Technische Universität München

III. Medizinische Klinik am Klinikum rechts der Isar

Azacitidine combined with crenolanib abrogates niche protection and expansion of residual leukemic stem cells (LSC) in *FLT3*-ITD^{+} acute myeloid leukemia (AML) with concurrent gene mutations in *NPM1*, *DNMT3A* or *TET2*

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1 Introduction

1.1 Hematopoietic stem cells (HSC) and hematopoiesis

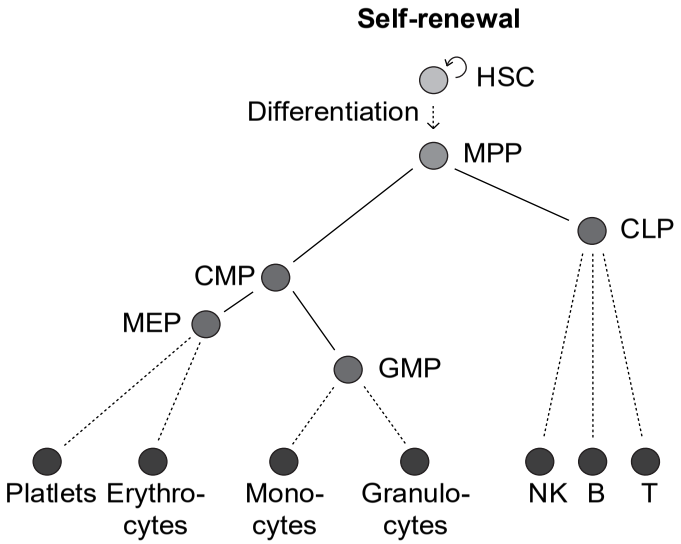
Blood is a highly regenerative, fluidic tissue comprised of red blood cells (erythrocytes), white blood cells (leukocytes: granulocytes, monocytes and lymphocytes), platelets and plasma (intercellular substance). Hematopoiesis (from Greek αἷμα, "blood" and ποιεῖν "to make") is a highly hierarchical system controlled by intrinsic and extrinsic factors. Only a small population of so-called hematopoietic stem cells (HSC) gives daily rise to about 10^{12} differentiated blood cells that fulfill essential functions such as transport of oxygen, hormones or nutrients as well as immunity or tissue remodeling (Figure 1).

In the 1960s, Till and McCulloch coined the term "stem cell". They demonstrated that murine bone marrow (BM) cells with radiation-induced chromosomal aberrations formed colonies in the spleen of recipient mice within 1 to 2 weeks post-transplant (Till and McCulloch, 1961). These colonies were of clonal nature, since they carried identical cytogenetic markers as the "cell of origin" (Becker, McCULLOCH and Till, 1963). Isolated colonies contained self-renewing cells that formed new colonies after re-injection into mice and non-colony forming cells with features of more differentiated cells (Siminovitch, McCulloch and Till, 1963)(Wu *et al.*, 1968). Thus, HSC were identified as cells with I) long-term self-renewing capacity and II) the ability to give rise to functionally differentiated blood cells.

Within the last six decades, research of normal and malignant hematopoiesis has exploded. Thanks to the development of ingenious techniques such as multiparameter flow cytometry (MPFC), xenotransplantation studies in immune-deficient mice, colony-forming-cell (CFC) assays or next generation sequencing (NGS), hematopoietic cell populations from mice and men could be isolated and functionally analyzed *in vitro* and *in vivo* (Eaves, 2015).

1.1.1 Pre-natal hematopoiesis

Just when the embryo has grown as big as it cannot any longer be supplied with oxygen by diffusion, primitive hematopoietic cells generate in extraembryonic (yolk sac, allantois and placenta) anatomical sites. Next, HSC generate in intraembryonic aorta-gonad-mesonephros (AGM) and soon migrate to the fetal liver, where they mature and expand. Finally, fetal HSC migrate to the spleen and to the BM, ready to engraft and maintain hematopoiesis after birth (Mikkola, 2006).



Cell type	Immunophenotype
Hematopoietic stem cell	Lin-CD34+CD38-CD90+CD45RA-(CD49f+)
Multipotent progenitors	Lin-CD34+CD38-CD90-CD45RA-(CD49f-)
Common lymphoid progenitor	Lin-CD34+CD38-CD10+(CD7+)
Common myeloid progenitor	Lin-CD34+CD38-CD123dimCD135+CD45RA-
Megakaryocyte-erythrocyte progenitor	Lin-CD34+CD38+CD123-CD135-CD110+
Granulocyte/monocyte progenitor	Lin-CD34+CD38+CD123dimCD135+CD45RA+
(Lineage-restricted progenitor)	Lin+/-CD34-
Functional blood cell	Lin+CD34-

Figure 1. Model of the hematopoietic system. At the apex of the hierarchically ordered hematopoietic system sit multipotent HSC with long-term self-renewing capacity. Activated HSC may progressively differentiate into more lineage-committed progenitors and eventually functional blood cells. The immunophenotype of each differentiation stage is depicted according to current knowledge: HSC(Shlush *et al.*, 2014)(Majeti, Park and Weissman, 2007)(Notta *et al.*, 2011), multipotent progenitor (MPP) (Shlush *et al.*, 2014)(Majeti, Park and Weissman, 2007)(Notta *et al.*, 2011), common lymphoid progenitor (CLP) (Shlush *et al.*, 2014)(Hao *et al.*, 2001), common myeloid progenitor (CMP) (Shlush *et al.*, 2014)(Manz *et al.*, 2002), megakaryocyte-erythroid progenitor (MEP) (Shlush *et al.*, 2014)(Doulatov *et al.*, 2010)(Liu *et al.*, 2006), granulocyte-macrophage progenitor (GMP) (Shlush *et al.*, 2014)(Manz *et al.*, 2002)(Doulatov *et al.*, 2010).

1.1.2 Adult hematopoiesis

During adulthood, multipotent HSC with long-term self-renewing capacity sit at the apex of the hierarchically ordered hematopoietic system. Multipotency gets lost and cells become more and more lineage-committed during step-wise differentiation into progenitors and eventually functional blood cells (Figure 1).

Under steady-state conditions, the majority of HSC reside in the BM in a relative quiescent state (dormancy; G0 phase of the cell cycle) (Cheshier, Morrison and Liao, 1999)(Yamaguchi *et al.*, 1998) ensuring their survival, long-term proliferation capacity (Passegue, 2005)(Glimm, Oh and Eaves, 2000) and genomic integrity (Walter *et al.*, 2015). These resting cells are true long-term (LT) HSC. Only a small proportion of HSC enter cell cycle (G1;S;G2/M phase) to proliferate via asymmetric or symmetric cell division towards differentiation or self-renewal. The number of mobilized and dividing HSC can increase in order to counteract stress, for example infection, blood loss or cytotoxic agents. Intrinsic and extrinsic stimuli control the appropriate balance between resting and active HSC (Figure 2).

HSC intensively interact with their BM microenvironment and induce a “niche” that protects and supports stem cell properties (Schofield, 1978) by direct cell contact or secreted factors. Throughout the bone marrow, quiescent HSC preferentially reside in a perivascular niche meaning in close proximity to blood vessels (sinusoids), sympathetic nerves, mesenchymal stromal cells (MSC) and endothelial cells. Activated HSC and progenitor cells are rather located in sinusoidal niches (Figure 2) (Morrison and Scadden, 2014)(Boulais and Frenette, 2015).

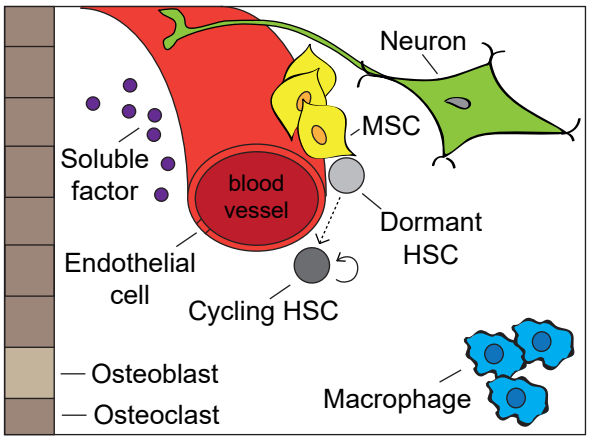


Figure 2. The stem cell niche. The BM microenvironment comprises several physiological niches for hematopoietic stem and progenitor cells. The niche is a complex and dynamic system comprising cellular and acellular factors. Depending on the resting or active state, HSC respectively locate to the perivascular (around arteriols) or sinusoidal (cells pass in and out of circulation) niche.

1.1.3 Ageing hematopoiesis and leukemia

Ageing fundamentally impacts HSC biology. HSC increase in number but are functionally impaired. They are less quiescent and show decreased homing capacity due to an altered interaction behavior with niche cells. HSC accumulate reactive oxygen species (ROS) which induce DNA damage. DNA and histone methylation patterns are changed. Moreover, aged hematopoiesis is characterized by an increased number of myeloid but decreased number of erythroid and lymphoid lineage cells which goes along with the induction of immunosenescence (Geiger, de Haan and Florian, 2013). Consequently, the skewed hematopoietic system becomes more susceptible to leukemogenic events.

Leukemia is defined according to the affected white blood cell type as well as the pace of the disease. Acute leukemia is characterized by rapid outgrowth of malignant immature blasts, whereas with chronic leukemia often more mature but incompletely functional leukemic cells accumulate slowly. When lymphoid lineage cells are affected one speaks of lymphoid/lymphocytic leukemia. When myeloid cells are transformed the disease is called myeloid/myelogenous leukemia.

1.2 Acute myeloid leukemia (AML)

Acute myeloid leukemia (AML), the most frequent form of acute leukemia, is characterized by rapid clonal expansion of immature myeloid blasts resulting in severe BM failure that leaves the body susceptible to anemia and infection. AML can evolve *de novo* or from other hematologic disorders such as myelodysplastic syndromes (MDS) or myeloproliferative neoplasms (MPN) (secondary AML; sAML). AML can also be induced by chemo- or radiation therapy (therapy-induced AML; tAML). However, AML is predominantly a disease of older patients (median age 70 years) with adverse prognosis.

1.2.1 Diagnosis and standard treatment

AML is a very heterogeneous disease and diagnosis is based on morphologic, immunophenotypic, cytogenetic and molecular genetic analysis of bone marrow aspirates (Döhner *et al.*, 2010). Sub-categorization is done using the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemias, recently revised in 2016 (Table 1) (Arber *et al.*, 2016).

Overall, AML still has a poor prognosis and treatment options remain limited especially for fragile and older patients. Only 35 to 40% of patients < 60 years old and 5 to 15 % of patients > 60 years old can be cured by standard treatment regimens (Figure 3). Patient-related (e.g. age and general fitness) as well as AML-related factors (e.g. white blood cell count, cytogenetic and molecular ge-

netic aberrations, Table 2) individually determine prognosis and treatment.

Table 1. WHO classification of myeloid neoplasm and acute leukemia (Arber *et al.*, 2016).

Acute myeloid leukemia (AML) and related neoplasms
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22.1);*RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);*CBFB-MYH11*
APL with *PML-RARA*
AML with t(9;11)(p21.3;q23.3);*MLLT3-KMT2A*
AML with t(6;9)(p23;q34.1);*DEK-NUP214*
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2*, *MECOM*
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);*RBM15-MKL1*
Provisional entity: AML with BCR-ABL1
AML with mutated *NPM1*
AML with biallelic mutations of *CEBPA*
Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not other specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia

Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (TAM)

The mainstay of intensive induction chemotherapy, which aims to induce remission (defined as < 5% of blasts in the BM) is 3 days of anthracycline and 7 days of cytarabine infusional therapy. If complete remission (CR, defined as <5% blasts in the BM) is achieved post-remission therapy including chemotherapy with high dose cytarabine (HiDAC) or allogeneic hematopoietic stem cell transplantation (alloSCT) is applied to eliminate minimal residual disease (MRD) and the risk of relapse. Treatment choice is made according to the patient's risk profile. However, although the majority of patients achieve complete remission, most patients will eventually relapse from MRD. At relapse or in primary refractory disease, only intensive salvage therapy as a bridge to alloSCT can offer a long-term perspective, and only in few cases. For older or frail patients not eligible for intensive treatment regimens clear therapy guidelines are currently missing. Thus, the remaining options such as best supportive care, hypomethylating agents (see 1.3.4) or low-dose cytarabine must be considered individually (Döhner *et al.*, 2010)(Longo *et al.*, 2015).

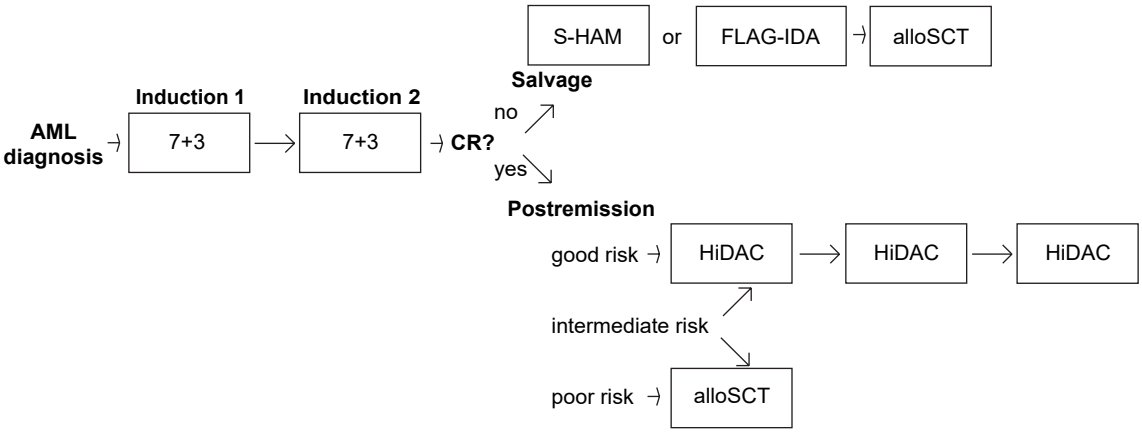


Figure 3. Standard treatment options in AML. Induction "7+3" indicates 3 days of an anthracycline (e.g. daunorubicine or idarubicin or mitoxantrone 45-60 mg/m^2) and 7 days cytarabine (100-200 mg/m^2 continuous IV); CR, complete remission; HiDAC consolidation: high-dose cytarabine (1-3 g/m^2 every 12 hours [q12h] on days 1 - 6); alloSCT: allogeneic stem cell transplantation; S-HAM: sequential HiDAC (1g/m2 per q12h on days 1, 2, 8, and 9) and mitoxantrone (10 mg/m2 on days 3, 4, 10, and 11); FLAG-IDA: combination of fludarabine, Ara-C, and idarubicin.

Table 2. Current Stratification of Molecular Genetic and Cytogenetic Alterations, European leukemia network (ELN) Recommendations (Longo *et al.*, 2015).

Risk Profile	Subset
Favorable	t(8;21)(q22;q22); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD (normal karyotype)
	Biallelic mutated *CEBPA* (normal karyotype)
Intermediate-I	Mutated *NPM1* and *FLT3*-ITD (normal karyotype)
	Wild-type *NPM1* and *FLT3*-ITD (normal karyotype)
	Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse‡
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *GATA2–MECOM(EVI1)*
	t(6;9)(p23;q34); *DEK-NUP214*
	t(v;11)(v;q23); *KMT2A* rearranged
	−5 or del(5q); −7; abnl(17p); complex karyotype§

1.2.2 Clonal evolution in AML

Recently, next-generation sequencing (including whole-genome-sequencing, whole-exome-sequencing, targeted sequencing, RNA sequencing and DNA-methylation analysis) in large AML cohorts and functional validation in xenotransplantation studies have shed more light on transforming events, clonal architecture and cause of relapse in AML.

Around 50% of patients carry a normal karyotype (CN-AML) without structural chromosome abnormalities (Bullinger *et al.*, 2010)(Walter *et al.*, 2009)(Suela *et al.*, 2007). In CN-AML, only a few recurring gene mutations drive pathogenesis. Each of them can be categorized according to its biologic function (Table 3) (Cancer Genome Atlas Research Network, 2013). Not a single, but two or more non-synonymous, cooperating driver mutations can induce full-blown leukemia. In fact, the mutational landscape in AML is very heterogeneous. It is a matter of ongoing investigations and discussions which combination of mutations can induce leukemia.

At diagnosis, the AML bulk is a complex conglomerate of several passenger mutations but only a few driver mutations (Welch *et al.*, 2012). The mutational history of AML bulk can be dissected on the basis of variant allele frequencies (i.e. rela-

tive proportion of bulk cells/sequencing reads that exhibit a certain somatic mutation). Low variant allele frequencies (VAF) indicate mutations that have either appeared at a late disease stage or are passenger mutations, which do not contribute to pathogenesis and therefore do not expand. Only a few driver mutations that have occurred and expanded early during leukemogenesis (founding clone) have high VAF. Accordingly, it was shown that mutations in *DNMT3A*, *NPM1* and *FLT3* evolve sequentially and cooperate during leukemogenesis (Figure 4).

Table 3: Categorization of driver gene mutations in AML (Cancer Genome Atlas Research Network, 2013).

Category (frequency)	Gene mutations
Transcription-factor fusion (18%)	*PML-PARA*, *MYH11-CBFB*, *RUNX1-RUNX1T1*, *PICALM-MLLT10*
Gene-encoding nucleophosmin 1 (27%)	*NPM1*
Tumor-suppressor genes (16%)	*TP53*, *WT1*, *PHF6*
DNA-methylation related genes (44%)	*DNMT3A*, *DNMT3B*, *DNMT1*, *TET1*, *TET2*, *IDH1*, *IDH2*
Activated signaling (59%)	*FLT3*, *KIT*, Other Tyr kinases, Ser-Thr kinases, *KRAS*/*NRAS*, *PTP*
Myeloid transcription factors (22%)	*RUNX1*, *CEBPA*, other myeloid transcription factors
Chromatin modifiers (30%)	*MLL*-X fusions, *MLL-PTD*, *NUP98-NSD1*, *ASXL1*, *EZH2*, *KDM6A*, other modifiers
Cohesion complex genes (13%)	
Spliceosome-complex genes (14%)	

NGS analysis of 200 *de novo* AML genomes revealed non-synonymous somatic gene mutations.

Epigenetic alterations in HSC due to mutations in epigenetic regulator genes (e.g. *DNMT3A*, *TET2* or *IDH1/2*) often precede the onset of leukemia. Such mutated HSC are called pre-leukemic stem cells (pre-LSC) as they clonally expand but still give rise to normal hematopoiesis. Pre-LSC can accumulate further driver mutations (e.g. lesions in *NPM1* or *FLT3*) and transform into fully leukemic stem cells (LSC) (Corces-Zimmerman and Majeti, 2014)(Shlush *et al.*, 2014)(Jan *et al.*, 2012). Similar to HSC, LSC have self-renewing and proliferating potential, are enriched in the $CD34^+$ $CD38^-$ BM compartment and sit at the apex of a heterogeneous leukemic bulk that does not have stem cell like properties (Dick, 2008) (Goardon *et al.*, 2011). Further, LSC intensely interact with stromal BM niche cells and induce a leukemic niche to gain a selective growth advantage over normal hematopoiesis and to resist therapy (Ishikawa *et al.*, 2007)(Schepers, Campbell

and Passegué, 2015)(Parmar *et al.*, 2011).

In some cases the mutational composition at diagnosis and relapse differs. At relapse, re-occurring leukemic blasts may arise from I) non-eradicated therapy-resistant LSC (founding clone), II) LSC with additional resistance conferring mutations (subclone) or III) remaining pre-LSC that acquire new driver mutations (Ding *et al.*, 2012)(Welch *et al.*, 2012)(Klco *et al.*, 2014). Hence, only drugs that eradicate LSC and pre-LSC but do not substantially impair normal hematopoiesis will be curative. The development of such therapeutics remains highly challenging because of profound immunophenotypic and functional similarities between normal HSC and LSC as well as the immense cytogenetic and molecular genetic heterogeneity in AML.

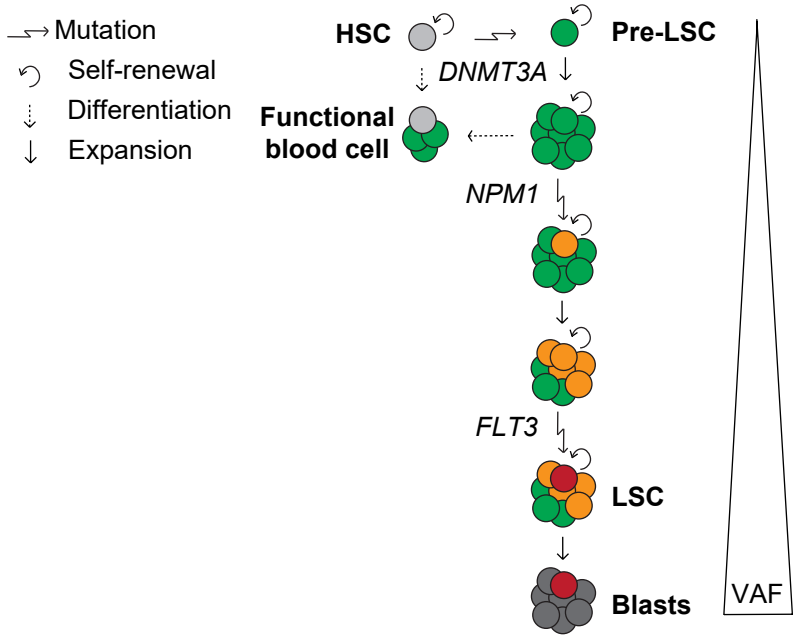


Figure 4. Model of clonal architecture in *FLT3*-ITD⁺ AML. Lesions in epigenetic drivers (e.g. *DNMT3A*) may induce transformation of HSC into pre-leukemic stem cells (Pre-LSC) that expand clonally. Pre-LSC are clinically silent and can differentiate into functional blood cells. They may gain several passenger mutations, but only one (e.g. *NPM1*mut) or two (e.g. *FLT3*-ITD) cooperating driver mutations can fully transform them into leukemic stem cells (LSC). LSC expand and give rise to many immature myeloid blasts.

1.3 Epigenetic modifiers in AML

Epigenetics are defined as heritable changes of genetic transcription and expression that do not change the DNA code. The epigenome is altered and maintained due the interplay of DNA methylation (by DNA methyltransferases, DNMTs), histone modification (by histone deacetylases, HDAC and histone methyltransferases, HMT) and nucleosome remodeling (by nucleosomal remodeling factors,

NURF). Epigenomic mechanisms play crucial roles in tissue development and differentiation. Thus, mutations in genes that encode epigenetic regulators (gate keepers) often herald the onset of malignant tissue transformation (Sharma, Kelly and Jones, 2010). In AML, two classes of epigenetic mutations were found: alterations in post-translational histone modifiers (i.e. ASXL1, EZH2, MLL) and regulators of DNA methylation (i.e. DNMT3A, TET2 and IDH1/2; Figure 5) (Cancer Genome Atlas Research Network, 2013)(Papaemmanuil *et al.*, 2016)(Metzeler *et al.*, 2016). Such epigenetic lesions are age-related and associated with skewed myelopoiesis (Busque *et al.*, 2012)(Xie *et al.*, 2014). Pre-leukemic epigenetic lesions may represent gate keeper mutations that induce genetic instability, which eases acquisition of further driver mutations and subsequent LSC expansion (Corces-Zimmerman and Majeti, 2014)(Shlush *et al.*, 2014)(Wakita *et al.*, 2013) (Jan *et al.*, 2012).

1.3.1 *DNMT3A* mutations

In mammals, gene silencing mainly occurs in CpG-rich DNA stretches (CpG islands, often found at the gene promotor region) through covalent methylation of cytosine bases at the 5'position (5mC). Less is known about non-CpG island methylation. Non-CpG island methylation is reversible whereas methylation of CpG-islands persists during cell cycle. Methylation patterns are generated by *de novo* methyltransferase DNMT3A and B (Okano *et al.*, 1999) and maintained by DNMT1 (Song *et al.*, 2011) (Figure 5). In AML, recurrent somatic missense (hot spot at arginine 882, R882; 60% of *DNMT3A*mut cases), nonsense, frameshift and splice-site mutations of *DNMT3A* were found (Ley *et al.*, 2010)(Yan *et al.*, 2011). Occurring *DNMT3A* mutations are mostly heterozygous and reduce or disrupt enzymatic activity in a dominant-negative fashion (Kim *et al.*, 2013) (Russler-Germain *et al.*, 2014). *DNMT3A*-deficent murine HSC have increased self-renewal capacity but impaired differentiation potential over serial transplantation in comparison to wild-type HSC. Further, *DNMT3A*-deficiency induces global hypomethylation, but also hypermethylation at specific gene loci. Some of the hypomethylated genes are frequently overexpressed in leukemia (Challen *et al.*, 2011). *DNMT3A*mut seems to facitlitate *FLT3*-ITD occurrence due to reduced methylation of hematopoietic enhancer regions. Further expression of *FLT3*-ITD in murine *DNMT3A*$^{-/-}$ and *DNMT3A*$^{+/-}$ HSC induces lymphoid and myeloid leukemia (Yang *et al.*, 2016).

