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The role of the guanine nucleotide exchange factors DOCK2 and DOCK5 in acute myeloid leukemia and their interactions with epigenetic regulation

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“Imagination governs the world.”

– Napoléon Bonaparte

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1. Introduction

The “Father of Medicine“, Hippocrates of Kos, was the first to refer to solid malignant tumors as *carcinos* (Καρκίνος, Greek for “crab”). He derived this from haptic and visual characteristics he observed in superficial tumors which his patients displayed. In the 2nd century AD, Galen introduced the word *oncos* (ὄγκος, Greek for “bulk/mass”) for tumorous lesions in general. He discriminated *oncos* against Hippocrates’ word *carcinos* which Galen solely applied to malignant tumors (Karpozilos and Pavlidis, 2004). These terms are still present in modern-day medicine through the use of “cancer” and “oncology”. And even though, these founding fathers of scientific medicine were pioneers of their time, mankind still had a long path before itself towards the understanding of malignant diseases.

Regarding hematologic malignancies it took centuries for man to develop an understanding of these specific types of diseases. This was especially due to lacking knowledge of the composition of blood and physiological hematopoiesis. After microscopy was invented and applied for biological studies, human red blood cells were detected for the first time in 1647 (Piller, 2001). Before leukemia was identified, alterations of blood composition and color were often interpreted as a sign of inflammation and pus. The turning point was when, in the middle of the nineteenth century, various physicians started to recognize leukemia as an autonomous disease entity and separated it from the concepts of inflammation and pus. During this time, Rudolf Virchow introduced the term *leukemia* (Greek: *leukos* = “white”, *haima* = “blood”) to describe what he had observed in the postmortem of a leukemia patient – a reversed ratio between colorful and colorless blood cells (Kampen, 2012). However, effective treatment of leukemia remained unknown for another century. In 1947, Sydney Farber recognized the possible therapeutic value of folic acid antagonists in leukemia. Based on this insight, the first chemotherapy was introduced. Therapy regimens continuously developed and led to improving remission rates.

Despite advances, induction therapy for acute myeloid leukemia has not changed significantly since 1973 (Luger, 2017). Consequently, major hurdles in treatment of acute myeloid leukemia (AML) have still not been overcome. Relapse rates are high and overall survival for patients with relapsed disease and elderly patients remain poor (Medeiros, 2018). Although, major milestones in the understanding of the heterogenous genetic landscape of AML have been reached, satisfactory translation of this knowledge into new clinical treatment options is still in its early stages (Döhner et al., 2015).

1.1. Acute Myeloid Leukemia (AML)

Acute myeloid leukemia is a hematologic malignancy which is characterized by uncontrolled clonal proliferation of abnormally or poorly differentiated cells of the hematopoietic system. These cells infiltrate the bone marrow, blood and other tissues (Döhner et al., 2015). The age-adjusted incidence rate per 100 000 is of ~ 4.3 in the United States (Shallis et al., 2019). In Germany, there is an incidence of ~ 4100 new cases per year with no significant change in incidence rates during the past 10 years (Kraywinkel and Spix, 2017). AML is the most common form of acute leukemia in adults accounting for approximately 80 % of the cases in this group (Yamamoto and Goodman, 2008). In age distribution, AML clearly shows two epidemiological peaks with one in early childhood and one in later adulthood. Patients with newly diagnosed AML have a median age of 65 years (Deschler and Lübbert, 2006). Gender distribution in incidence rates is roughly the same (Kraywinkel and Spix, 2017).

1.1.1. Pathophysiology and genomic landscape

The physiological hematopoiesis is a sophisticated process which results in the production of various blood cell types with functions ranging from oxygen transport to immunity. The starting point of this differentiation is the hematopoietic stem cell (HSC), localized in the bone marrow, that gives rise to committed progenitor cells, which in turn proliferate and differentiate into a functional end cell (Fey, 2007).

Pathophysiologically, AML can be divided into primary or “de novo” AML and secondary AML. Secondary AML can arise in patients because of an underlying hematological malignancy such as myelodysplastic syndrome (MDS) or following exposure to myelotoxic influence – e.g., chemotherapy with alkylating agents, topoisomerase-II-inhibitors or ionizing radiation (Sill et al., 2011). Regardless of its etiology, the pathogenesis of AML involves a differentiation block and subsequent clonal hyperproliferation of immature myeloid progenitor cells (De Kouchkovsky and Abdul-Hay, 2016). Most commonly, the origin of this pathological proliferation of clonal myeloid cells is the highly proliferative pool of progenitor cells and more infrequently, the pool of stem cells. Recent findings show that – in addition to the main clone – approximately 50 % of AML patients feature one or multiple subclones (Welch, 2013). Recurrent chromosomal aberrations and somatic mutations play a pivotal role and are therefore used in diagnostics as well as prognostic markers and as (potential) therapeutic targets (Cancer Genome Atlas Research Network et al., 2013). It is assumed that > 97 % of AML patients carry at least one genetic mutation (Patel et al., 2012). Along the way, AML has diverged from the concept of being a single malignant disease entity. Although AML

is considered a genetically heterogenic disease, AML genomes have fewer mutations than most other adult cancers with an average of 13 mutations found in genes (Cancer Genome Atlas Research Network et al., 2013). The Cancer Genome Atlas Research Network analyzed 200 genomes of adult de novo AMLs and could show that > 99 % of AMLs carried a genetic mutation in one out of nine biologic-functional categories. These categories include the following: (I) proteins for transcription-factor fusions, (II) the gene encoding nucleophosmin (NPM1), (III) tumor-suppressor genes, (IV) DNA-methylation–related genes, activated signaling genes, (V) chromatin-modifying genes, (VI) myeloid transcription-factor genes, (VII) cohesin-complex genes, (VIII) and spliceosome-complex genes (Cancer Genome Atlas Research Network et al., 2013). Recurrent structural and numerical chromosomal aberrations are essential drivers of leukemogenesis, as well. With the large-scale establishment of cytogenetic diagnostics, a large number of balanced and unbalanced chromosomal abnormalities (e.g., t(15;17), t(8;21), inv(16), t(9;21), t(9;11), del5, del7, etc.) have been discovered which are strongly associated with individual risk stratification and prognosis (Welch et al., 2012). However, nearly 50 % of AML patients have a normal karyotype (Walter et al., 2009). Through widespread efforts and new techniques like whole-genome sequencing, a set of recurring mutations have been identified which have broad therapeutic and prognostic implications. These include mutations of FMS-like tyrosine kinase 3 (FLT3), NPM1, the tyrosine kinase KIT, CCAAT/enhancer-binding protein alpha (CEBPA), Tet methylcytosine dioxygenase 2 (TET2), and the epigenetic enzymes DNA (cytosine-5)-methyltransferase 3A (DNMT3A) and Isocitrate dehydrogenase 1 (IDH1) (Welch et al., 2012).

FLT3 mutations are the most frequent genetic lesion in acute myeloid leukemia with a prevalence of approximately 25 % (Konig and Levis, 2015; Stirewalt and Radich, 2003). FLT3 is a gene which encodes a membrane-bound receptor tyrosine kinase. The most common form of FLT3 mutation is an internal tandem duplication (ITD) of the juxtamembrane region which occurs in 15 - 35 % of patients with AML and 5 -10 % of patients with MDS (Stirewalt and Radich, 2003). A FLT3-ITD mutation is an important negative prognostic factor in all age groups (Kottaridis et al., 2001). Another form of FLT3 mutation, namely point mutations in the activation loop of the kinase domain (FLT3-tyrosine kinase domain (TKD)), does not seem to influence the outcome of AML patients significantly (Bacher et al., 2008). Functionally, a FLT3-ITD leads to constitutive activation of proliferative downstream signaling cascades, including Ras/MAPK kinase (MEK)/ERK pathway and PI3K/Akt pathway. This further leads to Rac1-Guanosine-5'-triphosphate (GTP) binding and is associated with increased levels of reactive oxygen species (Takahashi, 2011). These mechanisms result in promotion of aberrant cell growth and enhancing of leukemogenesis.

Clinically, the pathophysiological mechanics of AML lead to the extrusion of physiological hematopoiesis, resulting in life-threatening conditions such as anemia, thrombocytopenia and severe immunosuppression (Behrmann et al., 2018).

1.1.2. Diagnosis and classification

The diagnosis of AML has several pillars. One is morphology: at least 200 leukocytes on blood smears and 500 nucleated cells on spiculated marrow smears should be counted. A bone marrow or blood blast count of ≥ 20 % is required, except for AML with t(15;17), t(8;21), inv(16), or t(16;16), which are diagnosed independently from blast count (Döhner et al., 2017). Further diagnostics include immunophenotyping. Expression of cell-surface and cytoplasmic markers is determined through flow cytometry, immunohistochemistry, or cytochemistry. These are used to establish the diagnosis of AML as well as determining specific myeloid lineage markers on cell surfaces (e.g., Cluster of Differentiation (CD)34, CD117, CD33, CD13, Human Leukocyte Antigen (HLA)-DR for myeloid precursor cells/blasts) (Döhner et al., 2017). Diagnostic work-up is completed by molecular genetic testing and cytogenetic diagnostics. Cytogenetic analysis is conducted by different methods including karyotyping and fluorescent in-situ hybridization, through which structural and numerical chromosomal aberrations can be detected. Molecular genetic testing is carried out through screening for mutations in commonly mutated genes which have prognostic value. This involves NPM1, CEBPA, RUNX1, FLT3-ITD, TP53 and Additional Sex Combs-Like 1 (ASXL1) (Döhner et al., 2017).

Throughout history, the classification of AML has evolved steadily. The first attempt of establishing a uniform system of classification was made by the French-American-British (FAB) Cooperative Group. The FAB classification is based on acute leukemia and MDS cells' morphological and cytochemical attributes and classifying them into the subtypes M0 - M7 (Bennett et al., 1976). The steadily improved understanding of the molecular pathogenesis and biology of hematologic malignancies, and AML in particular, led to the introduction of the World Health Organization (WHO) classification of tumors of the hematopoietic and lymphatic tissue in 2001 (Vardiman, 2010). The WHO classification integrates genetic, immunophenotypic, biological, and clinical features to define specific disease entities and therefore accounts for the cytogenetic and molecular diversity in AML (Walter et al., 2013). The WHO classification was last updated in 2016 and classifies AML in eight subtypes. Table 1 shows the WHO classification of AML.

Table 1. WHO classification of AML (adapted from Arber et al., 2016)

Subtype	Specification
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 RUN
AML with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
AML, not otherwise specified (NOS)	AML with minimal differentiation AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic/monocytic leukemia Pure erythroid leukemia Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis (TAM) Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic neoplasm	
Acute leukemias of ambiguous lineage	Acute undifferentiated leukemia Mixed phenotype acute leukemia (MPAL) with t(9;22)(q34.1;q11.2); BCR-ABL1 MPAL with t(v;11q23.3); KMT2A rearranged MPAL, B/myeloid, NOS MPAL, T/myeloid, NOS

1.1.3. Therapy and prognosis

The backbone of AML therapy has not undergone significant change over the last few decades. At diagnosis, an initial assessment is conducted to determine whether a patient is fit for intensive induction chemotherapy and to evaluate the risk for treatment-related mortality (TRM) (Burnett et al., 2011). The general approach for AML therapy consists of induction chemotherapy, followed by consolidation therapy. The standard therapy regimen for induction is “7 + 3”: a continuous 7-day infusion of cytarabine, followed by 3 days of anthracycline infusion (e.g., Daunorubicin or Idarubicin) (Döhner et al., 2015). The therapeutic goal of induction therapy is to achieve a complete remission (CR) which is defined as bone marrow blast count < 5 %; absence of circulating blasts/blasts with Auer rods, absence of extramedullary disease and hematologic recovery (neutrophil count ≥ 1000/µl, platelet count ≥ 100 000/µl) (Röllig, 2019). CR is achieved in approximately 60 - 70 % of patients (Wiernik et al., 1992). After achieving CR, consolidation therapy is essential to eliminate minimal residual disease (MRD) and prevent relapse. Standard postremission strategies include conventional chemotherapy as well as allogenic stem cell transplantation (aSCT) (Döhner et al., 2015). The selection of postremission therapy is made upon different patient- and disease-related factors: age, genetic risk profile, TRM and molecular aberrations which can be therapeutically targeted (Döhner et al., 2015; Morra et al., 2009). Depending on the individual patient’s risk for relapse, patients ≤ 60 years receive 3 to 4 cycles of intermediate or high dose cytarabine. For eligible patients with intermediate or adverse risk genetic profile, aSCT is recommended (Döhner et al., 2017; Stelljes et al., 2014). Overall, allogenic stem cell transplantation is the most potent postremission therapy for AML and is particularly effective for patients < 60 years of age and/or those with high-risk cytogenetics (Koreth et al., 2009; Li et al., 2015; Stelljes et al., 2014). Major complications of aSCT include relapse, acute and late-onset Graft-versus-host-disease, organ failure (kidneys, liver, lungs) and opportunistic infections due to excessive immunosuppression through chemoradiotherapy conditioning in the course of aSCT (Döhner et al., 2015; Gooley et al., 2010).

Novel therapies

The recent progress in understanding the molecular mechanisms which are driving leukemogenesis as well as the poor survival rates in elderly patients with adverse risk profile implicate the pressing need for new therapies. This led to the introduction of multiple new drugs in the last years that target different cellular processes on the molecular and epigenetic level. The frequency and prognostic relevance of FLT3 mutations have led to the introduction of FLT3 inhibitors into AML treatment algorithms.

FLT3 inhibitors are receptor tyrosine kinase inhibitors and, so far, two generations of FLT3-inhibitors have been approved by the American Food and Drug Administration. First-generation FLT3 inhibitors include midostaurin and sorafenib. Second-generation inhibitors include quizartinib and gilteritinib, which presumably possess higher specificity and potency and are therefore less toxic due to off-target effects (Majothi et al., 2020). Although, there is evidence supporting the use of FLT3 inhibitors in clinical practice, their role in the broader treatment approach and impact on clinical endpoints still need to be determined (Majothi et al., 2020). The second-generation inhibitor gilteritinib was FDA-approved based on the ADMIRAL trial (Perl et al., 2019). It showed that patients with relapsed or refractory AML had significantly longer survival and had higher remission rates compared to salvage chemotherapy. However, future trials will show whether the second-generation FLT3-inhibitors will be able to overcome shortcomings of first-generation compounds like drug resistance and elevated toxicity profiles (Döhner et al., 2015). Another drug class which recently has received lots of attention are epigenetic modifiers. These compounds include DNA hypomethylating agents, IDH1/IDH2-inihibitors and bromodomain inhibitors. Further compounds are the B-cell Lymphoma 2 pathway inhibitor venetoclax and new signaling pathway inhibitors like the Hedgehog pathway inhibitor vismodegib or Polo-like kinase 1 inhibitors like volasertib. While the rapid developments in understanding the heterogeneity of AML with subsequent new compounds in the pipeline are encouraging, recent findings highlight that inhibition of a single molecule or pathway does not result in substantial improvements in the major clinical endpoints (Shafer and Grant, 2016).

Until 50 years ago, AML was considered incurable. Meanwhile, AML is cured in 35 - 40 % in patients < 60 years of age and in 5 - 15 % in patients > 60 years of age (Döhner et al., 2015). One of the main reasons for the significantly worse prognosis in older patients is that this patient cohort is often ineligible for intensive induction chemotherapy – due to low performance status and comorbidities – which is decisive for clinical outcomes (Stirewalt et al., 2001). Prognostic factors to consider can be subdivided into patient-related factors and those factors related to the respective AML clone. Patient-related prognostic factors are age, performance status and comorbidities. AML-related prognostic factors include white blood count, existence of prior MDS, previous cytotoxic therapy for another disorder and cytogenetic and molecular genetic changes in the leukemic cells at diagnosis (Döhner et al., 2010). Prognosis for primary refractory disease and early relapsed – within the first 6 months after induction – AML have the worst prognosis (Döhner et al., 2010). The European LeukemiaNet has developed guidelines to allocate patients into three risk groups (low- intermediate-, high-

risk), to correlate cytogenetic alterations and molecular mutations with clinical outcome (Mrózek et al., 2012).

