOylates NPM1 switching ribosomal biogenesis on (134, 177). We investigated

the effect of EAPB0503 on SENP3 and the SUMOylation status of NPM1c. EAPB0503 induced degradation of SENP3 starting 24h post treatment to reach a highly significant degradation 48h post-treatment (figure 23A).

We then analyzed the effect of EAPB0503 on endogenous SUMO2/3 conjugation with wt-NPM1 or NPM1c in Hela transfected cells, using proximity ligation Duolink assays (PLA). We first confirmed the nucleolar localization of wt-NPM1 and the cytoplasmic localization of NPM1c in Hela transfected with PE-GFP wt-NPM1 and NPM1c plasmids respectively (Figure 23B). Importantly, our results demonstrate that EAPB0503 induces NPM1c SUMOylation by SUMO2/3 at 6 hours post-treatment, while no effect was noticed on Hela cells expressing wt-NPM1 (Figure 23B). These results demonstrate that EAPB0503 induces SUMOylation of NPM1c, and degrades SENP3. These results suggest that SENP3 degradation may lead to the inhibition of ribosomal biogenesis in these cells.



B

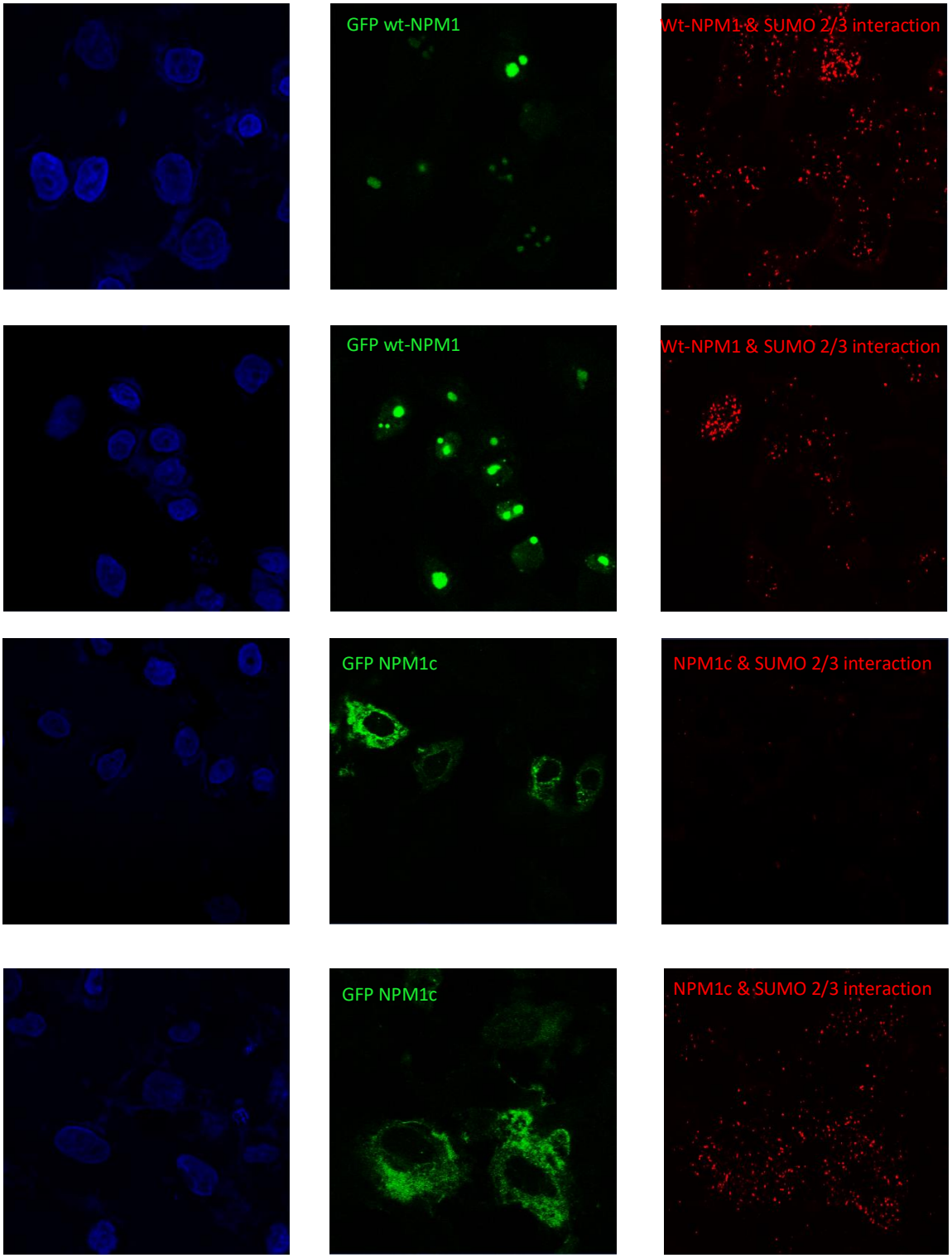

Untreated
GFP wt-NPM1
Wt-NPM1 & SUMO 2/3 interaction
EAPB0503 6h
GFP wt-NPM1
Wt-NPM1 & SUMO 2/3 interaction
Untreated
GFP NPM1c
NPM1c & SUMO 2/3 interaction
EAPB0503 6h
GFP NPM1c
NPM1c & SUMO 2/3 interaction

Figure 23. EAPB0503 degrades SENP3 and enhances SUMOylation of NPM1c**.**

(A) Western blot analysis of SENP3 in OCI-AML3 and OCI-AML2 treated with EAPB0503 for 6-, 24-, 48 hours as indicated, depicted from one representative experiment. Black histograms and red histograms represent the densitometry of the ratio SENP3/actin of untreated or EAPB0503-treated OCI-AML3 at 6-, 24-, or 48h in three independent experiments. The t-test was performed to validate significance. *, ** and *** indicate p values $\leq$ 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant. **(**B) Confocal microscopy analysis of maximal projections of Z-stacks of NPM1 and SUMO2/3 interaction by PLA assay, in Hela cells transfected with PE-GFP wt-NPM1 or PE-GFP NPM1c after their treatment with EAPB0503 for 6 hours (as indicated). Duolink red dots represent endogenous SUMO2/3 interactions with wt-NPM1 or NPM1c. Nuclei were stained with DAPI (blue).

J. EAPB0503 induces c-Myc oncoprotein degradation in OCI-AML3

It was reported that c-Myc oncoprotein is upregulated in cells expressing NPM1c due to the delocalization of its E3 ligase Fbw7ɣ to the cytoplasm favoring its degradation (146). We tested the effect of EAPB0503 on c-Myc oncoprotein. Interestingly, our data demonstrates that EAPB0503 induces a significant c-Myc oncoprotein degradation 24h post-treatment in OCI-AML3 ($p<0.001$). But the effect of EAPB0503 on c-Myc expression is not exclusive to OCI-AML3; it also affects OCI-AML2 c-Myc expression levels upon treatment. This suggests the effect of the drug on c-Myc activity is not related to NPM1c which requires further investigations in this pathway (Figure 24).

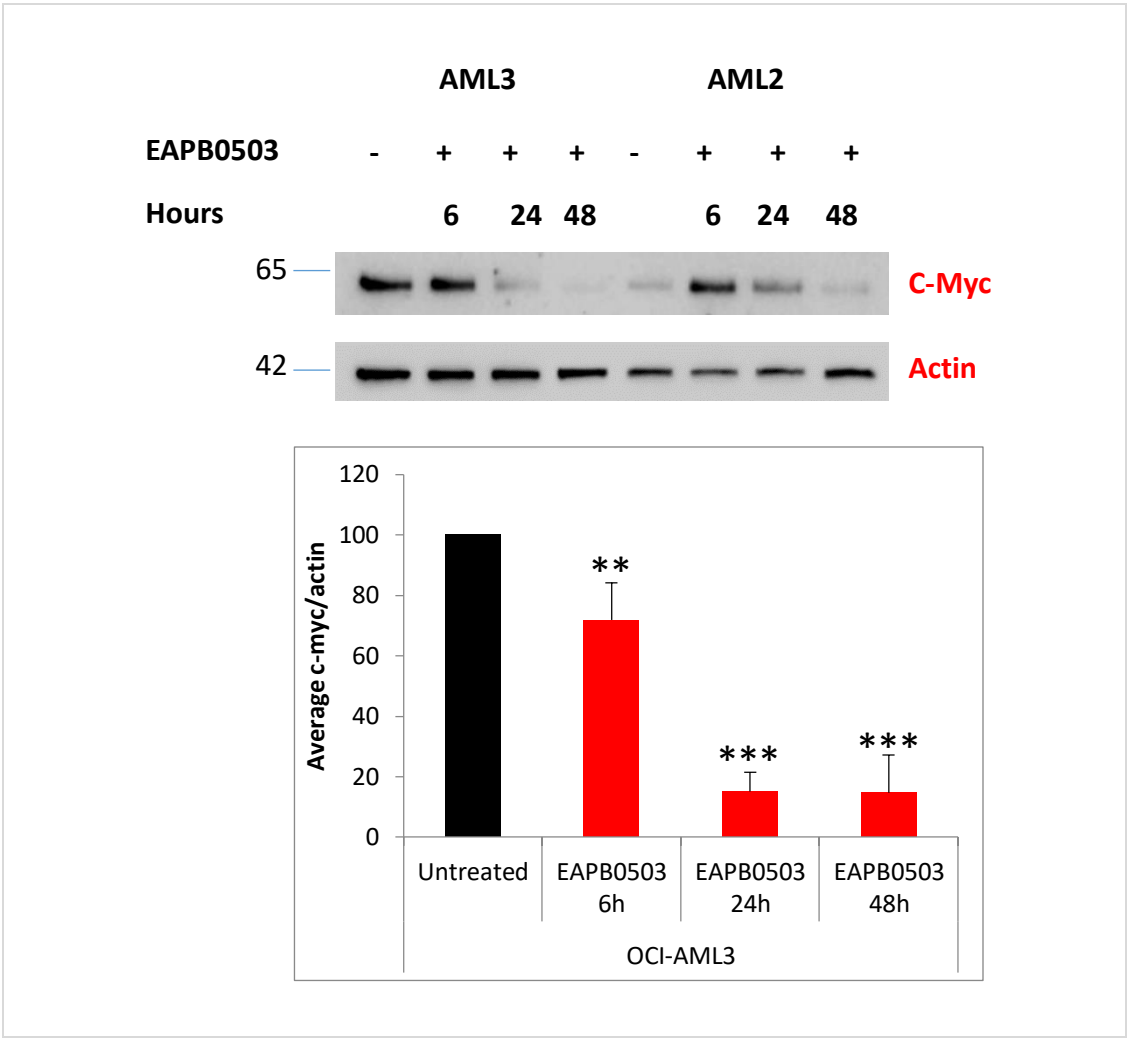


Figure 24. EAPB0503 induces c-Myc oncoprotein degradation in OCI-AML3 cell line**.**