1.3.2 *TET2* mutations

Ten-eleven translocation (TET) family of proteins TET1, TET2 and TET3 are DNA demethylases. TET catalyze oxidation of 5mC to 5-hydroxymethylcytosine (5-hmC) in a Fe(II)- and α-ketoglutarate (a-KG)-dependent manner. DNMT1 which

usually copies 5mC-DNA marks during replication does not recognize 5-hmC resulting in loss of methylation marks (passive DNA demethylation) (Valinluck and Sowers, 2007)(Ko *et al.*, 2010). 5-hmC is processed to unmodified cytosine, 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) (active DNA demethylation) (He *et al.*, 2011)(Ito *et al.*, 2011) (Figure 5). Moreover, TET promotes chromatin modification by binding to O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT), which transfers O-GlcNAc groups to serine (Ser) and threonine (Thr) residues of cytosolic or nuclear proteins. OGT target proteins include histone 2B (H2B) (Sakabe, Wang and Hart, 2010) or host cell factor 1 (HCF1) (Deplus *et al.*, 2013). H2B O-GlcNAc marks activated gene transcription and HCF1 O-GlcNAc may also affect histone modification since HCF1 is part of a H3K4 methyltransferase complex.

About 10 to 20% of AML cases carry somatic *TET2* deletions, missense, or nonsense mutations. Most *TET2* lesions are heterozygous and presumably affect methylation in a dominant negative manner (Quivoron *et al.*, 2011)(Abdel-wahab *et al.*, 2009)(Tian *et al.*, 2014)(Patel *et al.*, 2012). *TET2* mutations disrupt the catalytic demethylation activity characterized by low 5-hmC levels and hypermethylation of CpG sites (Ko *et al.*, 2010). Several mouse models have demonstrated that *TET2*-deficiency enhances hematopoietic stem/ progenitor cells (HSPC) proliferation and skews hematopoiesis towards myelopoiesis (Moran-crusio *et al.*, 2011)(Quivoron *et al.*, 2011)(Ko *et al.*, 2011)(Hao *et al.*, 2001)(Kunimoto *et al.*, 2012)(Shide *et al.*, 2012). TET2 mutations are associated with adverse outcome, especially in *FLT3*-ITD$^+$ AML (Patel *et al.*, 2012)(Tian *et al.*, 2014)(Hou *et al.*, 2014). Multipotent *TET2*-deficient *FLT3*-ITD$^+$ murine stem/progenitor cells can propagate AML in secondary recipient mice and are refractory to chemotherapy and FLT3-targeted tyrosine kinase inhibitor (TKI) therapy (Shih *et al.*, 2015).

1.3.3 *IDH1/2* mutations

As enzymes of the tricarboxylate acid cycle (TCA), isocitrate dehydrogenase 1 and 2 (IDH1/2) catalyze the NADP$^+$-dependent decarboxylation of isocitrate via the unstable intermediate oxalsuccinate to α-KG (also known as 2-oxoglutarate (2-OG)), NADPH and CO_2 (Figure 5). IDH1 is the cytosolic enzyme and IDH2 is expressed in mitochondria (Losman and Kaelin, 2013). *IDH1/2* lesions are found in around 15% of AML patients, enriched in CN-AML cases. Mutations occur almost always heterozygous at three highly conserved arginine residues: *IDH1*R132, *IDH2*R140 and *IDH2*R172 (Mardis *et al.*, 2009)(Gross *et al.*, 2010) (Ward *et al.*, 2010). In AML, *IDH1/2* lesions are mutually exclusive with *TET2* loss-of-function mutations. Both mutations induce similar hypermethylation signatures and consequently likely exhibit similar pro-leukemogenic mechanism in AML (Figueroa *et al.*, 2010).

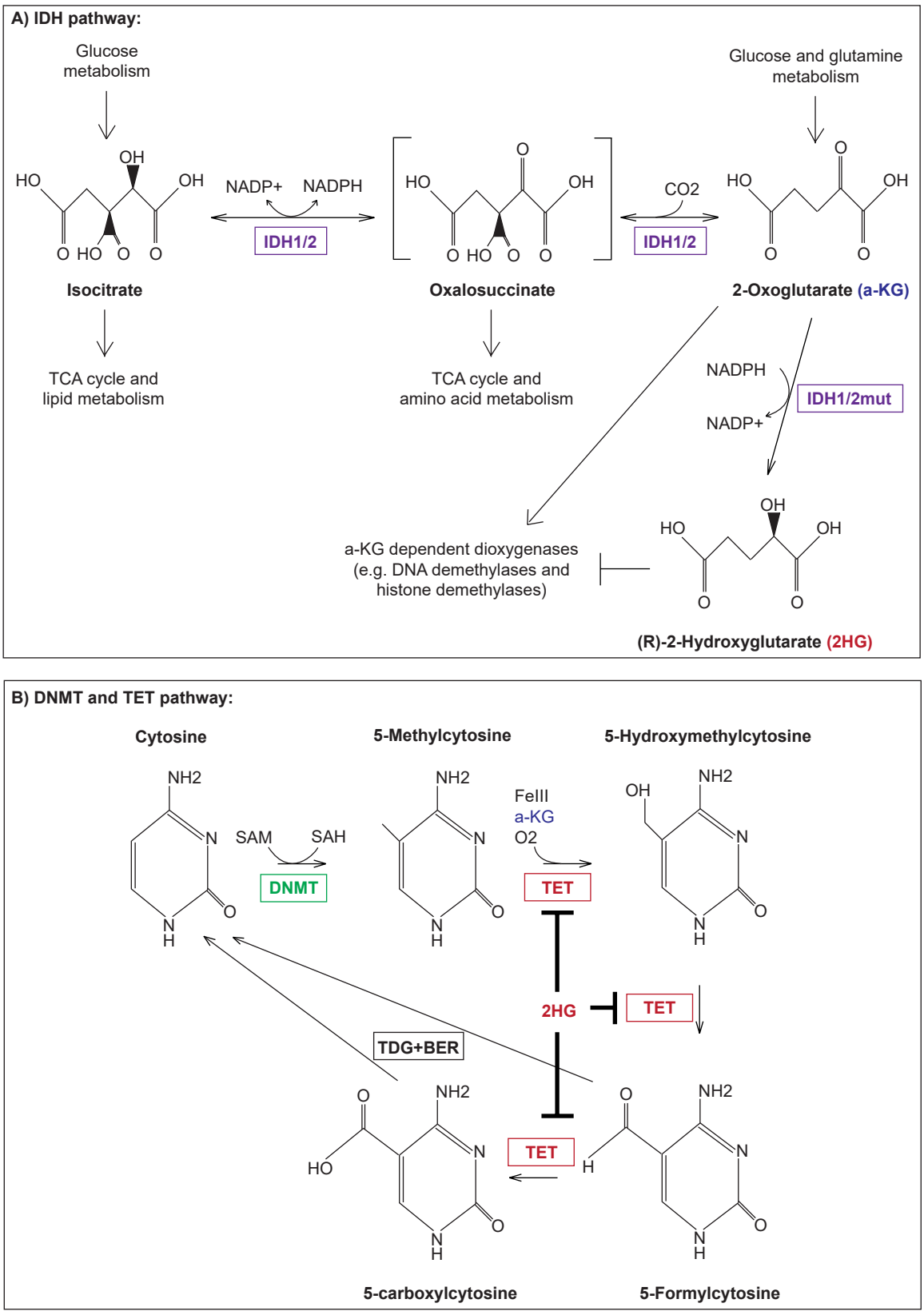


Figure 5. Reactions catalyzed by IDH1/2 or DNMT and TET. IDH1/2 wild-type catalyze the two-step decarboxylation of isocitrate to α-KG. IDH1/2 reduces α-KG to 2HG in a single-step reaction using NADPH (upper panel). Cytosine methylation and demethylation pathway involving *de novo* methyltransferase (DNMT), ten-eleven translocation (TET), thymine DNA glycosylase (TDG) and base excision repair (BER). 2-HG inhibits DNA demethylation mediated by a-KG dependent TET (lower panel).

IDH1/2 mutations are gain-of-function mutations that enable one-step conversion of isocitrate to (R)-2-hydroxyglutarate ((R)-2HG) in a NADPH-dependent manner. (R)-2HG is structurally similar to α-KG but cannot catalyze function of α-KG-dependent dioxygenases including TET2 and Jumonji-c domain containing histone demethylase (JHDM) (Figure 5) (Losman and Kaelin, 2013)(Losman *et al.*, 2013) (Xu *et al.*, 2015). Further, *IDH1/2* mutations agonize the α-KG-dependent EglN prolyl hydroxylase (Losman *et al.*, 2013)(Koivunen *et al.*, 2012). Conditional knock-in of *IDH1*R132 in mice increases the HSPC compartment and induces leukemic epigenetic changes similar to that of human *IDH1/2* mutant AML (Sasaki *et al.*, 2012).

1.3.4 Hypomethylating agents (HMA)

Cancer epigenomes are roughly characterized by local CpG-island hypermethylation (including promotor regions of tumor-suppressor genes) and global hypomethylation (Sharma, Kelly and Jones, 2010). Aberrant epigenomes are theoretically reversible by targeted inhibition of epigenetic regulators. Azanucleosides (AZN) are pyrimidine analogues of the nucleoside cytidine and can therefore be incorporated into DNA and/or RNA (Figure 6). Accordingly, it was shown that AZN process anti-neoplastic effects by direct cytotoxicity, activation of DNA damage pathways and hypomethylation through inhibition of DNMT. Two AZN have been approved for the treatment of MDS and AML: 5-azacytidine (azacitidine, AZA) and 5-aza-2-deoxycytidine (decitabine, DAC) (Table 4) (Diesch *et al.*, 2016).

Table 4. Approval status of hypomethylating agents in MDS and AML (Diesch *et al.*, 2016).

	Azacitidine		**Decitabine**	
	MDS	**AML**	**MDS**	**AML**
USA (FDA)	All subtypes	AML 20-30% blasts (formerly RAEB-t)	All subtypes	AML<30% blasts (formerly RAEB-t)
Europe (EMA)	INT2/high-risk MDS according to IPSS, CMML 10-29% blasts, not eligible for alloSCT	AML≥65 years regardless of blast counts, not eligible for alloSCT	Not approved	AML≥65 years not candidates for standard induction chemotherapy

FDA, Food and drug administration; EMA, European Medicines Agency; RAEB-T, refractory anemia with excess of blasts-in transformation; IPSS, International Prostate Symptom Score.

Due to two different metabolic pathways upon cellular uptake, DAC solely interacts with DNA whereas AZA is incorporated by 10-20% into DNA and by 80-90%

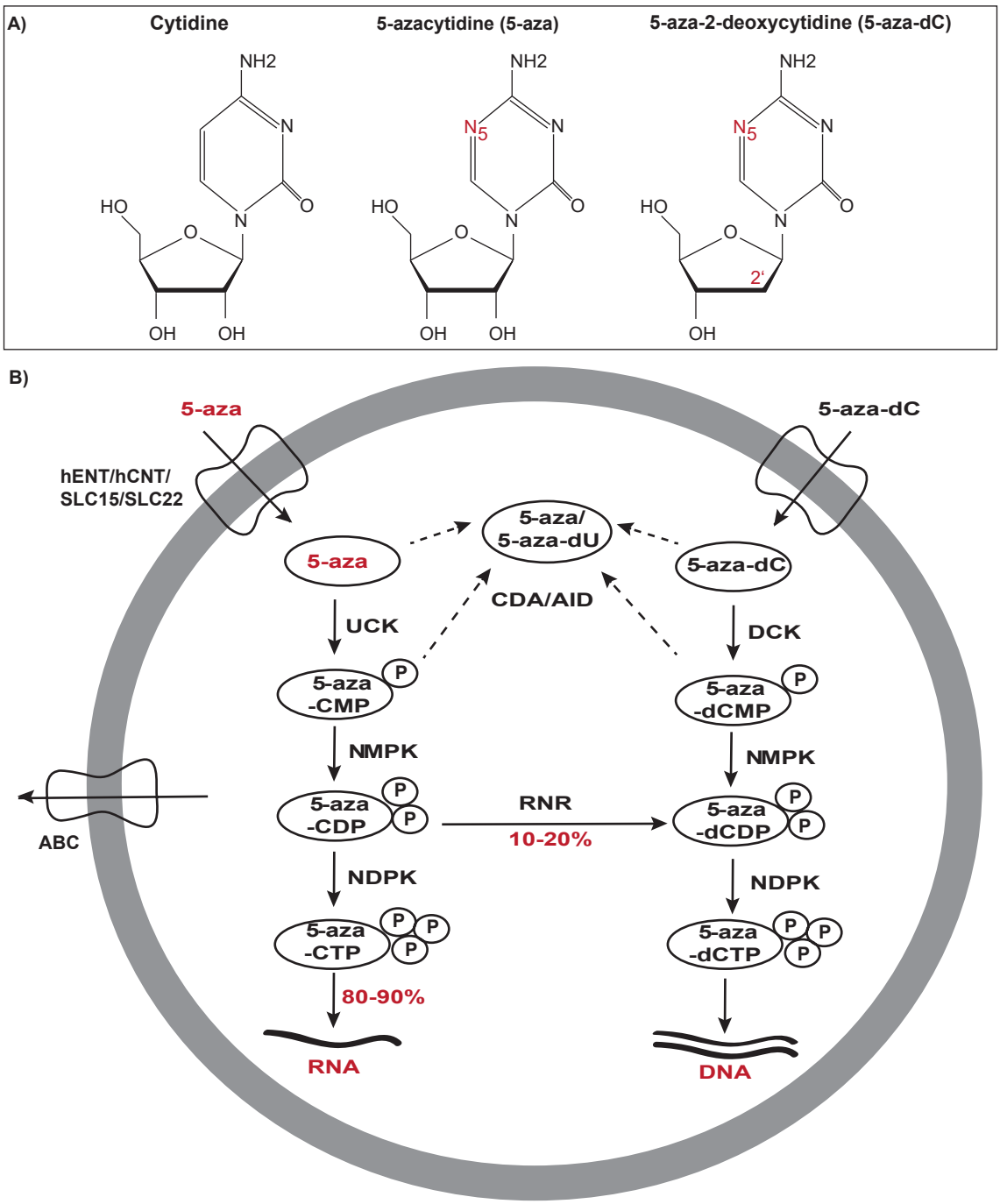
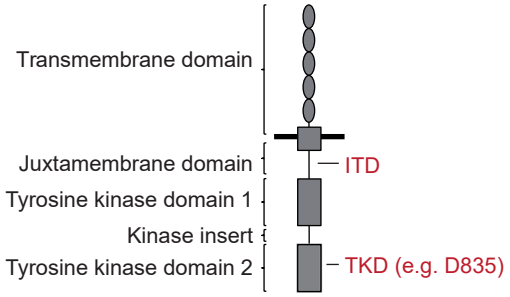


Figure 6. Chemical structure and metabolism of azacitidine and decitabine (Diesch *et al.*, 2016). Chemical structure of cytidine nucleoside and structural changes in azanucleosides are highlighted in red (A). Human equilibrative and concentrative nucleoside transporters (hENT/SLC29A and hCNT/SLC28A, respectively) and the SLC15 and SLC22 transporter families mediate AZN (5-aza and 5-aza-dC) uptake. Once inside the cell, the drugs are activated through consecutive ATP-dependent phosphorylation steps: the first one is mediated by uridine-cytidine kinase (UCK) for 5-aza and by deoxycytidine kinase (DCK) in the case of 5-aza-dC; the enzyme nucleoside monophosphate kinase (NMPK) incorporates the second phosphate group in both drugs; then, ribonucleotide reductase (RNR) partly converts (10–20 %) 5-aza-CDP into its deoxy form 5-aza-dCDP. Finally, nucleoside diphosphate kinase (NDPK) adds the third phosphate group and 5-aza-CTP is incorporated into RNA while 5-aza-dCTP is incorporated into DNA (B).

into RNA (Figure 6). Since DAC and AZA are potent inhibitors of DNMT, they are often referred to as hypomethylating agents (HMA), which may abrogate silencing of tumor-suppressor genes. However, a clear relation between demethylation of DNA and response to AZN could not be demonstrated thus far. Further, conflicting data from retrospective analyses have been reported about the prognostic relevance of recurrent epigenetic gene mutations in AML and MDS regarding clinical response to AZN (Diesch *et al.*, 2016).

1.4 *FLT3* with internal tandem duplication (*FLT3*-ITD) in AML

During normal hematopoiesis mainly early myeloid and lymphoid progenitors (Figure 1) express FMS-like tyrosine kinase 3 (FLT3) receptor (Figure 7). Together with other growth factors FLT3 receptor signaling regulates proliferation, differentiation and apoptosis of HSPC. FLT3 is often overexpressed in AML cells, which are naturally blocked at the HSPC stage (Carow *et al.*, 1996). Wild-type FLT3 becomes glycosylated and transferred as an inactive monomer to the cell membrane. FLT3-ligand (FL) binding induces FLT3 dimerization leading to phosphorylation of the TKD and subsequent downstream signaling. The activated receptor quickly becomes internalized and degraded. Almost all tissues express FL and paracrine as well as autocrine FL feedback loops control FLT3-WT receptor activation. FL also promotes growth of AML cells (Stirewalt and Radich, 2003).



578 598 578

FLT3-WT 578 to 621: 5'...**M**VQVTGSSDNEYFYVDFREY**E**YDLKWEFPRENLEFGKVLGSGA**F**...3'

599

FLT3-ITD 598/599(12): 5'...MVQVTGSSDNEYFYVDFREYE**N**EYFYVDFREYEYDLKWEFPRENLEFGKVLGSGAF...3'

Figure 7. *FLT3*-WT and -ITD receptor composition. FLT3 encodes a receptor tyrosine kinase (RTK; subclass III family) composed of a five immunoglobulin-like extracellular domain, a transmembrane domain (TMD), a juxtmembrane domain (JMD) and two intracellular tyrosine kinase domains (TKD) linked by kinase-inserts (Abu-Duhier *et al.*, 2001) (Agnès *et al.*, 1994). Exemplified amino acid (aa) sequences of *FLT3*-WT and *FLT3*-ITD are shown. Here, the twelve aa long internal-tandem-duplication (ITD) is located after aa 598 as indicated in red (Arreba-Tutusaus *et al.*, 2016).

Mutations in *FLT3* are one of the most prevalent genetic lesion in AML. Two main mutation types exist. Approximately 30 % of AML patients (predominantly CN-

AML cases) express *FLT3* with internal tandem duplication (*FLT3*-ITD) in the JMD sequence (exon 14 and 15) (Thiede *et al.*, 2002)(Schlenk *et al.*, 2002). The ITD can range from 3 to 400 base pairs (Figure 7) (Schnittger *et al.*, 2002). Around 7% of AML patients carry a missense point mutation in exon 20 of the TKD. The most common TKD alteration is an aspartatic acid to tyrosine substitution in codon 835 (D835Y). *FLT3*-ITD and -TKD mutations always maintain the original reading frame but induce conformational changes that render the receptor constitutively FL-independently active. *FLT3*-TKD does not seem to significantly impact AML outcome (Bacher *et al.*, 2008), whereas *FLT3*-ITD has high clinical relevance. FLT3-ITD signaling is characterized by aberrant AKT, ERK and STAT5 activation that promotes aggressive growth of AML cells (Stirewalt and Radich, 2003).

FLT3-ITD can be a “late” event during AML leukemogenesis (see above) and is sometimes lost at relapse. Nevertheless, several lines of evidence coming from experimental as well as clinical studies suggest that FLT3 may also be a crucial LSC initiating event (i.e. founding clone). For example, transgenic expression experiments using normal human $CD34^+$ BM cells showed that *FLT3*-ITD partially blocks differentiation and promotes proliferation as well as survival of normal HSPC (Li, Piloto and Kim, 2007). Moreover, several mouse models have demonstrated that *FLT3*-ITD drives myeloproliferative disorders, although it cannot alone induce full-blown leukemia (Li, Piloto and Kim, 2007)(Lee *et al.*, 2005) (Kelly *et al.*, 2002). Finally, two independent studies indicate the presence of *FLT3*-ITD in LSC-enriched $CD34^+$ AML BM subsets, although these data have to be interpreted with caution since the cell sorting technologies that had been used bear a high risk of false positive signals (Levis *et al.*, 2005)(Pollard *et al.*, 2006). At relapse, the *FLT3*-ITD/WT ratio often increases indicating a dominant relapse clone that is dependent on *FLT3*-ITD signaling (Schnittger *et al.*, 2002)(Shih *et al.*, 2002).

1.4.1 *FLT3*-ITD$^+$ AML subtypes

FLT3-ITD is most frequently associated with CN-AML (32%), but also often seen in acute promyelocytic leukemia (APL) and AML with a t(6;9)(p23;q34) translocation (Fröhling *et al.*, 2002)(Kottaridis *et al.*, 2001)(Schnittger *et al.*, 2002)(Thiede *et al.*, 2002)(Gale *et al.*, 2005)(Oyarzo *et al.*, 2004). *FLT3*-ITD is less often seen in AML with *CEBPA* lesions (5% to 10%) and AML with poor risk cytogenetics (3 to 7%). In CN-AML, *FLT3*-ITD$^+$ AML patients show elevated white blast cell counts (leukocytosis), bear an increased risk for relapse and have a dismal overall survival (OS) rate compared to non-mutated patients (Fröhling *et al.*, 2002) (Kottaridis *et al.*, 2001)(Schnittger *et al.*, 2002)(Thiede *et al.*, 20102)(Santos *et al.*, 2011). Overall, *FLT3*-ITD confers poor prognosis to CN-AML with an intermediate risk profile, but has no additional prognostic value in good-risk or poor-risk

AML subtypes (Fröhling *et al.*, 2002)(Schlenk *et al.*, 2008)(Santos *et al.*, 2011) (Patel *et al.*, 2012) (Table 2). Based on integrated mutational analysis on risk stratification in 398 *de novo* AML patients, Patel *et al*. showed that re-occurring gene mutations (Figure 8) can further predict outcome in *FLT3*-ITD$^+$ intermediate-risk AML and *FLT3*-ITD$^+$ CN-AML: I) poorest prognosis (14.5 % 3-year OS are *FLT3*-ITD$^+$ patients with mutant *TET2*, *DNMT3A*, *MLL*-PTD or trisomy 8 without mutant CEBPA II) intermediate prognosis (35.2% 3-year OS) are *FLT3*-ITD$^+$ patients with wild-type *CEPA*, *TET2*, *DNMT3A*, and MLL-PTD and III) best outcome (42% 3-year OS) are *FLT3*-ITD$^+$ AML patients with *CEPA* mutations (Patel *et al.*, 2012).

In *NPM1* mutated AML, OS rates significantly decrease in patients with *FLT3*-ITD/WT ratios >0.05 (Pratcorona *et al.*, 2013)(Schneider *et al.*, 2012). The most prevalent genetic lesion in CN-AML is *FLT3* with internal tandem duplication (FLT3-ITD). *FLT3*-ITD significantly often co-occurs with mutations in *NPM1* and *DNMT3A*. *FLT3*-ITD$^+$/*NPM1*mut/*DNMT3A*mut AML represents a distinct AML subtype with dismal prognosis and a specific mRNA, miRNA and DNA methylation (extensive DNA methylation loss) pattern (Metzeler *et al.*, 2016)(Cancer Genome Atlas Network, 2013)(Papaemmanuil *et al.*, 2016). Likewise, *TET2* mutations confer unfavorable prognosis to *FLT3*-ITD$^+$/*NPM1*mut CN-AML patients (Patel *et al.*, 2012)(Tian *et al.*, 2014)(Hou *et al.*, 2014). The presence of *FLT3*-ITD at relapse is more likely in patients with any of the epigenetic-modifying gene mutations *TET2*, *IDH1/2* and *DNMT3A* (Wakita *et al.*, 2013).