1.2. Leukemic stem cells (LSCs)

Leukemic stem cells are defined as a rare subpopulation of leukemic cells which are responsible for initiating and maintaining the disease and exhibiting properties of self-renewal, cell cycle quiescence, and chemoresistance (Thomas and Majeti, 2017). 25 years ago, the first seminal works by Lapidot et al. and Bonnet and Dick were published, which introduced the concept of a cancer stem cell model. Lapidot et al. showed through serial xenotransplantation of severe combined immunodeficient (SCID) mice that $CD34^+/CD38^+$ human leukemic blast cells were able to initiate leukemia in vivo (Lapidot et al., 1994). Bonnet and Dick first postulated the – somewhat similar to physiological hematopoiesis through hematopoietic stem cells – hierarchical organization of AML, with the LSC at the apex. They showed that engrafting a single $CD34^+/CD38^-$ LSC in xenotransplantation assays was enough to generate millions of leukemic cells through clonal proliferation (Bonnet and Dick, 1997). Recent studies indicate that, before the actual onset of AML, functionally normal HSCs sequentially acquire epigenetic and molecular mutations which ultimately leads to the transformation of these pre-leukemic HSCs into LSCs which serve as drivers of AML (Corces-Zimmerman et al., 2014). The origin as well as specific attributes of LSCs have fundamental implications for improving therapeutic outcomes in AML, since it is assumed that LSCs function as a reservoir for leukemic cells. The combination of LSCs' enormous proliferative abilities and their specific tumor microenvironment in the bone marrow niche (see 1.3.) make them drivers of leukemogenesis, relapse and chemoresistance (Jan et al., 2012). As a consequence, targeting LSCs and preleukemic HSCs and effectively eliminating them in the future is possibly a turning-point towards curing AML in patients who currently have a dismal prognosis (Thomas and Majeti, 2017).

1.3. Bone marrow niche and the tumor microenvironment

The bone marrow is the location of hematopoiesis which is the process by which the cellular components of the blood are formed and fed into the peripheral circulation. The HSC represents the apex of the hierarchically organized hematopoiesis. The HSC is characterized by its ability to self-renew and differentiate into various progenitors which then mature into final blood cells (Laurenti and Göttgens, 2018). The HSC microenvironment in the bone marrow is also called the "niche" and is composed of

several cellular and non-cellular components (Ghobrial et al., 2018). The bone marrow niche is further subdivided into two distinct niches: the endosteal and the perivascular niche (Yu and Scadden, 2016). The vascular niche is described as the site for mobilization of HSCs, proliferation and differentiation whereas the endosteal niche (localized at the inner surface of the bone cavity) is the main site for HSC homing (Ghobrial et al., 2018). The niche concept is an approach to explain the dependence between HSCs and their microenvironment (Schofield, 1983). In the past decades, various cell types, including osteoblasts, perivascular stromal cells, endothelial cells, macrophages as well as multiple cytokines and signaling pathways were implicated for their roles in maintaining HSC quiescence (Behrmann et al., 2018). The orchestration of these mechanisms and components is highly complex and disorders within this delicate homeostasis can lead to alterations in the HSCs' proliferative behavior and it is assumed that LSCs may "hijack" these homeostatic mechanisms, find protection in the niche during chemotherapy, and consequently contribute to eventual disease relapse for which a single LSC can be sufficient (Lane et al., 2009). However, it is not entirely clear whether the niche is a leukemogenic driver or rather a facilitator of malignancy, although these two concepts are not entirely mutually exclusive (Méndez-Ferrer et al., 2020). It has been shown that malignant cells transform and modulate the niche to optimize their own tumor microenvironment to the expense of normal HSCs. Specific niche stromal cells influenced by malignant cells are mesenchymal stem cells (MSCs), osteoblasts, endothelial cells, adipocytes and peripheral neurons (Méndez-Ferrer et al., 2020). The heterogenous group of MSCs seem to play a critical role for the physiological and pathological bone marrow niche, despite their rare occurrence in the bone marrow. Of particular importance are the CXCL12-abundant reticular cells (CAR cells) and nestin^{+} cells which are mainly located in the perivascular niche (Goulard et al., 2018; Tormin et al., 2011). CAR cells have been shown to significantly produce CX chemokine ligand 12 (CXCL12) which binds to its receptor C-X-C chemokine receptor type 4 (CXCR4). Sugiyama et al. showed that induced deletion of CXCR4 in adult mice led to significant reduction in HSC numbers and increased myelotoxicity of chemotherapy agents (Sugiyama et al., 2006). Several data suggests that the close relationship between MSCs, LSCs and the CXCR4/CXCL12 axis are one of the key factors protecting the LSCs from apoptosis and chemotherapy (Goulard et al., 2018). CXCR4/CXCL12 interaction also leads to up-regulation of vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) expression which support interaction of leukemic with endothelial cells and subsequent infiltration of the niche by malignant cells (Avecilla et al., 2004; Poulos et al., 2014). There is a broad range of further molecules and subsequent remodeling mechanisms of the components of the bone marrow niche

whose detailed discussion would exceed the scope of this study. In their entirety, they all lead to a molecular ecosystem which enables LSCs to be sheltered and proliferate in the sanctuary of the remodeled bone marrow niche. In an attempt to translate the enormous increase in knowledge on the basic science level into clinical practice, a significant number of pathways and molecules which are known to play a role in developing and sustaining the tumor microenvironment are currently under investigation as potential therapeutic targets (Kuek et al., 2021).

1.4. Guanine nucleotide exchange factors DOCK2 and DOCK5

1.4.1. Guanine nucleotide exchange factors (GEFs)

Guanine nucleotide exchange factors act as regulators of small guanosine triphosphatases (GTPase) by replacing guanosine diphosphate (GDP) by GTP. Small GTPases are GTP-binding proteins acting as molecular switches, commonly found in eukaryotic cells. They are essential in multiple processes of cellular homeostasis like cytoskeletal reorganization, migration, cell polarity, cell cycle progression and many others (Song et al., 2019). Figure 1 illustrates how small GTPases act as binary molecular switches that cycle between an inactive GDP-bound and an active GTP-bound state (Vetter and Wittinghofer, 2001). Activation of small GTPases is catalyzed by GEFs whereas the inactive state is catalyzed and stabilized by GTPase-activating proteins (GAP) and guanine nucleotide dissociation inhibitors (GDIs), respectively. GAPs enhance the catalytic activity of small GTPases and hereby support the hydrolyzation of GTP into GDP (Zalcman et al., 1996). The third type of small GTPase-regulating proteins are GDIs which act in opposition to GEFs. GDIs regulate inactivation and activation of Rho-GTPases by their localization in the cytosol or at the plasma membrane. The inactive Rho-GTPase is bound to the GDI in a complex and remains in the cytosol. When the inactive Rho-GTPase now dissociates from the GDI, it is translocated to the plasma membrane, where it can interact with a GEF and be activated (Garcia-Mata et al., 2011). The small GTPases constitute a protein superfamily, whose most prominent member is the Ras superfamily (Heider et al., 2010). The Ras superfamily is categorized into five subfamilies: Ras, Rho, Rab, Ran and Arf (Colicelli, 2004). For the present doctoral thesis, the focus lies on the Rho-family since DOCK2 and DOCK5 act as regulators for Rho-family proteins. The Rho-GEFs are subdivided into two families: the Dbl family and the more recently discovered Dedicator of Cytokinesis (DOCK) family (Wennerberg and Der, 2004). DOCK-GEFs specifically act as activators of the Rho-superfamily GTPases Rac and Cdc42 (Kunimura et al., 2019).

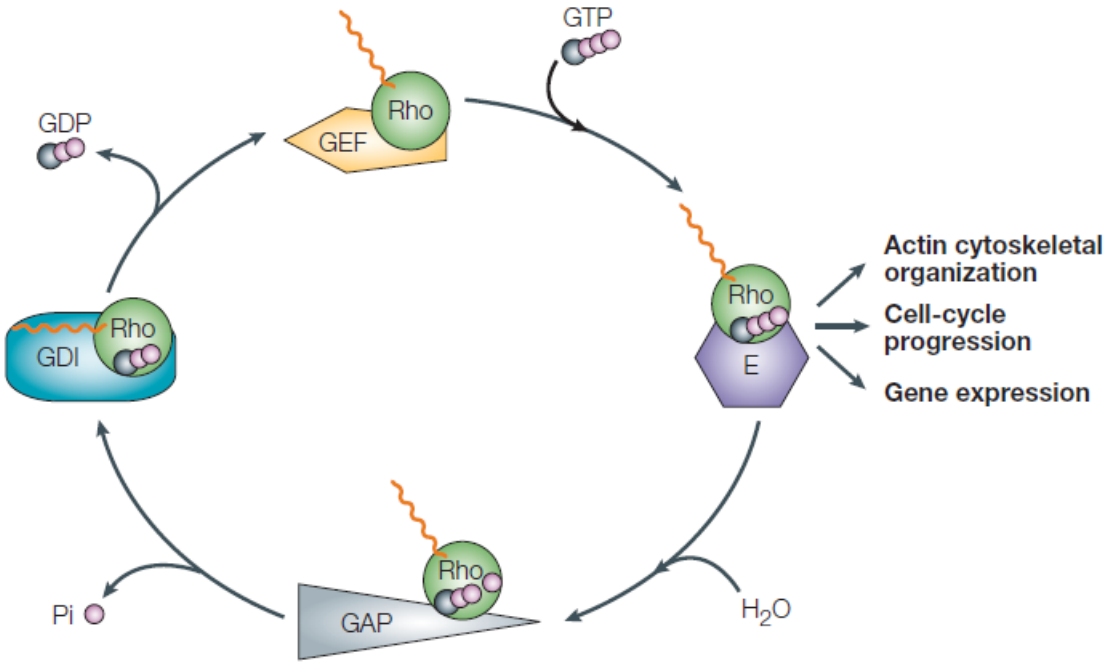


Figure 1. Schematic illustration of the Rho-GTPase cycle and regulation. Small GTPases are in equilibrium between a GTP-bound active state, and a GDP-bound inactive state. Activation of Rho-GTPases results in crucial cellular processes such as cytoskeleton reorganization, cell cycle progression and gene expression. Regulators of GTPase activity include GEFs which catalyze the exchange of GDP for GTP. GDIs which mainly bind the switch regions and the C-terminal isoprenyl moiety (orange wavy line) of Rho-GTPases to dissociate them into the cytosol. GAPs enhance the intrinsic hydrolytic activity of Rho GTPases to promote GDP-bound forms and terminate signaling. GTPase: guanosine triphosphatases, GTP: guanosine trisphosphate, GDP: guanosine diphosphate, E: effectors, GAP: GTPase-activating proteins, GDI: guanine nucleotide dissociation inhibitors, Pi: inorganic phosphate (Figure and text from (Rossman et al., 2005).

DOCK family GEFs mediate the GDP-GTP exchange through their characteristic and evolutionarily conserved DOCK homology region-2 (DHR-2) (Côté and Vuori, 2002). The DHR-1 domain acts as phosphatidylinositol (3,4,5)-triphosphate (PIP_3)-interacting domain which is important for cell elongation and migration. Côté et al. could show that a DHR-1-deficient DOCK is able to load GTP in Rac, but is incapable of promoting directional cell movement (Côté et al., 2005). Rho-GTPases have essential roles in the physiological maintenance of the cellular life cycle and cytoskeletal dynamics. Rac and Cdc42 in particular, are often dysregulated in malignant diseases due to hyperactivation by GEFs which, as a consequence, have increasingly emerged as key players in

oncological research (del Mar Maldonado and Dharmawardhane, 2018; Singh et al., 2004; Stengel and Zheng, 2011).

1.4.2. Physiology and pathophysiology of DOCK2/DOCK5

DOCK proteins constitute a family of evolutionarily conserved guanine nucleotide exchange factors which activate small GTPases of the Rho family. The DOCK family has 11 members which are further subdivided into four subfamilies – DOCK A, DOCK B, DOCK C, DOCK D – each of which has unique functions depending on the expression pattern and the substrate specificity (Kunimura et al., 2019). The present thesis seeks to determine the role of the DOCK-isoforms DOCK2 and DOCK5 in AML. Together with DOCK1, DOCK2 and DOCK5 form the DOCK A subfamily (Namekata et al., 2020).

DOCK2 mediates the activation of small G protein Rac1/ 2 through stabilizing its nucleotide-dissociated state (Nishikimi et al., 2013). Functional effects of Rac activation include cell proliferation/apoptosis, migration, T cell development and chemokine secretion (Chen et al., 2018). The Src-homology 3 (SH3) domain of DOCK2 interacts with engulfment and cell motility factor 1 (ELMO1) which is required for Rac activation (Sanui et al., 2003). The DHR-1 domain of DOCK2 is then used to bind PIP_3 or PIP_3 kinase (PIP_3 kinase can catalyze production of PIP_3) which presumably leads to an accumulation of PIP_3 at the leading edge of the cell. This results in reciprocal activation and subsequent promotion of cell polarity and motility (Kunisaki et al., 2006). Subsequently DOCK2 is translocated from the cell membrane to the cytosol where, ultimately, Rac is activated through interaction with the DHR-2 domain of DOCK2. Further stabilization and DOCK2 accumulation are promoted through binding of phosphatidic acid (PA) at the polyamino acid (PAA) region of DOCK2 (Nishikimi et al., 2009). Figure 2 illustrates the molecular signaling of DOCK2.

Analogous to DOCK2, DOCK5 also forms a complex with ELMO1 to activate Rac1 (Kukimoto-Niino et al., 2021). As DOCK5 is the least studied member of the DOCK A family, cellular effects of DOCK5 are not entirely clear, but Vives et al. could show that DOCK5 plays an essential role in osteoclast regulation and osseous metabolism through regulation of actin dynamics (Vives et al., 2011). Furthermore, DOCK5 is linked to motile and invasive capacities of epithelial cells as well as metastasis (Frank et al., 2017). In addition, a recent study implicated a link between obesity and DOCK5 by altering energy balance and hepatic insulin sensitivity (Lai et al., 2020).

Alterations of DOCK2 activity have been linked to inflammation, cancer and immunodeficiency (Chen et al., 2018; Kunimura et al., 2019). DOCK2 is specifically

expressed on hematopoietic cells and regulates activation and migration of immune cells through activation of Rac. Fukui et al. could show that DOCK2-deficient lymphocytes showed significantly decreased levels of chemokine-induced Rac activation and actin polymerization (Fukui et al., 2001). More recently, DOCK2-deficiency has been associated with combined T and B cell immunodeficiency (Moens et al., 2019). Further evidence suggests the involvement of DOCK2 in multiple inflammatory diseases such as acquired immunodeficiency syndrome, *Leishmania major* infections and allergic diseases (Chen et al., 2018). DOCK2 mutations have been linked to development of multiple malignant entities. Exome and whole genome sequencing identified DOCK2 mutations in esophageal adenocarcinoma and colorectal cancer (Dulak et al., 2013; Yu et al., 2015). Another study found high expression levels of DOCK2 in B cell and follicular lymphomas and could show that inhibition of DOCK2 is associated with lower proliferation (Wang et al., 2010).