Western blot analysis of c-Myc, and actin in OCI-AML3 and OCI-AML2 treated with EAPB0503 for 6, 24 48 hours as indicated, depicted from one representative experiment. Black histograms and red histograms represent the densitometry of the c-Myc/actin of untreated or EAPB0503-treated OCI-AML3 at 6-, 24-, or 48h in three independent experiments. The t-test was performed to validate significance. *, **and *** indicate p values $\leq$ 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant

DISCUSSION AND CONCLUSION

NPM1 mutations characterize one third of AML patients (137). Although NPM1c alone does not induce leukemia, however it mediates malignancy as observed in transgenic or knock-in mice (150). Recent studies showed that therapies targeting NPM1c, inducing its degradation lead to inhibition of proliferation and cell death of leukemic cells (153, 163, 171, 178). In line with these findings, we demonstrated that EAPB0503 selectively degrades NPM1c and restores the intracellular localization of wt-NPM1 in OCI-AML3 cells, degrades NPM1c in AML blasts, and reduce tumor burden in the BM of OCI-AML3 xenograft mice (171).

Imidazoquinoxalines are promising anticancer drugs based on their activities on T-cell leukemia, melanoma, CML, and AML (165, 169, 171). In this study, we deciphered the molecular mechanisms associated with NPM1c degradation and its subsequent induced cancer cell death. We showed that EAPB0503 leads to a progressive degradation of NPM1c as early as 6h post-treatment. This seems to be involved in the triggering of mechanisms, ultimately leading to cell death of NPM1c-expressing cells. We broadened our findings on p53 activation, to characterize the time point and the other important players in the p53 pathway in EAPB0503-treated cell lines and *ex-vivo* blasts. While NPM1c degradation started at 6h, p53 activation through MDM2 degradation was not observed before 24h post-treatment. Interestingly, p21 activation is concomitant with the early degradation of NPM1c, suggesting a p53-independent activation of this protein (Table 8, Figure 25).

NPM1 SUMOylation and de-SUMOylation regulate ribosomal biogenesis through a tight balance of interaction with either $p14^{ARF}$ or SENP3 (134). Our study reveals that

EAPB0503 is inducing degradation of SENP3 in OCI-AML3 and enhancing the SUMOylation of NPM1c by SUMO2/3 in Hela cells expressing NPM1c. Although those observations were made utilizing reporter (GFP) in Hela cells, different sources of antibodies against $p14^{ARF}$ did not lend themselves to the *in vitro* experiments. One lot of antibody obtained initially, was utilized in the in vivo experiments and revealed the upregulation of $p14^{ARF}$, soon after which the antibody became corrupted. Attempts to replenish it were frustrated by the fact that it was discontinued and antibodies from other sources have negligible if any activity. Based on the observed sequential regulation of SUMO2/3, degradation of SENP3 and upregulation of $p14^{ARF}$, it can be deduced that EAPB0503 may exert its effect on ribosomal biogenesis and protein synthesis in *NPM1c* AML (Table 8, Figure 25).

What strengthens these data in our obtained *in vivo* results, whereby EAPB0503 significantly prolonged survival and alleviated leukemia burden in both BM and livers of *NPM1c* xenograft mice, following NPM1c degradation and $p14^{ARF}$ upregulation.

This ongoing project will soon benefit from the acquisition of different NPM1c expressing cells, in addition to the tools required to Crisper SENP3 and observe its mediated effect on NPM1c degradation and the ribosomal biogenesis pathway. Exploring those pathways might identify other key players to be used in targeted therapy and enhance the efficacy of the drug.

Since SUMOylation followed by ubiquitylation mediates proteasomal degradation of proteins, it is essential to study EAPB0503 NPM1c-induced proteasomal degradation by investigating other post-translational modifications, mainly ubiquitylation.

Furthermore, performing several molecular investigations on patient-derived blasts will cement our in vitro observation, especially when coupled to co-culture experiments to mimic the *in vivo* niche and the potential effect of the drug on the mesenchymal stem cells.

Table 8. Effect of EAPB0503 on p53/MDM2 axis and NPM1c/SENP3/SUMOylation interplay in NPM1c AML

Time (hours) / **Protein**	**6**	**24**	**48**
p53 upregulation	basal level	basal level	basal level
p-p53 upregulation	basal level	++	basal level
p21 upregulation	+	++	++
MDM2 degradation	+	++	+++
NPM1c degradation	+	++	+++
SENP3 degradation	+	++	+++
NPM1c SUMOylation by SUMO2/3	++	basal level	basal level

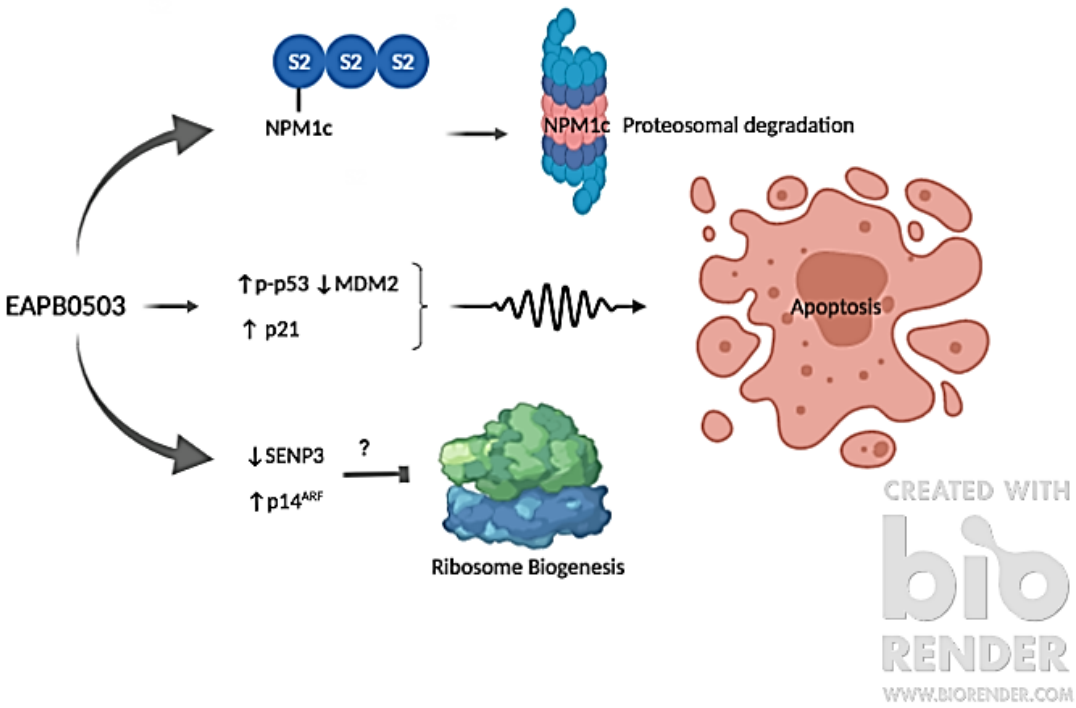


Figure 25. Suggested Mode of action of EAPB0503 in AML.

EAPB0503 leads to proteasomal degradation of NPM1c. It activates p53 through degradation of MDM2 and activates p21, leading to apoptosis. EAPB0503 perturbs the balance SENP3/P14^{ARF}/NPM1c presumably affecting ribosomal biogenesis.

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Review

A Critical Review of Animal Models Used in Acute Myeloid Leukemia Pathophysiology

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Abstract: Acute myeloid leukemia (AML) is one of the most frequent, complex, and heterogeneous hematological malignancies. AML prognosis largely depends on acquired cytogenetic, epigenetic, and molecular abnormalities. Despite the improvement in understanding the biology of AML, survival rates remain quite low. Animal models offer a valuable tool to recapitulate different AML subtypes, and to assess the potential role of novel and known mutations in disease progression. This review provides a comprehensive and critical overview of select available AML animal models. These include the non-mammalian *Zebrafish* and *Drosophila* models as well as the mammalian rodent systems, comprising rats and mice. The suitability of each animal model, its contribution to the advancement of knowledge in AML pathophysiology and treatment, as well as its advantages and limitations are discussed. Despite some limitations, animal models represent a powerful approach to assess toxicity, and permit the design of new therapeutic strategies.