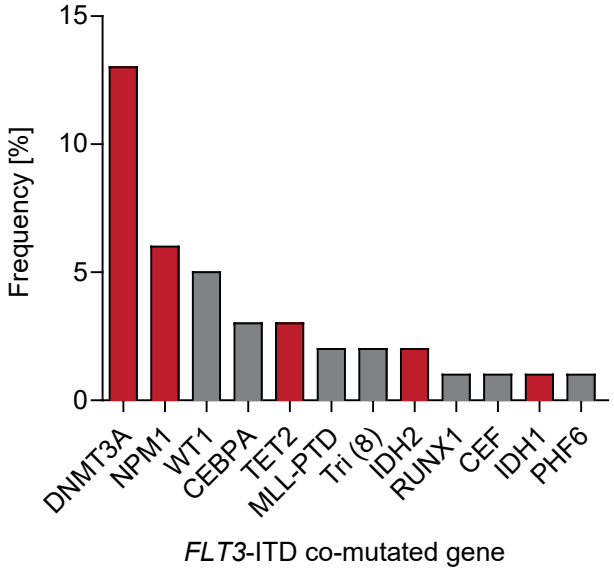


Figure 8. Frequency of co-occurring somatic gene mutations in *FLT3*-ITD$^+$ AML. Mutational analysis was performed in 398 *de novo* AML patients (median age 46.5 years) from the Eastern Cooperative Oncology Group E19000 clinical trial. Overall frequency of *FLT3*-mutant was 37% (30% ITD and 7% TKD). Graph represents frequencies for each co-occurring somatic gene mutations in the *FLT3*-ITD$^+$ cohort (Patel *et al.*, 2012).

1.4.2 Small molecule tyrosine kinase inhibitors (TKI) in *FLT3*-ITD$^+$ AML

Given its high incidence and oncogene potential, *FLT3*-ITD has become a prominent target in AML and many efforts have been made in the development of small molecule tyrosine kinase inhibitors (TKI) against the RTK. First-generation FLT3-active TKI (e.g. midostaurin, sunitinib (SU11248), sorafenib (sora) and lestaurtinib) were not specifically developed against FLT3 but several other RTK. They therefore are rather unspecific multikinase inhibitors that often cause unwanted off-target effects. Later, more FLT3-selective small molecule inhibitors were tested in library-based screens. Developers of these so-called second-generation TKI (e.g. quizartinib) promised higher selectivity towards mutated FLT3 and improved pharmacokinetics *in vivo*. Current FLT3-targeting TKI are type I or type II competitive ATP inhibitors (Table 5). Type I TKI directly block the ATP-binding site of the activated RTK (DFG-in conformational state), whereas type II inhibitors also bind to an adjacent hydrophobic binding site of the inactive RTK (DFG-out conformational state).

Only recently, for the first time a large global randomized phase III trial (RATIFY) demonstrated that combining the multikinase inhibitor midostaurin with intensive chemotherapy may bring a survival benefit for *FLT3*-ITD$^+$ AML patients. However, as single agents or in combination with chemotherapy, most TKI showed rather disappointing clinical phase I/II results in *FLT3*-ITD$^+$ AML with only transient responses (Table 5).

The limited clinical success of TKI in *FLT3*-mutated AML is not explainable solely in poor pharmacokinetic terms (e.g. short plasma half-life). Diverse intrinsic and extrinsic AML cell mechanisms can account for TKI resistance. Prolonged treatment with TKI leads to emergence of secondary kinase resistance mutations (Smith *et al.*, 2012)(Smith *et al.*, 2015) or activation of alternative signaling pathways. Autocrine feedback loops in AML cells induce increased expression of FLT3 ligand (FL) (Knapper *et al.*, 2006)(Kindler, Lipka and Fischer, 2010)(Sato *et al.*, 2012). Further, FL expression is inducible by chemotherapy (Sato *et al.*, 2011). Importantly, it was shown that TKI are more effective in patients with high *FLT3*-ITD/WT ratios meaning those with a dominant *FLT3*-ITD$^+$ clone. Vice versa, TKI therapy of *FLT3*-ITD patients with low *FLT3*-ITD/WT ratios may promote the formation of a resistant and aggressive refractory/relapse clone (Thiede *et al.*, 2002)(Pratz *et al.*, 2010)(Schlenk *et al.*, 2014). Accordingly, prevention of relapse requires complete eradication of rare LSC enriched in the CD34$^+$CD38$^-$ BM compartment and responsible for disease propagation. We have previously shown that LSC in *FLT3*-ITD$^+$ AML persist and may even expand during treatment with first-generation TKI despite effective inhibition of FLT3 phosphorylation owing to

their selective protection by niche cells (Parmar *et al.*, 2011). Therefore, it remains questionable whether FLT3-ITD-selective next generation TKI alone can eliminate LSC in their niche, especially since LSC can harbor additional mutations such as *DNMT3A*, *NPM1* and *TET2* which may contribute to treatment resistance.

Table 5. Results of clinical trials using first and second generation FLT3-active TKI.

Drug: Targets	Clinical response
Midostaurin (PKC412) Indolocarbazole alkaloid; type I inhibitor: PKC-α, VEGFR2, KIT, PDGFR, and FLT3	**Global randomized phase III trial (RATIFY)(Stone *et al.*, 2015):** 3279 previously untreated AML Midostaurin plus standard chemotherapy improved OS of younger (median age: 48) *FLT3*-mutated AML patients (TKD or ITD; low or high FLT3mut burden) in comparison to chemotherapy alone No difference in CR rate between the two study arms Beneficial effects are likely not just *FLT3*-selective
Lestaurtinib (CEP-701) Indolocarbazole alkaloid; type I inhibitor: JAK2, TrkA, TrkB, TrkC, and FLT3	**Phase 2 study (Knapper *et al.*, 2006):** Monotherapy in untreated older (mean age: 73) AML patients not considered fit for intensive chemotherapy Only transient reduction of BM blasts in 23% (5/22) *FLT3*-WT patients and only transient reduction of PB blasts or hematologic response (longer periods of transfusion independence) in 60% (3/5) patients with *FLT3*-ITD or TKD mutations **Phase 2 study (Levis *et al.*, 2011):** *FLT3*-mutant AML at first relapse No difference in remission rates or OS between salvage chemotherapy alone or salvage chemotherapy followed by lestauritinib treatment Only a small portion of patients achieved sustained FLT3 inhibition *in vivo*
Sorafenib (BAY 43-9006) Bis-aryl urea derivate; type II inhibitor: VEGFR, PDGFR, Raf kinase, and FLT3	**(Man *et al.*, 2012):** Monotherapy in relapsed or chemorefractory *FLT3*-ITD$^+$ AML patients (mean age: 45) Clearance or near clearance of BM blasts in 12/13 patients with evidence of leukemia cell differentiation Relapse after 72 days although the FLT3 downstream effectors remained repressed **Phase 1/2 study (Ravandi *et al.*, 2010):** Younger AML patients (<65 years old)

	Combination of sorafenib and idarubicin and cytarabine 100% response rate in *FLT3*-mutated AML (CR 16/18, CR with incomplete platlete revovery 2/18) More than half of patients relapsed despite the absence of TKI resistance mutation, unknown resistance mechanism **Phase 2 study (Serve *et al.*, 2013):** AML patients received either sora or placebo between the chemotherapy Cycles and subsequently for up to 1 year after the beginning of therapy Treatment in the sora arm did not result in significant improvement in EFS or OS regardless of the *FLT3* status Results of induction therapy were worse in the sorafenib arm, with higher treatment-related mortality and lower CR rates
Quizartinib (AC220), Bis-aryl urea derivate; type II inhibitor: FLT3, PDGFR, and KIT	**Phase 1 (Cortes *et al.*, 2013):** Monotherapy in relapsed or refractory AML patients (median age: 60) irrespective of *FLT3*-ITD mutation status 9/17 *FLT3*-ITD$^+$ patients achieved PR of CR (53%) 5/37 *FLT3*-WT patients (14%) Relapse occurred after a median duration of 13.3 weeks; median survival was 14.0 weeks **Phase 2 study (Levis *et al.*, 2012):** AML Patients ≥ 60 years old relapsed in <1 year or refractory to first-line chemotherapy Composite complete remission (CRc) rate included CR, complete remission with incomplete platelet recovery (CRp), and complete remission with incomplete hematologic recovery (CRi) For *FLT3*-ITD$^+$ patients the CRc rate was 54% (0 CR, 3% CRp, and 51% CRi), with a median duration of response of 12.7 weeks and median overall survival of 25.3 weeks Of those refractory to their last AML therapy, 39% achieved a CRc with quizartinib For *FLT3*-WT patients the CRc rate was 32% (2% CR, 2% CRp, and 27% CRi), with a median duration of response of 22.1 weeks and median overall survival of 19.0 weeks Of those refractory to their last AML therapy, 44% achieved a CRc

1.5 Research objectives and goals

Eradication of LSC in *FLT3*-ITD$^+$ AML by *FLT3*-TKI has thus far remained an elusive goal. Developing more effective TKI and combining TKI with other novel

agents such as epigenetic drugs are strategies currently being pursued in *FLT3*-ITD$^+$ AML (Ravandi *et al.*, 2013)(Strati *et al.*, 2015)(Gill *et al.*, 2015)(Chang *et al.*, 2016).

Crenolanib (creno), a benzamidine quinolone derivative originally developed as an inhibitor of platelet-derived growth factor receptor (PDGFR) demonstrated type I inhibition of *FLT3*-ITD as well as secondary TKD point mutants. The latter are known to confer resistance against type II FLT3-inhibitors. *In vitro* studies have found creno to have superior on-target effects compared to sorafenib or quizartinib and the compound is currently being evaluated in multiple phase II trials in relapsed or refractory *FLT3*-ITD$^+$ AML (Choy *et al.*, 2014)(Zimmerman *et al.*, 2013)(Galanis *et al.*, 2014).

AZA has emerged as a promising drug for the treatment of elderly AML and is also being evaluated as an add-on to intensive induction chemotherapy (Tschanter *et al.*, 2016)(Seymour *et al.*, 2015). AZA is a potent hypomethylating agent and it had been suggested that its therapeutic function in AML may be linked to epigenetic mutations in *DNMT3A* and *TET2* genes (Itzykson *et al.*, 2011)(Bejar *et al.*, 2014)(Traina *et al.*, 2014). Pre-emptive treatment with AZA can delay or prevent hematological relapses in AML with MRD, indicating it may have activity towards LSC (Platzbecker *et al.*, 2012).

Taking the promising features of creno and AZA into account, this study was undertaken to gain a better understanding and improve therapeutic intervention of LSC resistance in *FLT3*-ITD$^+$ AML. The specific research questions were:

I) Is *FLT3*-ITD present in the LSC compartment and therefore a valid LSC target?

II) Does monotherapy with the next generation TKI crenolanib eliminate LSC in *FLT3*-ITD$^+$ AML despite protective BM niche cells?

III) Can TKI efficacy be improved by addition of the hypomethylating agent AZA?

IV) Do cooperating gene mutations affect response to creno or AZA?

V) Are there specific mechanisms of stromal resistance that become altered by creno and/or AZA treatment?

2 Materials and methods

2.1 Materials

2.1.1 Biological resources

2.1.1.1 Animals

Animal studies were performed in agreement with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), in compliance with German law on the protection of animals, and with approval of the responsible regional authorities. NOD.Cg-Prkdcscid IL2rgtm1Wjl/Sz mice (NSG; The Jackson Laboratory) were maintained at the animal facility of the TUM.

2.1.1.2 Cell lines

MV4-11, MOLM-13, OCI-AML3 and RS4;11 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DMSZ).

The stromal cell line EL08-1D2 was generated from the fetal liver of murine embryos transgenic for the SV40 Tag immortalizing gene (Oostendorp *et al.*, 2002). EL08-1D2 cells strongly express IGFBP-4, IL-6 and osteopontin suggesting that this cell line might represent an osteoblastic cell (Oostendorp *et al.*, 2005).

2.1.1.3 Bone marrow samples

BM samples were obtained from newly diagnosed AML patients recruited to the German AML Study Group (AMLSG) trials. Written informed consent in accordance with the Declaration of Helsinki was obtained from all patients according to protocols approved by the Ethics Committee of the TUM.

2.1.1.4 Patient derived xenograft (PDX) cells

FLT3-ITD^{+} AML patient derived xenograft (PDX) cells and transgenic (t-)PDX cells which express firefly luciferase were generated by serial passaging in NSG mice as described (Vick *et al.*, 2015).

2.1.2 Instruments and general handling material

Animal blood counter	Scil vet academy, Germany
Blood lancets supra	Megro GmbH & Co KG, Germany

Cell culture dish, 10 mm	TPP, Switzerland
Cell culture flasks	Greiner Bio-One, Germany
Cell culture plates	Greiner Bio-One, Germany
Centrifuge	Heraeus Instruments, Germany
Charge-coupled device camera	OrcaII ER, Hamamatsu
Cryocontainer	Sigma-Aldrich
Cryotubes	Corning Inc.
CyAn ADP Lx P8	Beckman Coulter
Electrophoresis chamber	BioRad
ELx800 Universal Microplate reader	BIO TEK Instruments
FACS Aria II	Becton Dickinson
Filter tips	Starlab, Germany
Filter vacuum driven bottle top filter	Millipore, USA
Hamilton Syringe	Hamilton, Switzerland
Humidified incubator	Sanyo
Ice-maschine	Ziegra, Germany
Kodak films	Thermo Fisher
Laminar flow hood	BIOHIT, Germany
Light microscope	Carl Zeiss, Germany
MACS LD Columns	Miltenyi
MACS Midi Separator	Miltenyi
MACS Mini Separator	Miltenyi
MACS MS Columns	Miltenyi
Microcentrifuge	Hettich Zentrifugen
Microcentrifuge safe-lock tubes	Eppendorf
MiSeq sequencer	Illumina
Monoject blunt cannula needles	Kendall Healthcare, USA
NanoDrop	NanoDrop Technologies Wilmington, USA
Needles	Braun, Germany
Neubauer chamber	Paul Marienfeld GmbH, Germany
Olympus BX53 microscope	Olympus, Germany
Olympus DP26 camera	Olympus, Germany
Polybrene-centrifuge tubes	Greiner Bio-One, Germany
PVDF membranes	Millipore
Radiation unit	Gulmay, USA
Rocker platform	Biometra
Serological pipets	BD Falcon
SimplePCI software	Hamamutsu
S-Monovette blood collection System	Sarstedt, Germany
Sonificator	Bandelin Sonopuls

Sterile filter	Millipore
Vortex	Werke&Co, Germany
Water bath	Memmert
Wet-transfer device	BIO RAD
Whatman paper	GE Healthcare

2.1.3 Software

Microplate Manager 5.2. software

FlowJo software

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation

Gene expression omnibus database (GEO)

GraphPad Prism software

ImageJ software

IonTorrent PGM

R packages gplots version 3.0.1

R version 3.2.0

RcolorBrewer version 1.1-2

Sequence Pilot software package

The SimplePCI software

Visualization and Integrated Discovery (DAVID) version 6.8 Beta

2.1.4 Chemicals and reagents

α-MEM	Thermo Fisher
Benzamidine	Sigma-Aldrich
Biocoll Separating solution	Biochrom
BIT 9500 Serum Substitute	Stemcell technologies
Bovine serum albumin (BSA)	Carl Roth
Bromophenol blue	Sigma-Aldrich
$C_2H_4O_2$ (100%, acetic acid)	Merck Millipore
Ciprofloxacin	Frisenius Kabi, Germany
Deoxyribonuclease (DNase) I Solution (DN-25)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Serva

Dimethyl-thiazole diphenyl tetrazolium bromide (MTT)	Promega
Dithiothreitol (DTT)	Sigma-Aldrich
D-Luciferin, sodium salt *UltraPure Grade* | CAS	Biomol
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal calf serum (FCS)	PAA, Cölbe, Germany
Gelatin	Sigma-Aldrich
Glutamine	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
HBSS	Thermo Fisher
Horse serum (HS)	BioWhittaker, Denmark
HEPES	Thermo Fisher
Hydrocortison	Sigma-Aldrich
IMDM + Glutamax I	Thermo Fisher
Methanol (100%)	Millipore
MyeloCult H5100	StemCell technologies
Na_2VO_4	Sigma-Aldrich
$Na_4P_2O_7$	Sigma-Aldrich
NaCl	Sigma-Aldrich
NaF	Sigma-Aldrich
NaN_3	Sigma-Aldrich
PBS	Thermo Fisher
Penicillin/Streptomycin (Pen/Strep)	Thermo Fisher
PMSF	Sigma-Aldrich
protease inhibitor cocktail	Sigma-Aldrich
RPMI 1640	Thermo Fisher
SDS	Sigma-Aldrich
Skim Milk Powder	Sigma-Aldrich
ß-Mercaptoethanol	Thermo Fisher
HCl	Millipore
Triton X-100	Sigma-Aldrich
Trypan blue	Thermo Fisher
Tween	Sigma-Aldrich

2.1.5 Cytokines

FLT3-Ligand, IL-3, IL-6, LDL, and SCF were purchased from R&D Systems.

2.1.6 Drugs

Creno, Sora (both Selleckchem) and AZA (Celgene Corp) were freshly prepared directly before use following the manufacturers' instructions.

Medetomidine-midazolam-fentanyl (MMF)	Pfizer GmbH
Aipamezole-flumazenil-naloxone (AFN)	Pfizer GmbH
Isofluroran	Cp-pharma

2.1.7 Buffer and growth media composition

Amido black stain:	100 ml methanol (100%) 100 ml $C_2H_4O_2$ (100%) 800 ml H_2O
Annexin V/PI buffer:	5 ml Hepes 1M pH 7.4 28 ml NaCl 2.5 M 0.18 g $CaCl_2$ 467 ml H_2O 0.22 µm steril filtered
Cell culture medium for EL08-1D2:	400 ml α-MEM 15% h.i. FCS 5% h.i. HS 1% Penicillin/Streptomycin 10 µM β-Mercaptoethanol 0.22 µm steril filtered
Cell culture medium for MV4-11 and MOLM-13:	90% RPMI 1640 10% h.i. FCS 0.22 µm steril filtered
Cell culture medium for RS4;11, OCI-AML3:	90% alpha-MEM 10% h.i. FCS 0.22 µm steril filtered
Gelatin 0.1%:	5 g gelatin 500 ml H_2O
$HF2^+$ buffer:	100 ml HBSS 10X 20 ml FCS 10 ml Hepes 10 ml PenStrep 860 ml H_2O 0.22 µm steril filtered
LTC medium:	100% Myelokult 10 ng/ml FLT3-Ligand 20 ng/ml TPO

	Hydrocortison 1:250 ciprofloxacin 1:100 glutamin 0.22 µm steril filtered
Na-azid 200X:	4% NaN_3 (2 g on 50 ml H_2O)
Protein loading dye:	200 mM DTT 0.1 M TRIS HCl 4% SDS 0.05 % bromophenol blue 20% glycerol
Protein lysis buffer:	10 mM Tris/HCl (ph 7.4) 5 mM EDTA 130 mM NaCl 1% Triton X-100 protease inhibitor cocktail 10 mM $Na_4P_2O_7$ (ph=7–7.5) 5 mM NaF 1 mM PMSF 50 nM Benzamidine 1 mM Na_3VO_4 phosphate buffer (ph =7.0)
Running buffer 10X:	288g glycine 60 g TRIS 20 g SDS On 2 L H2O
Running buffer 1X:	1:9 10X Running buffer:H2O
Serum-free medium with five growth factors (SFM+5GF):	20 % BIT9500 80% IMDM Glutamax 10 µM ß-Mercaptoethanol 1:250 ciprofloxacin 4 ug/ml LDL 100 ng/ml SCF 100 ng/ml FLT3-Ligand 25 ng/ml TPO 10 ng/ml IL-3 10 ng/ml IL-6

	0.22 µm steril filtered
Tris-buffered saline (TBS) 1X:	1:9 dilution 10X TBS:H2O
TBS-Tween (TBS-T):	1 L 1X TBS 1 ml Tween
Transfer buffer:	.5 L H_2O 1 L methanol 0.5 L 10x running buffer
TBS 10X:	84 g NaCl 24.2 g Tris On 1 L H_2O Adjust pH = 7.6

2.1.8 Commercial kits

2.1.8.1 DNA

MiSeq Reagent Kits V3	Illumina, Chesterford, UK
QIAamp DNA Micro Kit	Qiagen
TruSight Myeloid Assay	Illumina, Chesterford, UK
ZR Viral DNA Kit™	Zymo Research

2.1.8.2 RNA

RNeasy Mini Kit	Qiagen

2.1.8.3 Protein

Protein ladder 10 to 250 kDa	Thermo Fisher
4-15 % Mini Protean TGX stain free gels	BioRad
DC Protein Assay Kit II	BioRad
SuperSignal West Dura Chemiluminescent	Thermo Fisher
SuperSignal West Femto Substrate	Thermo Fisher
SuperSignal West Pico Chemiluminescent Substrate	Thermo Fisher
Human FLT3 Ligand ELISA	R&D
Mouse FLT3 Ligand ELISA	R&D
Mouse on Mouse kit	Abcam

2.1.8.4 Cells

Human CD34 MicroBead Kit (MACS)	Miltenyi Biotech
ACK lysis buffer	Thermo Fisher

APC BrdU Flow Kit	BD Pharmingen
Methylcellulose (StemMACS HSC-CFU complete + Epo)	MiltenyiBiotech

2.1.9 Antibodies

2.1.9.1 Immunoblotting

A/G PLUS-agarose (sc-2003):	Santa Cruz
Anti-rabbit IgG-agarose conjugate (sc-2345):	Santa Cruz
FLT3/FLK2 (sc-480):	Santa Cruz
Purified mouse anti-phosphotyrosine (PY20):	BD Biosciences
Anti-phosphotyrosine (4G10):	Millipore
pFLT3 (Tyr591):	Cell signaling
ERK1 (K-23):	SantaCruz
pERK (E-4):	SantaCruz
Stat5 (3H7):	Cell signaling
pStat5 (Tyr 694) (D47E7):	Cell signaling
AKT (#9272):	Cell signaling
pAKT (Ser473) (193H12):	Cell signaling
ß-actin (AC-15):	Sigma-Aldrich
Anti-rabbit IgG ECL HRP-linked secondary antibody:	GE Healthcare
Anti-mouse IgG ECL HRP-linked secondary antibody:	GE Healthcare

2.1.9.2 Flow cytometry

Anti-human CD4 (RPA-T4):	Biolegend
Anti-human CD8a (RPA-T8):	Biolegend
Anti-human CD19 (HIB19):	Biolegend
Anti-human CD41 (MEM-06):	Sigma
Anti-human CD235alpha (HIR2):	eBioscience
Anti-human CD56 (B159):	BD Pharmingen
Anti-human CD34-FITC (581):	BD Pharmingen
Anti-human CD90-PE (5e10):	BD Pharmingen
Anti-human CD33-PC5.5 (D3HL60):	Beckmann Coulter
Anti-human CD45RA-APC Cy7 (H1100):	BD Pharmingen
Streptavidin-eFluor 450:	eBioscience
Anti-human CD38-APC (HB7):	BD Pharmingen
Anti-human CD45-APC-Cy7 (2D1):	BD Pharmingen
Anti-mouse CD45-APC (30-F11):	eBioscience
Cell Trace Violet:	Thermo Fisher
Propidium iodide (PI)	Thermo Fisher

2.2 Methods

2.2.1 General cell culture

2.2.1.1 Cell lines

MV4-11, MOLM-13 and RS4;11 cell lines were propagated as suggested by the DMSZ and passaged for <6 months after receipt. Growth media are listed in 2.1.7.

EL08-1D2 stromal cells were used up to passage 12 and cultured as described (Oostendorp *et al.*, 2002) in stroma medium on 0.1% gelatin-coated cell culture plates. For co-culture experiments, EL08-1D2 cells were grown 80% confluent in stroma medium. The stromal layer was washed with PBS before addition of co-culture cells in their respective media. For long-term cultures (LTC) EL08-1D2 cells were irradiated (30 gy) at 80% confluency. Cells were allowed to recover for 7 days before LTC were set up.

All cells were propagated in a humidified incubator at 37°C and 5% CO_2.