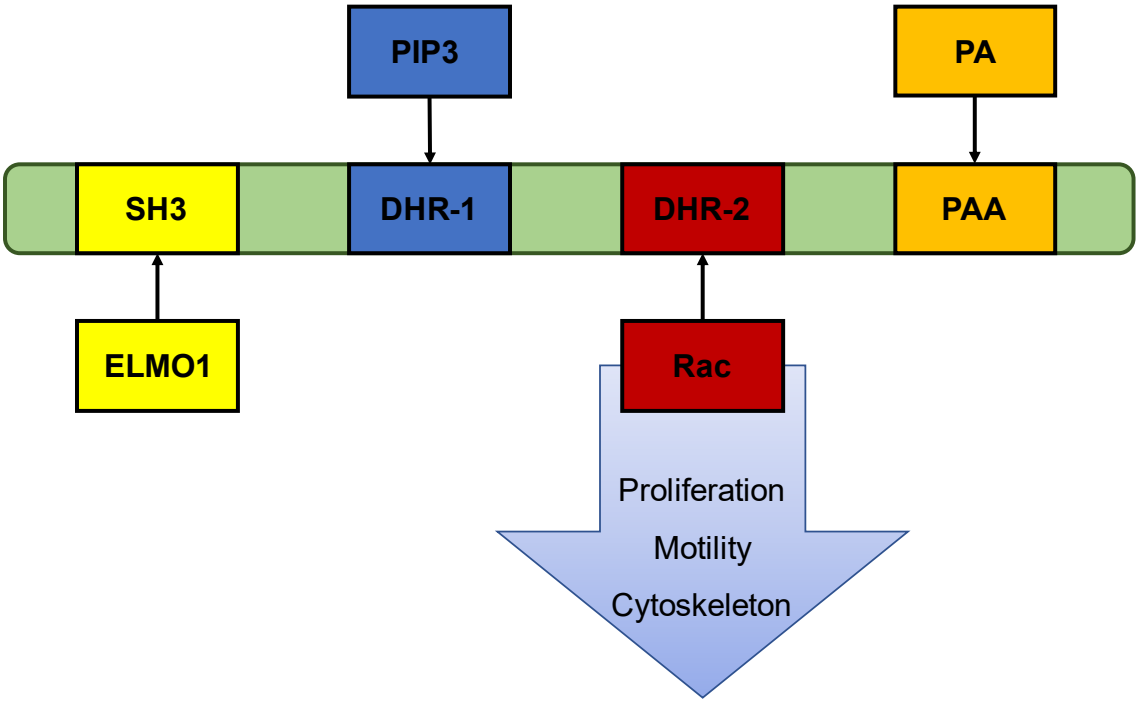


Figure 2. Dynamics of DOCK signaling. The SH3 is necessary to interact with ELMO1 which enables Rac-DOCK-interaction. The DHR-1 domain binds phospholipids while the DHR-2 domain interacts with Rac to activate downstream signaling and functional effects in cellular homeostasis. The PAA region binds PA which promotes accumulation and further stabilization of activated Rac. SH3: Src-homology 3, ELMO1: engulfment and cell motility factor 1, DHR-1/2: DOCK homology region, PAA: polyamino acid, PA: phosphatidic acid (Guo and Chen, 2017; Nishikimi et al., 2009).

1.4.2.1. DOCK2 and DOCK5 in AML

Recent studies implicate a role of DOCK2 in leukemogenesis. Wu et al. showed that the recurrent FLT3-ITD is commonly interacting with DOCK2, therefore associated with a poor prognosis. Furthermore, they showed that knockdown of DOCK2 resulted in decreased cell proliferation in vitro and prolonged survival in a DOCK2 knockdown-xenograft mouse model (Wu et al., 2017). In addition, the same group could show that FLT3-ITD-carrying AML cells showed higher resilience towards chemotherapy agents because of higher DNA damage response factor activity. DOCK2 expression showed to be proportional to this mechanism which translated into higher chemosensitivity in DOCK2 knockdown cells (Wu et al., 2019).

The role of DOCK5 in AML is relatively opaque with only one publication specifically addressing the role of DOCK5 in this malignant entity. Biswas and colleagues investigated the role of DOCK5 in the context of epigenetic aberrations in AML. Specifically, they found that deficiency in the DNA methylation protein Methyl-CpG-binding domain protein 3 (MBD3) is associated with elevated expression of DOCK5. However, it remains to be elucidated how this observation needs to be placed in the molecular context of leukemogenesis and how this translates into advances in clinical therapies (Biswas et al., 2019).

1.5. Epigenetics

The terms “epigenetics” and “epigenotype” were established by Conrad Waddington in 1942, which he introduced to explain differences in phenotypes without changes in genotype (Waddington, 2012, 1959). Nowadays, the definition of epigenetics has become more sophisticated, often along the semantic trajectory of a “stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence.” (definition from the 2008 Cold Spring Harbor Epigenetics meeting; Berger et al., 2009). Epigenetic mechanisms are deeply intertwined and orchestrated with transcription. They influence DNA transcription by covalent modification of DNA bases (e.g., methylation, acetylation), (posttranslational) modification of histones and nucleosome remodeling (Dawson and Kouzarides, 2012; Dupont et al., 2009). All these pathways lead to dynamic reorganization of the chromatin structure and altered accessibility of the DNA for regulatory factors and transcriptional polymerases (Nebbioso et al., 2018). Genes encoding epigenetic machinery are often mutated in a range of malignant entities where they drive tumorigenesis by promoting the hallmarks of cancer on the cellular level (proliferative signals, cell death impairment, inactivation of growth suppressors, angiogenesis, replicative independence leading to immortality, and

acquisition of cancer progression features such as invasion and metastasis) (Hanahan and Weinberg, 2000; Nebbioso et al., 2018). Our understanding of these mechanisms, their role for tumorigenesis and possible therapeutic implications have been amplified through innovations like global proteomic and genomic technologies (Dawson and Kouzarides, 2012).

1.5.1. Epigenetics in AML

During the last decade, a great wealth of knowledge about epigenetic changes that crucially contribute to some of the most aggressive forms of leukemia, lymphoma, and myelodysplastic syndromes has been discovered (Blecua et al., 2020). These mutations include proteins that are involved in DNA cytosine residues and enzymes which catalyze posttranslational modifications of histones and hence, alter the epigenetic regulation of transcription (Abdel-Wahab and Levine, 2013). Among the frequently reported epigenetic mutations in AML patients are the genes for DNMT3A, TET2, IDH1/ 2, and ASXL1 (Abdel-Wahab and Levine, 2013). Accordingly, recent evidence shows that aberrant hypermethylation is recurrently present in hematologic malignancies. Figuera and colleagues found that patients with MDS and secondary AML displayed more extensive aberrant DNA hypermethylation and were responsive to DNA methyltransferase inhibitors (Figueroa et al., 2009).

Another example is a recent study which connects loss of the histone 3 lysine 27 demethylase Lysine-specific demethylase 6A (KDM6A) to acquired chemoresistance in AML patients and relapse-specific loss of the protein (Stief et al., 2020). Another illuminating finding is that several groups could show that epigenetic mutations happen early on in leukemogenesis. The implication of this could be that targeting altered epigenetic pathways may be a major tool in the armament against decade-old challenges in AML therapy which are thought to be connected to the genomic heterogeneity of the leukemic main clones (i.e., LSCs) (Brunetti et al., 2017; Papaemmanuil et al., 2016; Goodell and Godley, 2013).

The steady inflow of new findings, connecting epigenetic/transcriptional alterations to leukemogenesis, is very encouraging. However, one of the major challenges does not lie solely in identifying epigenetic mutations but rather integrating and interconnecting this knowledge with data on other genomic aberrations, clinical correlates, prognosis and possible therapeutic implications.

2. Aims of the dissertation

The present study sought to investigate the role of DOCK2 and DOCK5 in AML and to put these findings into context with the epigenetic regulation and dysregulation in AML. Ultimately, we tried to evaluate the therapeutic potential of these pathways as innovative approaches in the treatment of AML. To analyze whether DOCK proteins and/or epigenetic pathways are viable therapeutic targets we aimed to answer the following questions:

1) What is the effect of pharmacological inhibition of DOCK2 and DOCK5 in AML cell lines?

2) What is the effect of a short hairpin RNA (shRNA) based DOCK2 and DOCK5 knockdown in AML cell lines and how do pharmacological and molecular biological inhibition of DOCK proteins correlate?

3) Does pharmacological epigenetic inhibition have antileukemic effects in vitro?

4) Does the combination of epigenetic inhibition and inhibition of DOCK proteins display synergistic effects in AML cell lines?

3. Materials and methods

3.1 Methods

All procedures involving the usage of cells were performed under sterile conditions using laminar flow hoods, suitable for working under S1 and S2 biosafety conditions.

3.1.1. Cell culture

General handling and conditions

All used AML cell lines were stored and cultivated in a CO2-Incubator at 37.5°C, 95 % humidity and 5 % O2. Cells were passaged 2 - 3 times a week. Depending on the growth behavior of the cell line, a portion of the cell suspension was replaced with new culture medium. The medium was previously warmed up in a 37°C water bath. Cells were cultivated in 25 - 75 cm^2 roux cell culture flasks, in 8 -15 ml of respective medium. To estimate growth density and viability of the cells qualitatively, before passaging them, they were assessed under the light microscope. To determine cell concentration and cell viability the trypan blue exclusion method was used. For this purpose, an automated cell viability analyzer was used (Vi-CELL™ XR Cell Viability Analyzer, Beckman Coulter).

Used cells lines were purchased either at the American Type Culture Collection (ATCC, USA) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Table 12 gives an overview of used cell lines and the respective cell media which were used to cultivate them. In case, the purchase dated back a longer period, the authenticity of the cell line was verified by the Multiplex human Cell line Authentication Test (Multiplexion GmbH, Heidelberg).

3.1.2. Molecular biological methods

3.1.2.1. Storing cells in RNAlater and RNA isolation

To store cells for later utilization (especially isolation of ribonucleic acid (RNA) for further experimental use), cells were suspended in RNAlater according to the manufacturers' instructions. For isolation of RNA, the innuPREP RNA Mini Kit was used according to the manufacturer’s instructions. RNA was either stored at - 80°C or immediately used to synthesize complementary desoxyribonucleic acid (cDNA) (see 3.1.2.2.). RNA concentration was detected using a NanoDrop™ spectrophotometer.

3.1.2.2. cDNA synthesis

1 µg of previously isolated (see 3.1.2.1.) RNA was used to synthesize cDNA with the PrimeScript™ RT Master Mix kit. The kit was used according to the manufacturer's instructions. Synthesized cDNA was diluted 1 : 5 in deionized diethylpyrocarbonate (DEPC)-treated water with a total volume of 100 µl.

3.1.2.3. Real time-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was used to measure the expression of DOCK2 and DOCK5 genes in DOCK2 and DOCK5 knockdown cells. Before performing RT-qPCR, RNA from target cells was isolated and reverse-transcribed to cDNA (see 3.1.2.2.). The device, used for analysis, was the Light Cycler96®. For preparation of reaction compounds, the SYBR® Premix Ex Taq™ II kit was used. The fluorescent dye used in this reaction was SYBR Green I, a DNA intercalating agent. Experiments were performed using 96-well PCR plates. All samples were measured in triplicates. Every experiment included a negative control (RNase free water) and a cDNA standard curve. cDNA for standard curves was synthesized from available AML cell lines and serial diluted 1 : 5, 1 : 50, 1 : 500 and 1 : 5000. See table 2 for the SYBR Green RT-qPCR protocol.

Table 2. Reaction protocol for SYBR Green RT-qPCR.

Step	Temperature	Duration	Number of Cycles
Preincubation	95°C	45 s	1
3 Step Amplification			40
• Step 1	95°C	5 s	
• Step 2	60°C	5 s	
• Step 3	72°C	26 s	
Melting			1
• Step 1	95°C	5 s	
• Step 2	65°C	60 s	
• Step 3	95°C	1 s	
Cooling	37°C	30 s	1

3.1.2.4. Calculation of relative gene expression from RT-qPCR experiments

To normalize gene expression of target genes, RT-qPCR had to be performed with a house keeping gene, first. In this case, glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) was used as a reference gene. For analysis, raw data from experiments was exported from the LightCycler® 96 SW 1.1 software to Microsoft Excel. To perform relative quantification of experimental raw data, the following mathematical model, introduced by Paffl, was applied:

$$ratio = \frac{(E_{target})^{\Delta CP_{target(control-sample)}}}{(E_{ref})^{\Delta CP_{ref(control-sample)}}}$$

E_{target} = real-time PCR efficiency of target gene transcript

E_{ref} = real time PCR efficiency of reference gene transcript

Δ**CP_{target}** = crossing point deviation of control − sample of the target gene transcript

Δ**CP_{ref}** = crossing point deviation of control − sample of the reference gene transcript

The Crossing Point (CP) is defined as the point at which measured fluorescence rises significantly above the background fluorescence (Pfaffl, 2001).

3.1.2.5. shRNA cloning and sequencing

shRNAs were purchased from Sigma Aldrich (St. Louis, USA). For each, DOCK2 and DOCK5, the manufacturer provided 5 shRNAs with verified target gene knockdown efficiency. Two shRNAs for DOCK2 and DOCK5, respectively were chosen for experiments, based on their knockdown capacity. Numeration of shRNAs is based on the numbering, they had initially received by the manufacturer. Transduction and knockdown efficiency of the initial 5 shRNAs were determined by cloning the plasmids into pLKO.1 vectors, transducing AML cell lines, and quantifying knockdown capacities through RT-qPCR (see section 3.1.2.3. and 3.1.2.4.). These experiments were carried out by fellow colleagues from the research group Wellbrock/Fiedler at the University Medical Center Hamburg-Eppendorf.

For shRNA based lentiviral transduction of AML cell lines, the shRNAs listed in Table 7 were cloned into a lentiviral gene ontology vector (LeGO) expressing the enhanced

Green Fluorescent Protein (eGFP) and a selection site for the antibiotic puromycin (see Figure 3 for an illustration of the LeGo G/puro vector). Cloning was performed by Dr. Frauke Fuchs from the lab group Wellbrock/Fiedler. To verify that re-cloned LeGO G/puro vectors contained the target plasmid with a scrambled/knockdown shRNA DNA-sequencing was performed. Sequencing was carried out by the company Eurofins Scientific (Hamburg). For alignment sequencing the Basic Local Alignment Search Tool (BLAST) was used. Ultimately, the following constructs were used for transfection and transduction of target AML cell lines:

hDOCK2 sh1-LeGO G/puro+

hDOCK2 sh2-LeGO G/puro+

hDOCK5 sh2-LeGO G/puro+

hDOCK5 sh5-LeGO G/puro+

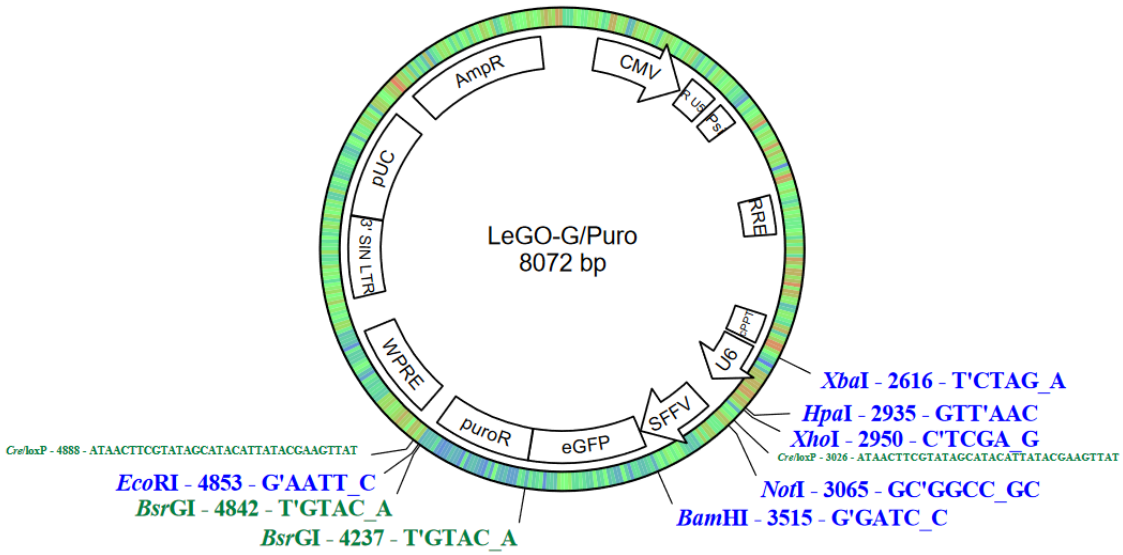


Figure 3. Lentiviral Gene Ontology Vector, LeGO-G/Puro with important sites (Weber et al., 2010).