Keywords: Zebrafish; Drosophila; rats; mice; NPM-1; FLT3 ITD; ETO-1; IDH1/2

1. Introduction

Acute myeloid leukemia (AML) is an aggressive and heterogeneous hematological group of neoplasms characterized by increased proliferation of myeloid progenitor cells and a reduced capacity to differentiate. This results in the accumulation of myeloblasts in the bone marrow (BM), which negatively impacts hematopoiesis and leads to BM failure [1]. AML is one of the most common acute leukemia in adults [2]. Its incidence rate is 2.5 per 100,000 cases/year and the median overall survival (OS) is approximately nine months [3]. AML treatment and prognosis largely depend on the patients' age [4–6]. AML was historically divided into eight major groups according to cell morphology and immune phenotype (M0 to M7) [7]. This classification has been revised several iterations since then [8–12]. Exome sequencing in AML patients led to the current classification through identification of more than 20 driver recurrent mutations [13]. These mainly include *Nucleophosmin-1* (*NPM1*), *DNA methyltransferase 3A* (*DNMT3A*), *Fms-like tyrosine kinase-3* (*FLT3*), *isocitrate dehydrogenase* (*IDH*), *Ten–Eleven Translocation 2* (*TET-2*), *Runt-related transcription factor* (*RUNX-1*), *CCAAT enhancer binding protein α* (*CEBPA*), *additional sex comb-like 1* (*ASXL1*), *mixed lineage leukemia* (*MLL*), tumor protein *p53*

(*TP53*), *c-KIT* [14]. These mutations dictate the response to treatment, rates of complete remission, disease-free survival, overall survival, and classify AML into three prognostic risk factors (favorable, intermediate, and adverse) (Table 1).

Animal models provide an excellent tool to understand the biology of pathological mechanisms involved in human diseases. Diverse animal species were used to answer pivotal questions related to disease progression, genetic mutations, immunity, and response to treatment. Among these models, Zebrafish was exploited to generate different mutations mimicking several subtypes of human AML.

Table 1. 2017 European LeukemiaNet (ELN) prognostic groups according to genetic abnormalities of acute myeloid leukemia (AML) [12].

Prognostic Group	Genetic Mutations and Abnormalities
Favorable	• t(8;21)/RUNX1-RUNX1T1 • inv(16) or t(16;16)/*CBFB-MYH11* • Mutated *NPM1* without *FLT3*-ITD • or with *FLT3*-ITD ^{low} * • Biallelic mutated *CEBPA*
Intermediate	• Mutated *NPM1* and *FLT3*-ITD ^{high} * • Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD ^{low} * • t(9;11)/MLLT3-KMT2A • Cytogenetic abnormalities not classified as favorable or adverse
Adverse	• t(6;9)/ DEK-NUP214 • t(v;11q23.3)/*KMT2A* rearranged • t(9;22)/*BCR-ABL1* • inv(3) or t(3;3)/*GATA2,MECOM(EVI1)* • Complex karyotype • Monosomal karyotype • Wild-type *NPM1* and *FLT3*-ITD ^{high} * • Mutated *RUNX1* † • Mutated *ASXL1* † • Mutated *TP53*

* ^{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.

2. Zebrafish: Characteristics and Relevance to Human Blood Malignancies

Danio rerio, commonly known as Zebrafish, shares genetic and molecular mechanisms of hematopoiesis with humans [15]. This model offers many advantages, including low-cost, optically transparent embryos, high fecundity, rapid embryogenesis, and short gestation time. The genome editing in zebrafish was known since 1970s, when the first transgenic zebrafish was generated by inserting naked linear DNA [16]. Since then, the genetic manipulation of this model evolved to include clustered regularly interspaced short palindromic repeats (CRISPR) technology [17], which renders zebrafish an attractive model for studying specific gene involvement and for drug screening in blood malignancies [18–20].

During normal zebrafish hematopoiesis, both the primitive and definitive waves arise from the mesoderm germ layer under the control of the Transforming Growth Factor beta (TGF-β) superfamily proteins, known as bone morphogenic proteins (BMP such as bmp2b and bmp7) [21–23]. The generated transient primitive erythroid and myeloid cells are essential for the embryonic development, while the hematopoietic stem cells (HSCs) and progenitor cells (HSPCs) produce blood lineages in the adult fish [24]. In the below section, we will provide an overview of AML models of Zebrafish (summarized in Table 2).

2.1. AML Models of Zebrafish

2.1.1. Spi-1: MYST3/NCOA2-EGFP

MYST3 (MOZ) is a member of the MOZ, YBF2, SAS2, TIP60 (MYST) family of histone acetyl-transferases (HAT), while NCOA2 (TIF2) is a member of the p160 HAT family [25–28]. The first AML model in Zebrafish was created by expressing the fusion protein, MYST3/NCOA2 (MOZ/TIF2). This fusion targets hematopoietic cells under the control of *spi-1 (pu.1)*, an early myeloid promoter [29]. pu.1 is an ETS-domain transcription factor expressed in both immature lymphoid/hematopoietic cells and myeloid cells during zebrafish hematopoiesis [30]. Cells expressing pu.1 differentiate into myeloid progeny, whereas cells with low pu.1 expression shift to the erythroid fate [31]. After an extended latent period, a small percentage of transgenic fish developed AML [29]. These animals presented with an extensive invasion of kidneys by myeloid blast cells, proving the oncogenic potency of *MYST3/NCOA2* fusion gene [29]. Although this model is useful as a chemical library screen, especially for compounds that target epigenetic regulation of gene expression [29], the long latency and low incidence waned the enthusiasm for its use.

2.1.2. hsp70: AML1-ETO

A chromosomal translocation between chromosomes 8 and 21 (t(8;21)(q22;q22)) occurs in 12–15% of AML patients [32]. This chromosomal rearrangement yields a fusion transcription factor encoding AML1 (RUNX1) linked to ETO, forming the AML1-ETO fusion product [33–35]. This translocation was introduced under the control of the heat shock promoter *hsp70* in zebrafish embryos (*hsp70: AML1-ETO*). Transgenic Zebrafish recapitulated the human AML features, at both the cytological and transcriptional levels [36]. The expression of this fusion protein led to the accumulation of non-circulating hematopoietic cells, whereby the intermediate cell mass was enriched with myeloperoxidase positive neutrophils and morphologically immature hematopoietic blasts [36]. The disruption of definitive hematopoiesis led to switching the cells fate from the erythroid to the myeloid lineage [36]. Overexpression of the transcription factor reversed the observed phenotypes, implicating scl, as major player downstream of AML1-ETO [36]. This model enabled the screening of a small molecule library and discovery of compounds that antagonize the activity of AML1-ETO in the hematopoietic progenitor cells (HPCs) [36]. Inhibition of COX-2 and β-catenin signaling antagonized AML1-ETOs effects on HPCs differentiation and may have implications in human AML [37].

2.1.3. MYCN: HSE: EGFP

MYCN (N-myc) proto-oncogene is upregulated in many types of hematological malignancies [38,39] including 20 to 40% of pediatric AML patients [40]. To unravel the molecular and transcriptional networks by which MYCN induces malignancy, Shen et al. established a transgenic embryonic zebrafish model, Tg (*MYCN: HSE: EGFP*), expressing the murine MYCN under a heat shock promoter [41]. MYCN overexpression induced immature myeloid blast cell expansion and reprogrammed the hematopoietic cell fate through MYCN downstream-regulated gene 1b (ndrg1b) and other lineage-specific hematopoietic transcription factors regulation [41]. The primitive hematopoiesis was enhanced through scl and lmo2 upregulation. Furthermore, erythroid differentiation was blocked through downregulation of gata1, while myelopoiesis was promoted by pu.1 overexpression [41]. This model presents a high AML incidence (~75% of transgenic zebrafish) and a rapid onset occurrence, providing a platform for whole-organism chemical suppressor screens, to identify compounds that can reverse MYCN function in vivo [41].

2.1.4. FLT3-ITD and NPM1c+ Models in Zebrafish

FLT3-ITD and NPM1 are two major players in defining the prognosis and response to treatment in AML patients. FLT3 is a tyrosine kinase receptor that plays a major role in hematopoiesis through the regulation of proliferation, differentiation, and apoptosis of HPCs [42]. It is highly expressed on

leukemic blasts of 70–100% of AML patients [43,44]. Several mutations occur in the FLT3 receptor, the most common of which leads to an internal tandem duplication (ITD) [45]. FLT3-ITD occurs in 20% of AML patients and is strongly associated with poor prognosis [46,47]. NPM1, a shuttling protein between the nucleoplasm and the cytoplasm, plays several roles, notably ribosomal biogenesis [48,49]. NPM1 is mutated (NPM1c+) in around 30% of AML patients with normal karyotype [50]. NPM1c+ is continuously translocated to the cytoplasm contributing to leukemogenesis [50].

FLT3-ITD plays a role in embryonic primitive and definitive hematopoiesis in zebrafish. Transgenic zebrafish embryos with human FLT3-ITD showed expansion and clustering of myeloid cells [51]. Thus far, the impact of FLT3-ITD on adult zebrafish remains underexplored.

Bolli et al. generated a transgenic zebrafish model expressing NPM1c+, which perturbed primitive hematopoiesis by promoting the early expansion of pu.1+ myeloid cells [52]. This phenotype was even more pronounced in a p53-deficient background [52]. An increase in the number of gata1+/lmo2 indicating expansion of erythro-myeloid progenitors (EMPs) was also observed. These EMPs highly expressed both c-myb and CD41 but not RUNX1, suggesting a disruption of definitive hematopoiesis where these cells could be the main target of NPM1c+. This model provides a tractable in vivo system for the study of the mechanisms through which hematopoietic development is perturbed in the presence of NPM1c+ [52].

Transgenic zebrafish models expressing either human FLT3-ITD or NPM1 proteins under the control of *pu.1* promoter were also generated [53]. For that purpose, *spi-1*: FLT3-ITD-2A-EGFP/CG2 expressing mutant FTL3-ITD and *spi-1*: NPM1-Mut-PA/CG2 expressing mutant NPM1 constructs were designed. This double mutant transgenic fish (FLT3-ITD/NPM1.Mut) exhibited an accelerated rate of myeloid leukemogenesis [53]. By the age of six months, around 66% of the transgenic fish produced significantly increased precursor cells in the kidney marrow along with dedifferentiated myeloid blasts [53].