2.2.1.2 Bone marrow samples

Mononuclear cells (MC) were isolated from BM aspirates directly after receipt using biocoll separating solution. In brief, heparinized whole blood and IMDM were mixed (1:1) and carefully applied over 12 ml biocoll separating solution. After centrifugation at 1200 x g for 20 minutes, the layer of enriched MC (between plasma and biocoll) was carefully taken with a 10 ml pipette. Cells were washed twice in IMDM and frozen as described in 2.2.1.4 until usage.

Patient samples were screened for presence of *FLT3*-ITD, *IDH1*, *IDH2*, *DNMT3A*, *NPM1* and *TET2* mutations as described (Schlenk *et al.*, 2008).

2.2.1.3 Cell counting

Viability and count of cells was estimated by trypan blue exclusion in a Neubauer chamber under a light microscope.

2.2.1.4 Freezing and thawing

For long term storage, up to $1x10^8$ cells were resuspended in 1 ml h.i. FCS supplemented with 10% DMSO and transferred into cryotubes. The cell suspension was slowly frozen (-1°C/minute) to -80°C using a cryocontainer.

Frozen cells were thawed in a 37°C water bath. Quickly, the cell suspension was transferred into a 15 ml Falcon and washed twice with PBS (cell lines) or

IMDM (patient material). BM samples and PDX cells were incubated with 200 ug/ml DNase for 5 minutes at RT to reduce clumping. Cells were again washed in IMDM.

2.2.2 Drug activity assays on leukemia cell lines

2.2.2.1 Drug dose-response curve

Dose-dependent cytotoxicity was determined by colorimetric quantification of MTT according to the manufacturer's instructions.

2.2.2.2 Analysis of apoptosis

To determine cell death and necrosis using flow cytometry, cells were washed and stained in Annexin V/PI buffer and APC-Annexin V as well as PI were added.

2.2.2.3 Analysis of cell cycle

Cell cycle analysis was performed using the APC BrdU Flow Kit according to the manufacturer's instructions.

2.2.2.4 SDS-PAGE and immunoblotting

Cells were collected and washed in ice-cold PBS containing 1 mM Na_2VO_4 and shock frozen in liquid nitrogen. Cell pellets were thawed on ice and incubated in lysis buffer. After 15 minutes, lysates were sonicated. Cell debris was removed by centrifugation at full speed for 15 minutes at 4°C in a microcentrifuge. Supernatants were collected and the protein concentration was estimated using the DC Protein Assay Kit II following the manufacturers' instructions. Protein absorption was measured at 750 nm using the ELx800 Universal Microplate reader and the Microplate Manager 5.2. software.

30 µg protein were taken up in loading dye, boiled at 95°C for 10 minutes and separated in 4-15 % Mini Protean TGX stain free gels by SDS-PAGE in 1X Running Buffer in an electrophoresis chamber. The gel was blotted onto PVDF membranes in transfer buffer in a wet-transfer device. After blocking in 5% BSA or milk in TBS-T, membranes were incubated overnight at 4°C with the following primary antibodies: FLT3/FLK2 (1:1000), pFLT3 (Tyr591) (1:1000), ERK1 (1:1000), pERK (1:1000), Stat5 (1:1000), pStat5(Tyr 694) (1:1000), AKT (1:1000), pAKT (Ser473) (1:1000), ß-actin (1:2000).

After washing with TBS-T, anti-rabbit or anti-mouse IgG ECL HRP-linked secondary antibodies were added for 20 minutes at RT. Membranes were washed with TBS and visualized on Kodak films using the enhanced chemiluminescence (ECL)-method (SuperSignal West). Signal intensity was analyzed using the Im-

ageJ software. Antibodies were stripped off the membranes using Amido Black Stain solution.

2.2.3 Characterization of leukemic driver mutations in *FLT3*-ITD$^+$ AML BM compartments

2.2.3.1 Flow cytometric cell sort (FACS) of leukemic blasts, progenitor and stem cell compartments

MC from *FLT3*-ITD$^+$ BM samples #1-10 were stained in HF2 buffer for lineage markers using biotinylated antibodies: CD4, CD8a, CD19, CD41, CD235α, CD56. Cells were then stained with the following flourochrome-conjugated antibodies: CD34-FITC, CD90-PE, CD33-PC5.5, CD45RA-APC Cy7, Streptavidin-eFluor 450, CD38-APC, CD45-APC-Cy7. PI was added as live/dead marker. Cell sorting was performed on a FACS Aria II. Sorting purity of >98% was routinely obtained.

2.2.3.2 Targeted sequencing of AML bulk and re-sequencing of FACS-sorted BM subpopulations

Genomic DNA was isolated from AML bulk and FACS-sorted AML subpopulations using the ZR Viral DNA Kit™ for < 2 x 10^5 cells and the QIAamp DNA Micro Kit for > 2 x 10^5 cells according to the manufacturers' instructions.

Molecular alterations in the gDNA from AML bulk samples were evaluated by targeted sequencing using the TruSight Myeloid assay which covers the following 54 genes or gene hotspots related to myeloid neoplasms: *BCOR*, *BCORL1*, *CDKN2A*, *CEBPA*, *CUX1*, *DNMT3A*, *ETV6*, *EZH2*, *IKZF1*, *KDM6A*, *PHF6*, *RAD21*, *RUNX1*, *STAG2* and *ZRSR2* and oncogenic hotspots of *ABL1*, *ASXL1*, *ATRX*, *BRAF*, *CALR*, *CBL*, *CBLB*, *CBLC*, *CDKN2A*, *CSF3R*, *FBXW7*, *FLT3*, *GATA1*, *GATA2*, *GNAS*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KIT*, *KRAS*, *MLL*, *MPL*, *MYD88*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PTEN*, *PTPN11*, *SETBP1*, *SF3B1*, *SMC1A*, *SMC3*, *SRSF2*, *TET2*, *TP53*, *U2AF1* and *WT1*. For preparation of target enrichment libraries, 50 ng of genomic DNA were used and prepared as recommended in the manufacturers' protocol (TruSight Myeloid Sequencing Panel Reference Guide 15054779 v02, Illumina). Samples were paired-end sequenced (2x225 bp) on a MiSeq sequencer using MiSeq Reagent Kits V3. Sequence data alignment of demultiplexed FastQ files, variant calling and filtering was done using the Sequence Pilot software package with default settings and a 5% VAF mutation calling cut-off. Detection of large insertions and deletions was performed using PINDEL algorithm following BWA-MEM mapping with default settings (Li and Durbin, 2010)(Ye *et al.*, 2009). Human genome build HG19 was used as reference genome for mapping algorithms.

Analysis in gDNA extracted from sorted AML subpopulations was done using targeted resequencing based on individual amplicons generated specifically for the affected regions. Deep sequencing was performed on MiSeq or IonTorrent PGM platforms with a minimum coverage of 10,000 reads.

2.2.4 Drug activity assays on primary *FLT3*-ITD$^+$ CD34$^+$ cells

2.2.4.1 Standardized 4-day co-culture of CD34$^+$ LSC and stromal niche cells

MC from FLT3-ITD$^+$ AML BM samples were enriched for CD34$^+$ cells by magnetic MACS bead separation according to the manufacturer's instruction.

CD34$^+$ enriched FLT3-ITD$^+$ AML cells were cultured on EL08-1D2 in SFM+5GF. Co-cultures were treated once with DMSO (1:1000), 100 nM creno, 10 µM AZA or simultaneously with 100 nM creno and 10 µM AZA for 4 days.

2.2.4.2 Analysis of apoptosis in CD34$^+$ cells

After 4 day co-culture, harvested cells were stained in HF2$^+$ buffer with CD45 APC-Cy7, CD34-FITC, CD33-PC5.5 antibodies to separate leukemic cells from murine stroma cells. Then, cells were washed and stained in Annexin V/PI buffer and APC-Annexin V and PI were added.

2.2.4.3 ELISA of human and murine FLT3-ligand (FL)

Cell culture supernatants were harvested from suspension and co-cultures as indicated. FL levels were measured using murine- and human-specific ELISA kits according to the manufacturer's instructions. Optical density was determined using the ELx800 Universal Microplate reader and the Microplate Manager 5.2. software.

2.2.4.4 Colony forming cell (CFC) assays

After 4 day co-culture, CD34$^+$ cells were harvested and remaining short-term and long-term proliferation and differentiation capacity was assayed by colony-forming cell (CFC) assays in methylcellulose. After 14 days, CFC were scored using standard criteria. To determine short-term leukemic cells (colony-forming units (CFU)) corresponding to leukemic progenitor cells, cells were directly plated in methylcellulose after 4 day co-culture. Long-term LSC (long-term cells (LTC)) corresponding to early leukemic progenitor and stem cells were assessed by CFC assays after co-culture on irradiated (30gy) EL08-1D2 for 6 weeks in LTC medium.

2.2.5 Patient derived xenograft (PDX) mouse model

2.2.5.1 PDX cell generation

Patient derived xenograft (PDX) cells were generated by serial passaging of one AML BM samples in NSG mice. One sample was genetically manipulation to express the T2A-linked transgenes firefly luciferase (effluc) and the red fluorochrome mCherry on an equimolar basis (transgenic (t-)PDX cells) as described (Vick *et al.*, 2015).

2.2.5.2 PDX transplantation into NSG mice

PDX or t-PDX cells were cultured on EL08-1D2 cells and treated with DMSO, AZA, creno or both for 4 days *in vitro*. Cells were harvested and viable cells were estimated by trypan blue exclusion. A total of 2 x 10^5 viable cells (per mouse) in 200 µl PBS were injected into the tail vein of non-irradiated 6 to 16-week-old NSG mice.

2.2.5.3 Analysis of PDX engraftment

To monitor PDX cell engraftment, peripheral blood (PB) samples were collected every other week beginning week 6 after PDX injection from the facial vein of NSG mice and analyzed by flow cytometry. Engraftment was defined as ≥0.1% murine $CD45^-$/ $CD45^+$/$CD33^+$ cells.

Transgenic-PDX engraftment in NSG mice was repetitively monitored by bioluminescence *in vivo* imaging (BLI). Mice were narcotized by intra peritoneal (IP) application of 70 µl MMF. 300 µl of a 15 mg/ml stock solution D-Luciferin were injected IP to activate the recombinant luciferase. Bioluminescence Images were taken using a charge-coupled device camera, equipped with an image intensifier for 10-120 seconds, bin size 2, gain 900 as previously described (Seidler *et al.*, 2008). The SimplePCI software was used to display and quantify BLI signals. 150 µl AFN were injected IP as well as subcutaneously into mice to reverse anesthesia.

Mice were sacrificed when PB sampling or BLI indicated systemic AML in the DMSO-treated mice. PDX cells were re-isolated from PB, femurs, tibiae and spines. After erythrocyte lysis, cells were stained with the following antibodies for flow cytometry: CD45 APC-Cy7, CD34-FITC, CD33-PC5.5, murine CD45-APC, Cell Trace Violet.

2.2.5.4 Immunohistochemistry

Tissue sections were deparaffinized. Antigen retrieval was carried out by pres-

sure cooking in citrate buffer (pH 6) for 7 minutes. Anti-human CD45 was detected after 1 hour incubation at RT by the Mouse on Mouse kit according to the manufacturer's protocol. Slides were evaluated using an Olympus BX53 microscope and images taken with an Olympus DP26 camera.

2.2.6 RNA sequencing and target gene validation

Total RNA was isolated from murine EL08-1D2 and human PDX cells using RNeasy Mini Kit (Qiagen). RNA sequencing (RNAseq) library preparation and data processing was performed according to the Unique Molecular Identifiers (UMI) sequencing protocol with the exception that pre-amplification was performed using 12 cycles (Parekh *et al.*, 2016). Briefly, RNA was reverse transcribed with oligo-dT primers carrying sample barcodes and unique molecular identifiers (UMIs). Next, cDNA of all samples was pooled, unincorporated primers digested by Exonuclease I digest and pre-amplified. After quality control of amplified cDNA, the sequencing library was constructed using the Nextera XT Kit (Illumina) and a custom primer enriching for 3' ends of transcripts. The library was sequenced on an Illumina HiSeq1500 with 16 cycles on the first read to decode sample barcodes and UMIs and 50 cycles on the second read into the cDNA fragment.

All samples were mapped against concatenated human (hg19) and mouse (mm10) genomes with Ensembl gene models (v75). Gene expression of PDX samples (reads mapped to human) and EL08 samples (reads mapped to mouse) was analyzed separately. 1/4 replicates of co-cultured EL08 cells that had been treated with crenolanib had very few mapped reads (3,508 in total) in comparison with all other samples (> 150,000 each) and was therefore excluded from analysis.

R version 3.2.0 was used on a x86_64-pc-linux-gnu (64-bit) platform under Ubuntu 14.04.2 LTS for gene expression analysis and visualization. Differential gene expression analysis was performed using DESeq2 (Love, Huber and Anders, 2014). DESeq2 uses library-size-corrected read count data to find differentially expressed genes and is error model based on the negative binomial distribution for the read counts. For gene expression analysis, the fits of the negative binomial with a generalized linear model were analyzed. Coefficients (interpreted as the log2-fold changes) were tested using the Wald test. The false discovery rate according to Benjamini-Hochberg was used for multiple testing corrections (Benjamini and Yoav, 1995). All conditions (DMSO, AZA, crenolanib, AZA + crenolanib) were compared to monoculture (EL08) or the condition after sorting (PDX). The hundred genes with the highest average gene expression difference between all treatments (AZA, crenolanib, AZA + crenolanib) compared to DMSO are shown

in heatmaps (main Figure 6B). The genes were sorted by log2 fold change for the DMSO samples in ascending order. The heatmaps were drawn with the R packages gplots version 3.0.1 and RcolorBrewer version 1.1-2.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation were done with the Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8 Beta (https://david-d.ncifcrf.gov/, May 2016, last accessed: August 18, 2016) for these two sets of genes (Dennis, Sherman and Hosack, 2003).

Raw and processed RNA sequencing data were deposited at NCBI's GEO under accession number GSE86021.

2.2.7 Data analysis

2.2.7.1 Flow cytometry

Flow cytometric analysis was performed on a CyAn ADP Lx P8. Data were analyzed with FlowJo software (TreeStar Inc).

2.2.7.2 Statistical Analysis

Statistical analyses were performed by Student's t-test or one-way ANOVA followed by Turkey's multiple comparisons using GraphPad Prism software (GraphPad Inc). P values are presented in the figures where a statistically significant difference was found: *, $P < .05$; **, $P < .01$; ***, $P < .001$.

3 Results

3.1 Patient samples

A total of 32 different BM samples from untreated *de novo FLT3*-ITD$^+$ AML patients were used in this study. Only one BM sample (#27) was taken from a *secondary* AML patient with a history of MDS (Table 6).

3.2 Validation of *FLT3*-ITD as potential LSC target

The prerequisite for durable responses in AML therapy is the successful elimination of LSC in the BM. Although *FLT3*-ITD is considered a late event in leukemogenesis, it may be the crucial initiator for LSC expansion (Chan and Majeti, 2013) and distinctly characterizes an aggressive AML subtype. Only limited experimental data exist indicating presence of *FLT3*-ITD in LSC-enriched CD34$^+$/CD38$^-$ BM subsets and sophisticated sorting was not performed in these studies (Levis *et al.*, 2005)(Pollard *et al.*, 2006). Thus, to assess occurrence of *FLT3*-ITD in distinct leukemic stem/progenitor subpopulations in relation to pre-leukemic and early leukemic hits, we analyzed ten *FLT3*-ITD$^+$ AML samples (five CD34$^+$ and five CD34$^-$) by amplicon based resequencing of 54 genes followed by flow cytometric sorting and targeted resequencing of identified alterations within leukemic stem/progenitor subpopulations (Figure 9A).

The mutational profile of our cohort was representative as it revealed a mean of 3.3 driver mutations per patient at diagnosis, concurrent with recent reports on the mutational landscape of AML (Metzeler *et al.*, 2016)(Papaemmanuil *et al.*, 2016). *NPM1* mutations were found in 7/10 patients. CD34 expression was typically (Falini *et al.*, 2005)(Haferlach *et al.*, 2009)(Chou *et al.*, 2006) low in the majority (5/7) of *NPM1*-mutated samples. In 7/10 patients, epigenetic mutations in either *TET2*, *IDH1*, *IDH2* or *DNMT3A* were found. *IDH1/2* lesions appeared exclusive, whereas two patients (#4 and #9) with *DNMT3A* mutations also carried a *TET2* mutation. Only one patient (#5) had *FLT3*-ITD as the sole mutation, but showed an aberrant t(6;9) karyotype (Figure 9B, Table 6 #1-10).

Next, we separated blasts, committed progenitors and early stem cell compartments by multiparameter flow cytometry based on the expression of CD33 as a known marker aberrantly expressed on LSC (Walter *et al.*, 2012)(Taussig *et al.*, 2005)(Hauswirth *et al.*, 2007). We found that the early leukemic stem cell compartment (Lin$^-$/CD33$^{(+)}$/CD45$_{dim}$/CD34$^+$CD38$^-$) in most CD34$^+$ AML samples (# 2, 3, 4, 5) was dominated by multilymphoid progenitors (MLP, CD45RA$^+$), whereas in CD34$^-$ AML, we also found multipotent progenitors (MPP, CD45RA$^-$/CD90$^-$) and

Table 6. AML patient sample characteristics.

ID	Age	Sex	Cytogenetics	*FLT3*-ITD/ WT ratio	*NPM1*	*DNMT3A*	*TET2*	Other	CD34
1	58	m	46, XY, del13(q)	0.166	wt	wt	wt	*IDH2*, *RUNX1*, *SF3B1*, *STAG2*	pos
2	81	m	46, XY	0.713	mut	wt	wt	neg	pos
3	47	f	46, XX	0.62	mut	wt	wt	*RAD21*	pos
4	35	f	46, XX	0.848	wt	mut	mut	*STAG2*, *GATA2*, (*MLL-PTD*)	pos
5	63	f	46, XX, t(6;9)	0.23	wt	wt	wt	neg	pos
6	68	m	46, XY	0.18	mut	wt	mut	neg	neg
7	31	f	46, XX	0.63	mut	wt	wt	*CEBPA*, *IDH1*	neg
8	69	m	49, XY, +Y, +21, +22	0.302	mut	wt	wt	*IDH1*	neg
9	63	f	46, XX	0.559	mut	mut	mut	*WT1*	neg
10	74	f	46, XX	0.98	mut	wt	mut	*NPM1*, *TET2*	neg
11	36	m	46, XY	5.141	mut	mut	wt	n.a.	pos
12	59	m	46, XY, i(14)(q10)	1.707	wt	wt	wt	n.a.	pos
13	43	f	46, XX, add(19)(13.1)	0.582	wt	wt	wt	n.a.	pos
14	41	f	46, XX, t(9;11)(p22;q23)	0.789	wt	wt	wt	n.a.	pos
15	59	f	46, XX	0.799	mut	wt	mut	n.a.	neg
16	66	m	46, XY	8.911	mut	mut	wt	n.a.	n.a.
17	64	m	n.a.	0.625	mut	mut	wt	n.a.	n.a.
18	70	m	n.a.	0.095	wt	wt	mut	n.a.	n.a.
19	51	m	n.a.	0.861	wt	wt	wt	n.a.	n.a.
20	46	f	46, XX	1.687	mut	mut	wt	n.a.	neg
21	63	f	46, XX, i(14)(q10) 46, idem, t(X;3)(p11;p13)	0.854	wt	mut	wt	n.a.	neg
22	78	f	46, XX	0.552	mut	wt	mut	n.a.	neg
23	72	f	46, XX	0.4	mut	n.a.	n.a.	n.a.	pos
24	75	f	46, XX	0.8	wt	mut	wt	*JAK2*	pos
25	59	f	46, XX	0.513	mut	mut	wt	n.a.	neg
26	35	m	46, XY	0.836	mut	wt	wt	n.a.	neg
27	61	f	46, XX	0.649	wt	wt	wt	RUNX1, NRAS	pos
28	66	m	46, XY	0.151	mut	mut	wt	n.a.	neg
29	77	f	46, XX	0.237	mut	wt	mut	n.a.	neg
30	83	f	47,XX,+4	0.937	mut	mut	mut	n.a.	pos
31	84	m	46,XY	0.885	mut	wt	mut	n.a.	neg
32	76	m	46, XY,complex	1.294	wt	mut	mut	n.a.	pos

hematopoietic stem cells (HSC, $CD45RA^{-}/CD90^{+}$, #7), consistent with published reports (Figure 9C) (Goardon *et al.*, 2011).

NPM1 mutations were present in all analyzed leukemic stem/progenitor compartments (Table 7). *DNMT3A*, *TET2*, *IDH1* mutations were present in early and late leukemic compartments at equally high allelic frequencies (Figure 9D, Table 7), concurrent with the concept that these are founder mutations (Chan and Majeti, 2013)(Corces-Zimmerman and Majeti, 2014). Of note, in all 5/10 AML samples where we were able to detect and sort leukemic HSC and MPP populations, *FLT3*-ITD was present in these fractions and allelic frequencies hierarchically increased from there (Figure 9E, Table 7), indicating dominant clonal hematopoiesis.

In summary, we demonstrate that *FLT3*-ITD, *NPM1*, *TET2* and *DNMT3A* mutations frequently co-occur within the most primitive $Lin^-/CD33^{(+)}/CD45_{dim}/CD34^+CD38^-$ putative LSC compartment and thus likely cooperate in leukemic expansion (founding clone). We can therefore ascertain that *FLT3*-ITD is indeed a potential target for LSC elimination in AML. However, co-occurrence of *NPM1*, *TET2* and *DNMT3A* mutations in this same compartment may influence response to *FLT3*-mutated LSC to TKI.

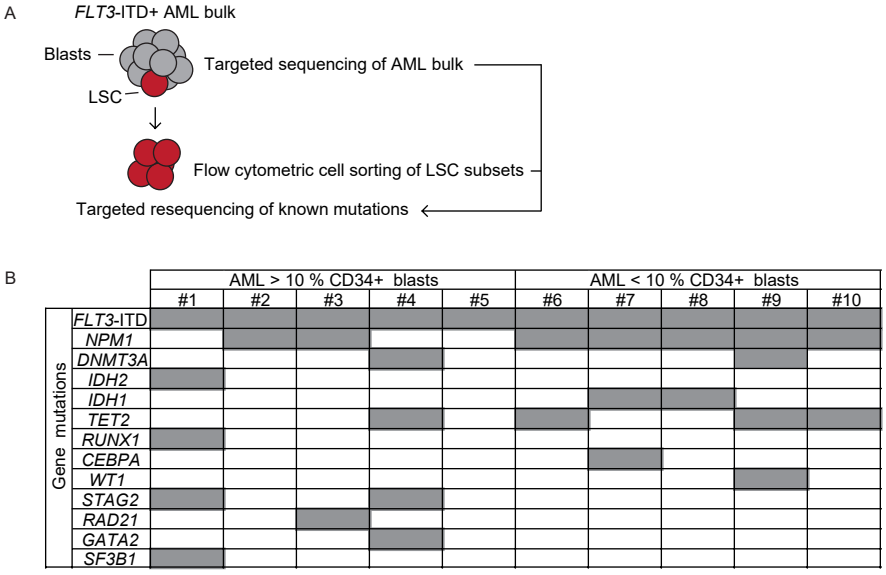


Figure 9. Detection of *FLT3*-ITD and concurrent gene mutations in leukemic stem/progenitor compartments. Experimental design (A). Mutational landscape of 10 unfractionated *FLT3*-ITD^+ AML BM samples with CD34 blast expression >10% or <10% assessed by amplicon based resequencing of 54 genes (B). MPFC gating strategy for sorting of $CD34^+$ and $CD34^-$ AML BM samples. Blasts ($Lin^-/CD33^{(+)}CD34^-$), committed progenitors ($Lin^-/CD33^{(+)}/CD45_{dim}/CD34^+CD38^+$), and early stem cell compartments ($Lin^-/CD33^{(+)}/CD45_{dim}/CD34^+CD38^-$) i.e. MLP ($CD45RA^+$), MPP ($CD45RA^-CD90^-$) and HSC ($CD45RA^-CD90^+$). Representative plots for a $CD34^+$ AML (upper panel) and $CD34^-$ AML sample (lower panel) are shown (C, page 47). gDNA was isolated from sorted compartments and mutations in *FLT3*, *NPM1*, *DNMT3A*, *TET2* and *IDH1* genes were detected by targeted resequencing. VAF (%) of *FLT3*-ITD (D, page 48) and epigenetic mutations (E, page 48) in sorted populations are shown. n.a., not available (e.g. population not found).