3.1.3. Generation of DOCK2 and DOCK5 knockdown AML cell lines

To transduce AML cell lines with a lentiviral vector, the calcium phosphate co-precipitation technique was used (Pfeifer et al., 2000). The transduction protocol involved the following steps: (I) engineering of the recombinant virus carrying the transgene, (II) (II) amplification of recombinant viral particles in a packaging cell line, (III) purification

and titration of amplified viral particles, (IV) and subsequent transduction of AML cell lines. Procedures using virus or other potentially hazardous biological agents were performed under biosafety level (S2) conditions.

3.1.3.1. Transfection of lentiviral vectors

In a first step, recombinant lentiviruses were produced for which lentiviral systems of the 3rd generation were utilized. HEK 293T cells were used as a packaging cell line since they ensure high transfectability and support high levels of viral protein expression. The plasmids were mixed with 2 M CaCl2 (final concentration: 0.25 M) and HEPES-buffered saline transfection buffer (HBS buffer) to form calcium-phosphate-DNA-co-precipitates, which can be taken up by the target cells through endocytosis.

First, 5 x 10^6 HEK 293T cells were plated in four 10 ml Petri dishes. Later the same day, a DNA-$CaCl_2$ compound for transfection of the HEK293T cells was produced. For this purpose, 10 µl of the lentiviral insert plasmid, encoding the insert of interest (either scrambled control shRNA or DOCK2/DOCK5 knockdown shRNAs) were mixed with 10 µl of packaging plasmids, 2 µl + 5 µl of two different envelope plasmids, 437.5 µl distilled H_2O and 62.5 µl $CaCl_2$ (for detailed information regarding vectors/plasmids used for transfection, refer to Table 9). Then, 2 x 500 µl HBS were pipetted into reaction tubes. The DNA-$CaCl_2$ compound was then titrated dropwise into the HBS buffer while air was carefully insufflated using a serological pipette. The solution was incubated at room temperature for 10 - 20 min. Meanwhile, medium for the HEK 293T cells was replaced with a solution, containing 10 ml of fresh culture medium (DMEM + 10 % FBS) and 1 : 1000 diluted chloroquine. The DNA-$CaCl_2$-HBS solution was pipetted dropwise into the Petri dish containing the cells. The cells were incubated in a CO_2-incubator overnight. The next day, the virus-containing supernatant was harvested. The supernatant was filtered through a sterile syringe filter, aliquoted (8 x 1 ml) and stored at - 80°C.

3.1.3.2. Transduction of target AML cell lines

For transduction, 1 x 10^6 cells of the target cell line were seeded in triplicates in a 12-well culture plate in their respective culture medium, mixed with 10 mg/ml concentrated transfection reagent polybrene. Subsequently, every well was treated with 15 µl of virus. This volume was determined iteratively by repeating transduction with ascending virus concentrations up to 500 µl virus per well. 15 µl virus per well turned out to be the optimal volume for achieving the target transduction efficiency of 15 - 40 %. The culture plate was centrifuged for 1 h at 2000 rpm and room temperature. Afterwards,

the plate was incubated overnight at 37.5°C. On the second day, the plate was centrifuged at 300 g for 5 min and cells were resuspended in 2 ml of fresh culture medium. After verifying transduction efficiency (target efficiency ~ 15 - 40%) via flow cytometry (for further information regarding flowcytometric transduction validation, see section 3.1.4.) transduced cells were put under selection by adding puromycin (0.002 mg/ml) to the culture medium. On the 11^{th} day after transduction, transduction efficiency was measured again via flow cytometry and compared to transduction efficiency on the 4^{th} day. After keeping the cells under puromycin selection for at least one week, they could be handled at S1 biosafety level, instead of S2. After verifying efficiency of transduction and selection, 3 - 4 x 10^6 transduced cells were cryoconserved and another 4 x 10^6 cells were stored in RNAlater for subsequent RNA isolation, cDNA synthesis and in vitro assays.

3.1.4. Flow cytometry

Flow cytometry is a technology which offers the possibility of isolating subpopulations of cells of interest with high recovery and high degree of purity from heterogeneous cell mixtures, based on light scattering and fluorescent characteristics, like expression of eGFP on the cell surface (Picot et al., 2012). Through hydrodynamic focusing, individual cells of a cell suspension can be successively passed through the laser beam one after the other. The resulting scattered light or fluorescence signal is evaluated by a detector. A simultaneous analysis of the relative cell size (in forward scatter (FSC)), the granularity (in the side scatter (SSC)), as well as different fluorescence-stained cell antigens is possible.

Flow cytometry experiments were performed with the FACSCalibur™ and the FACSCanto™ flow cytometers. Software used for analyses were CellQuest™ Pro 5.2.1 and FlowJo X 10.0.7 r2. Before analyzing target cells in the flow cytometer, cell suspensions had to be prepared the following way: suspensions with 5 x 10^5 - 8 x 10^5 (depending on cell density) shRNA-transduced cells plus one wild type control were pipetted into tubes. Tubes were centrifuged at 300 g for 5 min. The resulting cell pellet was suspended in a 4 % paraformaldehyde solution (Formafix 4 %) and incubated at room temperature for 30 min. After incubation, the suspension was centrifuged and washed with PBS twice. During the last step, the pellet was resuspended in 500 µl PBS. In this study, flow cytometry was used to determine transduction efficiency in transduced cell lines and to select them for further handling. Successfully transduced cells expressed eGFP on their cell surface. During flowcytometric analysis, this specific fluorescence signal was measured.

3.1.5. In vitro assays

3.1.5.1. Proliferation assays and half maximal inhibitory concentration (IC_{50})

Proliferation assays were performed to evaluate growth behavior of cells and compare functional effects in differently treated cells (e.g., knockdown cells, cells treated with inhibitors) with a control. Depending on cell line-specific differences in their growth behavior, 1 x 10^5 to 5 x 10^5 cells in 500 - 1000 µl were seeded as triplicates in a 24-well culture plate. After 3 days of incubation at 37.5°C viable cell numbers were detected using an automated hemocytometer. Depending on the specific experiment, 200 µl of remaining cell suspension of each well was resuspended in 800 µl medium, re-plated as triplicates in a 24-well culture plate and viable cell counts were measured on day 3 and 6.

Proliferation assays with the small molecule inhibitors CPYPP/TBOPP and epigenetic inhibitors

Kasumi-1, Molm-13, UKE-1 and TF-1 wild type AML cell lines were mono-treated with the small molecule inhibitors CPYPP and TBOPP as well as the epigenetic inhibitors GSK126, GSKJ4, SGI-1027 and ZEN-3365. The cells were seeded according to the previously described protocol with a density of 3 x 10^5 cells/ml for Molm-13 and 5 x10^5 cells/ml for the other cell lines. Cell suspensions were treated with ascending concentrations of inhibitor compounds which were dissolved in DMSO. The concentrations ranged from 0.1 nM - 100 µM. The individual concentration range for each compound was determined using available data by the manufacturers and/or from the literature. As a control, each assay contained a DMSO-treated triplicate. Cells were counted on day 3, split, re-plated and counted on day 6, again.

Proliferation assays with DOCK2 and DOCK5 knockdown cells

For the assays, transgenic cell lines Kasumi-1, Molm-13, UKE-1 and TF-1 were used. The cell lines used for the assays contained one of the following constructs each:

Scrambled sh-LeGO G/puro

hDOCK2 sh1-LeGO G/puro+

hDOCK2 sh2-LeGO G/puro+

hDOCK5 sh2-LeGO G/puro+

hDOCK5 sh5-LeGO G/puro+

Molm-13 cells were seeded with a density of 3 x 10^5 cells/ml, the remaining cells were seeded with a density of 5 x 10^5 cells/ml. Cells were counted on day 3, and day 6 after plating. To uphold antibiotic selection of the transduced cell population, the cells were treated with 2 µg/ml puromycin, every day.

Calculation of IC_{50}s

The IC_{50} is the concentration of a drug required for 50% inhibition in a biological process. It is among the most widely used variables which is indicative of a drug's efficacy. The software AAT Bioquest® IC_{50} Calculator was used to calculate the IC_{50}s for each cell line and the respective inhibitor. A prerequisite for valid IC_{50}s is a wide range of used concentrations and therefore at least 8 different data points which can be used to calculate the IC_{50}. We accounted for this by covering a wide concentration range with each inhibitor and cell line as well as narrowing the concentration steps around the supposed IC_{50} (based on available information from the literature and by manufacturers).

3.1.5.2. Colony formation assays

Colony formation assays were performed to evaluate AML cell line's ability to form colonies and assess their functional integrity after in vitro manipulation (Wylie and Bowen, 2007). For this reason, a specific semi-solid, growth factor-carrying medium was used (MethoCult™ H4230) in which cells were cultivated.

3 cm diameter Petri dishes were filled with 1 ml cell suspension, each. Depending on the specific growth behavior of the AML cell line, the final cell concentration was 2,750 - 13,750 cells/ml. Each aliquot was equivalent to one reaction mixture and plated in triplicates. The small 3 cm Petri dishes were placed into a larger 10 cm square Petri dish. Petri dishes were cultivated at 37.5°C. Colonies were counted and their growth pattern was assessed under the light microscope, after 6 - 11 days.

3.1.5.3. Synergy assays and combination index (CI)

For synergy assays Molm-13 and UKE-1 cell lines, either transgenic or wild type, were used. The decision to continue with these cell lines was based on the more visible effects observed so far in these two cell lines compared to Kasumi-1 and TF-1.

Combination of DOCK knockdown cells and epigenetic inhibitor compounds

Molm-13 and UKE-1 cells, transduced with the two DOCK2/DOCK5 knockdown shRNAs and a scrambled control (see 3.1.5.1. for overview of used constructs), respectively, were used to perform proliferation assays with different concentrations of three epigenetic inhibitor compounds. The selection of inhibitor drugs was based on their presumed therapeutic potency which was derived from their IC_{50}s (see 4.5.). Transduced Molm-13 and UKE-1 cells were seeded in triplicates and treated with ascending concentrations of inhibitor. The concentrations for the inhibitors were selected based on their IC_{50}s. Each assay covered the IC_{50} and a concentration range of at least two decimal powers below and above the respective IC_{50} range. Approaches were seeded in a 24-well culture plate. Each well contained 500 µl volume with 1.5 x 10^5 cells per well. For each combination, 5 conditions were plated in triplicates including a DMSO control. 5 µl of inhibitor (or DMSO as control) plus 1.25 µl puromycin for selection were added to each well. Cells were counted once after 3 days since we observed no significant knowledge benefit in counting the cells a second time after 6 days. Resulting data was used to calculate IC_{50}s (through AAT Bioquest® IC_{50} Calculator) which were then compared to the respective IC_{50}s of AML wild type cells, acquired before.

Combination of small molecule inhibitors and epigenetic inhibitor compounds

For drug combination assays, Molm-13 and UKE-1 wild type cells were used to perform proliferation assays under combination treatment with a small molecule inhibitor and an epigenetic inhibitor compound, respectively. Again, the approaches were seeded in triplicates, using 8 different conditions (including DMSO control). The concentrations for small molecule inhibitor and epigenetic inhibitor were determined by using the ratio of $IC_{50[\text{small molecule inhibitor}]}$: $IC_{50[\text{epigenetic inhibitor}]}$ and sustaining this ratio along all concentration points. The selected concentrations covered the respective IC_{50} and at least two decimal powers below and above the respective IC_{50} range. Particular caution was applied with calculation of the necessary inhibitor concentrations since every inhibitor gets more diluted by pipetting it into the final cell suspension (in this case 1 : 200) which was accounted for by calculating the concentrations for inhibitor approaches, accordingly. Cells were counted only once, after 3 days, since we observed no significant knowledge benefit in counting the cells a second time, after 6 days.

Calculation of combination indexes (CI)

To analyze and quantify the effects of a combined treatment of AML cell lines with epigenetic inhibitors and small molecule inhibitors, we used the Chou-Talalay method to calculate CI values for each combination using the CompuSyn 1.0 software. The

concepts of CI and multi-drug-effects are based on the median-effect principle and the isobologram technique described by Chou (Chou, 2010; Chou and Talalay, 1984). Based on this theoretical foundation, the CompuSyn software can computerize proliferation assay data and translate it into a CI value for each combination. CI values are interpreted the following way: CI < 1: synergism, CI = 1: additive effect, CI > 1: antagonism. To help evaluate the quality of the data points, the r value is computerized as well, with an r value = 1 indicating perfect accuracy of the input data. For in vitro experiments, an r value of > 0.97 should be aimed at to ensure sufficient validity of the resulting CI value. Noteworthy is the fact, that synergy should not be mistaken with an enhancement of a particular drug efficacy. By definition, synergy is a reciprocal form of interaction between two compounds whereas enhancement is unilateral (Chou, 2018, 2010).

3.1.6. Statistical analysis

Data is presented as mean ± standard deviation (SD). Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test, two-tailed paired t-test or one-way repeated measures ANOVA with Geisser-Greenhouse correction, followed by Dunnett's test. P values < 0.05 were considered statistically significant. Levels of significance were defined as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.2. Materials

3.2.1. Equipment

The equipment used in this study is listed in Table 3.

Table 3. Equipment

Equipment	Model	Company
Camera	AxioCam MRc	Carl Zeiss (Oberkochen)
Cell counter	Vi-CELL™ XR Cell Viability Analyzer	Beckmann Coulter (Brea, USA)
Centrifuges	Centrifuge 5415 D Centrifuge 5424 Centrifuge 5804 D Centrifuge 5810 R	Eppendorf (Hamburg)
	Labofuge™ 400 R	Heraeus (Hanau)
	myFuge™ Mini Centrifuge	Benchmark Scientific (Sayreville, USA)
CO_2 Incubators	Labotect Incubator C200	Labotect (Göttingen)
	Forma Scientific 3165	Thermo Fisher Scientific (Waltham, USA)
Flow cytometer	FacsCalibur™ FacsCanto™	Becton Dickinson (Franklin Lakes, USA)
Freezing container	MrFrosty™	Thermo Fisher Scientific (Waltham, USA)
Inverted microscope	Axiovert 25	Zeiss (Jena)
Liquid nitrogen tank	Cryoplus™	Thermo Fisher Scientific (Waltham, USA)
Laminar flow hoods	Heraeus Laminair® HB 2448	Heraeus (Hanau)

	Heraeus HERAsafe® HS 9	
Micropipettes	Various volumes ranging from 2,5 µl to 1000 µl pipettes	Eppendorf (Hamburg)
Pipette Filler	pipetus® 100-240 V	Hirschmann Laborgeräte (Eberstadt)
RT-qPCR cycler	Light Cycler96®	Roche (Basel, Switzerland)
Spectrophotometer	NanoDrop™ ND-1000	Thermo Fisher Scientific (Waltham, USA)
Thermocycler	T1 Thermocycler	Analytik Jena (Jena)
Vortex mixer	VF-2	Janke & Kunkel/IKA (Staufen)
Water bath	Thermomix ME	B. Braun (Melsungen, DE)

3.2.2. Chemicals, agents and supplements

The chemicals, agents and supplements used in this study are listed in Table 4.

Table 4. Chemicals, agents and supplements

Chemicals, agents and supplements	Company
Bovine Serum Albumin Fraction V	Carl Roth (Karlsruhe)
Calciumchlorid ($CaCl_2$)	Sigma-Aldrich (St. Louis, USA)
Cloroquine	Sigma-Aldrich (St. Louis, USA)
Deionized, diethylpyrocarbonate (DEPC)-treated water	Thermo Fisher Scientific (Waltham, USA)
Dimethyl sulfoxid (DMSO)	Sigma-Aldrich (St. Louis, USA)
Fetal bovine serum (FBS)	Biochrom (Berlin)
Formafix 4%, buffered	Grimm med. Logistik GmbH (Torgelow)
Gibco™ Dulbecco's Phosphate buffered saline (DPBS), pH 7,4	Thermo Fisher Scientific (Waltham, USA)
Gibco™ Horse Serum (HS)	Thermo Fisher Scientific (Waltham, USA)

Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF); humane, recombinant	Peprotech (Hamburg)
HEPES-Buffered Saline (HBS), pH 7,4	Sigma-Aldrich (St. Louis, USA)
Hexadimethrine bromide (Polybrene)	Sigma-Aldrich (St. Louis, USA)
Hydrocortisone	Sigma-Aldrich (St. Louis, USA)
Phosphate-Buffered Saline (PBS)	Thermo Fisher Scientific (Waltham, USA)
Polybrene Transfection Reagent	Merck KGaA (Darmstadt, DE)
Puromycin dihydrochloride	Sigma-Aldrich (St. Louis, USA)
RNAlater RNA Stabilization Reagent	Qiagen (Venlo, Netherlands)

3.2.3. Kits and enzymes

The kits and enzymes used in this study are listed in Table 5.