2.1.5. Spi-1: CREB-EGFP

The cAMP response element binding protein (CREB) plays a major role in hematopoiesis through the regulation of proliferation and differentiation of myeloid progenitor cells [54]. Overexpression of CREB is associated with immortalization, growth factor-independent proliferation and blast-like phenotype in BM progenitor cells [55]. CREB is highly expressed in BM samples of both adult and pediatric AML patients [56]. Tregnago et al. generated a transgenic zebrafish model (*spi-1: CREB-EGFP*) expressing the *CREB* gene downstream *pu.1* promoter in the myeloid cell lineage. CREB overexpression resulted in upregulation of erythroid and myeloid genes, altering primitive hematopoiesis. Among adult transgenic zebrafish, 80% of the fish developed AML after 9–14 months through the blockage of myeloid differentiation [57]. These fish showed aberrant expression of a set of 20 genes in common with pediatric AML. The most intriguing is the CCAAT-enhancer-binding-protein-δ (C/EBPδ) that acts downstream CREB. It resulted in impaired myeloid differentiation that could be reversed through inhibition of the CREB-C/EBPδ axis. These findings are complementary with the data obtained by screening for CREB and C/EBPδ in pediatric AML patients, offering an opportunity to test for novel therapeutics through this model [57].

2.1.6. Spi-1: SOX4-EGFP

SOX4 is a transcription factor belonging to the SOX (Sry-related high-mobility groupbox) family [58]. In AML patients, SOX4 overexpression results in poor prognosis and short overall survival [59]. SOX4 was reported to contribute to the leukemic phenotype of C/EBPα mutant AML in murine models as well as in human AML. C/EBPα protein typically inhibits the self-renewal of leukemic cells and restores cellular differentiation. SOX4 overexpression results in C/EBPα inactivation, enabling leukemic cells proliferation and AML development [60,61].

Lu et al. generated a transgenic zebrafish model Tg (spi-1:SOX4-EGFP) expressing SOX4 protein downstream the spi-1 myeloid promoter. Early developmental stages of transgenic zebrafish did not

reveal a difference of expression of SOX4. However, by the age of five months, Tg (spi-1:SOX4-EGFP) zebrafish kidneys started showing mild vacuoles in the renal tubule which evolved into effacement, distorted structure, and increased infiltration of myeloid cells by the ages of 9 and 12 months. A higher number of myeloid progenitor cells and excess blast cells with focal aggregation were observed in the kidney marrow blood cells of 9-, 12-, and 15-months old fish but not younger ones, highlighting that myeloid transformation is age-dependent [59].

2.1.7. IDH 1/2 Mutation

Mutations identified in a family of enzymes involved in the citric acid cycle, isocitrate dehydrogenases 1/2 (IDH1/2), account for 16% of AML patients [62]. These mutations substitute arginine residue almost exclusively at codon 132 in IDH1 (IDH1-R132H) and codons 140 and 172 in IDH2 [62]. To study the involvement of IDH in AML, *zidh1* was either suppressed or deleted and resulted in the blockage of differentiation and accumulation of early myeloid progenitor cells, while decreasing macrophage and natural killer progenitor cells [63]. The importance of IDH1 mutation was asserted when plasmids of IDH1-R132H were injected into zebrafish embryos [63]. An increase in 2-hydroxyglutarate (2-HG) level, a reduction of 5-Hydroxymethylcytotsine (5-hmC), and an expansion of myelopoiesis were obtained in these embryos. A human IDH1-R132H–specific inhibitor significantly ameliorated both hematopoietic and 2-HG responses in human but not zebrafish IDH1 mutant expression [63]. This result is not surprising and highlights some of the drawbacks using Zebrafish as a model for human diseases. On the other hand, studies on *zidh2* were restricted to the regulation of embryonic hematopoiesis in zebrafish but with no relevance to the human AML [63].

Even with the drawbacks of not possessing many mammalian-like organs, zebrafish still provides an excellent, affordable, and rapid platform for evaluating several aspects of AML. The variations in the biological microenvironment might impede drug delivery and performance in humans. Additionally, zebrafish are ectothermic (cold-blooded), so their physiology is not identical to humans, which might affect enzyme kinetics and metabolism. The genetic diversity detected between individual zebrafish belonging to the same strain confounds data and could be misleading [64]. The sparsity of reagents to study zebrafish at the molecular level is contrasted by the abundance of mouse-specific reagents.

3. Rodent Models

Due to the complexity and heterogeneity of AML in humans, rodent models have been instrumental in providing a platform for answering pivotal questions related to AML pathogenesis, disease progression, and developing new effective therapeutic approaches. Among these models, rats and mice represent the closest accepted mammalian models to AML.

3.1. Rats

Several transplantable leukemia rat models were established using carcinogens, radiations, and pollutants [65–67].

Transplantable Rat Models

Acute Myeloid Leukemia/ Chronic Meylogenous Leukemia (AML/CML) leukemia: Repeated intravenous injections of 7, 12-dimethylbenz (a) anthracene (DMBA) into WOP/H-Onc strain or Wistar/H-Onc strain, induced leukemia in 10% of the rats in 5–9 months. This leukemia has myeloid characteristics as revealed by hematological and histological examination, as well as infiltration of myeloid blasts into several organs (BM, liver, spleen, and lymph nodes). This myeloid nature showed similarities with both human CML (as demonstrated by high peroxidase and Sudan black B positive cells and reduction in alkaline phosphatase positivity) and human AML (non-specific esterase activity, highly reduced in the peripheral blood but slightly reduced in BM). These findings do not support the use of these rats as an exclusive AML model [68].

Brown Norwegian Myelogenous Leukemia (BNML): The transplantable promyelocytic leukemia in BN rat (BNML) was first described in 1971. This slow growing leukemia shares many common characteristics with AML, including the disappearance of normal hematopoiesis [69]. Similarities in in vitro colony forming assays between AML patients and BNML rats validated it as a model for AML [70,71]. Several therapeutic modalities were optimized using this model; these include the combination of anthracyclines, [72,73] Ara-C, [74,75], 4′-(9-acridinylamino) methanesulfon-m-anisidide (AMSA) [76], and other therapeutics [77–79]. One of the most significant advantages in the BNML model is its contribution to the improvement of minimal residual disease (MRD) detection by karyotyping [80] and multidimensional flow cytometry [81,82].

3.2. Mice

Mice offer an invaluable model due to their small size, cost-effectiveness, and easy maintenance, availability of research tools, and ease of manipulation to produce and recapitulate several human diseases, including cancer. Since hematopoiesis in mice has been well characterized, they provide a reasonably reproducible model to study AML pathogenesis and potential therapies. Murine AML models include induced, transgenic animals, and humanized mouse models (Table 3) among others.

3.2.1. Chemically-Induced Model

AML models were generated using the L1210 and p388 cell lines, isolated from DBA/2 mice chemically exposed to the carcinogen 3-methylcholantrene [83]. These models were transplantable and provided a platform for testing chemotherapeutic drugs, studying their kinetics, and evaluating their anti-leukemic effectiveness [84]. The L1210 model was used to screen anthracyclines [85] and antimetabolites [86,87] including Cytarabine [88]. The p388 model was used to investigate the efficacy of natural products as topoisomerase II inhibitors [89]. These models allowed significant improvement in the treatment of AML, including the currently used Cytarabine [90]. The main limitation of using these animal models is the induction of more lymphoid than myeloid leukemia, and the needed prolonged exposure to those carcinogens to develop leukemia [91].

3.2.2. Radiation-Induced Model

The correlation between radiation and leukemia was established in patients exposed to x-rays, and survivors of nuclear attacks. Among this cohort of subjects, children presented mostly with ALL, whereas adults were more prone to CML and AML [92–95]. All established radiation-induced AML models carry deletions on chromosome 2, where the hematopoietic transcription factor *Sfpi1/pu.1* is located [96].

RF Model

The RF strain was developed by Furth in 1933 at the Rockefeller Institute [97]. In this model, myeloid leukemia was developed following exposure to fission neutron irradiation or gamma irradiation [98]. In the RF model, a single dose of ionizing radiation-induced myeloid leukemogenesis in 4–6 months, with symptoms reminiscent to human AML [99]. Flt3-ITD mutations were identified in 10% of RF mice [100], which correlates with the occurrence of this mutation in human AML [101].

SJL/J Model

This model is characterized by high spontaneous frequency of reticulum cell neoplasm type B at an early age [102]. The radiation-induced AML in this model is similar to the secondary human AML occurring after irradiation of Hodgkin disease patients [103]. The efficient development of AML required the addition of promoting factors, such as corticosteroids and growth factors, colony stimulating factor CSF-1, known to be high in AML patients [104].

C3H/He and CBA Models (CBA/Ca, CBA/Cne, and CBA/H)

These models were generated in 1920, by cross-breeding Bragg albino with DBA mice. While C3H/He was specifically selected for the high incidence of mammary tumors [105], CBA was selected for a lower incidence of mammary tumors. The C3H/He was detected 24 h after irradiation in BM cells; this indicates that chromosomal 2 alteration is responsible for the initiation of myeloid leukemogenesis [106]. CBA showed chromosome 2 and 4 aberrations [107,108]. Moreover, an 8% decrease in DNA methylation was observed after exposure to radiation. This hypomethylation played a role in leukemogenesis [109]. The CBA model is considered the most favorable model in radiation-inducedAML because of low spontaneous leukemia incidence (0.1 to 1%), high incidence of AML after exposure to radiation or benzene, with lower latency, compared to other models, and more importantly, it mimics human AML at the cytological, histopathological, and molecular levels.