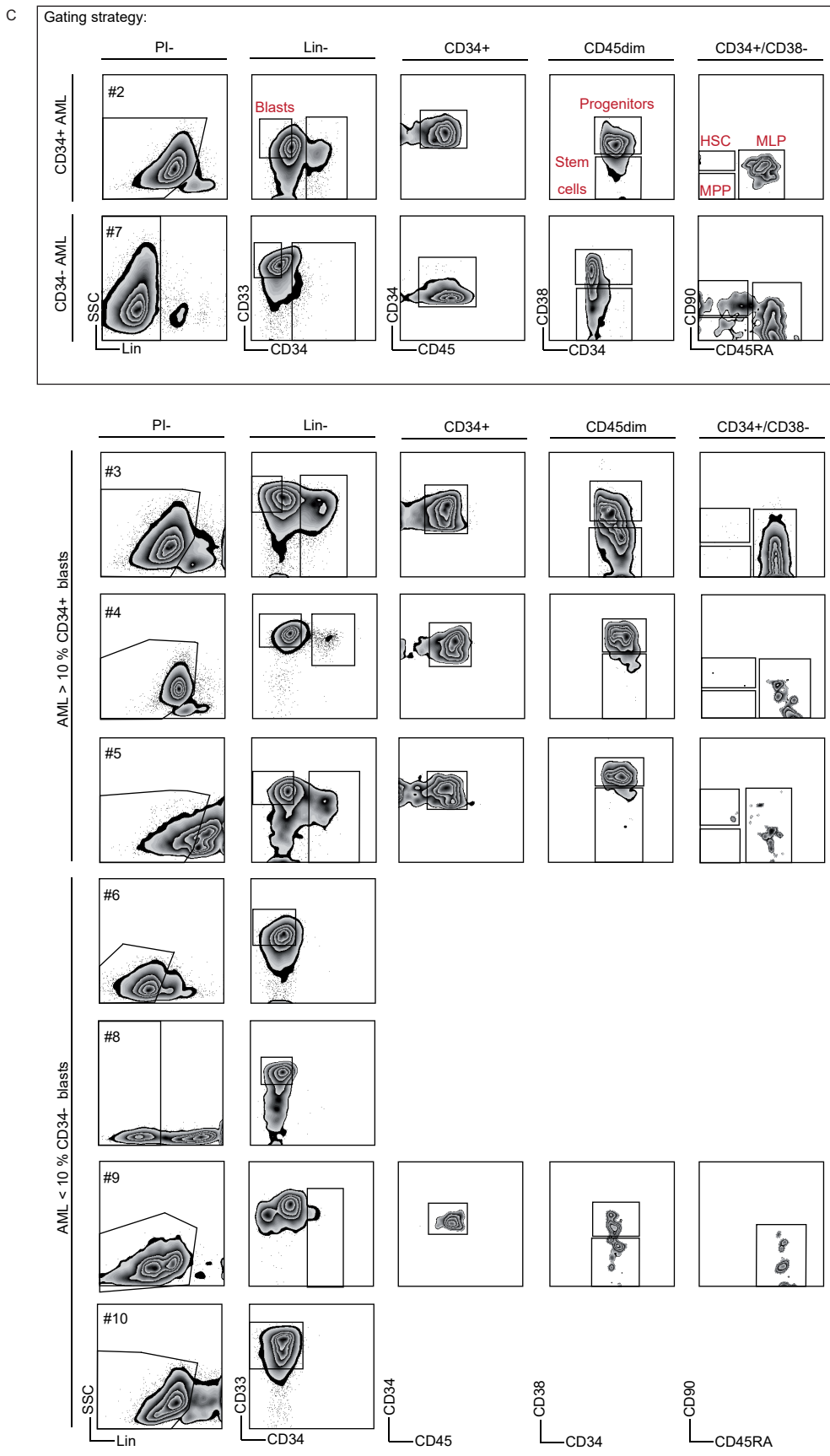


Figure 9C. Detection of *FLT3*-ITD and concurrent gene mutations in leukemic stem/ progenitor compartments.

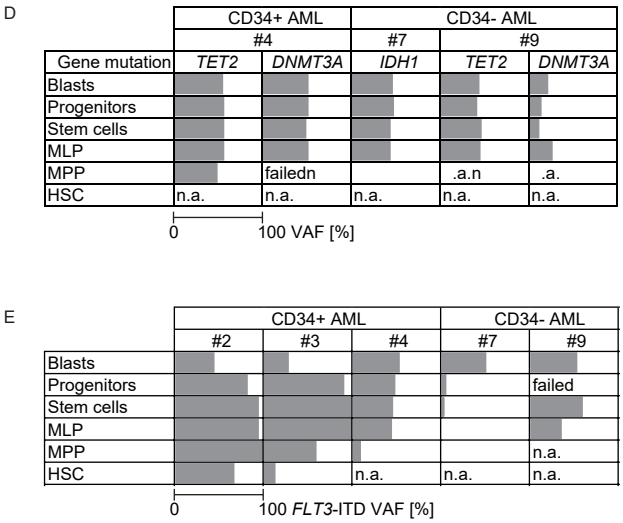


Figure 9D and E. Detection of *FLT3*-ITD and concurrent gene mutations in leukemic stem/progenitor compartments.

Table 7. Analysis of co-occurring mutations in leukemic stem/progenitor cell compartments of *FLT3*-ITD$^+$ AML BM samples at diagnosis.

		BM compartment					
ID	**Mutation**	**Blasts**		**Stem cells**	**MLP**	**MPP**	**HSC**
#2	FLT3-ITD/WT ratio	0.72	3.9	11.3	12.9	39.2	1.8*
	NPM1mut	pos	pos	pos	pos	pos	pos*
#3	FLT3-ITD/WT ratio	0.35	7.7	25.1	31.5	1.3	0.11
	NPM1mut	pos	pos	pos	pos	pos	pos*
	RAD21mut VAF [%]	55%	62%	61%	60%	60%	45%
#4	FLT3-ITD/WT ratio	1.04	0.83	0.74	0.71	0.07	n.a.
	NPM1mut	wt	wt	wt	wt	wt	n.a.
	TET2mut VAF [%]	54%	56%	56%	56%	48%	n.a.
	DNMT3Amut VAF [%]	51%	51%	48%	51%	failed	n.a.
#6	FLT3-ITD/WT ratio	0.25	n.a.	n.a.	n.a.	n.a.	n.a.
	NPM1mut	pos	n.a.	n.a.	n.a.	n.a.	n.a.
	TET2mut VAF [%]	55%	22%	n.a.	failed	n.a.	n.a.
#7	FLT3-ITD/WT ratio	0.93	0.02	0.003	0	0*	n.a.
	NPM1mut	pos	pos	pos	pos	pos*	n.a.
	IDH1mut VAF [%]	45%	47%	43%	43%	0.5%	n.a.
	CEBPAmut VAF [%]	40%	30%	34%	21%	16%	n.a.
#9	FLT3-ITD/WT ratio	1.05	failed	1.33	0.5	n.a.	n.a.
	NPM1mut	pos	pos	pos	pos	n.a.	n.a.
	TET2mut VAF [%]	43%	40%	46%	44%	n.a.	n.a.
	DNMT3Amut VAF [%]	20%	12%	9%	25%	n.a.	n.a.
#10	FLT3-ITD/WT ratio	0.98	0.77	n.a.	n.a.	n.a.	n.a.
	NPM1mut	pos	pos	n.a.	n.a.	n.a.	n.a.
	TET2mut VAF [%]	51%	52%	n.a.	n.a.	n.a.	n.a.

3.2 Efficacy of creno as single agent and in combination with AZA against *FLT3*-ITD$^+$ AML cells

We have previously shown that CD34$^+$ LSC in *FLT3*-ITD$^+$ AML persist and expand during treatment with first- (SU5614) and second-generation TKI (sora) owing to their selective protection by stromal cells (Parmar *et al.*, 2011). Nevertheless, the development of new TKI such as creno promising improved pharmacokinetics and increased efficacy in *FLT3*-ITD$^+$ AML is ongoing. Hence, we ask whether creno as a single agent can target FLT3-ITD$^+$ AML cells in contact with stroma more effectively than TKI of older generations, such as sora. Since the combination of sora and AZA demonstrated clinical benefit to relapsed *FLT3*-ITD$^+$ AML patients (Ravandi *et al.*, 2013), we also tested whether addition of AZA to sora or creno can modulate stromal resistance of *FLT3*-ITD$^+$ AML cells.

3.2.1 TKI and AZA titration in *FLT3*-ITD$^+$ AML cell lines

First, we compared the *FLT3*-ITD selective effects of sora and creno in leukemia cell lines. Whereas RS4;11 (FLT3-WT) cells were not affected, sora and creno were similarly cytotoxic to leukemia cell lines MV4;11 (*FLT3*-ITD heterozygous) and MOLM-13 (*FLT3*-ITD homozygous) in a dose-dependent manner. Sora- and creno-induced apoptosis was maximal at a concentration of 100 nM at 96 hours in *FLT3*-ITD$^+$ cells (Figure 10). Thus, in line with recent reports, we used both TKI at a concentration of 100 nM (Parmar *et al.*, 2011)(Galanis *et al.*, 2014) for further experiments.

Next, we tested the response of *FLT3*-ITD$^+$ cell lines to AZA. Dose titration revealed that MV4;11 and MOLM-13 were only marginally sensitive to AZA up to 10 µM (Figure 11), the dose corresponding to the maximum plasma concentration achieved in patients receiving AZA treatment (Stresemann and Lyko, 2008)(Hollenbach *et al.*, 2010). We used 10 µM AZA in the following experiments.

3.2.1 Stromal resistance of *FLT3*-ITD$^+$ AML cells

To test stromal resistance of *FLT3*-ITD$^+$ AML cells against TKI as single agent and in combination with AZA, we made use of an established *in vitro* co-culture system with the osteoblast-like murine embryonic cell line EL08-1D2 mimicking the BM niche[28] and known to support *FLT3*-ITD$^+$ leukemia cells (Parmar *et al.*, 2011).

Initially, we assessed the induction of apoptosis in MV4;11 and MOLM-13 cells after 4-day suspension and co-cultures with EL08-1D2 cells in the presence of TKI, AZA and their simultaneous combination (Figure 12A). Sora and creno as single agents induced significant apoptosis in *FLT3*-ITD$^+$ AML cells in suspension cultures but were completely ineffective against *FLT3*-ITD$^+$ AML cells in contact with

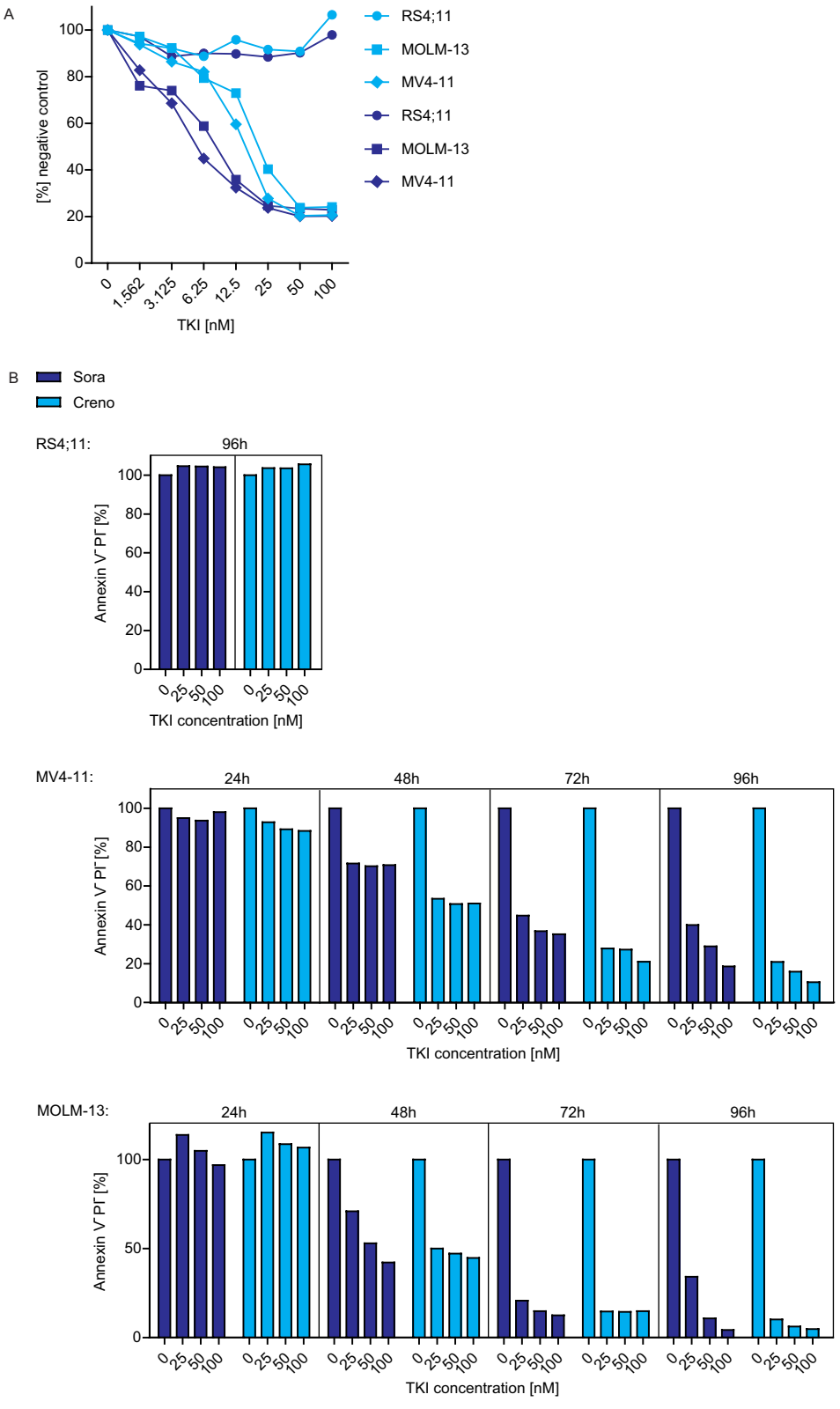


Figure 10. Dose response for *FLT3*-ITD⁺ leukemia cells treated with sora and creno. Dose-dependent TKI cytotoxicity was assessed by MTT assay in triplicates after 96 hours (A). Percentage of live cells was determined by Annexin V/PI flow cytometry after 24 to 96 hours at treatment with indicated TKI concentration (B).

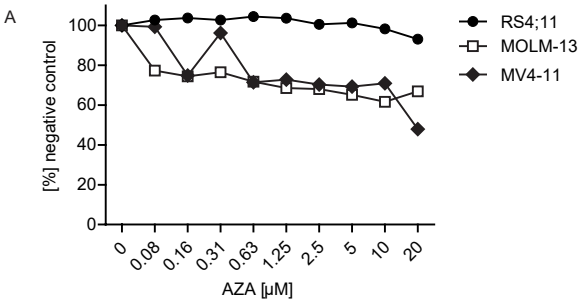


Figure 11. Dose response for *FLT3*-ITD⁺ leukemia cells treated with AZA. Cell viability of MV4-11 and MOLM-13 cell lines treated with indicated AZA concentrations was determined by MTT assay in triplicate after 96 hours, n=3 ± SEM.

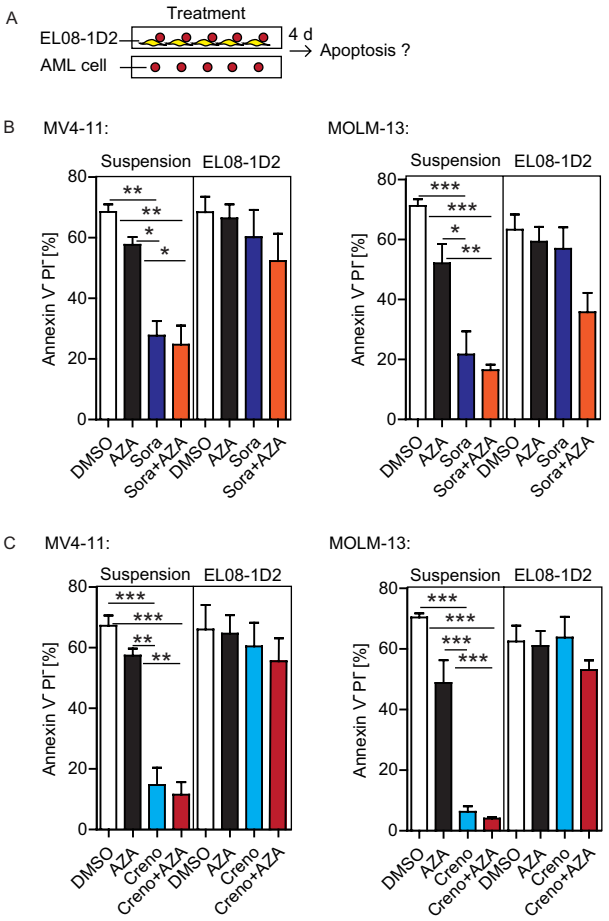


Figure 12. Efficacy of TKI as single agent and in combination with AZA in *FLT3*-ITD⁺ AML cells. MV4-11 and MOLM-13 cells were cultured in suspension or on stromal EL08-1D2 layers for 4 days. Cultures were treated with DMSO, AZA, creno or in simultaneous combination. Induction of cell death was measured by Annexin V/PI staining. Experimental design (A). Results for sora (B) and creno (C) are shown as mean of three independent experiments, n=3 ± SEM.

stroma. Simultaneous combination of TKI and AZA induced most pronounced apoptosis of *FLT3*-ITD⁺ AML cells although stromal resistance was not overcome within 4 days of treatment. Of note, results were similar for sora (Figure 12B) and

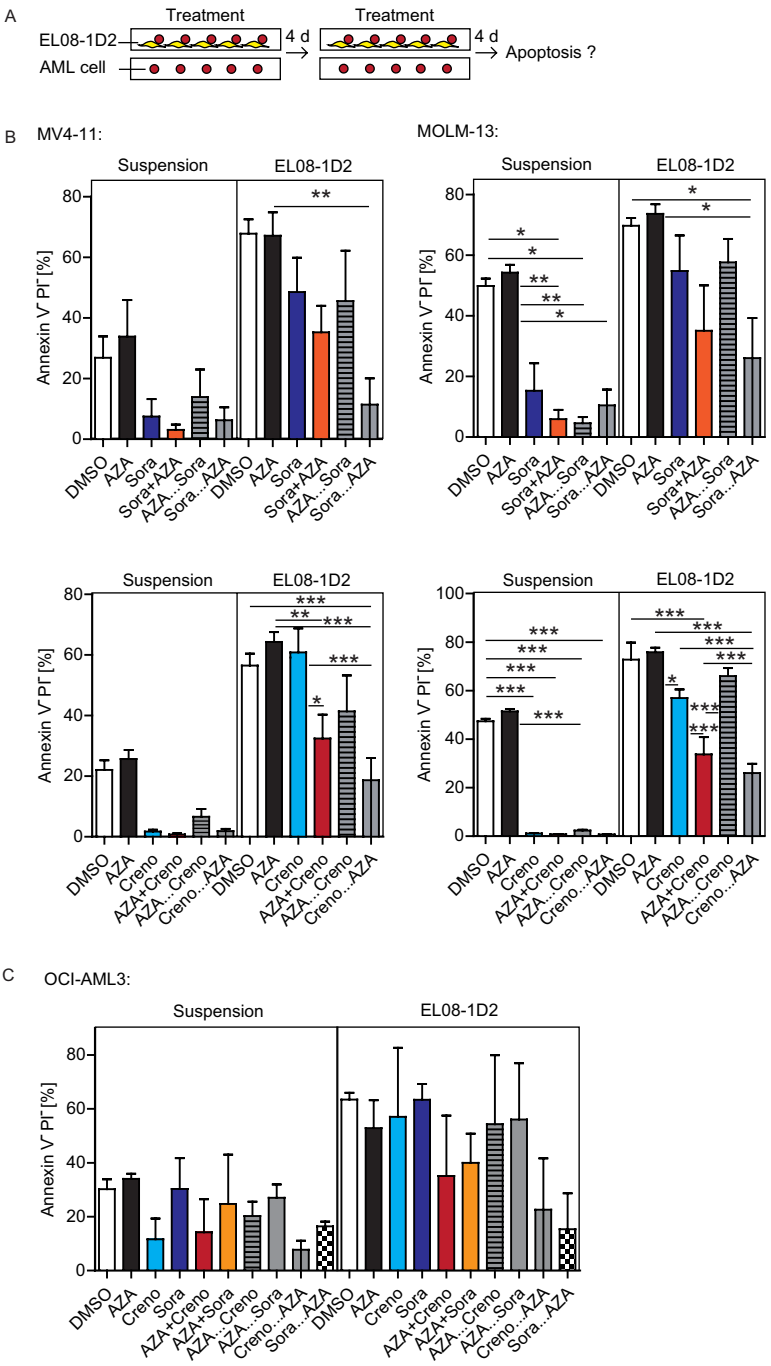


Figure 13. Anti-leukemic effects of TKI and AZA in *FLT3*-ITD⁺ and *FLT3*-WT AML cells. AML cell lines were cultured in suspension or on stromal EL08-1D2 layers for 8 days. Cultures were treated with DMSO, AZA, creno or in simultaneous or sequential combination. Induction of apoptosis was measured by Annexin V/PI flow cytometry. Experimental design (A). Results for sora (B) and creno (C) in MV4-11 and MOLM-13 cells are shown as mean of three independent experiments, n=3 ± SEM. Results for sora and creno in OCI-AML3 are shown as mean of two independent experiments, n=2 ± SEM.

creno (Figure 12C) in MV4;11 as well as MOLM-13 cells. Therefore, we asked whether combination therapy with AZA and TKI could be improved by prolonged drug exposure and sequential drug application (Figure 13A). *FLT3*-ITD⁺ cell lines

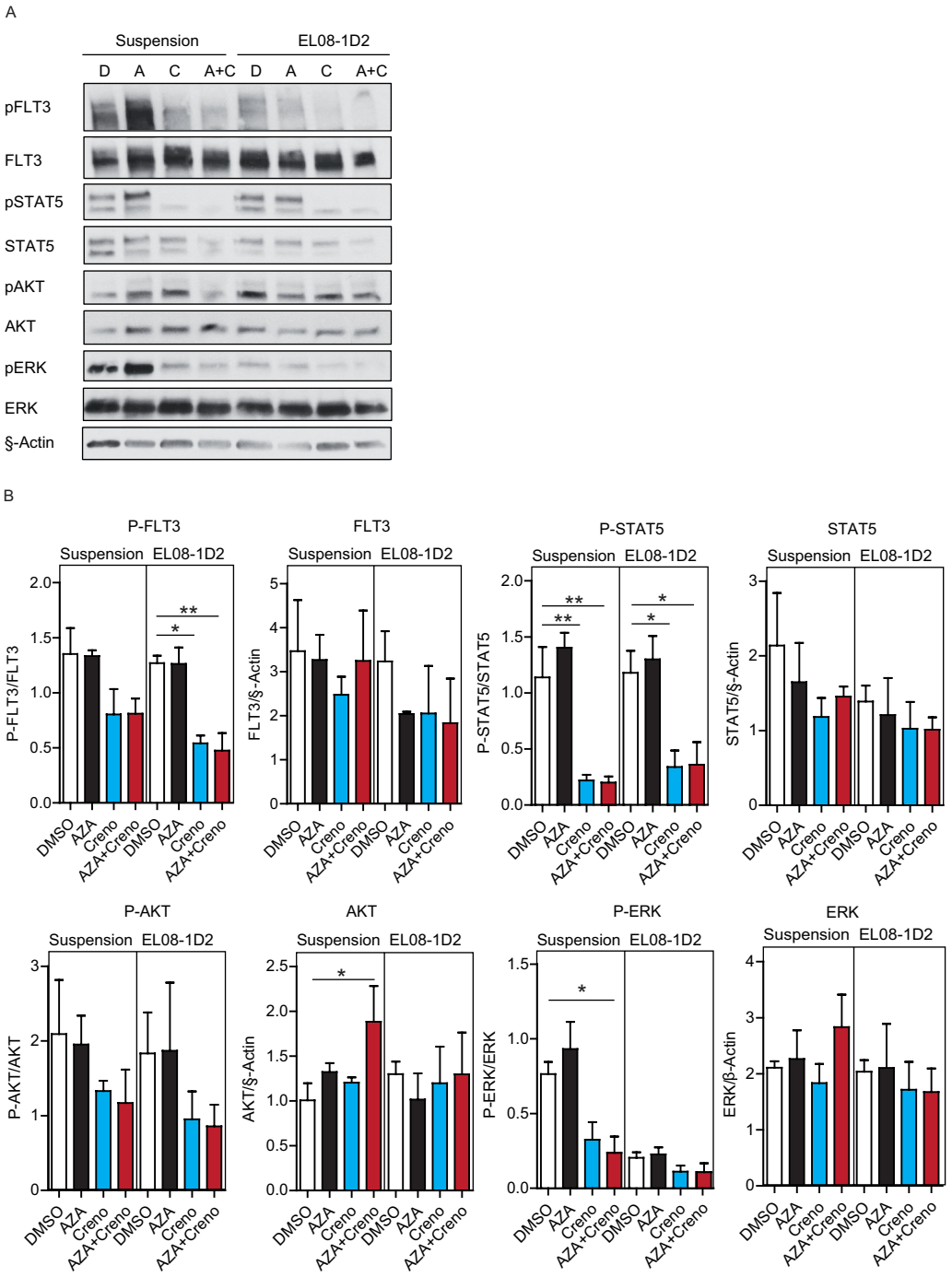


Figure 14. Stromal TKI resistance is independent of *FLT3*-ITD signaling. FLT3-ITD signaling was analyzed by immunoblot after treatment with AZA, creno or their combination for 1h. A representative blot is shown (A). Signal intensities of three independent experiments were quantified using Image J software; results are shown as mean n=3 +/- SEM (B).

remained resistant against AZA or TKI as single agents. Of note, AZA and TKI in simultaneous combination or even more when given sequentially (i.e. TKI followed by AZA) significantly increased apoptosis (Figure 13B) of *FLT3*-ITD$^+$ AML cells after eight days. Conversely, when AZA was added before TKI the effect was less pronounced. Thus, improved response could not be ascribed to a cell-divi-

sion dependent AZA effect in *FLT3*-ITD$^+$ AML cells.