Table 5. Kits and enzymes

Kits and enzymes	Company
innuPREP RNA Mini Kit	Analytik Jena (Jena)
PrimeScript™ RT Master Mix	Takara Bio (Shiga, Japan)
SYBR® Premix Ex Taq™ II	Takara Bio (Shiga, Japan)

3.2.4. Culture media

The culture media used in this study are listed in Table 6.

Table 6. Media

Media	Company
Gibco™ DMEM	Thermo Fisher Scientific (Waltham, USA)
Gibco™ IMDM	Thermo Fisher Scientific (Waltham, USA)
Gibco™ RPMI Medium 1640	Thermo Fisher Scientific (Waltham, USA)
Gibco™ Horse Serum, heat inactivated	Thermo Fisher Scientific (Waltham, USA)

MethoCult™ H4230	STEM CELL Technologies (Vancouver, Canada)

3.2.5. Nucleic acids

3.2.5.1. shRNAs

The shRNAs used in this study are listed in Table 7.

Table 7. shRNAs

shRNA	Source	Sequence (5' → 3')
Scrambled shRNA	#1864 Addgene (Watertown, MA, USA), subcloned	CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG
DOCK2 shRNA 1	TRCN0000010479 MISSION® shRNA, Sigma-Aldrich (Taufkirchen, DE)	CCGGCAAGGAAGTGACAGTTGAGAACTCGAGTTCTCAACTGTCACTTCCTTGTTTTTG
DOCK2 shRNA 2	TRCN0000010480 MISSION® shRNA, Sigma-Aldrich (Taufkirchen, DE)	CCGGCATACAGACAGATGTCCATCACTCGAGTGATGGACATCTGTCTGTATGTTTTTG
DOCK5 shRNA 2	TRCN0000113802 MISSION® shRNA, Sigma-Aldrich (Taufkirchen, DE)	CCGGCGAGTGCTCTACTTGAGATTTCTCGAGAAATCTCAAGTAGAGCACTCGTTTTTG
DOCK5 shRNA 5	TRCN0000113802 MISSION® shRNA, Sigma-Aldrich (Taufkirchen, DE)	CCGGCGAGTGCTCTACTTGAGATTTCTCGAGAAATCTCAAGTA

	GAGCACTCGTTTTT G

3.2.5.2. Primers

The primers used in this study are listed in Table 8.

Table 8. Primers

Primer	Sequence (5' → 3')
hDOCK2 f600	AGAAATGTCAAAAGACCAGCCA
hDOCK2 r759	TATGACCGTTTGCTTGTTGGG
hDOCK5 f274	ACGTCCACTCTGCGAGAATG
hDOCK5 r376	CGATCAGGCTGTACGTCATCT
hGAPDH f822	GTCAGTGGTGGACCTGACCT
hGAPDH r1066	TGCTGTAGCCAAATTCGTTG

3.2.5.3. Vectors and Plasmids

The vectors and plasmids used in this study are listed in Table 9.

Table 9. Vectors used for subcloning approaches and plasmids for lentiviral transfection

Vector/plasmid	Application	Source
LeGo G/puro$^+$ vector	Subcloning shRNA Reporter gene: eGFP Selection site: puromycin	Kindly provided by PD Dr. K. Riecken and Prof. Dr. B. Fehse, UKE (www.lentigo-vectors.de)
pCMV-VSV-G	Envelope plasmid for transfection	Addgene (Watertown, MA, USA)
pMDLg/pRRE	Packaging plasmid for transfection	Addgene (Watertown, MA, USA)

pRSV-Rev	Packaging plasmid for transfection	Addgene (Watertown, MA, USA)

3.2.6. Consumables

Consumables were purchased from the following companies: Beckman Coulter, Biorad, Braun, Corning, Dako, Eppendorf, Sarstedt, Whatman.

3.2.7. Inhibitors

The inhibitor compounds used in this study are listed in Table 10.

Table 10. Pharmacological inhibitors

Inhibitors	Molecular target	Company
CPYPP	Dedicator of cytokinesis (DOCK) 1/ 2/ 5	Kindly provided by Prof. Fukui (Division of Immunogenetics, University of Tokyo, Japan)
GSK126	Enhancer of zeste homolog 2 (EZH2)	Sigma-Aldrich (Taufkirchen, DE)
GSKJ4	KDM6A/KDM6B	Sigma-Aldrich (Taufkirchen, DE)
SGI-1027	DNA (cytosine-5)-methyltransferase 3A (DNMT3A)	Sigma-Aldrich (Taufkirchen, DE)
TBOPP	DOCK1	Kindly provided by Prof. Fukui (Division of Immunogenetics, University of Tokyo, Japan)
ZEN 3365	Bromodomain-containing protein 4 (BRD4)	Kindly provided by Zenith Epigenetics (San Francisco, USA)

3.2.8. Software

The software used in this study is listed in Table 11.

Table 11. Software

Software	Company
AAT Bioquest® IC_{50} Calculator	AAT Bioquest (Sunnyvale, USA)
CellQuest™ Pro 5.2.1	Becton Dickinson (Franklin Lakes, USA)
CompuSyn 1.0	ComboSyn (Paramus, USA)
FlowJo X 10.0.7 r2	Becton Dickinson (Franklin Lakes, USA)
GraphPad PRISM 7	GraphPad Software (La Jolla, USA)
LightCycler® 96 SW 1.1	Roche (Basel, Switzerland)
Microsoft Office 2016	Microsoft (Redmond, USA)
Nanodrop 2000	Thermo Fisher Scientific (Waltham, USA)
Zotero	Roy Rosenzweig Center for History and New Media, George Mason University (Washington DC, USA)

3.2.9. Cell lines

The cell lines and respective medium to cultivate them used in this study are listed in Table 12.

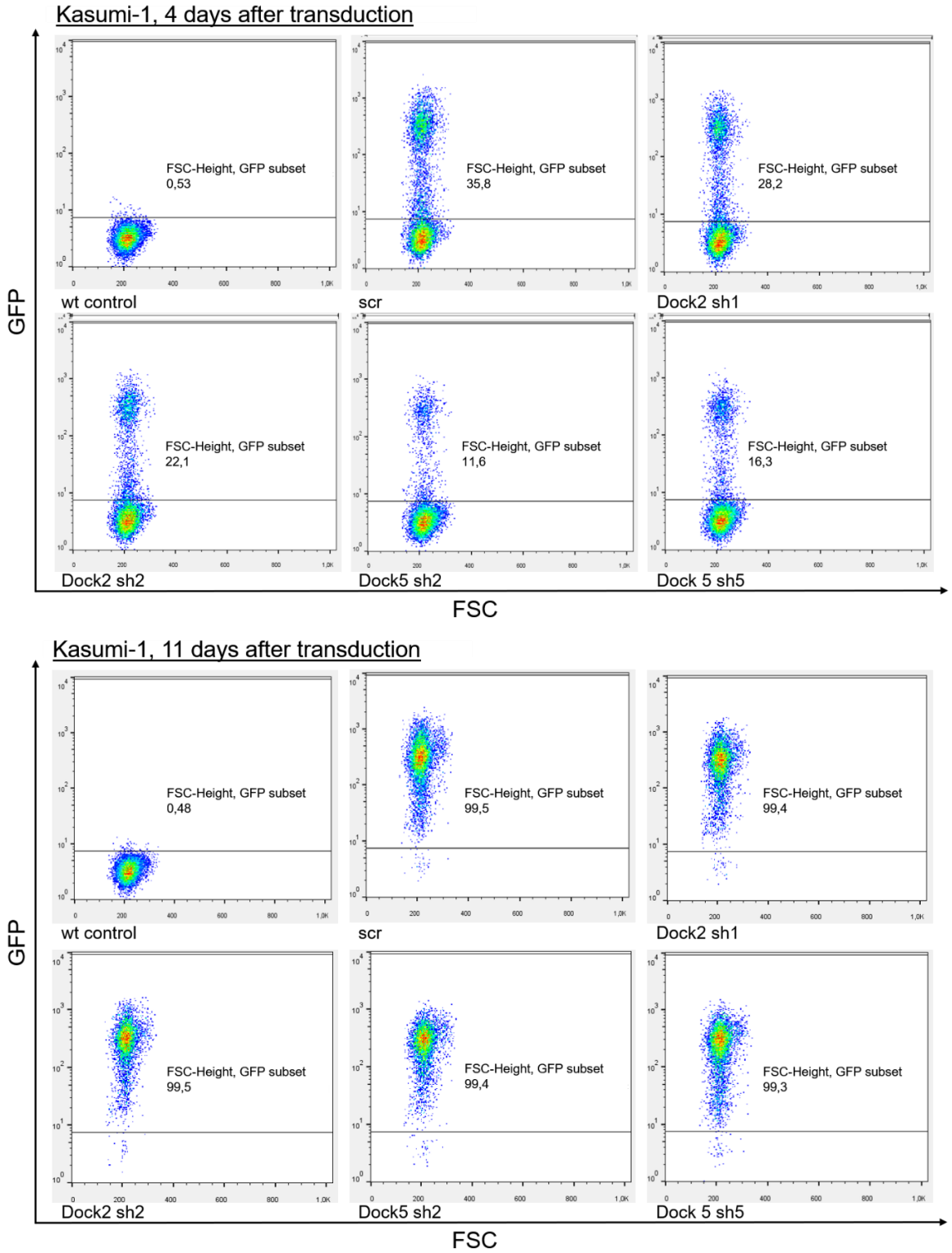
Table 12. Cell lines

AML cell line	Origin	Cell culture medium	Reference
HEK 293T	Human embryonic kidney	DMEM + 10 % FBS	Graham et al., 1977
Kasumi-1	Peripheral blood from a 7-year-old Japanese male with AML	RPMI 1640 + 20 % Fetal Bovine Serum (FBS)	Asou et al., 1991
Molm-13	Peripheral blood from a 20-year-old male with AML at relapse after initial MDS	RPMI 1640 + 10 % FBS	Matsuo et al., 1997
UKE-1	Patient with essential thrombo-cythemia, transformed into AML	IMDM + 10 % FBS + 10% Horse Serum + 1ml Hydrocortisone Sodium Succinate	Fiedler et al., 2000
TF-1	From bone marrow of a 35-year-old male with erythroleukemia	RMPI 1640 + 20 % FBS + 20µl GM-CSF	Kitamura et al., 1989

4. Results

4.1. Flowcytometric determination of transduction efficiency

To achieve a molecular downregulation of the genes DOCK2 and DOCK5, the AML cell lines Kasumi-1, Molm-13, TF-1 and UKE-1 were transduced with lentiviral vectors containing a scrambled control or encoding shRNAs directed against DOCK2 or DOCK5. Transduction efficiency was determined by flow cytometric analysis, 11 days after transduction. Whereas the first 4 days after transduction, no selection was carried out, the following 7 days an antibiotic selection (puromycin) was conducted. To differentiate successfully transduced cells from wildtype cells, used vectors contained GFP which allowed to sort $eGFP^+$ cells from $eGFP^-$ cells. Flow cytometry analyses showed that transduction efficiency after 4 days (without further selection of transduced cell populations) was relatively low (max. 30 % among living cells, see Figure 4). After one week of antibiotic selection and consecutive flow cytometric analysis, consistently high transduction rates of > 98 % could be achieved for all used cell lines. The gating strategy contained a live-dead gate to discriminate living from dead cells, following the sorting of GFP^+ cells. Figure 4 exemplarily shows flow cytometric determination of transduction efficiency at 4, respectively 11 days after transduction for Kasumi-1 cells.



GFP: Green Fluorescent Protein; FSC: Forward Scatter

Figure 4. Exemplary flow cytometric analysis of transduction efficiency in AML cell line Kasumi-1. Analyses were performed 4 days after transduction (**A**) as well as 11 days after transduction using an antibiotic selection (puromycin) (**B**). After gating for living cells, the percentage of eGFP$^+$ cells was determined. This number corresponds with successfully transduced viable cells. The results for AML cell line Kasumi-1 are displayed exemplarily. Similar results were achieved for cell lines Molm-13, TF-1 and UKE-1. wt = wild type; scr = scrambled, sh1/sh2/sh5 = shRNA 1/ 2/ 5.

4.2. DOCK2/DOCK5 knockdown verification through RT-qPCR

Knockdown capacity in all cell lines was determined 11 days after transduction (after 7 days of antibiotic selection, respectively) to ensure downregulation of DOCK2 and DOCK5 in the transduced cell lines. Additionally, knockdown capacity was determined repeatedly and at regular intervals throughout the period of cell cultivation for each cell line (further data is not shown in this dissertation). Before carrying out in vitro experiments with DOCK2 and DOCK5, knockdown consistency was verified. The relative messenger RNA (mRNA) expression for both genes was normalized to the housekeeping gene GAPDH which served as a control in transduced cells. Figure 5 A - C illustrates that a successful knockdown for DOCK2 and DOCK5 could be achieved for almost all cell lines and vectors. Exceptions from this include Kasumi-1 which does not express DOCK5 and is therefore not illustrated below as well as TF-1 which did not show a working DOCK5 knockdown with the second shRNA (relative expression ratio >1.0).

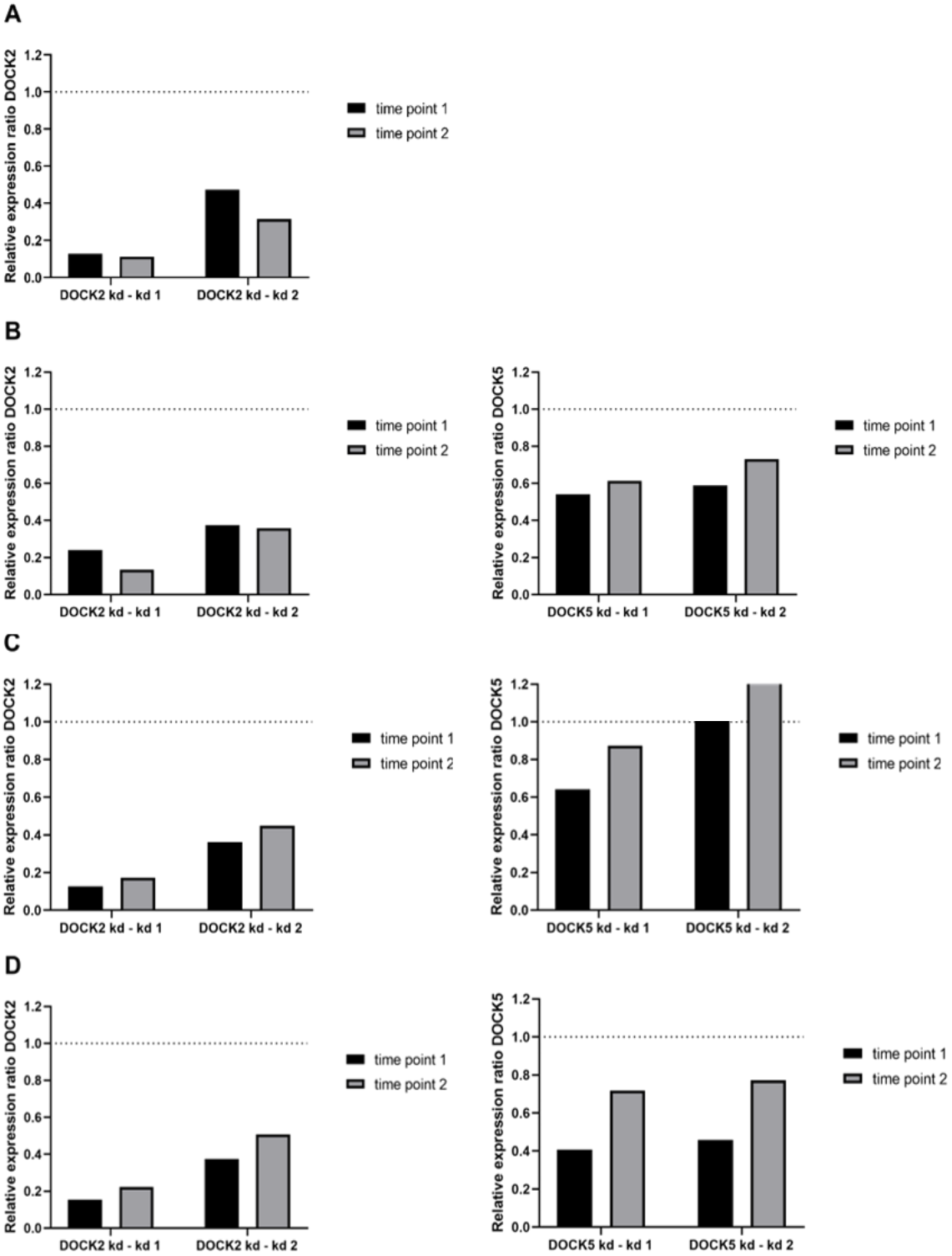


Figure 5. Verification of DOCK2 and DOCK5 knockdown capacity in transduced AML cell lines through RT-qPCR. Illustrated are the results for the AML cell lines **Kasumi-1 (A)**, **Molm-13 (B)**, **TF-1 (C)** and **UKE-1 (D)**. Knockdown (Kd) 1 and 2 refer to two different shRNAs. Time point 1: RT-qPCR was performed after 7 days of antibiotic selection and 11 days in total, after transduction. Time point 2: RT-qPCR was performed 6 weeks after transduction and regular antibiotic selection. Expression data of DOCK2 and DOCK5 are illustrated as a relative ratio to GAPDH expression. The results are normalized to the scrambled approach of the respective cell line (= black dotted line) which served as control.