3.2.3. Virally Induced Leukemia Models

Murine leukemia viruses (MuLV) induce non-B and non-T cell leukemia in mice [110,111] and are considered among the simplest retroviruses that shed light on the pathogenesis of leukemia [112,113]. A model was created by injecting cell-free filtrates, including replication-deficient spleen focus forming virus (SFFV) and a replication-competent Friend MuLV [114,115]. It was noticed that the same infection of MuLV induces several subtypes of AML (Table 4), resembling French–American–British (FAB) classification of human AML [116]. Furthermore, MuLV-induced AML led to the discovery of several genes with a significant role in the regulation of growth, death, lineage determination, and development of hematopoietic precursor cells [117]. MuLV induced AML is considered a critical landmark for understanding the pathogenesis of human AML, since it unraveled relevant unknown oncogenes to leukemogenesis (Table 4).

3.2.4. Transposon Models

Sleeping Beauty (SB) transposon is an insertional mutagenesis system, allowing overexpression or inactivation of specific genes depending on the transposon orientation and integration site [118,119]. SB consists of a mobilized piece of DNA, transposon, and a transposase enzyme [120]. In a transgenic animal with a humanized NPM1c+ knock-in allele, this system enhanced the incidence and onset of AML in NPM1c+ mice [121]. An advantage of this model was the identification of mutations in leukemia genes [121].

3.2.5. Transgenic Models: Single Mutation

PML-RARα t(15;17)

Acute promyelocytic leukemia (APL) is a subtype of AML, characterized by t(15;17) chromosomal translocation, resulting in the promyelocytic leukemia-retinoic acid receptor α (PML-RARα) fusion protein [122,123]. PML-RARα was expressed in three mouse models under the myeloid regulatory promoters. Under the *CD11b* promoter, transgenic mice showed abnormal myelopoiesis and increased radiation sensitivity, however, did not develop any leukemia [124]. Mice expressing the transgene under the human cathepsin G (*HCG*) and human MRP8 (*hMRP8*) promoters [124–126] developed APL phenotypes after a long period of latency [125,126]. These two models recapitulated the remissions seen after all trans-retinoic acid (ATRA) treatment in human APL [125,126].

AML1-Eight-Twenty One Oncoprotein

AML1-Eight-Twenty One oncoprotein (ETO) chimeric product, encoded by the t(8;21), occurs in around 12–15% of AML [32]. Knock-in mice expressing AML1-ETO is embryonic lethal due to the complete absence of liver-derived definitive hematopoiesis [127,128]. Embryonic livers contained dysplastic multilineage hematopoietic progenitors that had an abnormally high self-renewal

capacity in vitro, a phenotype typical of leukemic cells [129]. To bypass the embryonic lethality, inducible transgenic models were generated. These mice expressed AML1-ETO in their BM progenitor cells [130,131]. Although abnormal maturation and proliferation of progenitor cells were observed, mice failed to develop leukemia [130,131]. Expression of AML1-ETO under the control of *hMRP8* promoter was unable to develop AML until their exposure to a robust DNA-alkylating mutagen, *N*-ethyl-*N*-nitrosourea [132]. To further enhance AML development, this mouse model was modified by either the expression of other factors or mutations in tyrosine kinases such as c-KIT, FLT3-ITD, or the TEL- platelet-derived growth factor receptor β (PDGFbR) [133,134].

CBFB-MYH11

The beta subunit of the core binding complex (CBFB) is a heterodimeric core-binding transcription factor, with a critical role in hematopoiesis [135]. CBF products, due to chromosomal translocations, account for approximately 25% of pediatric and 15% of adult AML patients [136]. The translocation Inv(16) (p13;q22) is a result of the binding of CBFB subunit to the tail region of the smooth muscle myosin heavy chain (*SMMHC*) gene, MYH11 [137]. The resulting fusion protein (CBFB-MYH11) competes with the binding of CBF to target genes, disrupting transcriptional regulation, thus contributing to leukemic transformation [137]. Similar to embryos with homozygous mutations in AML1 [128], knock-in embryonic mice ($Cbfb^{+/Cbfb\text{-}MYH11}$) lacked definitive hematopoiesis and died during gestation [138]. Chemically or retrovirally induced mutations in heterozygous *CBFB-MYH11* adults led to AML development [138,139]. A conditional knock-in mouse model expressing *CBFB-MYH11* fusion protein in adult mice ($Cbfb^{+/56M}$) was also generated [140] and led to AML development in 90% of the mice within five months [140].

Mutant Nucleophosmin-1 (NPM1c+)

Mutations in the *Nucleophosmin-1 (NPM1)* gene represent one of the most frequent genetic aberrations in AML [141] and account for 30% of AML patients [50]. Transgenic mice harboring the *NPM1c+* mutation developed myeloproliferation in BM and spleen, supporting a role of NPM1c+ in AML [142]. Chou et al. generated a knock-in transgenic mouse model by inserting the most frequent mutation, TCTG called mutation A, in the C-terminus of wt-NPM1 [143]. Mice homozygous for the transgene encountered embryonic lethality, whereas one-third of the heterozygotes (*Npm1wt/c+*) developed the fetal myeloproliferative disease but not AML [143]. Conditional expression of *NPM1c+* with further genetic manipulations resulted in two models [121,144]. In one model, one-third of the transgenic mice developed leukemia after a long period of latency associated with AML features [144]. In the other model, the expression of humanized NPM1c+ in the hematopoietic stem cells caused *HOX* overexpression, enhanced self-renewal, and expanded myelopoiesis [121].

Fms-Related Tyrosine Kinase 3 Internal Tandem Repeats

The second most common genetic aberrations in de novo AML patients occur in the fms-related tyrosine kinase 3 internal tandem repeats (*FLT3-ITD*) gene on chromosome 13. These associate with poor prognosis and short overall survival (OS) [145]. A transgenic mouse model expressing FLT3-ITD under the *vav* hematopoietic promoter was created [146]. The majority of transgenic mice developed a myeloproliferative syndrome (MPS) characterized by megakaryocytic hyperplasia and thrombocytosis but not AML [146]. In FLT3-ITD knock-in mice, loss of FLT3 wild-type allele contributed to myeloid expansion and aggressiveness of the MPS disease [147]. Several other models expressing this mutation also revealed MPS but not AML [148,149].

Mixed Lineage Leukemia (MLL)

The translocation t(9;11)(p22;q23) produces the fusion product MLL-AF9 [150,151]. In one model, embryonic stem cells were generated from an in-frame fusion of AF9 with exon 8 of mouse MLL [152]. Other models conditionally expressed MLL-AF9 [153]. These models developed only

AML despite the widespread activity of the MLL promoter [152,153]. Conditional expression of MLL-AF9 in long-term hematopoietic stem cells (LT-HSC) produced aggressive AML with extensive tissue infiltration, chemo-resistance, and expressed genes related to epithelial-mesenchymal transition in solid cancers [154]. MLL early introduction results in abnormalities of myeloid cell proliferation and differentiation [155]. Moreover, HOXa9 was found to be essential for the MLL-dependent leukemogenesis in vivo [156].

The translocation t(4;11)(q21;q23) produces the fusion product MLL-AF4. This translocation is associated with pro-B-ALL and rarely AML [157]. Although several models have been established for this translocation, only few models resulted in AML. MLL-*AF4* models generated using both a knock-in [158] and *Cre*-inducible invertor model [159] produced large B-cell lymphoma rather than the immature acute leukemia observed in humans [158,159]. The MLL-AF4 expression in hematopoietic precursors, during mouse embryonic development, developed long latency B-cell lymphoma [159,160]. Furthermore, MLL-AF4 knock-in followed by in vitro inducible transduction generated mice with both AML and pre-B-ALL as well as a few MLLs [161].

Leukemia with the t(11;19)(q23;p13.3) translocation express MLL-ENL fusion proteins capable of malignant transformation of myeloid and/or lymphoid progenitor(s). Immortalized cells containing MLL-ENL proviral DNA or enriched primary hematopoietic stem cells transduced with MLL-ENL induced myeloid leukemia in syngeneic and SCID recipients [162]. Using an in vitro B-cell differentiation system, retroviral transduction of *MLL-ENL* generated a leukemia reminiscent of human MLL-ENL ALL [163]. Other models expressed MLL-ENL-ERTm, the ligand-binding domain of the estrogen receptor modified to specifically recognize synthetic but not endogenous estrogens, using retroviral transduction approach [164]. Several other models were generated encountering more mutation along with MLL-ENL [165,166].

IDH 1/2

A conditional knock-in mouse model was created by inserting the mutated human IDH1 (R132H) into the endogenous murine *idh1* locus. IDH1 (R132H) was expressed in all hematopoietic cells under the *vav* promoter (vav-KI mice) or specifically in cells of the myeloid lineage (LysM-KI mice) [167]. Transgenic mice showed increased number of early hematopoietic progenitors and developed splenomegaly and anemia with extramedullary hematopoiesis, characteristics of a dysfunctional BM niche, along with partial blockage in myeloid differentiation [167]. Moreover, LysM-KI cells have hypermethylated histones and changes to DNA methylation similar to those observed in human *IDH1*- or *IDH2*-mutant AML, demonstrating the induction of leukemic DNA methylation signature in the mouse model [167].

3.2.6. Transgenic Models: Compound Transgenic Mouse Models

K-RAS-G12D + PML-RARα

4% and 10% of APL patients with PML-RARα fusion had oncogenic *N-RAS* and *K-RAS* mutations, respectively [168,169]. The conditional expression of oncogenic K-RAS and PML-RARα in mice induced a rapid-onset and highly penetrant, lethal APL-like disease [170].