We repeated our 8-day experiment with *FLT3*-WT OCI-AML3 cells (doubling time: ca. 35-40 hours). In comparison to FLT3-ITD$^+$ AML cell lines, OCI-AML3 cells reacted more inconsistent to the tested treatment options as shown by higher standard errors of the means (SEM) so that differences between treatment options did not reach significance within 3 independent experiments. Nevertheless, in comparison to DMSO control and single agent treatment, simultaneous and sequential (i.e. TKI followed by AZA) combination of AZA and TKI reduced the mean viability of OCI-AML3 cells (Figure 13C). Thus, anti-leukemic effects of AZA and TKI were not entirely FLT3-ITD specific.

Efficacy of creno to inhibit *FLT3*-ITD was not disrupted by stromal contact, as shown by persistent inhibition of FLT3, STAT5, ERK and AKT phosphorylation in the presence of stroma (Figure 14). AZA alone or in combination with creno did not affect *FLT3*-ITD signaling in comparison to DMSO controls or creno monotherapy, respectively.

AZA and crenolanib induced cell cycle arrest of MV4-11 cells in suspension as single agents or in combination. Stromal contact abrogated proliferation arrest of *FLT3*-ITD$^+$ AML cells treated with AZA alone, but not in cells treated with creno (Figure 15).

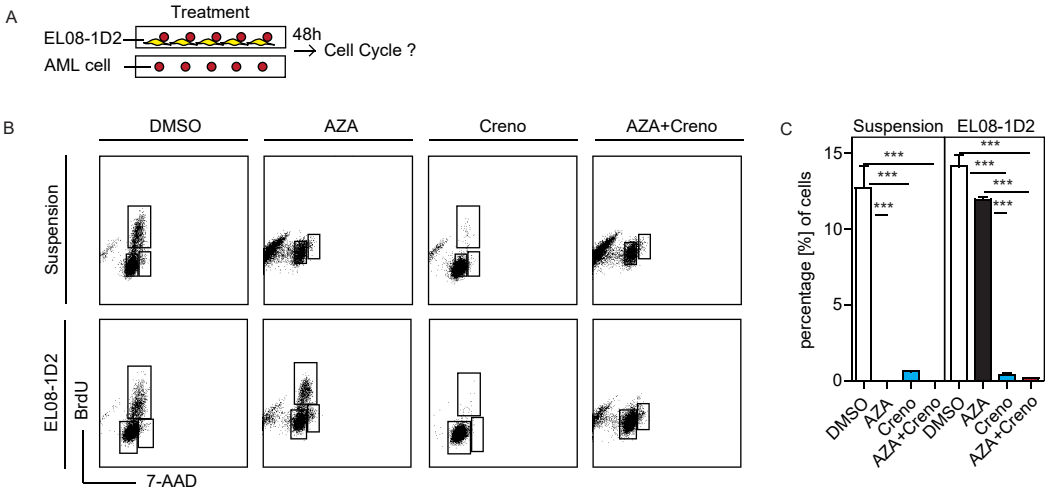


Figure 15. Crenolanib induces cell-cycle arrest of *FLT3*-ITD$^+$ AML cells despite stromal contact. MV4-11 cells were cultured in suspension or on stromal EL08-1D2 layers. Cultures were treated with DMSO, AZA, creno or in simultaneous combination for 48 hours. Experimental design (A). Cells were pulsed with BrdU for 30 minutes and then stained with 7-AAD and APC-conjugated anti-BrdU antibody for flow cytometric cell cycle studies. Representative FACS plots are shown (B). Statistical summary as mean of three independent experiments, n= 3 ± SEM (C).

Pre-treatment of EL08-1D2 cells with AZA did not sensitize MV4-11 cells to either

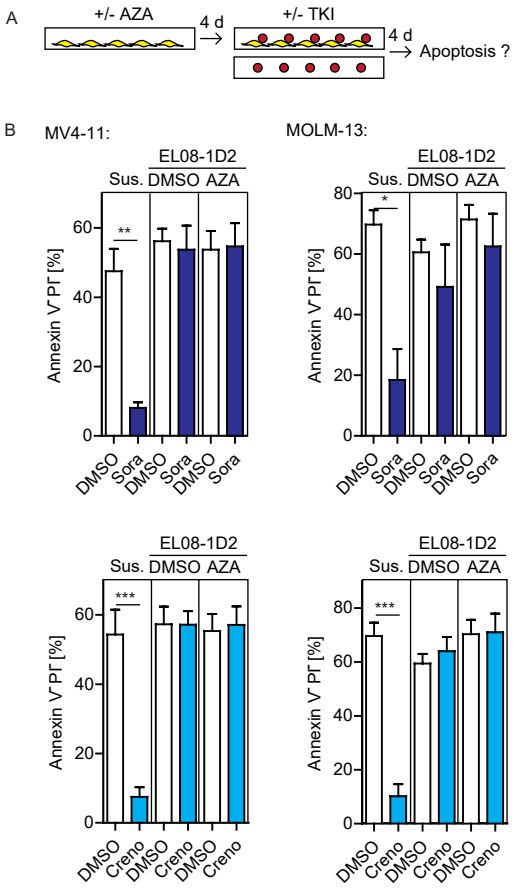


Figure 16. AZA does not impair supporting capacity of stromal cells. EL08-1D2 cells were cultured in the presence or absence of AZA (10 µM) for 4 days. The stromal cell layer was washed twice with PBS, MV4-11 cells were added and co-cultures were treated with creno or DMSO for 4 days. AML cells were harvested and apoptosis was measured by Annexin V/PI flow cytometry. Experimental design (A). Results are shown as mean of three independent experiments, n=3 ± SEM (B).

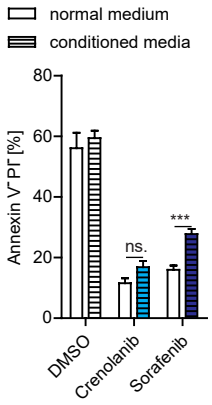


Figure 17. Stromal resistance is not mediated by stroma-derived soluble factors alone. MV4-11 cells were cultured in standard medium or medium pre-conditioned by EL08-1D2 cells for 4 days. Cells were treated with creno for 4 days and induction of apoptosis was measured by Annexin V/PI flow cytometry. Results are shown as mean of three independent experiments, n=3 ± SEM.

sora or creno (Figure 16), implying that AZA perturbs stromal protection by direct effects on the interaction between *FLT3*-ITD$^+$ cells and their niche.

Sora and creno were less effective against MV4-11 cells in medium that had previously been conditioned on EL08-1D2 cells in comparison to cultures with unconditioned medium. However, in comparison to DMSO controls, soluble stroma factors had no significant influence on TKI-induced apoptosis (Figure 17), indicating that direct stromal contact is necessary for complete protection of *FLT3*-ITD$^+$ AML cells from TKI.

3.3 Influence of concurrent *DNMT3A* and *TET2* mutations on creno and AZA response in *FLT3*-ITD⁺ AML

Primary *FLT3*-ITD⁺ AML is genetically more heterogeneous than leukemic cell lines and the efficacy of creno as well as AZA may depend on concurrent mutations present in LSC. Thus, to translate our findings back into situation in the patient, we tested stromal resistance of LSC-enriched CD34⁺ cells from primary *FLT3*-ITD⁺ AML (Table 6) in our *in vitro* BM model. A 4-day treatment period was chosen, within which LSC undergo at least one division but do not lose CD34 expression (Götze *et al.*, 2007).

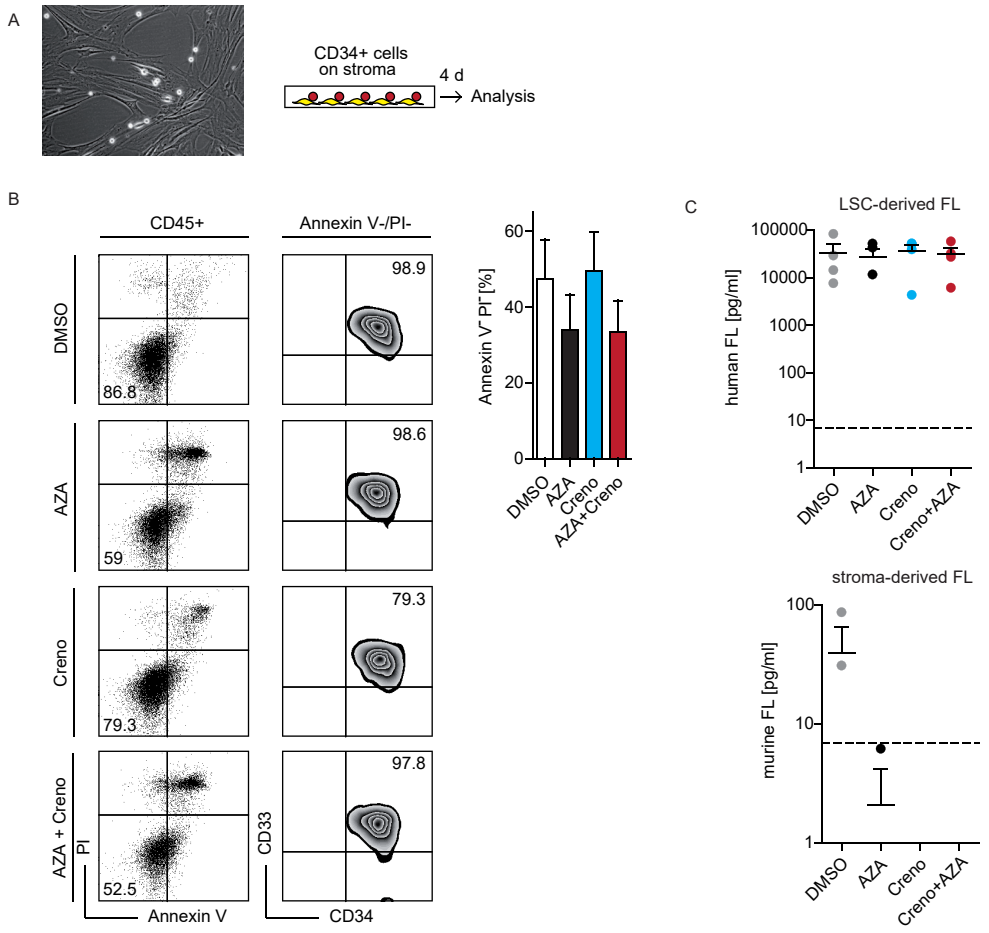


Figure 18. Treatment of primary CD34⁺ LSC from *FLT3*-ITD⁺ AML patients in a 4-day *in vitro* BM model. CD34⁺ *FLT3*-ITD⁺ BM cells were cultured on EL08-1D2 stroma in SFM + 5GF and treated with DMSO, AZA, creno or simultaneous combination thereof for 4 days (A). Cells were harvested and stained for flow cytometry with antibodies against human CD45, CD33 and CD34. Annexin V antibodies and PI were added to determine apoptotic and necrotic cells. A representative facs plot (patient #24) (upper panel) and statistical summary (Patient #23 -27) n=5 ± SEM (lower panel) is shown (B). Cell culture supernatants (patients #23-25) were collected and FL levels were measured using murine- and human-specific ELISA. Results are shown as mean n=3 ± SEM (C).

In comparison to AML cell lines, primary CD34⁺ *FLT3*-ITD⁺ AML cells were more

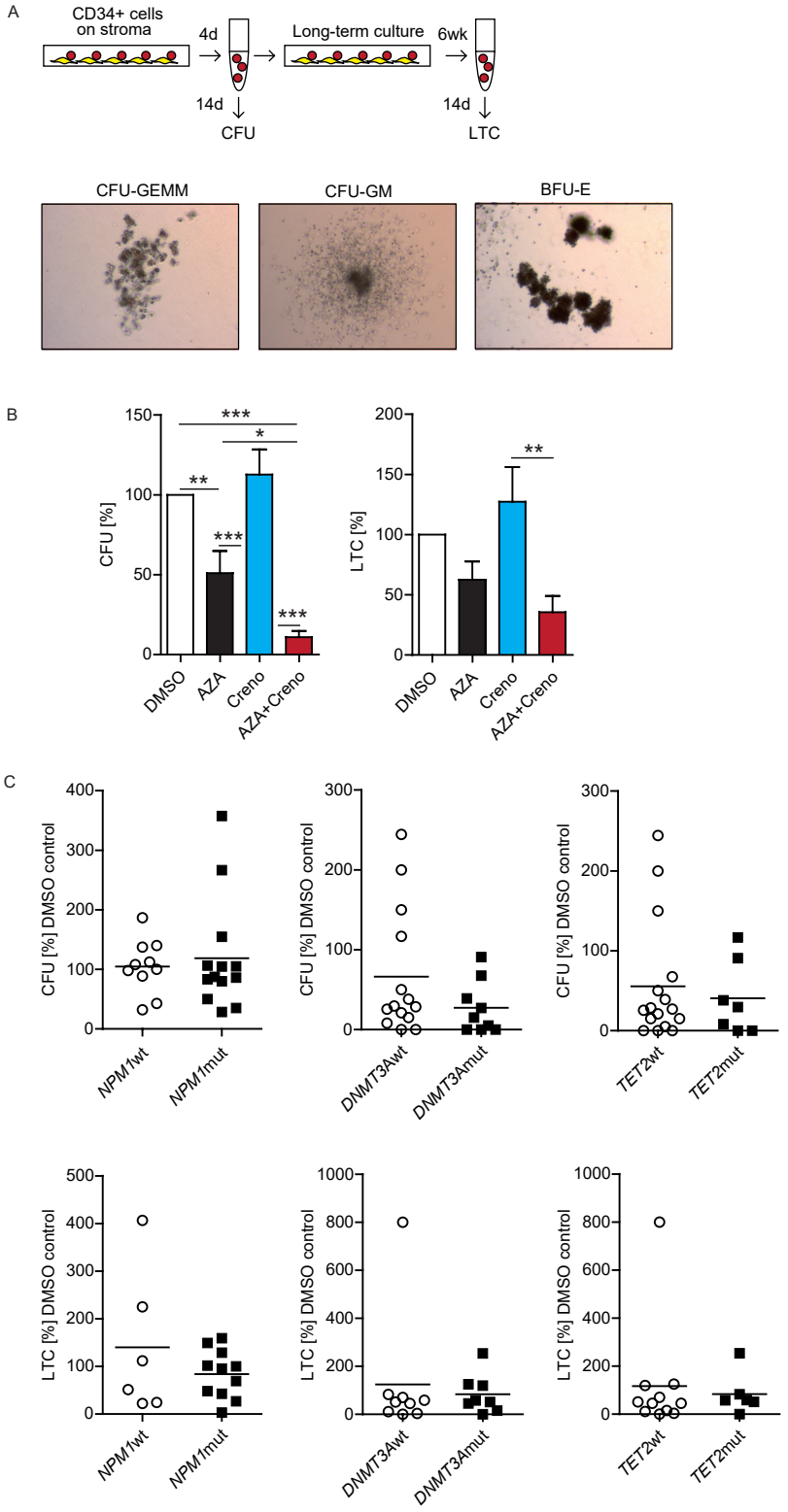
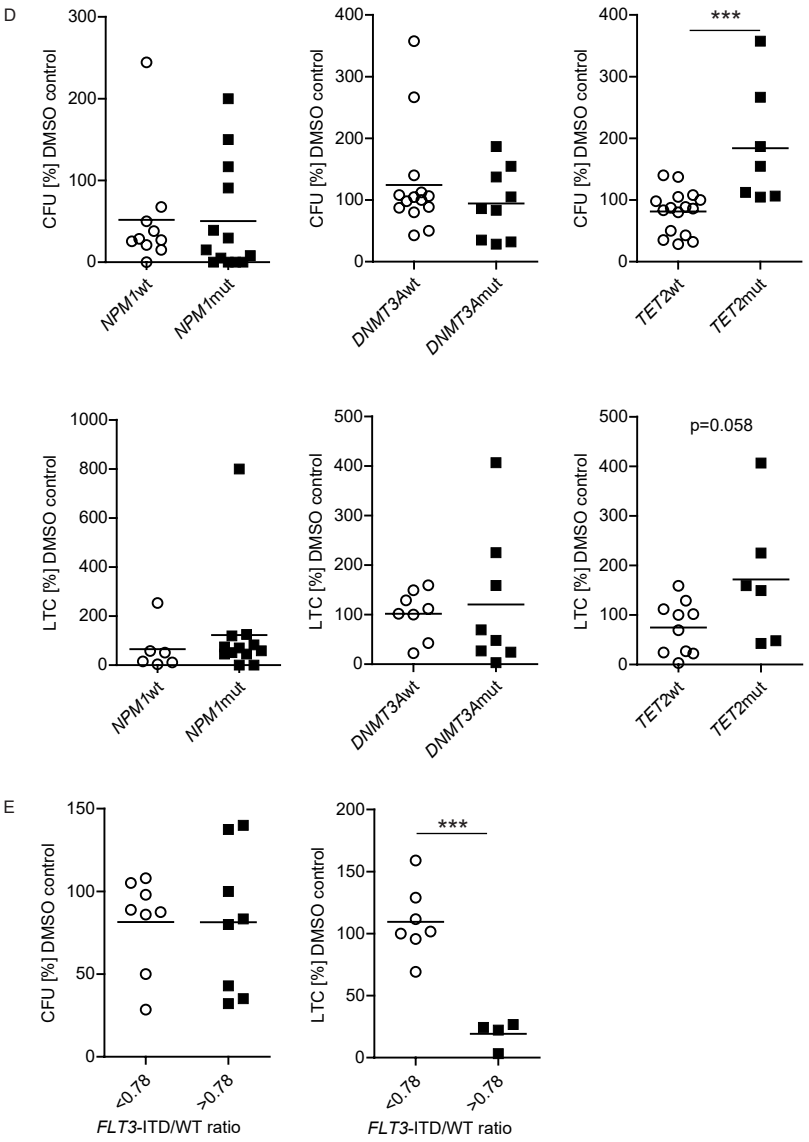


Figure 19. Sensitivity of primary *FLT3*-ITD⁺ LSC to AZA and creno is dependent on concurrent gene mutations. Experimental design: enriched CD34⁺*FLT3*-ITD⁺ BM cells were cultured on EL08-1D2 stroma in SFM + 5GF and treated with DMSO, AZA, creno or the combination thereof. Cells were harvested after 4 days. Progenitor activity was assessed by short-term colony-forming unit assay (CFU) in methylcellulose. Long-term LIC capacity (LTC) was assessed after 6 weeks on irradiated (30 Gy) EL08-1D2 cells followed by plating in methylcellulose (A). Colonies were scored after 14 days using stan



dard criteria. Results are shown as mean CFU (n = 23 ± SEM) and LTC frequencies (n = 17 ± SEM) in relation to DMSO controls (B). Response to AZA alone regarding co-mutations in *NPM1* (CFU n = 23; LTC n =18), *DNMT3A* (CFU n = 23; LTC n =17), *TET2* (CFU n = 23; LTC n =17) (C). Response to creno alone regarding co-mutations in *NPM1* (CFU n = 23; LTC n =18), *DNMT3A* (CFU n = 23; LTC n =16), *TET2* (CFU n = 23; LTC n =16) (D). Analysis of CFU (n = 16) and LTC (n = 11) capacity of *FLT3*-ITD^{+}/*TET2*WT samples in relation to *FLT3*-ITD/WT ratios after treatment with creno alone (E).

sensitive to AZA shown by an increased apoptosis rate after AZA and AZA + creno treatment. Creno alone was again completely inefficient against stroma protected *FLT3*-ITD$^+$ AML cells. Neither creno nor AZA, nor the combination induced differentiation of LSC as shown by identical CD45$_{dim}$/CD33$^+$/CD34$^+$ phenotypes (Figure 18A, B). Of note, stromal protection of *FLT3*-ITD$^+$ LSC towards inhibition by creno was not due to reactive FL secretion. Levels of stroma-derived FL were below detection threshold by ELISA regardless of treatment condition while levels of LSC-derived FL were unchanged (Figure 18A, C).

We further determined short- as well as long-term proliferation potential of pre-treated LSC in CFC assays (Figure 19A). In comparison to DMSO, AZA as a single agent reduced short-term and long-term colony growth of LSC by 49% and 37%, respectively (Figure 19B). As expected from our cell line data, creno alone did not prevent expansion of committed leukemic progenitors with short-term (CFU) or LSC with long-term proliferative potential (LTC) on stroma. Strikingly, the combination of AZA and creno eliminated 89% of short-term and 64% of long-term LSC despite stromal contact (Figure 19B).

Contrary to expectations, response to AZA as a single agent was not influenced by presence of *DNMT3A*, *NPM1* or *TET2* mutations as determined by short-term and long-term proliferative potential (Figure 19C). Most strikingly, *TET2* mutations conferred resistance to creno alone, and *FLT3*-ITD$^+$/*TET2*mut cells expanded significantly during creno treatment, as evidenced by increased CFC numbers compared to DMSO in *FLT3*-ITD$^+$/*TET2*mut samples (Figure 19D). In contrast, neither *DNMT3A* nor *NPM1* mutations had an influence on response to creno (Figure 19D).

Higher *FLT3*-ITD/WT ratios have been demonstrated to increase sensitivity towards TKI with published ratios ranging from 0.5-0.78 (Thiede *et al.*, 2002)(Pratz *et al.*, 2010)(Schlenk *et al.*, 2002). Accordingly, in *FLT3*-ITD$^+$ AML without *TET2* mutations only long-term LSC with a *FLT3*-ITD/WT ratio >0.78 were effectively targeted by creno while leukemic progenitors with a ratio <0.78 were not sensitive (Figure 19E). Importantly, the combination of AZA and creno effectively targeted *FLT3*-ITD$^+$ LSC regardless of *FLT3*-ITD/WT ratios or concurrent mutations.

3.4 AZA + creno reduces *in vivo* engraftment of *FLT3*-ITD$^+$ LSC

Finally, to translate our *in vitro* results with primary AML samples to an *in vivo* model, we made use of a patient-derived-xenograft (PDX) mouse model. PDX models faithfully mimic patient characteristics and therefore are a powerful tool to define drug efficacy and resistance in leukemia (Vick *et al.*, 2015)(Krupka *et al.*, 2016)(Townsend *et al.*, 2016). Thus, to prove the potential of AZA and creno alone or in combination to eliminate LSC in their niche, we tested the *in vivo* en-

graftment capacity of two separate PDX samples after their *ex vivo* treatment in our *in vitro* BM model (Figure 20). One primary *FLT3*-ITD$^+$/*NPM1*mut/*DNMT3A*mut and one *FLT3*-ITD$^+$/*NPM1*mut/*TET2*mut BM sample were serially transplanted in NSG mice, proving presence of long-term LSC. Targeted resequencing of PDX cells demonstrated that driver mutations present in the founding clone of the original AML were preserved during passaging (Table 8) (Vick *et al.*, 2015).