4.3. The antiproliferative effect of DOCK2/DOCK5 knockdown in AML cell lines

One of the trademarks of hematopoietic cells is their high cell turnover which becomes even more evident in case of their possible malignant transformation. To evaluate the effect of a lentiviral shRNA knockdown of DOCK2 or DOCK5 on the proliferative capacity of AML cell lines, in vitro proliferation assays were performed. Kasumi-1, Molm-13, UKE-1 and TF-1 knockdown cells were seeded in triplicates in a 96-well plate, with a constant density of 100 000 cells per well and the number of viable cells was determined after 3 days and 6 days. Figure 6 shows the relative proliferation rate of transduced DOCK2 and DOCK5 knockdown cells after 3 days of incubation. The number of viable cells was normalized to the respective scrambled control and is displayed as mean ± SD of at least three independent experiments.

Overall, the DOCK2 and DOCK5 knockdown showed weak effects on the proliferation of the observed cell lines, compared to the control cells. Across all cell lines, DOCK2 knockdown showed to be the most effective regarding cells' proliferative abilities. However, even with noticeably reduced proliferation rates in cells with DOCK2 knockdown, the effect failed to be statistically significant in two out of four observed cell lines (Molm-13 and UKE-1). TF-1 and Kasumi-1 cells with DOCK2 knockdown showed a statistically significant reduction of proliferation ($p < 0.05$). DOCK5 knockdown was significantly less effective than DOCK2 knockdown, with mostly marginal inhibition of relative proliferation rates.

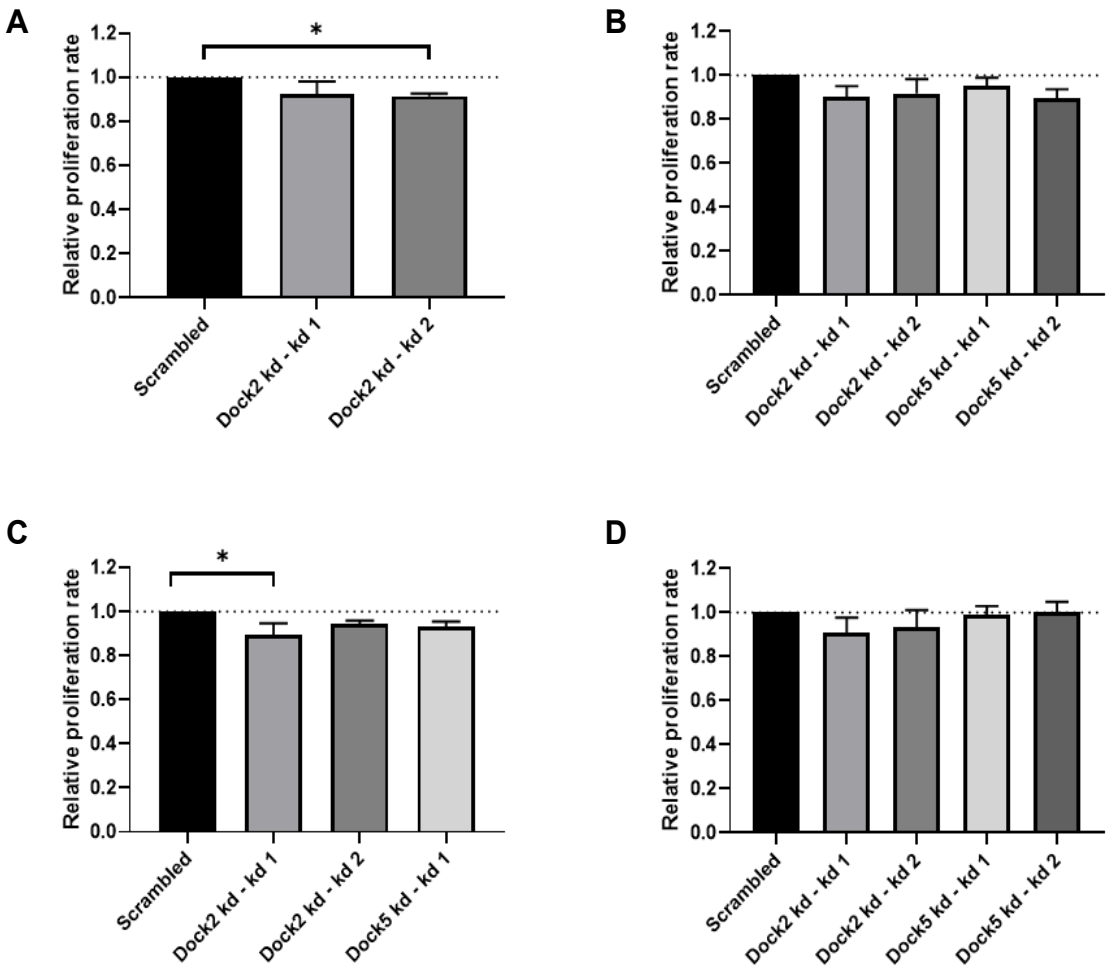


Figure 6. Proliferation assays with DOCK2 and DOCK5 knockdown in AML cell lines. Kasumi-1 (**A**), Molm-13 (**B**), TF-1 (**C**), UKE-1 (**D**). Results for AML cells after successfully establishing a lentiviral shRNA-knockdown of DOCK2/DOCK5 and seeding them with constant density. Knockdown (Kd) 1 and 2 refer to two different shRNAs. At 3 and 6 days after seeding, viable cells were counted using an automated cell viability analyzer. Presented data refers to the first time point (3 days after seeding). The numbers of viable cells are normalized to their respective scrambled control which is illustrated as black dotted line. Results are presented as mean ± SD (n = 3). Ordinary one-way ANOVA followed by Dunnett's multiple comparisons test was performed for statistical analysis. Kd = Knockdown. Levels of significance: ***** = $p < 0.05$.

4.4. Effect of a DOCK2 and DOCK5 knockdown on the clonogenicity of AML cell lines

Leukemic stem cells possess the ability to anchor in the bone marrow niche where they self-renew and produce molecular signaling factors so their daughter cells can differentiate and proliferate. To evaluate the effect of a DOCK2 and DOCK5 knockdown on cellular adhesion and colony formation, AML cell lines Kasumi-1, Molm-13, TF-1 and UKE-1 were seeded on a soft agar medium and incubated for 6 - 8 days. Subsequently, colony number and colony morphology were determined under an inverted microscope. Colony numbers were normalized to 100 using the respective scrambled control for each cell line. Results of colony formation assays are illustrated in Figure 7. Noteworthy is a relatively high standard deviation for DOCK5 knockdowns in Molm-13 and TF-1. To exclude the possibility of a dwindling knockdown strength as the reason for high standard deviations, every transduced cell line was tested for its knockdown efficiency through RT-qPCR after the first colony formation assay. Consecutive analyses showed no significant changes (compared to earlier knockdown verification experiments) regarding knockdown efficiency in each cell line.

DOCK2 knockdown resulted in a significant reduction ($p < 0.05$) of relative colony number in Kasumi-1, Molm-13 and TF-1 cells. Knockdowns in UKE-1 did not lead to a significant decrease in colony formation. DOCK2 knockdown in Molm-13 differentiated itself distinctly from the other transduced cell lines. Beyond the (semi-)quantitative difference in colony number, a qualitative difference in terms of colony morphology could be detected. Figure 47 A shows how DOCK2 knockdown correlated with a significantly reduced colony density, size and spatial dissemination of cells, compared to the respective scrambled control.

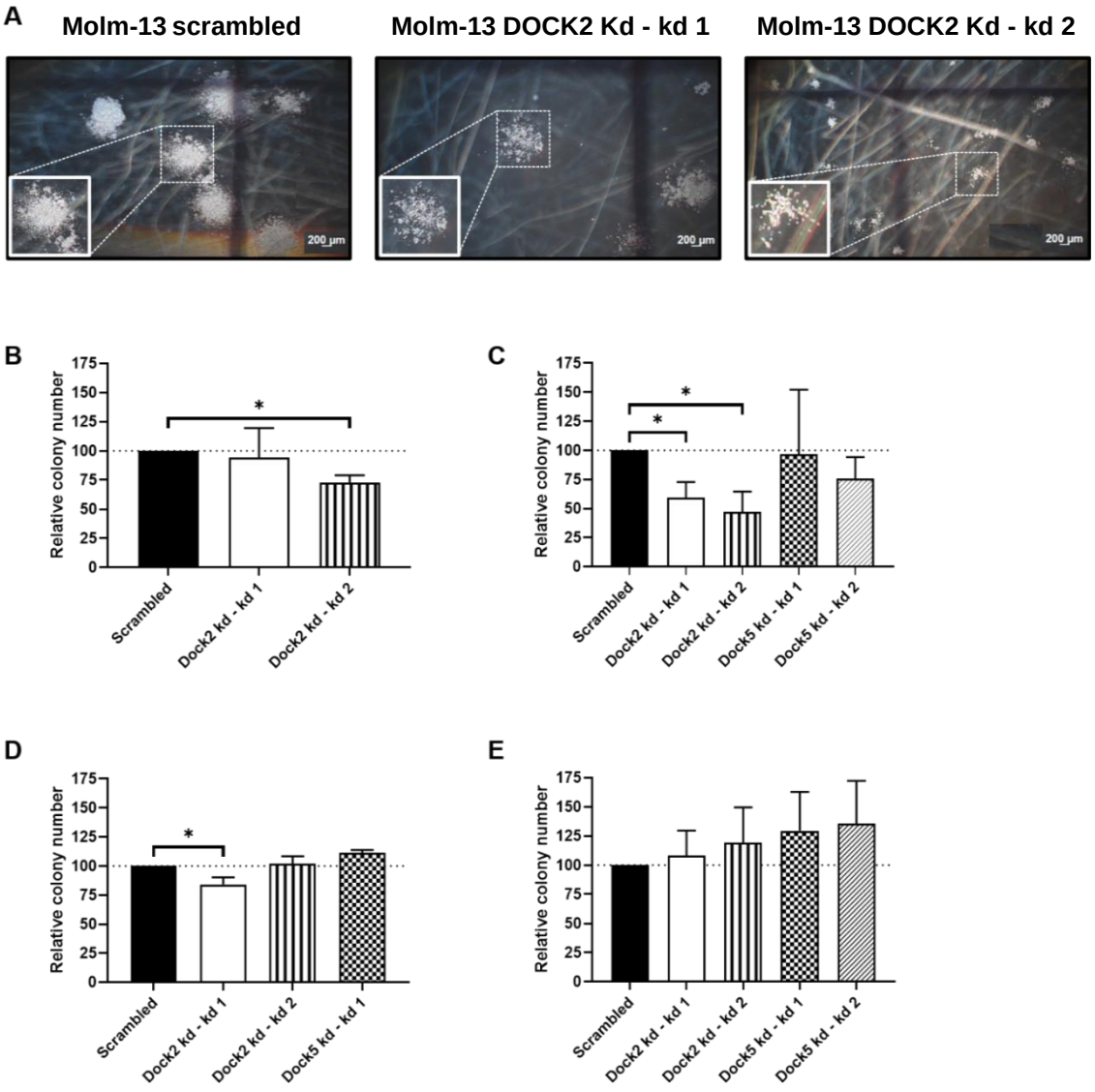


Figure 7. In vitro colony formation in DOCK2 and DOCK5 knockdown AML cell lines. (A) Representative light microscopic pictures of Molm-13 colonies, 6 days after seeding cells on a soft-agar medium. Illustrated is the comparison between Molm-13 scrambled control cells and two different DOCK2 shRNA knockdown approaches. Magnified detail is used to emphasize the differences in microscopic colony morphology and spatial expansion. Bright lines in the pictures are image artifacts corresponding to scratches on the agar plate surfaces. Column charts below show the relative number of colonies for **Kasumi-1 (B)**, **Molm-13 (C)**, **TF-1 (D)** and **UKE-1 (E)**. Colony numbers are normalized to their respective scrambled control (= black dotted line). Cells were counted through a light microscope 6 to 9 days after seeding them on a soft agar medium. Data is expressed as mean ± SD (n = 3). Statistical analysis included two-tailed paired t-test. Kd = Knockdown. Levels of significance: * = p <0.05.

4.5. The effect of pharmacological inhibition of DOCK proteins and epigenetic regulators on the proliferation of AML cells

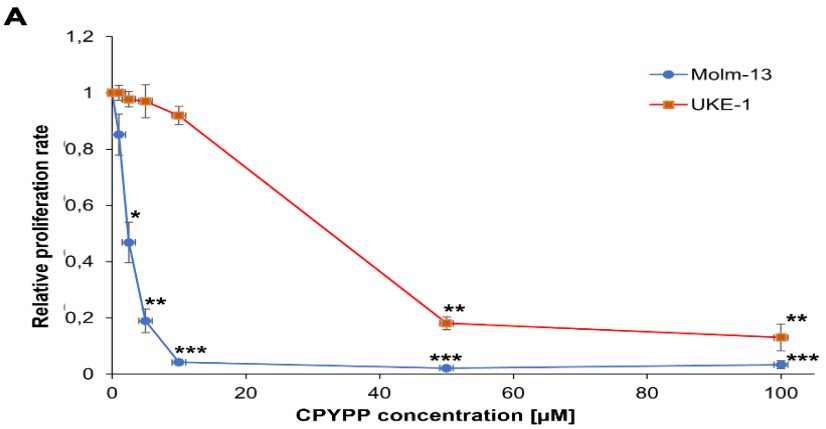
To investigate the role of DOCK2 and DOCK5 in the pathogenesis of AML, we transduced AML cell lines with a lentiviral vector containing a shRNA based DOCK2 and DOCK5 knockdown. Besides molecularly inactivating DOCK2/DOCK5 through a knockdown, we decided to investigate the effect of a pharmacological inhibition of the DOCK proteins. CPYPP is a small molecule inhibitor which acts by reversibly binding to the DHR-2 domain of DOCK2 (see 1.4.2.) and therefore inhibiting its catalytic activity for Rac. Within the DOCK family, CPYPP is not specific to DOCK2 but also shows cross-reactivity with DOCK5 as well as DOCK180 (Nishikimi et al., 2013). TBOPP is a selective DOCK1 inhibitor which does not impair DOCK2 and DOCK5 functionality levels (Tajiri et al., 2017). The molecular target of TBOPP is the catalytic DHR-2 domain. Both inhibitors were kindly provided by Professor Yoshinori Fukui (Kyushu University, Japan).