These mice may be used to test for the therapeutic efficacy of inhibitors of RAS post-translational modifications and RAS downstream signaling [170].

N-RASD12 + BCL-2

N-RAS, a protein belonging to the family of RAS GTP-ases, is mutated in patients at risk of leukemic transformation after chemotherapy and/or radiotherapy [171]. *N-RAS* mutation at codon 12 is the most frequent abnormality in myelodysplastic syndromes (MDS), associated with AML transformation and poor OS [172]. B-cell lymphoma 2 (BCL-2) protein is an apoptosis regulatory protein. BCL-2 is overexpressed in AML patients [173], which blocks the differentiation of myeloid

progenitors [174]. Both mutants have been previously identified as risk factors for AML in MDS patients [172].

Two murine models of initiation and progression of human MDS/AML were generated [175]. The transplantable model expressing hBCL-2 in a primitive compartment by mouse mammary tumor virus–long terminal repeat *(MMTVtTA /TBCL-2/NRASD12)* represents human MDS, whereas the constitutive *MRP8 [BCL-2/NRASD12]* model is closer to AML [175]. Both models showed expanded leukemic stem cell ($Lin^-/Sca\text{-}1^+/c\text{-}Kit^+$) populations. hBCL-2 is observed in the increased RAS-GTP complex within the expanded $Sca\text{-}1^+$ compartment [175]. The difference of hBCL-2 oncogenic compartmentalization associates with the pro-apoptotic mechanisms in MDS and the anti-apoptotic in AML mice [175]. Downregulation of hBCL-2 in MDS mice partially reversed the phenotype and prolonged survival; however BM blasts and tissue infiltration persisted [175]. This model revealed that the two candidate oncogenes *BCL-2* and mutant *N-RAS* can cooperate to give rise to malignant disease with a penetrance of around 80% and a latency period of 3 to 6 months [175].

Mixed Lineage Leukemia-Partial Tandem Duplication + FLT3-ITD

Mixed lineage leukemia-partial tandem duplication (*MLL*-PTD) is expressed in 5 to 7% of cytogenetically normal (CN)-AML patients [176,177]. Approximately 25% of these patients have constitutive activation of FLT3-ITD, conferring a poor prognosis [178]. To recapitulate the $Mll^{PTD/WT}$:$flt3^{ITD/WT}$ AML found in humans, a double knock-in mouse model was generated by expressing these two mutated genes under their respective endogenous promoters [179]. After a period of latency, this model developed AML with a short life span, extensive extramedullary involvement, and increased aggressiveness [179]. Reminiscent of this subtype of AML in humans, these transgenic mice have normal chromosomal structures, reduced *MLL-WT* expression, loss of *FLT3*-WT, and increased total *FLT3* expression [179–182]. Moreover, increased *HOXA9* transcript levels were observed, rendering this model valuable for the assessment of epigenetic modifying agents combined with tyrosine kinase inhibitors [179].

NUP98-HOXD13 + FLT3-ITD

The chromosomal translocation t(2;11)(q31;p15) leads to the fusion of Nucleoporin *(NUP98)*, a structural component of the nuclear pore complex, to the homeobox protein NHD13 (HOXD13), inducing leukemogenesis [183]. *NUP98-HOX* fusions are observed in human and murine MDS [184]. Clinical and experimental evidence demonstrated that high rate of FLT3-ITD mutations was observed in patients with NUP98 translocations [185]. High-level transcriptional expression of *NUP98-HOX* correlated with higher transcript levels of *FLT3* and an increased incidence of FLT3 activating mutations [185]. A novel model combining an FLT3-ITD mutation with NHD13 (HOXD13) was generated using their respective endogenous promoters [186]. Initially, these transgenic mice developed leukemia with both primitive myeloid and lymphoid origin. Later, strictly myeloid leukemia with minimal differentiation were monitored [186]. Indeed, *NHD13* transgene enhanced the overexpression of the *HOX* genes, *HOXA7*, *HOXA9*, *HOXB4*, *HOXB6*, *HOXB7*, *HOXC4*, and *HOXC6* [186], shown to play an important role in HSC self-renewal and are upregulated in acute leukemia [187–189]. Nevertheless, mice encountered a spontaneous loss of heterozygosity with a high frequency, resulting in the loss of WT *FLT3* allele, [186], a characteristic of patients with FLT3-ITD mutations [180]. These transgenic mice provide a model to study the molecular pathways underlying MDS-related AML [186].

NPM1c+/FLT3

NPM1c+ and FLT3-ITD double mutations are found in about 40% of AML patients [190]. A compound transgenic mouse model with a double mutation in NPM1 and FLT3 was generated by crossing conditional $Npm1^{flox-cA/+}$ with constitutive $Flt3^{ITD/+}$ mice [191]. Inducing recombination of $Npm1^{flox-cA}$ in hematopoietic stem cells was accomplished by crossing the double heterozygous mice into *Mx1-Cre* transgenic mice [191]. Double mutant mice developed AML and died by the age of 31–68 days. Peripheral blood showed increased leukocyte counts, reduced numbers of circulating

B and T lymphocytes along with a marked population of immature blasts, while BM cells exhibited increased self-renewal potential [191]. Solid organs were infiltrated with abnormal myeloid cells inducing splenomegaly and hepatomegaly by the time of death, highlighting the role of this double mutation in leukemogenesis [191].

N-RAS-G12D + CBFB-MYH11

A knock-in mice (*Nras*$^{LSL\text{-}G12D}$; *Cbfb*^{56M}) with an allelic expression of oncogenic N-RAS^{G12D} and CBFB-MYH11 developed leukemia in a cell-autonomous manner, with a short median latency and high leukemia-initiating cell activity [192]. Mice displayed an increased survival of pre-leukemic short-term HSCs and myeloid progenitor cells with a sustained blocked differentiation induced by the fusion protein [192]. *Nras*$^{LSL\text{-}G12D}$; *Cbfb*^{56M} leukemic cells were sensitive to pharmacologic inhibition of the MEK/ERK signaling pathway [192], highlighting the importance of this pathway in AML and proposing MEK inhibitors as potential therapeutic agents in inv16/ N-RAS^{G12D} AML [192].

NPM1c + N-RAS-G12D

One of the most common mutations with NPM1c+ is the *N-RAS* mutation occurring *in* 20% of NPM1c+ AML patients [190]. *NPM1* and *N-RAS* double mutant transgenic mice (*Npm1*$^{cA/+}$; *Nras*$^{G12D/+}$) developed high penetrance, enhanced self-renewal capacity in hematopoietic progenitors, and AML-like myeloid differentiation bias [193]. At the genomic level, frequent amplification of the mutant *N-RAS-G12D* allele was observed, along with other somatic mutations in AML driver genes [193]. Within the *HOX* genes, which were overexpressed, *HOXa* genes and downstream targets were crucial for the survival of the double-mutant mice [193].

WT1-R394W + FLT3-ITD

Wilms tumor 1 (WT1) is a zinc finger transcriptional regulator of target genes implicated in cell differentiation and quiescence [194]. Mutations in *WT1* occur in 10–15% of CN-AML, and it is frequently associated with mutations in several genes [194,195]. *FLT3-ITD* and *WT1* mutations, when present concomitantly, identify a group of AML patients that fail to respond to the standard induction chemotherapy, which results in poor OS [195,196]. Double mutant mice *Flt3*$^{+/ITD}$/*Wt1*$^{+/R394W}$ displayed manifestations of shortened survival, myeloid expansion in the BM, anemia, and erythroid dysplasia [197]. Although this model did not appear sufficient to consistently recapitulate human AML, it demonstrated that the combined mutations resulted in a more aggressive disease than either mutant genotype [197].

3.2.7. Humanized Models

Humanized mouse models, injected with AML cell lines or patient-derived AML blasts, offered a faster approach and were instrumental in studying different aspects of AML. Several models were attempted to study AML in Nude mice with little success [198,199]. This section will focus on promising models for AML studies.

SCID Mice

The severe combined immuno-deficient (*SCID*) mice lacking B and T cell immunity [200], represent essential humanized AML mouse models [201]. Indeed, patient-derived AML cells engraftment enabled the identification of leukemia-initiating cells (LIC), expressing CD34^{+} CD38^{-} surface markers, recapitulating the human HSCs signature [202]. Engraftment of AMLs from different FAB classes into *SCID* mice reflected their intrinsic biologic behavior, suggesting a clinical correlation to the growth and dissemination of these leukemic subtypes [203]. However, lack of species cross-reactivity of cytokines and the innate host immunity against human AML cells resulted in poor engraftment of the BM [204]. In an attempt to overcome these limitations, exogenous human cytokines and growth factors were

provided, which resulted in better engraftment of human cells [202,204–206]. One limitation of this model is the "leakiness" of the *SCID* mutation occurring in around 10% of the mice [207]. These mice present functional B and T cells, enhanced natural killer (NK) cell activity, and complement activation decreasing the engraftment efficiency [208]. An attempt to bypass this problem uses radiation and/or anti-asialo-GM1 antibody pretreatment. Unfortunately, it reduced the survival of the host, rendering this model unsuitable for human xenograft [209,210].

NOD/SCID Mice

To further improve tumor engraftment, a non-obese diabetic (NOD/*SCID*) model exhibiting further impairment of NK activity, reduced mature macrophage, and total lack of B and T cells was generated [211]. This model yielded higher engraftment rates with fewer human AML cells, yet with preserved morphological, phenotypical, and genotypical characteristics of the AML donors [212–215]. This model was used successfully in the screening for new therapeutics in AML [216]. In addition, human AML cells engraftment enabled the fractionation of LICs ($CD34^+$ $CD38^-$) into $CD34^+/CD71^-$/HLA-DR [217], CD34 Thy1 hematopoietic stem cells [218] and CD34/CD117 (or ckit) [219] subpopulations. Nevertheless, the NOD/*SCID* model presents the limitation by which higher engraftment rates required the supplementation of human cytokines or transplantation of growth-factor producing cells [220,221]. Moreover, long term engraftments (more than 8.5 months) were disabled due to the development of thymic lymphomas and restoration of NK cells activity during this period [211]. A variant with NOD/*SCID* background is the NSS model (N/S-S/GM/3) expressing Steel factor (SF), granulocyte macrophage-colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) human growth factors was generated [222]. NSS displayed enhanced engraftment of pre-leukemic myeloid cell cultures, as well as primary human AML samples, suggesting that the NSS mouse is a better host for at least a subset of AML samples [223].