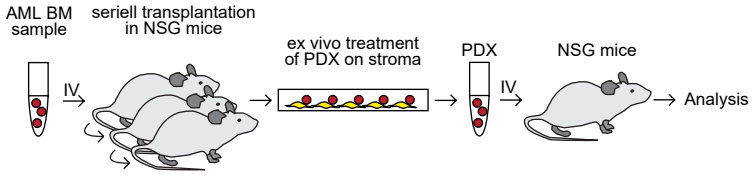


Figure 20. Patient-derived-xenograft (PDX) mouse model. AML BM cells were serially transplanted in NSG mice. PDX cells were re-isolated and treated *ex vivo* on EL08-1D2 stroma with DMSO, AZA, creno or the combination thereof for 4 days. PDX cells were harvested and 2 x 10^5 viable cells were injected IV into NSG mice (n=20 per experiment). Engraftment was monitored by weekly peripheral blood (PB) sampling or BLI.

Table 8. PDX sample characteristics (Vick et al., 2015).

					VAF of driver mutation in PDX			
ID	**Age**	**Sex**	**Disease stage**	**Cyto-genetics**	***FLT3*-ITD/WT ratio**	***NPM1***	***DNMT3A***	***TET2***
AML361	40	F	Initial	Normal	0.31	0.48	0.47	wt
AML602	40	F	Relapse	Aberrant complex	0.39	0.39	wt	0.39

In the first PDX cohort (*FLT3*-ITD$^+$/*NPM1*mut/*DNMT3A*mut), engraftment was monitored by serial PB sampling. At 7 weeks post-transplant, PDX cells (mCD45$^-$/hCD45$^+$/hCD33$^+$) were detected in PB of all mice in the DMSO cohort (mean 5.28%; range 3.33-7.14%), at which point all mice were sacrificed. PDX cells in PB were also detected in the AZA (mean 2.87%; range 1.16-5.74%), creno (mean 2,22%; range 0.85-4.41%) and AZA + creno (mean 1.29%; range 0.97-1.81%) groups. Strikingly, PDX infiltration of BM as assessed by FACS and IHC was significantly reduced in mice transplanted with AZA + creno-treated *FLT3*-ITD$^+$ cells compared to DMSO control or AZA alone (Figure 21).

In the second PDX cohort (*FLT3*-ITD$^+$/*NPM1*mut/*TET2*mut) stable expression of luciferase enabled monitoring of engraftment by non-invasive luciferin-induced bioluminescence (Figure 22A, B). BLI signals were first detected in the BM, extremities and sternum 4 weeks post-transplantation. Notably, during the first 35 days leukemic expansion of *TET2*mut PDX cells was significantly accelerated in the creno

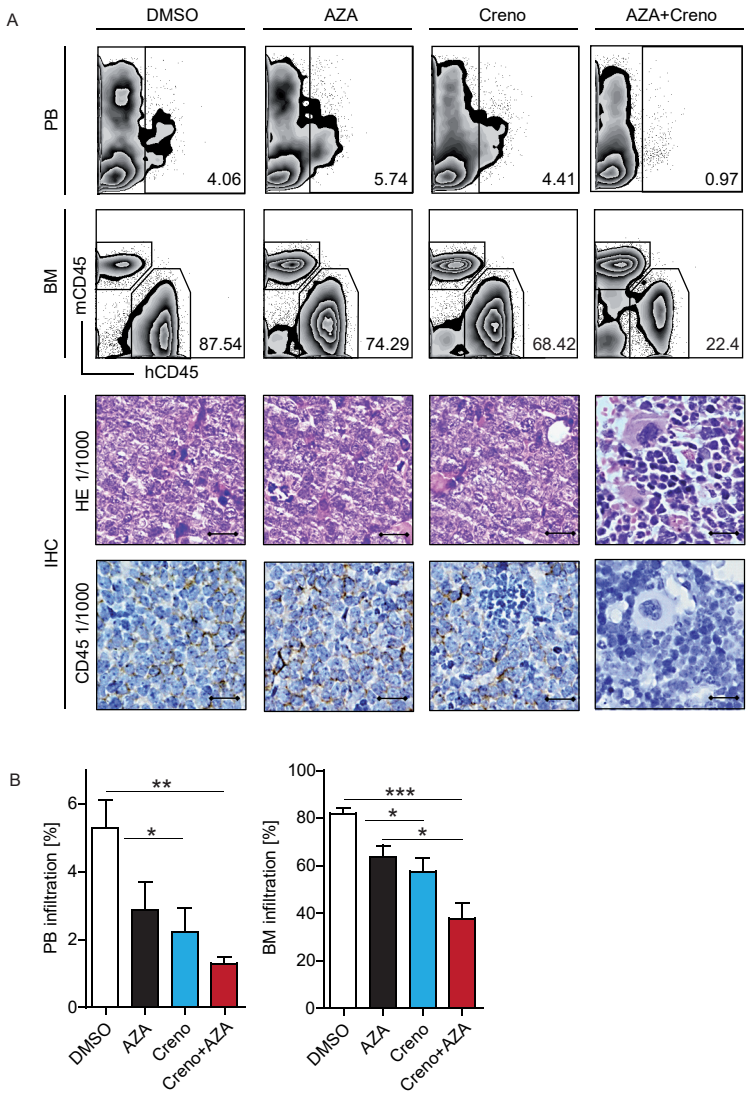


Figure 21. AZA + creno overcomes stromal protection of *FLT3*-ITD$^+$/*NPM1*mut/ *DNMT3A*mut LSC. Representative images of AML infiltration analyzed by flow cytometry of PB (top) as well as BM (bottom) and immunohistochemistry (IHC) (middle) of femurs visualized by Hematoxylin-eosin (H&E) staining or expression of human CD45 (hCD45); scale bar, 10 µM (A). Statistical summary of PB and BM infiltration in NSG mice (n=5 per condition) (B).

treatment group compared to DMSO controls (Figure 22C). After 8 weeks, leukemia was spread over the whole body in 5/5 mice in the DMSO group, in 3/5 mice in the AZA group and in 4/5 mice in the creno group, at which point all mice were sacrificed.

Quantity of leukemic cells in the BM of NSG mice was assessed using FACS and IHC. Corresponding to our CFC data (Figure 19D), BM infiltration by PDX cells was even increased in the creno cohort compared to DMSO control, while it was decreased in the AZA group and further severely reduced in the AZA + creno cohort, in which normal BM architecture was preserved as assessed by IHC (Figure 22D, E). Thus, our PDX data confirm that *TET2* mutations confer resistance to crenolanib.

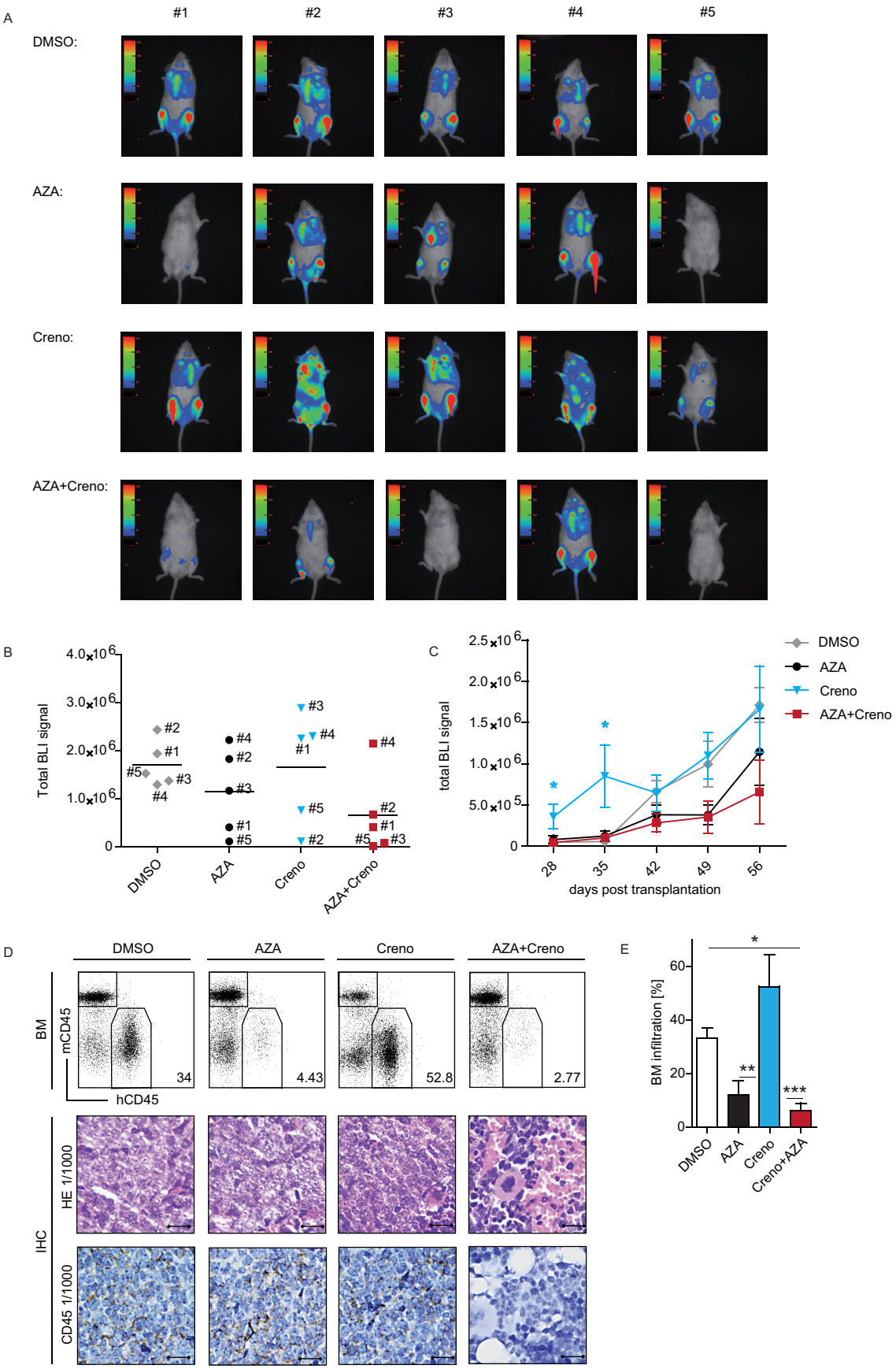


Figure 22. AZA + creno targets *FLT3*-ITD$^+$/*NPM1*mut/*TET2*mut LSC despite niche cells. T-PDX infiltration in NSG mice was monitored by noninvasive BLI *in vivo* imaging. BLI one day before sacrifice are shown (A). Time course of bioluminescence signals in

NSG cohorts (B). Statistical summary BLI signals on day 56 (C). Representative images of leukemic infiltration analyzed by flow cytometry of BM (top) as well as immunohistochemistry (IHC) (bottom) of femurs visualized by Hematoxylin-eosin (H&E) staining or expression of human CD45 (hCD45); scale bar, 10 µM (D). Statistical summary of BM infiltration in t-PDX transplanted NSG mice (n=5 per condition) analyzed by FACS (E).

In summary, as proven by reduced engraftment of two different PDX samples in NSG mice, the combination of AZA and creno effectively overcomes stromal resistance and prevents LSC expansion in *FLT3*-ITD$^+$ AML irrespective of cooperating genetic mutations.

3.5 AZA alters mechanism of stromal resistance

AZA incorporates into RNA as well as DNA and thus can alter gene expression via diverse mechanisms. To investigate in how far AZA modulates mechanisms involved in stromal resistance of LSC we evaluated the individual gene expression pattern by RNAseq of co-cultured *FLT3*-ITD$^+$/*NPM1*mut/*DNMT3A*mut PDX cells (Table 8) and EL08-1D2 cells after *in vitro* treatment with DMSO, AZA, creno or the combination (Figure 23A).

As expected, contact of PDX cells and EL08-1D2 cells altered gene expression patterns of both cell types in comparison to monoculture controls (Figure 23B, DMSO lane), illustrating active interaction between AML and stromal niche cells. Pathway analyses and functional GO annotation of the top 100 differentially expressed genes revealed that treatment with AZA and creno significantly affected regulation of genes involved in extracellular-matrix-receptor interaction of PDX cells (Figure 23C, Table 9). On the EL08-1D2 side, treatment primarily altered genes involved in signaling pathways well known to promote a leukemic microenvironment, e.g. NF-kappaB, HIF-1 and TNF signaling (Table 9). Of note, crenolanib induced a different gene expression pattern in EL08-1D2 and PDX cells compared to AZA or AZA + creno (Figure 23B), indicating a dominant and multifactorial AZA effect on gene expression modulating stromal resistance.

Table 9. Pathway analysis in PDX and niche cells.

KEGG Pathways	Significantly enriched genes
hsa04512:ECM-receptor interaction	*CD36, COL5A2, SPP1*
mmu04620:Toll-like receptor signaling pathway	*Tab2, Chuk, Cxcl10, Lbp*
mmu04064:NF-kappa B signaling pathway	*Tab2, Chuk, Lbp*
mmu04066:HIF-1 signaling pathway	*Serpine1, Egln1, Vegfa*
mmu04668:TNF signaling pathway	*Tab2, Chuk, Cycl10, Lbp*
mmu05200:Pathways in cancer	*Arhgef12, Chuk, Rock1, Egln1, Vegfa*
mmu04270:Vascular smooth muscle contraction	*Arhgef12, Npr2, Rock1*

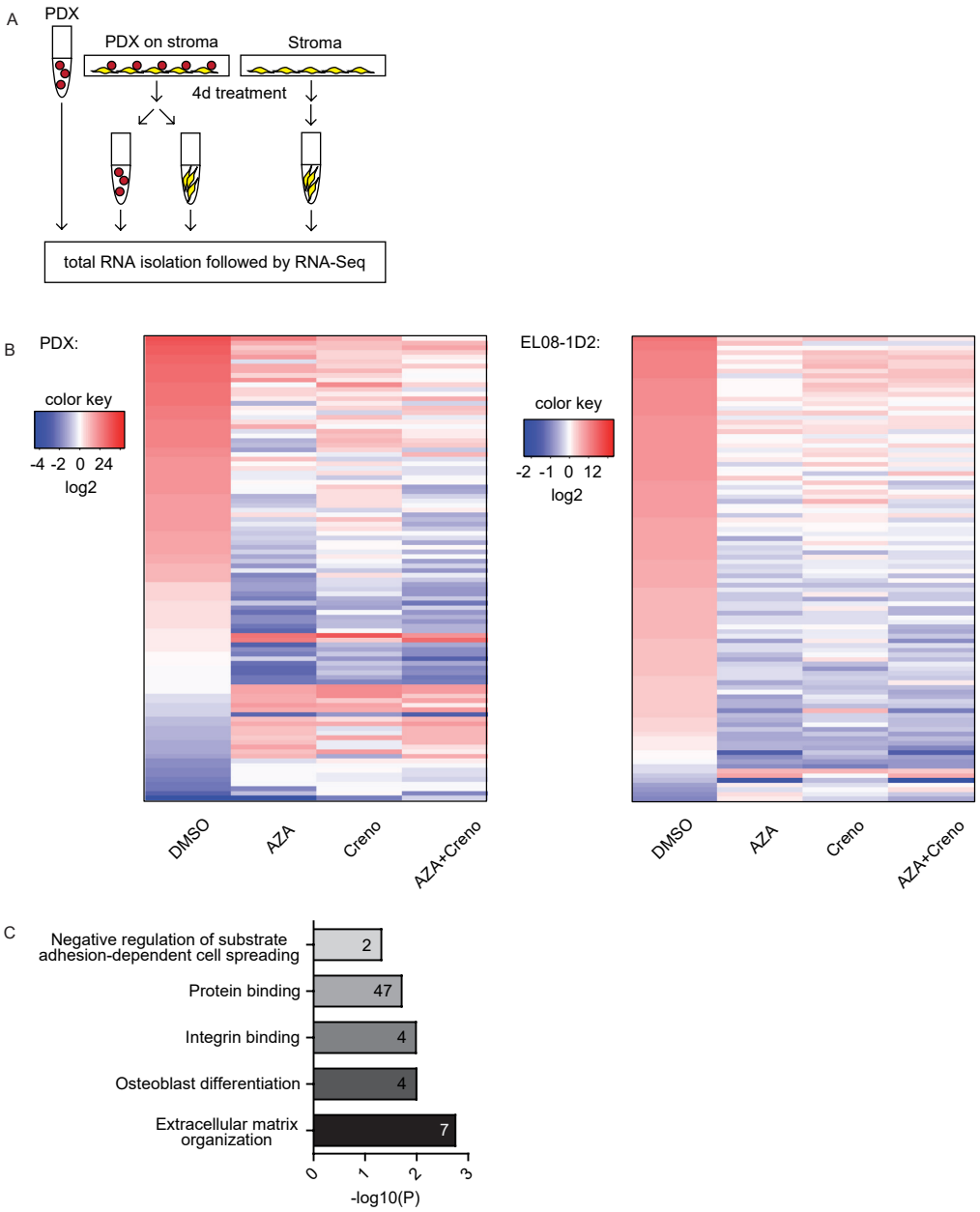


Figure 23. RNAseq analyses reveals changes in PDX and stromal cells by AZA and creno. Total RNA was isolated from human PDX and murine EL08-1D2 cells alone or from co-cultures that had been treated with DMSO, AZA, creno or the combination for 4 days in vivo. Four replicates were processed and analyzed for each condition (A). Heatmap of top 100 differentially expressed genes between DMSO-control and treatment conditions for PDX cells (left panel) and EL08-1D2 cells (right panel). Mean expression values are plotted in red and blue colors displaying up- and down-regulation relative to monoculture-controls (B). GO annotation based on the top 100 differentially expressed PDX genes between DMSO control and treatments. Signifantly enriched molecular functions and the corresponding gene counts are indicated (C).

4 Discussion

Standard treatment regimens in AML have not much changed for the last 30 years and relapse remains the major problem in AML therapy. Re-occurring leukemic blasts evolve from chemo-resistant, quiescent and self-renewing LSC enriched within the $CD34^+CD38^-$ population of the BM (Dick, 2008)(Ishikawa *et al.*, 2007). Hence, novel therapeutic agents that target and eliminate LSC are urgently needed. *FLT3*-ITD, which is highly prevalent and propagates one of the most aggressive AML subtypes, has become a prominent target in AML therapy but eradication of LSC in *FLT3*-ITD^+ AML by FLT3-TKI has thus far remained an elusive goal. Mechanism of resistance to FLT3-TKI may be of primary (inherent) or secondary (developed after treatment) nature.

This study was undertaken to gain a better understanding and improve therapeutic intervention of LSC resistance in *FLT3*-ITD^+ AML. We present *in vitro* and *in vivo* data from *FLT3*-ITD^+ AML cell lines and primary human AML bone marrow samples showing that

I) *FLT3*-ITD is present in the earliest $Lin^-/CD33^{(+)}/CD45_{dim}/CD34^+CD38^-$ potential LSC compartment of primary AML BM samples and therefore represents a valid target for LSC elimination in *FLT3*-ITD^+ AML.

II) Like previous FLT3-inhibitors, the novel TKI crenolanib as single agent is unable to target niche-protected *FLT3*-ITD^+ LSC.

III) Co-existence of *TET2* mutations confers complete resistance of *FLT3*-ITD^+ LSC to crenolanib and these cells even expand upon TKI monotherapy.

IV) Addition of AZA to creno abrogates stromal resistance and expansion of *FLT3*-ITD^+ LSC independent of coexisting genetic mutations in *DNMT3A*, *NPM1* and *TET2.*

V) Contrary to several published retrospective correlative clinical analyses, AML stem cells harboring *DNMT3A*, *TET2* or *NPM1* mutations were not more sensitive to AZA as a single agent than *FLT3*-ITD^+ stem cells without epigenetic mutations.

Although the mechanisms of synergistic anti-leukemic effects remains elusive thus far, our data provide strong evidence that combining AZA and creno represents a promising novel multimodal therapy approach to target residual *FLT3*-ITD^+ LSC in their niche.

4.1 *FLT3*-ITD is present in early and late leukemic BM compartments and thus represents a potential target for FLT3-TKI to eliminate LSC

So far, only two independent studies have directly analyzed the presence of *FLT3*-ITD in $CD34^+/CD38^-$ LSC-enriched BM compartments, which is a main prerequisite for long-during success of FLT3-TKI therapy. In 2005, Levis *et al.* sorted primary AML samples into stem cell-enriched $CD34^+/CD38^-$ fractions using immunomagnetic cell sorting technique (MACS, Miltenyi) and estimated the *FLT3*-ITD/WT ratio in sorted and unsorted cells using PCR and gene scan. They found similar *FLT3*-ITD ratios ranging from 0.67 to 0.9 in unsorted and sorted cell subsets (Levis *et al.*, 2005). However, these data have to be interpreted with caution since $CD34^+CD38^-$LSC separation using MACS bears a high risk of false positive signals from contaminating $CD34^+/CD38^+$ or $CD34^-$ non-LSC. In 2006, Pollard *et al.* analyzed the presence of *FLT3*-ITD in FACS-sorted $CD34^+/CD33^-$ and $CD34^+/CD33^+$ cell populations and resultant CFC units from 24 pediatric *FLT3*-ITD^+ AML samples by PCR or whole-exome sequencing. In 19/24 cases, *FLT3*-ITD was detected in the $CD34^+/CD33^-$ population, which correlated with worse clinical outcome of these patients (Pollard *et al.*, 2006). However, it remains unclear whether results obtained in children can be transferred to adults with *FLT3*-ITD+ AML, especially in light of the fact that the spectrum of cooperating mutations may differ (e.g. presence of DNMT3A is age-related).

Here, using contemporary and sophisticated MPFC in combination with targeted sequencing, we more precisely analyzed the clonal architecture of early and late leukemic BM compartments from adult *FLT3*-ITD^+ AML patients. We took into account that some of the hematopoietic cell surface markers can be aberrantly expressed in AML. Such as in the majority of AML >10% of BM cells are $CD34^+$, but CD34 expression is typically decreased to <10% in *NPM1*mut AML (Falini *et al.*, 2005)(Chou *et al.*, 2006)(Haferlach *et al.*, 2009). LSC activity may reside within $CD34^+$ and $CD34^-$ AML cell populations (Taussig *et al.*, 2010)(Sarry *et al.*, 2011), but is mostly enriched within the $CD34^+CD38^-$ BM compartment similar to normal HSC (Dick, 2008). Recently, Slush *et al.* (Shlush *et al.*, 2014) published a MPFC strategy to isolate phenotypically normal ($CD33^-$) but pre-leukemic (*DNMT3A*-mutated) hematopoietic stem and progenitor cell populations from AML BM samples. Of note, we did not exclude CD33 as a well-known LSC marker (Walter *et al.*, 2012)(Hauswirth *et al.*, 2007)(Taussig *et al.*, 2005) from our MPFC panel to prospectively isolate and analyze the leukemic cell composition along the hematopoietic hierarchy in $CD34^+$ and $CD34^-$ *FLT3*-ITD^+ AML cases. Strikingly, *FLT3*-ITD was present in the earliest definable $Lin^-/CD33^{(+)}/CD45_{dim}/CD34^+CD38^-$ BM compartment in 5/5 analyzed AML samples regardless of the

CD34 status. In CD34$^+$ AML samples, stem cell compartments where dominated by MLP with high *FLT3*-ITD levels. Goardan *et al.* recently demonstrated that the dominant MLP population in CD34$^+$ AML bears long-term self-renewing and leukemic engraftment capacity in NSG mice and sits at the apex of a leukemic hierarchy (Goardon *et al.*, 2011). Here, *FLT3*-ITD levels increasing from early leukemic to blast subsets indicate outgrowth of an oncogene driven dominant diagnostic clone in 3/5 samples (#4, 7 and 9). In 2/5 samples (#2 and 3), *FLT3*-ITD VAF increase from the HSC to the CD34$^+$CD38$^-$ stem cell stage but decreased in more mature leukemic compartments, indicating initial *FLT3*-ITD driven LSC expansion but also *FLT3*-ITD independent subclone formation during blast accumulation.