Further used substances target different epigenetic molecules and regulators including the EZH2 inhibitor GSK126, the KDM6A/KDM6B inhibitor GSKJ4, the DNMT3A inhibitor SGI-1027 and the BRD4 inhibitor ZEN 3365. These epigenetic modifiers are regularly mutated in genomic analyses of patients with leukemia, even though their exact role in the pathogenesis of hematological diseases needs to be further established.

To observe the antiproliferative effect of the inhibitor drugs, wild type AML cells (cell lines Molm-13 and UKE-1) were used to perform proliferation assays. Cells were seeded in triplicates and treated with ascending inhibitor concentrations. Viable cell count was conducted after 3 days of incubation. Results are normalized to the solvent control (DMSO) and presented as mean ± SD (n = 3). Subsequently, the IC_{50} was calculated using the Quest Graph™ IC50 Calculator by AAT Bioquest Inc.

Treatment with the DOCK2 inhibitor CPYPP led to a significantly decreased proliferation in both, Molm-13 and UKE-1, cell lines with a calculated IC_{50} of 2.5 µM (Molm-13) and 25 µM (UKE-1). GSK126 did show a statistically significant antiproliferative effect at high concentrations (IC_{50} Molm-13: 17.48 µM; UKE-1: 25 µM), though the overall effect of GSK126 is distinctly smaller, compared to CPYPP but is still considered statistically significant in both cell lines ($p < 0.05$). GSKJ4 led to a significant reduction of leukemic cell proliferation in Molm-13 and UKE-1 ($p < 0.01$). IC_{50} values for Molm-13 (7.64 µM) and UKE-1 (5.28 µM) reflect the potency of the antiproliferative effect of GSKJ4. SGI-1027 was very effective, particularly for Molm-13. Small doses of the DNMT3A inhibitor led to a highly significant reduction in cell proliferation (IC_{50} Molm-13: 414 nM; $p < 0.01$). Proliferation of UKE-1 cells was significantly reduced by SGI-1027 (IC_{50}: 7.7 µM; $p < 0.01$)., although the effect in Molm-13 cells was evidently stronger. The selective

DOCK-1 inhibitor TBOPP was more effective in Molm-13 (IC_{50}: 11.32 µM; $p < 0.01$) than in UKE-1 (IC_{50}: 43.23 µM; $p < 0.01$). This is surprising, considering that earlier findings by our research group through expression analyses indicate that Molm-13 is not expressing DOCK1, while UKE-1 does (Fuchs, 2020). The inhibitor of the transcription factor BRD4 – ZEN 3365 – proved to be the strongest antiproliferative agent among the observed ones. Treatment with 2.5 µM ZEN 3365 was sufficient to reduce the proliferation rate by approximately 70 %. The calculated IC_{50} for Molm-13 of 713.74 nM ($p < 0.001$) indicates a high therapeutic effectiveness and it led to a significant inhibition of cell proliferation rate which was, to a smaller extent, reproduceable in UKE-1 as well (IC_{50}: 2.63 µM; $p < 0.001$). Figure 8 aggregates and visualizes the acquired data.


A
Relative proliferation rate
1,2
1
0,8
0,6
0,4
0,2
0
0
20
40
60
80
100
CPYPP concentration [µM]
Molm-13
UKE-1
*
**
***
**
***
**
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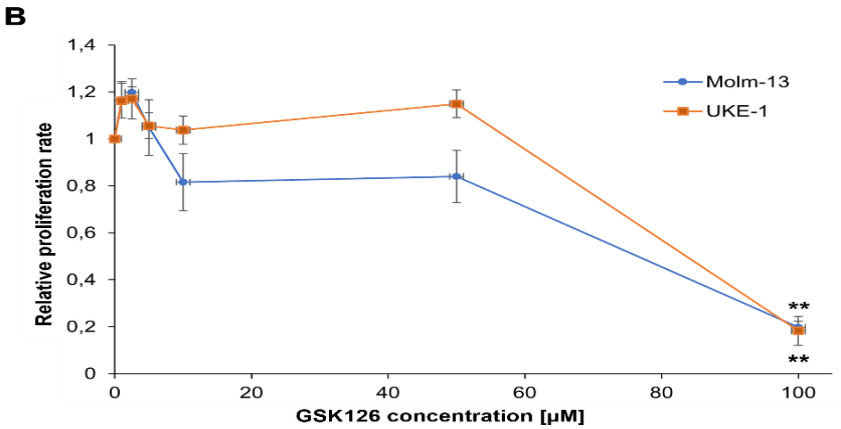
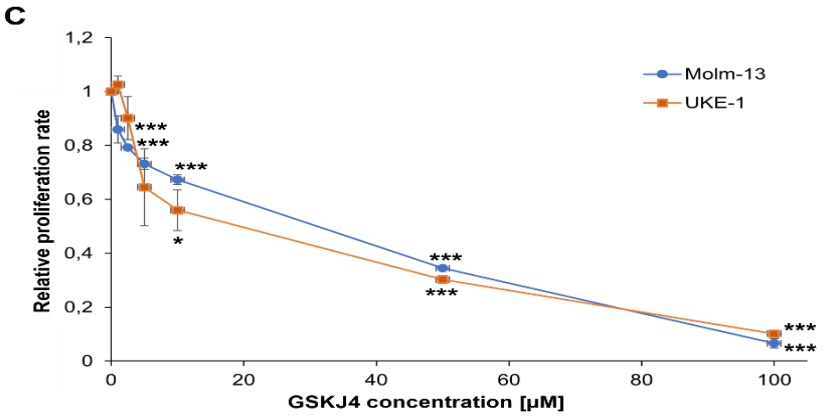

B
Relative proliferation rate
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
20
40
60
80
100
GSK126 concentration [µM]
Molm-13
UKE-1
**
**


C
Relative proliferation rate
1,2
1
0,8
0,6
0,4
0,2
0
0
20
40
60
80
100
GSKJ4 concentration [µM]
Molm-13
UKE-1
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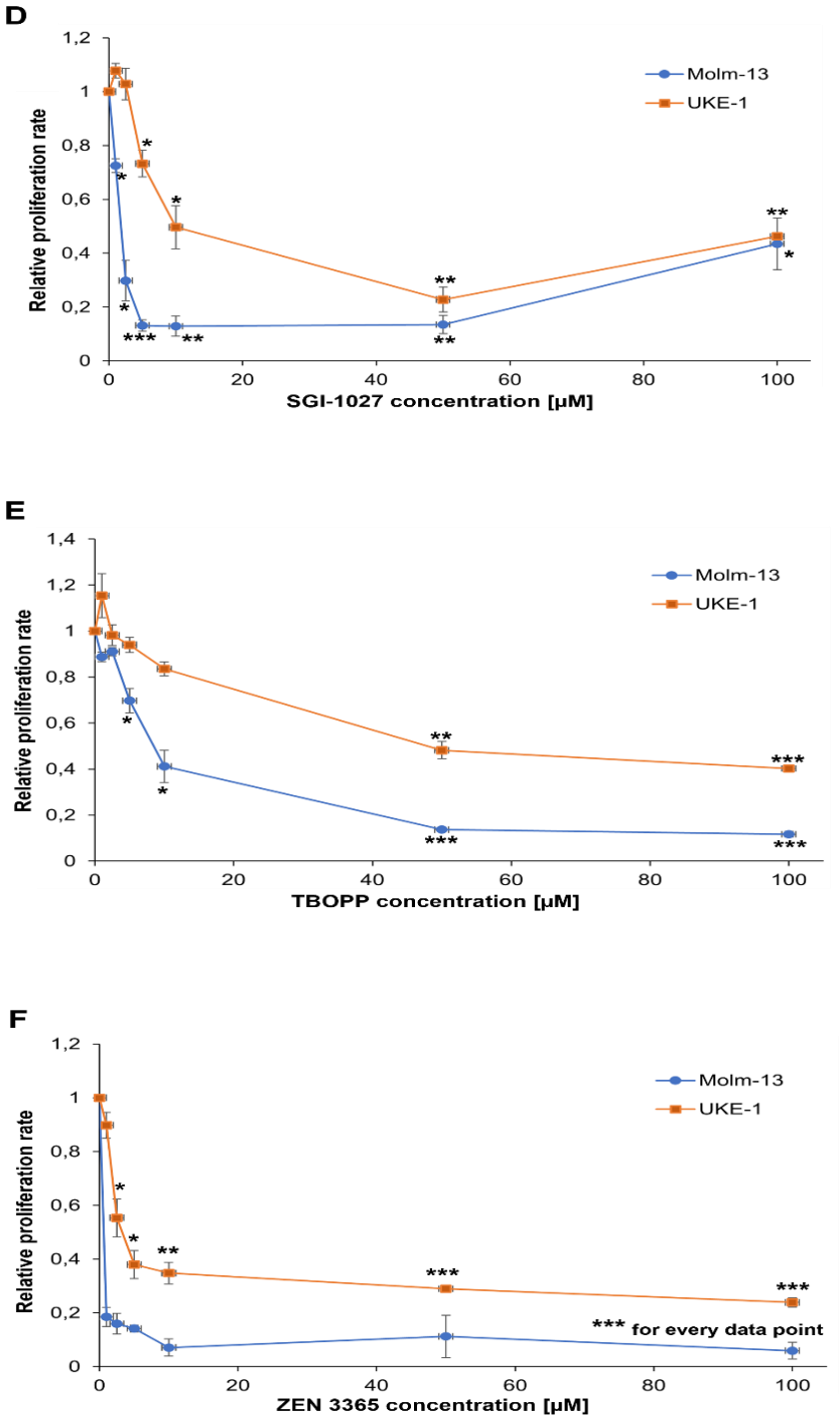


Figure 8. Proliferation of AML wild type cell lines Molm-13 and UKE-1 under treatment with different inhibitor drugs. (A): CPYPP, (B): GSK126, (C): GSKJ4, (D): SGI-1027, (E): TBOPP, (F): ZEN 3365. The relative proliferation of Molm-13 and UKE-1 under treatment with ascending concentrations of different inhibitors is normalized to the solvent control (DMSO). For reasons of clarity, proliferation data of very low concentrations (in the nanomolar range) is not illustrated in the above figure. The present data was used to calculate the respective IC_{50}s. Results are presented as mean ± SD (n = 3). Statistical analyses were performed as one-way repeated measures ANOVA with Geisser-Greenhouse correction, followed by Dunnett's test. Levels of significance: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

4.6. Combination of lentiviral shRNA based DOCK2/DOCK5 knockdown with epigenetic inhibitor compounds

Molm-13 cell line

Recent findings indicate that epigenetic changes and mutations of epigenetic regulator proteins play a role in the pathogenesis of leukemia and other hematological malignancies. This includes genes that are well-known for being recurrently mutated such as DNMT3A, as well as genes recently implicated in leukemogenesis such as EZH2 (Bullinger et al., 2017). Since there is a high level of interconnectivity on the molecular signaling level, we sought to investigate the effect of a combined inhibition of epigenetic regulator proteins with DOCK2 and DOCK5 on the proliferative abilities of AML cells. The goal was to evaluate whether a DOCK2 or DOCK5 knockdown amplifies the therapeutic potency of epigenetic inhibitors towards AML cells.

Data from our preceding experiments (proliferation assays and expression analyses through RT-qPCR) allowed us to select the most effective shRNAs for DOCK2 and DOCK5 knockdown, respectively. Molm-13 and UKE-1 cells, transduced with the according shRNAs, were then used to perform proliferation assays with different concentrations of three inhibitor drugs. The selection of inhibitor drugs was based on their presumed therapeutic potency which was derived from their IC_{50}s (see 4.5.). Transduced Molm-13 and UKE-1 cells were seeded in triplicates and treated with ascending concentrations of inhibitor. Concentrations were determined, based on the previously calculated IC_{50}s of the used inhibitors. Viable cell count was conducted after 3 days of incubation. Resulting data is presented as mean (n = 3) ± SD.

Figure 9 shows the results of proliferation assays of DOCK2/DOCK5 knockdown transduced Molm-13 cells in combination with GSKJ4, SGI-1027 and ZEN 3365. Statistical analysis was performed as two-way ANOVA with Geisser-Greenhouse correction followed by Dunnett's. It was used to compare the relative proliferation rates of the scrambled approaches to the corresponding approaches with DOCK2 and DOCK5 knockdowns cells. The differences in proliferation rates between scrambled and DOCK2 as well as DOCK5 knockdowns cells were statistically not significant across all inhibitors and all approaches. Table 13 shows the calculated IC_{50}s for the combinations of transduced Molm-13 cells and the different inhibitors. The differences between the IC_{50}s of scrambled : knockdown cells are not significant as well and, overall, do not give any indication of a significantly higher sensitivity of the cells for the inhibitors through combination with a DOCK2 or DOCK5 knockdown. Interestingly, the IC_{50}s for Molm-13 scrambled diverge from respective IC_{50}s in Molm-13 wild type cells (see 4.5.).

Since the scrambled vector acts as a control with no intrinsic knockdown activity, it should not alter the functionality of DOCK (or any other protein). Before starting proliferation assays, all approaches were tested for knockdown efficiency through RT-qPCR (data not shown). This way, the possibility of an aberrated DOCK2 or DOCK5 activity through the scrambled vector was excluded. Thus, a definite explanation for the discrepancies between the IC_{50} values for scrambled vs. wild type cells cannot be provided with the available information.

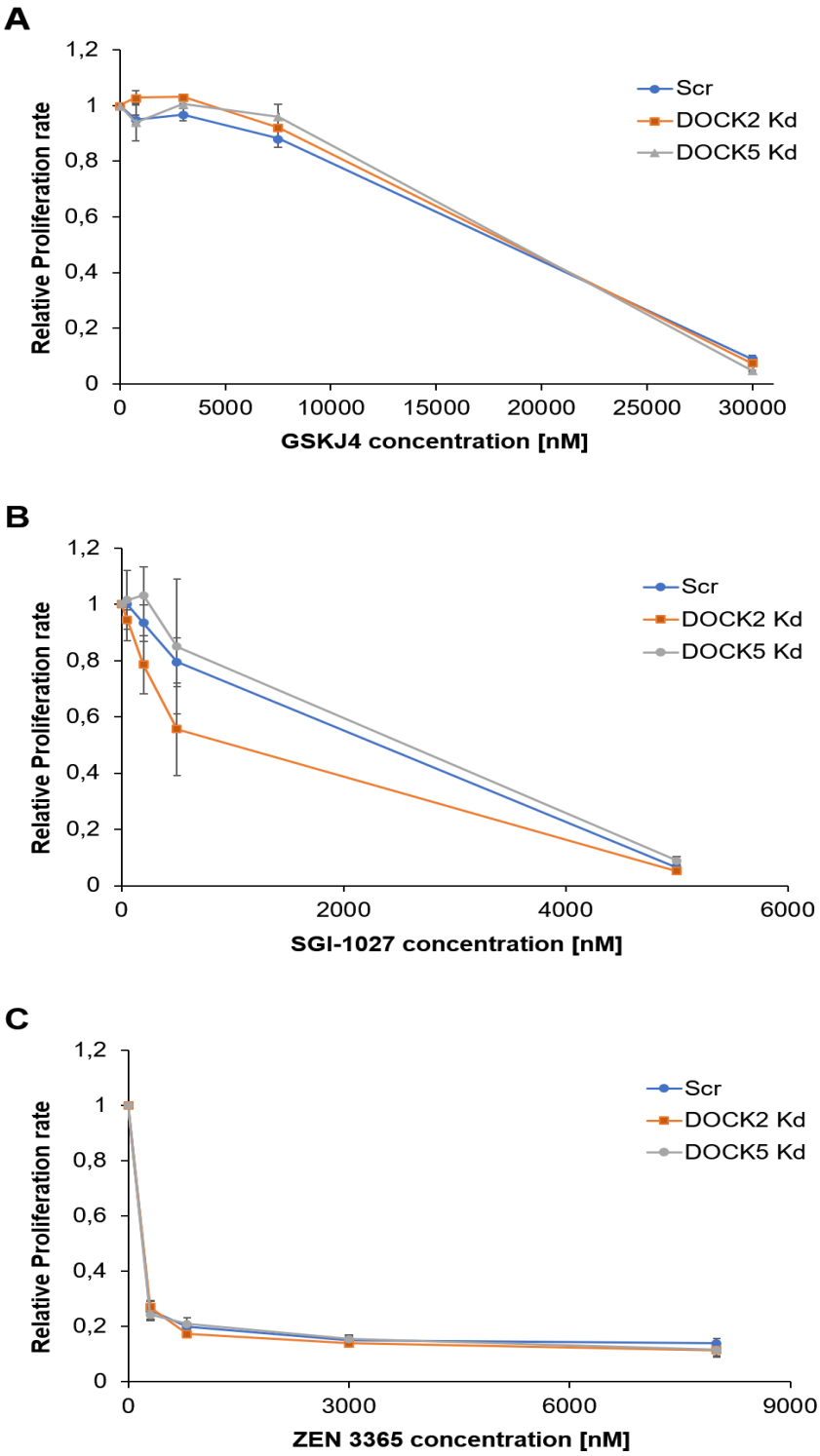


Figure 9. Effect of the combination of a DOCK2/DOCK5 knockdown in AML wild type cell line Molm-13 and treatment with epigenetic inhibitor drugs. (A) KDM6A/KDM6B inhibitor GSKJ4. (B) DNMT3A inhibitor SGI-1027. (C) BRD4 inhibitor ZEN 3365. Cells were seeded with a defined density and treated with different concentrations of inhibitors. Viable cells were counted after 3 days and results are normalized to the respective solvent control. Data is presented as mean ± SD (n =3). One-way repeated measures ANOVA with Geisser-Greenhouse correction and Dunnett's test were performed for statistical analysis. Scr = scrambled; Kd = knockdown.