NSG Mice

NOD/*SCID* mice were further immunosuppressed to generate the NOD/*SCID* b2-microglobulin null mice with a complete abolishment of the NK cell activity [224]. Importantly, a NOD/*SCID* IL2-R$\gamma^{-/-}$ or NSG model was generated by deletion or truncation of the gamma chain of IL-2R [225]. 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 γ (IFN-γ) upon stimulation [225,226]. Engraftment of newborn NSG mice with human $CD34^+$ HSCs leads to the generation of a complete hematopoietic system, including red blood cells and platelets [226]. Studies revealed a significantly higher potential of AML cells engraftment in adult NSG mice in comparison to previous immunodeficient hosts [227,228]. Attempts to create different subtypes of AML were successful in NSGs [228]. NSG mice xenotransplanted with five well-characterized AML cell lines established AML models of particular relevance and significance to drug-sensitivity studies [228]. These models were exploited to study the in vivo potency of an Imidazoquinoxalines immunomodulatory drug, EAPB0503, and showed its specific activity in NPM1c+ AML subtype [229]. The usability of NSG model allowed the evaluation of the effect of a synthetic retinoid ST1926, or its encapsulated form in nanoparticles (ST1926-NP). El-Houjeiri et al. demonstrated that ST1926-NP is more potent in NSG injected with THP-1 cells [230]. MOLM-13-injected NSG mice showed strong efficacy to chemotherapy (cytarabine, 50 mg/kg) and 5+3 regimen of daunorubicin (1.5 mg/kg) [231]. These models enabled the in vivo tracking of UCB-NK cells, demonstrating their capability to migrate to BM and inhibit progression of human leukemia cells. Administering a low dose of human IL-15 enhanced survival of these mice, emphasizing the role of innate immunity in AML outcome [232]. In that sense, utilization of NSG model enabled the assessment of the combination of HSPC-NK cell adoptive transfer with the hypomethylating agents (HMAs), azacitidine (AZA), and decitabine (DAC). Cany et al. signified that the therapeutic combination exerted a significant delay in AML progression in these mice [233].

Table 2. A summary of generated AML Zebrafish models and their contribution to the understanding of the disease.

Zebrafish Model	Zebrafish Manipulation	Model Features and Major Findings	References
spi-1: MYST3/NCOA2-EGFP	Transgenic expression of human MYST3/NCOA2 fusion under the spi-1/*pu.1* promoter	First AML model in zebrafish 1.1% of transgenic fishes expressing the transgene developed AML after long latency	[29]
hsp70: AML1-ETO	Transgenic expression of human AML1-ETO fusion under *hsp70* promoter	A phenotype similar to human AML Disruption of definitive hematopoiesis: the switch of cell fates from erythroid to myeloid through gata1 downregulation and pu.1 overexpression AML1-ETOs effects on HPCs differentiation was mediated through Cycloxygenase-2 (COX-2) and β-catenin signaling pathways	[36,37]
mRNA: NPMc+	mRNAs injection into 1-cell–stage embryos followed by morpholinos (MOs) targeting *npm1a* and *npm1b*	Perturbation of primitive and definitive hematopoiesis Alterations in the expression of major transcription factors (pu.1+, mpx+, csf1r+, c-myb, CD41, RUNX1)	[52]
HSE-MYCN-EGFP	Induction of murine *N*-myc gene through heat-shock promoter	AML development with high incidence and rapid onset Enhancement of primitive hematopoiesis through alteration of transcription factors (pu.1, gata1, scl, lmo2, p27kip and p21cip1) Activation of major cancer signaling pathways	[41]
IDH1/2 mutants	Knockdown of zebrafish *idh1* and *idh2* (*zidh1* and *zidh2*) by morpholino knockdown and Transcription activator-like effector nuclease (TALEN-)mediated mutagenesis	*zidh1* suppression/deletion is correlated with a blockage of differentiation of the myeloid lineage *zidh1* effects definitive hematopoiesis exclusively *zidh2* affects primitive hematopoiesis exclusively	[63]
	Transgenic expression of human IDH1 mutation	Embryos recapitulated the features of human AML	
FLT3-ITD-2A-EGFP spi-1: NPM1-Mut-PA spi-1:	Transgenic expression of human FLT3-ITD or/and NPM1 mutations under the *spi-1* promoter	Myeloproliferative neoplasm (MPN) development as a result of a single mutation. 66.6% of double mutant transgenic fish showed increased precursor cells in the kidney marrow along with dedifferentiated myeloid blasts.	[53]
spi-1: CREB-EGFP	Expression of *CREB-EGFP* under *spi-1* promoter in myeloid lineage	Alteration of primitive hematopoiesis in embryos AML development in 79% of adult fishes by 9–14 months Aberrant expression of 20 genes diagnosed in pediatric AML	[57]
Spi-1: SOX4-EGFP	Expression of *SOX4* protein downstream the *spi-1* promoter	Increase in the number of myeloid progenitor cells and blast cells in the kidney marrow Distortion of the kidney structure	[59]

Table 3. A summary of generated AML mice models and their contribution to the understanding of the disease.

Mouse Model		Manipulation	Outcomes and Major Findings	References
Chemically-Induced Model		Transplantable AML models were generated using the L1210 and p388 cell lines, isolated from DBA/2 mice chemically exposed to the carcinogen 3-methylcholantrene.	Provide a platform for testing chemotherapeutic drugs, studying their kinetics, and evaluating their anti-leukemic effectiveness (mainly Cytarabine)	[83,84,90]
Radiation- Induced Model	**RF model**	Myeloid leukemia was developed following exposure to fission neutron irradiation or γ irradiation	FLT3-ITD mutations were identified in 10% of RF-AML mice which correlates with the occurrence of mutation of human AML	[98,100,101]
	SJL/J model	The radiation induced AML (RI-AML) in this model, is similar to the secondary human AML occurring after irradiation of Hodgkin disease patients	The efficient development of AML in this model was achieved by adding promoting factors, corticosteroids and growth factors like colony stimulating factor CSF-1, known to be high in AML patients	[103,104]
	C3H/He and CBA models (CBA/Ca, CBA/Cne, and CBA/H)	These models were generated by cross breeding Bragg albino with DBA mice	CBA model is considered the most favorable model in RI-AML High incidence of AML after exposure to radiation or benzene with lower latency compared to other models, Mimics human AML at the cytological, histopathological, and molecular levels.	[107,108,234]
Virally-induced leukemia models MuLV		Murine leukemia viruses (MuLV) induce non-B and non-T cell leukemia in mice	Same infection of MuLV induces several subtypes of AMLthat resembles FAB classification Identifies unknown oncogenes contributing to leukemogenesis.	[112,113,116,117] + Table 2
Transposon models		Sleeping Beauty (SB) transposon is another insertional mutagenesis system, allowing overexpression or inactivation of specific genes depending on the transposon orientation and integration site	Identification of mutations in leukemia genes, which provided new pathogenetic insights and potential therapeutic targets in NPM1c+ AML	[118,119,121]

Table 3. *Cont.*

Mouse Model			Manipulation	Outcomes and Major Findings	References
Trans-genic models	Single mutation	**Promyelocytic Leukemia protein (PML)-RARα t(15;17)**	Expressing PML-RARα under *CD11b* promoter	Abnormal myelopoiesis and increased radiation sensitivity No AML development	[124]
			Expressing PML-RARα under human cathepsin G (*HCG*) promoter	APL phenotype after long latency period Remission seen after All Trans Retinoic Acid (ATRA) treatment in APL	[125]
			Expressing PML-RARα under human MRP8 (*hMRP8*) promoter	APL phenotype after long latency period Remission seen after ATRA treatment in APL	[126]
		AML1- Eight-Twenty One oncoprotein (ETO)	Knock-in of AML1-ETO into mouse embryos (AML1-ETO/+)	Absence of liver-derived definitive hematopoiesis Embryonic lethality	[127,128]
			Expressing AML1-ETO in adult bone marrow progenitor cells	Abnormal maturation and proliferation of progenitor cells No AML development	[130,131]
			Expressing AML1-ETO under human MRP8 (hMRP8) promoter	AML development after exposure to *N*-ethyl-*N*-nitrosourea	[132]
		CBFB-MYH11	Knock-in embryonic mice (Cbfb+/Cbfb-MYH11)	Lack of definitive hematopoiesis Embryonic lethality	[138]
			Chemical/ retroviral mutagens on heterozygous CBFB-MYH11 adults	AML development	[138,139]
			Conditional knock-in adult mice (*Cbfb+/56M)*	AML development in 90% of mice after 5 months	[140]
		Mutant Nucleophosmin-1 (NPM1c+)	Knock-in mice expressing NPM1 with mutation A (NPM1c+)	Homozygotes encountered embryonic lethality 1/3 of the heterozygotes (*Npm1wt/c+*) developed fetal myeloproliferative disease but not AML	[143]