In summary, our data ascertain FLT3-ITD as a TKI target to eliminate potential LSC subsets. However, FLT3-TKI alone will not eradicate *FLT3*-WT AML subclones, which may promote refractory disease or relapse (Thiede *et al.*, 2002) (Pratz *et al.*, 2010)(Schlenk *et al.*, 2014). In addition, cooperativity between *FLT3*-ITD and other early driver mutations in *NPM1*, *DNMT3A* and *TET2* may cooperate in LSC expansion (see 1.4.1) and treatment resistance as we demonstrate by our CFC and PDX data (see below).

4.2 A comment on our experimental design to test stromal resistance of residual *FLT3*-ITD$^+$ LSC against AZA and/or creno

In terms of nomenclature, LSC were originally defined by I) a unique immunophenotype (CD34$^+$CD38$^-$) that allows their prospective isolation and II) long-term tumor initiating capacity in immune-deficient mice (Dick, 2008). A recent study has strongly questioned this rigid nomenclature by demonstrating that not the founding clone but subclones preferentially define leukemic engraftment in immune-deficient mice presumably due to the selective pressure during xenotransplantation (Klco *et al.*, 2014). Thus, it was beyond the scope of our study to validate LSC identity of early and late leukemic *FLT3*-ITD$^+$ BM subsets by classical serial xenotransplantation assays. Further, since AML is a very heterogeneous disease regarding the immense diversity of possible founding clones (LSC compartments) and subclones, it would have been extremely laborious, expensive and impractical to profoundly test stromal TKI resistance of *FLT3*-ITD$^+$ LSC harboring diverse co-mutations in individualized xenograft models. Instead, we tested the efficacy of AZA and/or creno directly on stem/progenitor cells from 22 different primary *FLT3*-ITD$^+$ AML BM samples in contact with niche cells in CFC assays and then proved our findings in two distinct PDX re-transplantation models. The latter has been demonstrated to faithfully characterize drug efficacy across heterogeneous AML subtypes (Townsend *et al.*, 2016). Of note, our experimental read-out fo-

cused on the engraftment behavior of residual niche-protected LSC after drug treatment, which ideally models the patient's situation at post-remission. It is technically nearly impossible to model post-remission treatment *in vivo* (at least we are not aware of any such-like AML mouse model).

4.3 FLT3-TKI alone cannot target niche-protected *FLT3*-ITD$^+$ AML cells

Regardless of the clonal architecture in *FLT3*-ITD$^+$ AML or secondary resistance mechanism, we have previously demonstrated that long-term *FLT3*-ITD$^+$ AML LSC are maintained and protected by niche interaction and become quiescent but not apoptotic upon treatment with first (SU5614) and second generation TKI (sora) (Parmar *et al.*, 2011). Here, we show by direct comparison in our *in vitro* BM model with *FLT3*-ITD$^+$ AML cell lines that the next generation more selective TKI creno is as inefficient as sora against stroma-protected *FLT3*-ITD$^+$ AML cells, despite complete inhibition of FLT3-ITD signaling. In summary, our pre-clinical data are in line with accumulating disappointing clinical results and strongly suggest that any TKI monotherapy will fail to induce long-term clinical remission in *FLT3*-ITD$^+$ AML due in part to inherent stromal resistance of *FLT3*-ITD$^+$ LSC.

4.4 Addition of AZA to TKI abrogates stromal resistance of *FLT3*-ITD$^+$ AML cells

This study presents *in vitro* and *in vivo* data from *FLT3*-ITD$^+$ AML cell lines and primary human AML bone marrow samples showing that the combination of creno and AZA is a highly effective strategy to abrogate stromal protection and long-term engraftment potential of LSC. Our findings are supported by some clinical proof of principle studies. For example, the combination of AZA and sora induced an overall response of 46% in relapsed *FLT3*-ITD$^+$ AML patients, including patients that had failed prior therapy with *FLT3*-TKI (Ravandi *et al.*, 2013). Similarly, the combination of the multikinase inhibitor midostaurin and AZA induced an overall response of 26% in AML or high-risk MDS patients, especially those that were *FLT3*-mutated but had not been previously exposed to other *FLT3*-TKI (Strati *et al.*, 2015). Further, sora was effective as a re-induction agent and AZA consolidation prolonged median progression free survival (PFS) of patients with relapsed or refractory sAML in comparison to a historical cohort that had been treated with sora alone (Gill *et al.*, 2015).

Interestingly, we found that the sequential application of TKI (sora or creno) followed by AZA exhibits even advanced anti-leukemic effects in heterozygous and homozygous *FLT3*-ITD$^+$ AML cell lines. This stands in contrast to a recent report stating that simultaneous application of quizartinib and AZA was advantageous

over sequential treatment (Chang *et al.*, 2016). Divergent results could be explained by the use of different cell lines, TKI or AZA concentrations, and experimental read-out time-points. Unfortunately, we were unable to test sequential treatment effects of AZA and TKI in primary *FLT3*-ITD$^+$ LSC in our standardized 4-day culture period due to the fact that AML HSPC divide slowly and prolonged *in vitro* culture will lead to drug-independent loss of CD34$^+$ expression and viability, which we specifically aimed to avoid.

We found that sequential combination of AZA and creno or sora did reduce viability in the *FLT3*-WT AML cell line OCI-AML3, indicating that synergistic or additive drug effects were partly independent of *FLT3*-ITD characteristics. This matches the aforementioned clinical observation that *FLT3*-WT AML patients also respond to the combination of midostaurin and AZA (Strati *et al.*, 2015). Combination therapies bear a higher risk of unwanted toxicity, especially when two “dirty drugs” such as a multi-kinase inhibitor and HMA are combined. However, treatment with AZA + midostaurin or AZA + sora were generally safe with only a small percentage of patients showing ≥ grade 3 - 4 adverse hematologic effects (thrombocytopenia, neutropenia, and anemia, fever and infection), which were mainly attributable to the TKI (Ravandi *et al.*, 2013)(Strati *et al.*, 2015). At the time of writing, we have no clinical data about the safety profile of creno mono- or combination therapy in AML as ongoing Phase II trial results have not been published yet.

Synergistic effects between AZA and sora or quizartinib can induce differentiation of primary AML blasts (Chang *et al.*, 2016)(Gill *et al.*, 2015). Likewise creno can induce differentiation of AML blasts *in vitro* and *in vivo* (communication with Arog Pharmaceuticals). Induction of LSC differentiation which goes along with loss of stem cell properties such as stromal resistance is an often speculated and desired therapy effect. However, in our 4-day *in vitro* BM model neither AZA nor creno induced relevant differentiation of CD34$^+$*FLT3*-ITD$^+$ LSC as single agents or in combination. Of note, the time period of 4 days may be too short to observe any effect on differentiation.

4.5 Combination of AZA and creno targets *FLT3*-ITD$^+$ LSC in their niche despite concurrent mutations in *NPM1*, *DNMT3A* and *TET2*

Although LSC with higher *FLT3*-ITD/WT ratios were more sensitive to creno in CFC assays, this only held true for samples without *TET2* mutations. *FLT3*-ITD$^+$/*TET2*mut LSC were completely resistant to creno as a single agent in the presence of stroma and expanded upon TKI treatment with CFC results corroborated by our PDX transplant data. In support of our primary human data, *FLT3*-ITD$^+$/*TET2*$^{-/-}$ murine progenitors propagate AML in secondary transplants, induce aberrant DNA methylation pattern and are refractory to chemotherapy and quizartinib

as a single agent (Shih *et al.*, 2015). This *TET2*mut-dependent TKI-resistance phenotype warrants confirmation in a clinical trial as it may have important implications for tailoring of TKI therapy in *FLT3*-ITD$^+$ AML.

Since *IDH* mutations inhibit *TET2* function and induce a similar leukemic signature as *TET2* lesions (Figueroa *et al.*, 2010), it is tempting to speculate that *IDH* mutations may also confer resistance to TKI in *FLT3*-ITD$^+$ AML. Unfortunately, we were unable to investigate this due to the limited number of *IDH*-mutated AML samples in this study.

Strikingly, addition of AZA to creno effectively overcame niche protection of *FLT3*-ITD$^+$ LSC *in vitro* and resulted in significantly reduced expansion in CFC assays and severely impaired engraftment in NSG mice despite concurrent *NPM1*, *DNMT3A* or *TET2* mutations.

The conception that HMA may reverse epigenetic signatures such as hypermethylation of tumor suppressor genes led to the widely held assumption that especially patients with epigenetic driver mutations will respond to AZA treatment. In fact, conflicting data has been reported on the correlation between clinical response to AZA and the presence of *DNMT3A* or *TET2* mutations in retrospective analyses, but data from prospective trials have not been reported thus far (Tschanter *et al.*, 2016)(Seymour *et al.*, 2015)(Bejar *et al.*, 2014)(Traina *et al.*, 2014)(Metzeler *et al.*, 2012). In our cohort directly testing AZA on primary *FLT3*-ITD$^+$ LSC, the presence of *NPM1*, *DNMT3A* or *TET2* mutations did not confer a better response to AZA alone, arguing against a direct link between AZA efficacy and epigenetic mutations. Furthermore, despite many efforts, a clear correlation between DNA demethylation and response to AZA has not been able to be demonstrated. The fact that AZA is incorporated to a large part into RNA (up to 90%) and only to a smaller percentage into DNA (Diesch *et al.*, 2016)(Stresemann and Lyko, 2008), suggests that the observed effect of AZA on stromal resistance of *FLT3*-ITD$^+$ LSC may be mediated via the RNA level and not only by DNA demethylation.

To our knowledge, this is the first work studying the effect of TKI and AZA on different mutational genotypes in primary human *FLT3*-ITD$^+$ AML. We demonstrate that efficacy of TKI therapy in *FLT3*-mutated AML is highly dependent on the mutational landscape within each leukemia and provide a valuable framework for designing rational combination therapy in *FLT3*-ITD$^+$ AML. Although the recently reported encouraging results of the RATIFY trial shows that combining the unselective TKI midostaurin with intensive chemotherapy results in a survival benefit for *FLT3*-ITD$^+$ AML patients (Stone *et al.*, 2015) our data suggest that testing patients with *FLT3*-ITD$^+$ AML for TET2 mutations should be considered before initiation of TKI therapy and that the combination of AZA + TKI could be employed

as a means to suppress residual LIC.

4.6 AZA exhibits direct effects on the interaction between LSC and niche cells

Crosstalk between LSC and the niche is not dependent on one single mechanism but is a dynamic process mediated by diverse mechanisms including cellular and soluble mediators. Surely, we were not able to depict the entire complexity of stromal TKI resistance of LSC in our *in vitro* co-culture assays. However, at least in our experimental setup, TKI resistance was not mediated by reactive FL secretion as has been described previously (Sato *et al.*, 2011), or niche-derived soluble factors, but was dependent on direct association between LSC and niche cells. Our RNA-seq data suggest that not one dominant, but several equally important pathways involved in the complex interaction between LSC and their niche were actively disrupted by AZA, which warrant further investigation. To analyze and validate each of these pathways was beyond the scope of this project but will be performed in future work. In itself, the striking diversity of RNA and DNA dependent pathways by which AZA may exhibit its anti-leukemic activity argues that the multimodality of mechanisms is the key to effectively overcoming stromal resistance so as to enable creno to specifically target *FLT3*-ITD^{+} LSC in their niche. It therefore seems unlikely that targeting a singular pathway involved in stromal protection may recapitulate this effect. In this sense, “dirty drugs” such as AZA may be more advantageous than targeted therapies for achieving succes in AML treatment.

5 Abstract

Acute myeloid leukemia (AML) still has a poor prognosis. Even if complete remission (defined as <5% of leukemic blasts in the bone marrow (BM)) is achieved by conventional chemotherapy, most patients will eventually relapse. Re-occurring leukemic blasts originate from chemo-resistant, quiescent, and self-renewing leukemic stem cells (LSC). Similar to normal hematopoietic stem cells, LSC are enriched within the $CD34^+CD38^-$ cell population of the AML BM niche. Thus targeting LSC by novel therapeutic approaches remains a crucial goal for achievement of cure in AML. Constitutive activation of the FMS-like tyrosine kinase 3 (FLT3) by internal tandem duplication (*FLT3*-ITD) is found in approximately 20-30% of AML patients and drives oncogenesis. Therefore, it has become a prominent target in AML. However, despite entry of tyrosine kinase inhibitors (TKI) into clinical trials for *FLT3*-ITD^+ AML, it has so far not been demonstrated that inhibition of aberrant FLT3 signaling can actually eradicate LSC. Although inhibition of mutant FLT3 leads to clearance of leukemic blasts in the periphery, the BM often remains unchanged and remissions are usually short-lived. Here, using primary AML samples *in vitro* as well as in a patient-derived xenograft (PDX) model, we investigated whether the novel FLT3-selective TKI crenolanib (creno) alone or in combination with the hypomethylating agent azacitidine (AZA) can eliminate LSC in *FLT3*-ITD^+ AML and whether treatment efficacy depends on coexisting driver mutations. Using multi-parameter flow cytometry in combination with targeted sequencing, we show that *FLT3*-ITD is present within the most primitive Lin^-/$CD33^{(+)}$/$CD45_{dim}$/$CD34^+CD38^-$ LSC compartment of primary AML BM samples and is frequently accompanied by *NPM1*, *DNMT3A* or *TET2* lesions. However, the novel FLT3-TKI creno alone failed to eradicate *FLT3*-ITD^+ LSC in contact with stroma while addition of AZA overcame niche protection resulting in dramatically reduced clonogenic capacity of LSC *in vitro* and severely impaired engraftment capacity of LSC in immune-deficient (NSG) mice. Strikingly, *FLT3*-ITD^+ samples harboring concurrent *TET2* mutations were completely resistant to creno alone while neither *DNMT3A* nor *NPM1* mutations influenced response. Thus resistance of *FLT3*-ITD^+ LSC to TKI is linked to concurrent mutations. Contrary to several published retrospective correlative clinical analyses, primary AML LSC harboring *DNMT3A*, *TET2* or *NPM1* mutations were not more sensitive to AZA than LSC without epigenetic mutations. Hence, our data strongly argue against the common belief that AZA efficacy is directly linked to epigenetic mutations. However, AZA + creno effectively diminished *FLT3*-ITD^+ LSC irrespective of mutations in *NPM1*, *DNMT3A* or *TET2*, providing strong preclinical evidence for this combination as a means to suppress residual LSC in *FLT3*-ITD^+ AML.

6 Zusammenfassung

Die akute myeloische Leukämie (AML) hat noch immer eine sehr schlechte Prognose. Selbst im Falle erreichter kompletter Remission (<5% leukämische Blasten im Knochenmark (KM)) nach konventioneller Chemotherapie, erleiden die meisten Patienten ein Rezidiv. Wiederkehrende leukämische Blasten werden von chemo-resistenten, ruhenden und selbst-erneuernden leukämischen Stammzellen (LSZ), die sich in dem $CD34^+CD38^-$ Zellkompartiment des KM befinden, erzeugt. Daher ist die Eliminierung der LSZ das große Ziel neuer Therapieansätze und die essentielle Voraussetzung für Heilung der AML. Die konstitutive Aktivierung der Rezeptor-Tyrosinkinase (RTK) „FMS-like Tyrosin-kinase 3 (FLT3)" durch eine interne Tandem-Duplikation im *FLT3* Gen (*FLT3*-ITD) wird in 20-30% der AML Patienten gefunden und befördert die Onkogenese. Daher ist FLT3-ITD eine prominente Zielstruktur neuer Ansätze in der AML-Therapie. FLT3-abzielende Tyrosinkinase-Inhibitoren (TKI) konnten sich bisher jedoch in klinischen Studien nicht beweisen. Zwar eliminieren derzeitig getestete Inhibitoren leukämische Blasten in der Peripherie, aber nicht die LSZ im KM, sodass das Ansprechen der *FLT3*-ITD+ AML Patienten auf FLT3-TKI nicht von Dauer ist. In dieser Studie untersuchten wir, ob der neue FLT3-selktive TKI „Crenolanib" alleine oder in Kombination mit dem hypomethylierendem Agens Azacitidin (AZA) LSZ in *FLT3*-ITD^+ AML eliminieren kann und ob die Therapieeffizienz von koexistierenden Mutationen in *NPM1*, *DNMT3A* oder *TET2* abhängig ist. Wir zeigen mithilfe der Multi-Parameter Durchflusszytometrie und gezielter Sequenzierungsanalyse, dass *FLT3*-ITD bereits in dem primitivsten $Lin^-/CD33^{(+)}/CD45_{dim}/CD34^+CD38^-$ LSZ-Kompartiment auftritt – oft in Begleitung von Mutationen in *NPM1*, *DNMT3A* oder *TET2*. Crenolanib alleine vermag es nicht solche *FLT3*-ITD^+ LSZ zu eliminieren die in Kontakt mit KM-Stroma sind. Allerdings überwindet die Zugabe von AZA zu Crenolanib die stromale Resistenz der LSZ, was sich durch reduzierte Proliferation der LSZ *in vitro* sowie in immundefizienten (NSG) Mäusen belegen lies. Insbesondere *FLT3*-ITD^+/TET2-mutierte Proben waren absolut resistent gegenüber Crenolanib alleine, wohingegen die Koexistenz von *NPM1* oder *DNMT3* keinen Einfluss auf die Therapieresistenz nahm. TKI-Resistenz der *FLT3*-ITD^+ LSZ ist demnach abhängig von kooperierenden Genmutationen. Im Gegensatz zu mehreren retrospektiv korrelativen klinischen Analysen, waren primäre AML LSZ mit *DNMT3A*, *TET2* oder *NPM1* Mutationen nicht sensitiver gegenüber AZA als andere AML LSZ. Damit sprechen unsere Daten gegen einen direkten Link zwischen der AZA Effektivität und epigenetischen Mutationen. Nichtsdestotrotz vermag die Kombination aus Crenolanib und AZA *FLT3*-ITD^+ LSZ unabhängig von koexistierenden Mutationen in *NPM1*, *DNMT3A* oder *TET2* zu unterdrücken. Diese prä-klinischen

Befunde deuten darauf hin, dass die Kombination aus AZA und Crenolanib Potenzial zur Eliminierung von LSZ innerhalb der Postremissionstherapie hat.

7 Appendices

7.1 Abbreviations

2-OG	2-oxoglutarate
5-caC	5-carboxylcytosine
5-fC	5-formylcytosine
5-hmC	5-hydroxymethylcytosine
5mC	Methylation of cytosine bases at the 5’ position
a-KG	Alpha-ketoglutarate
aa	Amino acid
AGM	Aorta-gonad-mesonephros
AKT	AKT serine/threonine kinase 1
alloSCT	Allogeinic stem cell transplantation
AML	Acute myeloid leukemia
AMLSG	AML Study Group
APL	Promylocytic AML
ASXL1	Additional sex combs like 1, transcriptional regulator
AZA	Azacitidine
AZN	Azanucleosides
BCR-ABL1	BCR, RhoGEF and GTPase activating protein
BER	Base excision repair
BLI	Bioluminescence imaging
BM	Bone marrow
CBFB-MYH11	Core-binding factor beta subunit
CEBPA	CCAAT/enhancer binding protein alpha
CFC	Colony-forming cell assays
CFC	Colony forming cell assay
CFU	Colony forming units
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CN-AML	AML with chromosomal normal karyotype
CpG-island	DNA stretches where cytosine follow guanine nucle otides
CR	Complete remission
Creno	Crenolanib

DAC	Decitabine
DNA	Deoxyribonucleic acid
DAVID	Visualization and integrated discovery software
DEK-NUP214	DEK proto-oncogene
DMSO	Dimethyl sulfoxide
Dnase	Deoxyribonuclease I
DNMT	De novo methyltransferase
DSMZ	German collection of microorganism and cell cultures
DTT	Dithiothreito
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELN	European leukemia network
EMA	European Medicines Agency
ERK	Extracellular regulated MAP kinase
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FACS	Flow cytometric cell sort
FCS	Fetal calf serum
FDA	Food and drug administration
FL	FLT3-ligand
FLAG-IDA	Fludarabin/Idarubicin/Cytarabine
FLT3	FMS-like tyrosine kinase 3
G1; S; G2/M	Cell cyle phases
GATA2	GATA binding protein 2
GATA2-MECOM(EVI1)	GATA binding protein 2
gDNA genomic	DNA
GEO	Gene Expression Omnibus Database
GMP	Granulocyte-macrophage progenitor
GO	Gene ontology
H&E	Hematoxylin-eosin
H2B	Histone 2B
HCF1	Host cell factor 1
HDAC	Histone deacetylases
HiDAC	High-dose cytarabine
HMA	Hypomethylating agent
HMT	Histone methyltransferases

HS	Horse serum
HSC	Hematopoietic stem cells
HSPC	Hematopoietic stem and progenitor cells
IHC	Immunohistochemistry
IDH1/2	Isocitrate dehydrogenase 1 and 2
IGFBP-4	Insulin like growth factor binding protein 4
IPSS	International Prostate Symptom Score
ITD	Internal-tandem-duplication
JAK2	Janus kinase 2
JHMD	Jumonji-c domain containing histone demethylase (JHMD)
JMD	Juxtamembrane domain
KDM6A	Lysine demethylase 6A
KEGG	Kyoto encyclopedia of genes and genomes
KIT	KIT proto-oncogene receptor tyrosine kinase
KRAS/NRAS	KRAS proto-oncogene, GTPase
KTM2A	Lysine methyltransferase 2A
Lin	Lineage
LSC	Leukemic stem cell
LT-HSC	Long-term hematopoietic stem cells
LTC	Long-term culture
MC	Mononuclear cells
MDS	Myelodysplastic syndromes
MECOM	MDS1 and EVI1 complex locus
MEP	Megakaryocyte-erythroid progenitor
miRNA	microRNA
MLL-PTD	KMT2A lysine methyltransferase 2A
MLLT3-KMT2A	MLLT3, super elongation complex subunit
MPFC	Multiparameter flow cytometry
MPN	Myeloproliferative neoplasms
MPP	Multipotent progenitor
MRD	Minimal residual disease
mRNA	messenger RNA
MSC	Mesenchymal stromal cells
MTT	Dimethyl-thiazole diphenyl tetrazolium bromide
Mut	Mutated
NGS	Next generation sequencing

NIH	US National Institute of Health
NPM1	Nucleophosmin 1
NSG	NOD.Cg-Prkdcscid IL2rgtm1Wjl/Sz mice
NUP98-NSD1	Nucleoporin 98
NURF	Nucleosomal remodelling factors
O-GlcNAc	O-linked beta-N-acetylglucosamine
OGT	O-linked beta-N-acetylglucosamine transferase
OS	Overall survival
p	Phosphorylated
PB	Peripheral blood
PDGFR	Platelet-derived growth factor receptor
PDX	Patient-derived xenograft
Pen/Strep	Penicillin and streptomycin
PHF6	PHD finger protein 6
PICALM-MLLT10	Phosphatidylinositol binding clathrin assembly protein
PKC-alpha	Protein kinase C alpha
PML-RARA	Promyelocytic leukemia
Pre-LSC	Pre-leukemic stem cell
R-2HG	R-2-hydroxyglutarate
RAD21	RAD21 cohesin complex component
RAEB-t	Refractory anemia with excess of blasts-in transfor mation
Raf	Raf oncogene
RATIFY	Radomized phase III trial
RBM15-MKL1	RNA binding motif protein 15
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RUNX1	Runt related transcription factor 1
RUNX1-RUNX1T1	Runt related transcription factor 1
S-HAM	Sequential high-dose cytarabine
sAML	Secondary acute myeloid leukemia
Ser	Serine
SFM+5GF	Serum free medium with five growth factors
Sora	Sorafenib

STAT5	Signal transducer and activator of transcription 5
t-PDX	Transgenic patient derived xenograft
tAML	Therapy-induced acute myeloid leukemia
TBS	Tris buffered saline
TCA	Tricarboxylate acid cycle
TDG	Thymine DNA glycosylase
TET	Ten-eleven translocation family of proteins
Thr	Threonine
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TMD	Transmembrane domain
TP53	Tumor protein p53
TrkA	NAD-binding component of TrK potassium transporter
TrkB	Potassium uptake system protein TrkB
TrkC	Neurotrophic receptor tyrosine kinase 3
TUM	Technical University Munich
UMIs	Unique Molecular identifiers
VAF	Variant allele frequency
VEGFR2	Kinase insert domain receptor
WHO	World health organization
Wt	Wild-type
WT1	Wilms tumor 1

7.2 Figures

7.3 Tables

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9 List of previous publications

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