Table 3. *Cont.*

Mouse Model		Manipulation	Outcomes and Major Findings	References
		Expression of NPM1 with mutation A (NPM1c+) under the pCAG promoter	1/3 of the transgenic mice developed leukemia after a long period of latency	[144]
		Expression of humanized NPM1c+ in the hematopoietic stem cells	*HOX* overexpression Enhanced self-renewal Expanded myelopoiesis	[121]
	Fms-related tyrosine kinase 3 internal tandem repeats (FLT3-ITD)	Expressing FLT3-ITD under the vav hematopoietic promoter	Myeloproliferative syndrome (MPS) Megakaryocytic hyperplasia and thrombocytosis No AML development	[146]
		FLT3-ITD knock-in mice with lost FLT3 wild-type allele	Myeloid expansion and aggressiveness of the MPS disease No AML development	[147]
	Mixed Lineage Leukemia (MLL)	Embryonic stem cell formed by in-frame fusion of AF9 with exon 8 of mouse MLL	AML development	[152]
		Conditional expression of MLL-AF9 using programmed interchromosomal recombination	AML development	[153]
		Conditional expression of MLL-AF9 in LT-HSC	Aggressive AML Extensive tissue infiltration Chemoresistance Expression of genes related to epithelial-mesenchymal transition (EMT) in solid cancers	[154]
		Early introduction of MLL	Abnormalities of myeloid cell proliferation and differentiation	[155]
	IDH 1/2	Expressing IDH1/2 under the vav promoter (Vav-KI mice) or specifically in cells of the myeloid lineage (LysM-KI mice)	Increased number of early hematopoietic progenitors Splenomegaly Anemia Extramedullary hematopoiesis, characteristics of a dysfunctional BM niche and partial blockage in myeloid differentiation Induction of leukemic DNA methylation signature in mouse model	[167]

Table 3. *Cont.*

Mouse Model		Manipulation	Outcomes and Major Findings	References
Compound mutations	**K-RAS-G12D + PML-RARα**	Constitutive expression of K-RAS and PML-RARα	Rapid-onset and highly penetrant, lethal APL-like disease	[170]
	N-RAS12D + BCL-2	*MMTVtTA /TBCL-2/NRASD12* Expression of hBCL2 in a primitive compartment by mouse mammary tumor virus–long terminal repeat	MDS development Expanded leukemic stem cell ($Lin^-/Sca\text{-}1^+/c\text{-}Kit^+$) populations Increased apoptosis Malignant disease with a penetrance of around 80% and a latency period of 3 to 6 months	[175]
		MRP8 [BCL-2/NRASD12] Constitutive expression *of BCL-2* under human *MRP8* promoter	AML development Expanded leukemic stem cell ($Lin^-/Sca\text{-}1^+/c\text{-}Kit^+$) populations No apoptotic cells Malignant disease with a penetrance of around 80% and a latency period of 3 to 6 months	[175]
	MLL-PTD + FLT3-ITD	Expressing MLL-PTD and FLT3-ITD under their respective endogenous promoters	Latent AML with a short life span, extensive extramedullary involvement and increased aggressiveness Normal chromosomal structures Reduced *MLL*-WT expression Loss of *FLT3*-WT and increased total *FLT3* expression Increased *HOXA9* transcript levels	[179]
	NUP98-HOXD13 + FLT3-ITD	Expressing FLT3-ITD and NHD13 (HOXD13) under their respective endogenous promoters	Myeloid leukemia with minimal differentiation Overexpression of several *HOX* genes Spontaneous loss of heterozygosity with a high frequency, resulting in the loss of WT *FLT3* allele	[186]
	NPM1c+ - FLT3	Crossing conditional $Npm1^{flox-cA/+}$ with constitutive $Flt3^{ITD/+}$ mice	AML development Lethality by the age of 31-68 days Modified blood cell counts Immature blasts in BM Myeloid cells infiltration into organs Splenomegaly and hepatomegaly	[191]

Table 3. *Cont.*

Mouse Model		Manipulation	Outcomes and Major Findings	References
	N-RAS-G12D + CBFB-MYH11	Allelic expression of oncogenic N-RAS^{G12D} and CBFB-MYH11	Leukemia development in a cell-autonomous manner with a short median latency High leukemia-initiating cell activity Increased survival of pre-leukemic short-term HSCs and myeloid progenitor cells with blocked differentiation Leukemic cells were sensitive to MEK/ERK inhibitors	[192]
	NPM1c + N-RAS-G12D	Conditional expression of *NPM1c*+ and *N-RAS-G12D*	AML-like myeloid differentiation bias Hematopoietic progenitors with high penetrance and enhanced self-renewal capacity Frequent amplification of the mutant *N-RAS-G12D* allele Somatic mutations in AML driver genes Overexpression of *HOX* genes	[193]
	WT1-R394W + FLT3-ITD	Crossing *Flt3*$^{+/ITD}$ mice with *Wt1*$^{+/R394W}$ mice	MDS/MPN development Shortened survival Myeloid expansion in the BM, Anemia Erythroid dysplasia	[197]
Xenograft/humanized models	***SCID* mice**	Autosomal recessive mutation	Lack of B and T cells Retained innate immunity and cytokines Identification of leukemia initiating cells (LIC) Poor engraftment of human AML cells in the BM	[200]
	NOD/*SCID* mice	NOD/*SCID* model: Express additional mutations	Impairment of NK activity Reduced mature macrophages Total lack of B and T cells Fractionation of LIC into subpopulations	[211]
		NSS model (N/S-S/GM/3): variant of NOD/*SCID* mice expressing SF, GM-CSF and IL-3	Better host for a subset of AML	[222,223]
	NSG mice	Deletion or truncation of the γ chain of IL-2R	Defective production of major interleukins and IFN-γ Impairment of dendritic cells Complete abolishment of the NK cell activity Higher engraftment capacity of human AML cells than previous models	[224]

Table 4. Murine leukemia virus (MuLV) induced AML models: Major gene discoveries and their involvement in different French–American–British (FAB) AML subtypes.

MuLV Virus	Mouse Strain	AML Subtype	FAB Classification	Major Gene Discoveries	References
CasBrM-MuLV	NFS	Granulocytic	M1 or M2	*His-1*	[235,236]
CasBrE MuLV	NIH Swiss	Myeloid	M1 or M2	*Fli-1*	[237–239]
Endogenous ecotropic MuLV	AKXD-23	Granulocytic	M1 or M2	*Evi-1*	[240,241]
Friend-MuLV	C57BL/6	Granulocytic	M1 or M2	*Ccnd1*	[237,242,243]
Friend-MuLV	DBA/2	Myeloblastic	M1 or M2	*Evi-1, & c-myb*	[244–246]
M-MuLV	BALB/c	Promonocytic	M5	*c-myb*	[246,247]
B ecotropic MuLV	BXH-2	Myelomonocytic	M4	*c-myb*, *HOXa7*, *HOXa9*, *Meis1*, *CBFa1*, *SOX4*, *Hhex*, *Rarg*, *Sharp1*, *Ccnd3*, *Cdc25l*, *RASGRP*, *Clabp*, *Hmgcr*, *Nf1*, & *Il17r*	[248–255]

4. Drosophila Melanogaster

AML1-ETO

The chromosomal translocation t(8:21)(q22;q22) is frequent and common in AML. It represents up to 40% of AML subtype M2 of the FAB classification [256]. The fusion gene resulting in this translocation encodes for the chimeric protein AML1-ETO, which contains the N-terminus of AML1 (including its DNA binding domain) and most of the ETO protein [33,257], and inhibits the expression of AML1 target genes leading to leukemogenesis [258]. The detailed molecular mechanism governing this interference is poorly understood, which enticed the generation of several animal models to understand its mode of action. AML1-ETO alone is not sufficient to induce leukemia unless accompanied by secondary mutations [130,131,259]. The simplicity of genetics and ease of manipulation in *Drosophila* presents it as an attractive model to study this complex translocation. In addition, *Drosophila* hematopoiesis is comparable to that of mammals [260]. Two AML1-ETO models of genetically engineered *Drosophila* were generated. In the first model, AML1-ETO is a constitutive transcriptional repressor of AML1 target genes. In the second model, AML1-ETO dominantly interferes with AML1 activity by potentially competing for a common co-factor [261]. The transcription factor Lozenge (Lz) that is similar to human AML1 protein is necessary for the development of crystal cells, one of the major *Drosophila* blood cells, during hematopoiesis [262]. Using these models and by comparison with loss-of-function phenotypes of Lz, AML-1-ETO was shown to act as a constitutive transcriptional repressor [261]. Osman et al. reported that AML1-ETO inhibits the differentiation of crystal cell lineage, and induces an increase in the number of circulating LZ+ progenitors. Moreover, large scale RNA interference screen for suppressors of AML1-ETO in vivo showed that *calpainB* is required for AML1-ETO-induced leukemia in *Drosophila*. Surprisingly, calpainB inhibition in Kasumi-1 cells (AML patient cell line carrying t(8;21) translocation) leads to AML1-ETO degradation and impairs their clonogenic potential [263]. Another study identified pontin/RUVBL1as a suppressor of AML1-ETO. Indeed, PONTIN knock-down inhibits the proliferation of t(8;21) positive cells, and that PONTIN is essential for Kasumi-1 clonogenic potential and cell cycle progression [264]. Thus, AML1-ETO can be recapitulated in Drosophila blood for investigating its mechanism and identifying potential targeted therapeutics for this AML subtype.

Despite advances in our understanding of many molecular mechanisms, in vitro research falls short in determining overall effect of treatment modalities or drug discovery. AML is an intricate disease where culture consisting of a single cell line system, can never recapitulate the complexity of the disease. In the difficulty of obtaining primate models of AML, small rodents, zebrafish, and Drosophila with well characterized genetic background and relative ease of manipulation, are the backbone of current work where leukemic cells are interfaced with the host immunity, metabolic environment and importance of the niche ation. Not one model is sufficient to address all posed questions. However, collectively, these models have expanded our knowledge and understanding of several pathways and important players in AML pathogenesis.

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