Table 13. IC50s of different epigenetic inhibitor agents for transduced Molm-13 cells.

Inhibitor	IC_{50} scrambled	IC_{50} DOCK2 Kd	IC_{50} DOCK5 Kd
GSKJ4	13.02 µM	9.10 µM	15.54 µM
SGI-1027	1078 nM	460 nM	873 nM
ZEN 3365	12 nM	4 nM	5 nM

Data for calculation of IC_{50} values was obtained from proliferation assays (see 4.5.) from at least three independent experiments. The IC_{50} calculator by AAT Bioquest was used for calculation.

UKE-1 cell line

To further elucidate the role of epigenetic modifiers in combination with DOCK2 and DOCK5, transduced UKE-1 DOCK2/DOCK5 knockdown cells were used to perform proliferation assays. The experiments followed the same setup as assays with transduced Molm-13 cells. The used inhibitor concentrations were reiterated by orientating towards the respective IC_{50}s for UKE-1 wild type cells. Figure 10 illustrates the results. The data indicates that especially DOCK2 knockdown led to a higher antiproliferative potency of epigenetic inhibitor agents which translates into a lower proliferation rate across all DOCK2 knockdown approaches, as well as decreased IC_{50} values for every inhibitor (Table 14). However, the differences are statistically not significant. Across all inhibitors DOCK5 knockdown showed almost no difference compared to the scrambled approaches and, overall, did not noticeably reduce proliferation rates or IC_{50}s.

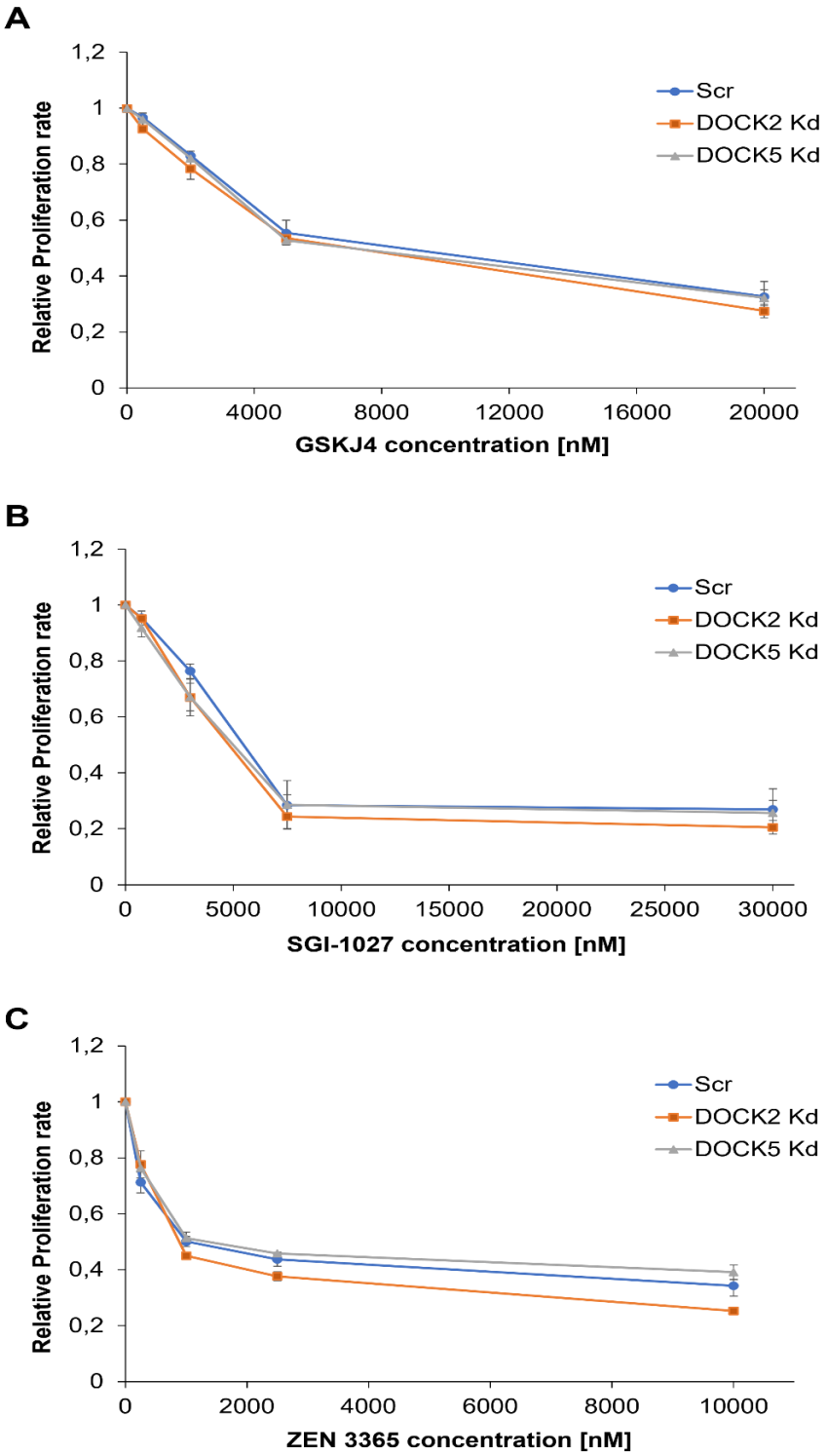


Figure 10. Effect of the combination of a DOCK2/DOCK5 knockdown in AML cell line UKE-1 and treatment with epigenetic inhibitor drugs. (A) KDMA6A/KDM6B inhibitor GSKJ4. **(B)** DNMT3A inhibitor SGI-1027**. (C)** BRD4 inhibitor ZEN 3365**.** Cells were seeded with a defined density and treated with different concentrations of inhibitors. Viable cells were counted after 3 days, and results are normalized to the respective solvent control. Data is presented as mean ± SD (n =3). One-way repeated measures ANOVA with Geisser-Greenhouse correction and Dunnett's test were performed for statistical analysis. Scr = scrambled; Kd = knockdown.

Table 14. IC_{50}s of different epigenetic inhibitor agents for transduced UKE-1 cells

Inhibitor	IC_{50} scrambled	IC_{50} DOCK2 Kd	IC_{50} DOCK5 Kd
GSKJ4	7,66 µM	6,51 µM	7,12 µM
SGI-1027	5,50 µM	3,97 µM	4,78 µM
ZEN 3365	1,47 µM	1,10 µM	1,36 µM

Data for calculation of IC_{50} values was obtained from proliferation assays (see 4.5.) from at least three independent experiments. The IC_{50} calculator by AAT Bioquest was used for calculation.

4.7. Drug combination of small molecule inhibitors CPYPP and TBOPP with epigenetic inhibitor agents

After combining transduced DOCK2 and DOCK5 knockdown cells with epigenetic inhibitors, we sought to investigate the effect of combinatorial pharmacological inhibition of DOCK2 and DOCK5 and epigenetic inhibitors. Molm-13 and UKE-1 wild type cells were seeded with a defined density and treated with a combination of either the DOCK A inhibitor CPYPP or the DOCK1 inhibitor TBOPP (= small molecule inhibitors) plus one of three epigenetic inhibitors. The number of viable cells was determined after three days of incubation. To determine the concentrations for synergy assays, IC_{50}s of small molecule and epigenetic inhibitors were used as an orientation mark. I.e., it was ensured that the used concentration range included the IC_{50} of both inhibitors with sufficient tolerance margins. Secondly, the initial ratio of $IC_{50[\text{small molecule inhibitor}]}$: $IC_{50[\text{epigenetic inhibitor}]}$ for the respective wild type cell was used to establish constant concentration ratios for the synergy assays. Table 15 (Molm-13 wild type cells) and Table 16 (UKE-1 wild type cells) give an overview of drug combinations and corresponding combination indexes. Calculations were carried out by using the Chou and Talalay method and the corresponding software CompuSyn Version 1.0. CI values are illustrated at the respective ED_{50} (“median effect dose”) and ED_{95}. CI values at high doses – thus high EDs – are particularly important when observing combination assays with antiproliferative/anticancer agents. Therefore, CI values <1 at the ED_{95} level have a wider range of therapeutic implications for cancer therapies. The linear correlation coefficient (“r value”) was calculated for every combination experiment to ensure the conformity of the data with principles of the mass action law (Chou, 2018). All drug combinations have an r value of 0.9 - 0.98 (data not shown) with r = 1 indicating perfect accuracy of the data.

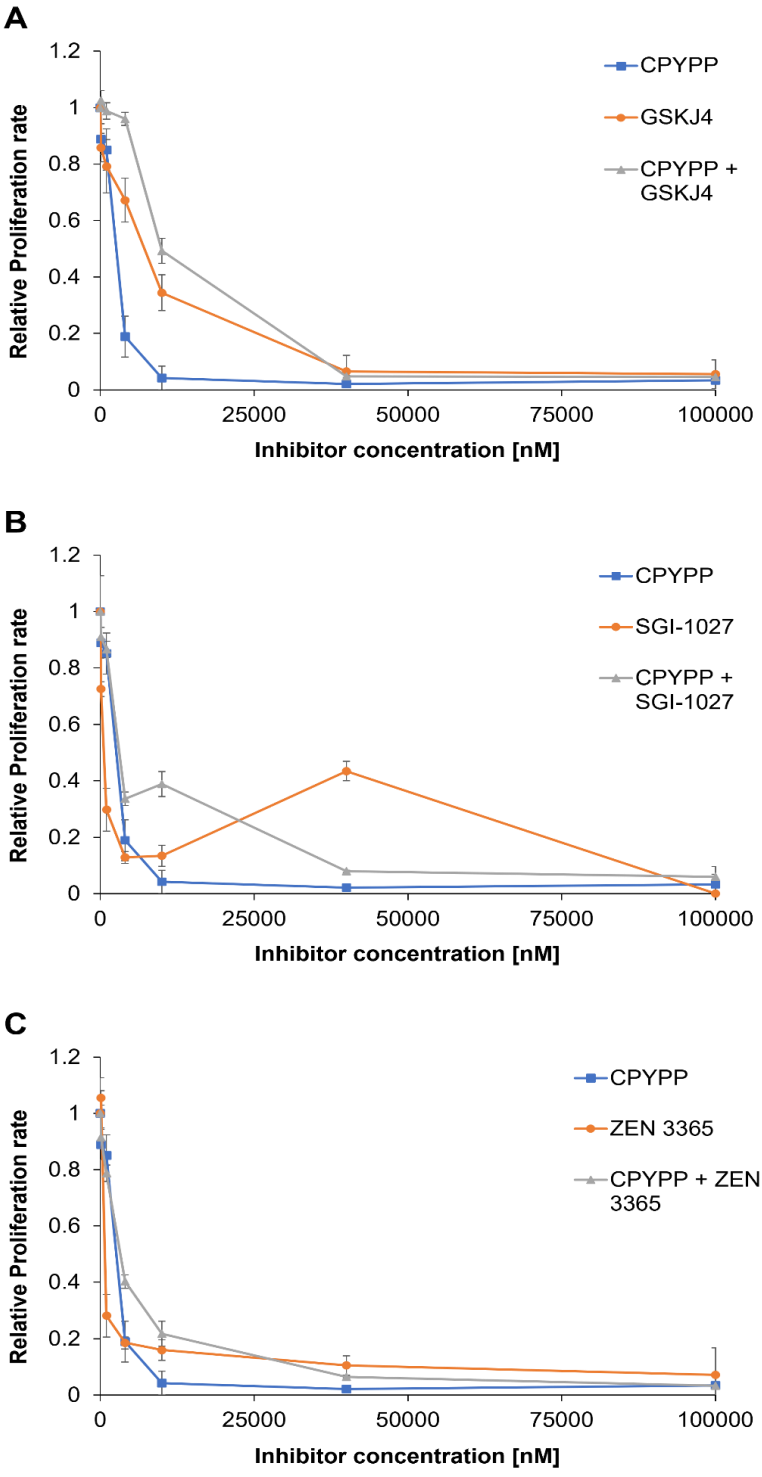
Molm-13 cell line

Molm-13 wild type cells were used to perform synergy assays according to the experimental setup described above. Table 15 shows the results of the drug combinations. To calculate the CI values, Molm-13 wild type cells were treated with combinations of a small molecule inhibitor and an epigenetic inhibitor. Underlying data represent the mean of at least three independent experiments performed in triplicates. Figure 11 illustrates the dose-effect relationships between Molm-13 cells, small molecule inhibitors and epigenetic inhibitors. To visualize diverging drug efficacy levels for monotherapy and combination therapy, every graph contains dedicated differentiation between proliferation data at individual inhibitor application as well as data from their respective combination.

Table 15. Combination index (CI) values for Molm-13 wild type cells at ED_{50} and ED_{95}.

Drug combination	Drug ratio	CI at ED_{50}	CI at ED_{95}
CPYPP + GSKJ4	1 : 3	4.92	1.64
CPYPP + SGI-1027	5 : 1	0.79	1.39
CPYPP + ZEN 3365	3.125 :1	0.60	0.98
TBOPP + GSKJ4	1.3 : 1	0.86	0.11
TBOPP + SGI-1027	20 : 1	0.79	0.18
TBOPP + ZEN 3365	13.3 : 1	0.14	0.28

AML cells were treated with ascending concentrations of inhibitor while adhering to the specific and fixed drug concentration ratio. CI values are indicated for the respective 50 % effective dose (ED_{50}) and the 95 % effective dose (ED_{95}). Underlying data represents the mean of at least three independent experiments performed in triplicates. CI < 1: synergism; CI = 1: additive effect; CI > 1: antagonism.


A
Relative Proliferation rate
1.2
1
0.8
0.6
0.4
0.2
0
0
25000
50000
75000
100000
Inhibitor concentration [nM]
CPYPP
GSKJ4
CPYPP + GSKJ4
B
Relative Proliferation rate
Inhibitor concentration [nM]
CPYPP
SGI-1027
CPYPP + SGI-1027
C
Relative Proliferation rate
Inhibitor concentration [nM]
CPYPP
ZEN 3365
CPYPP + ZEN 3365

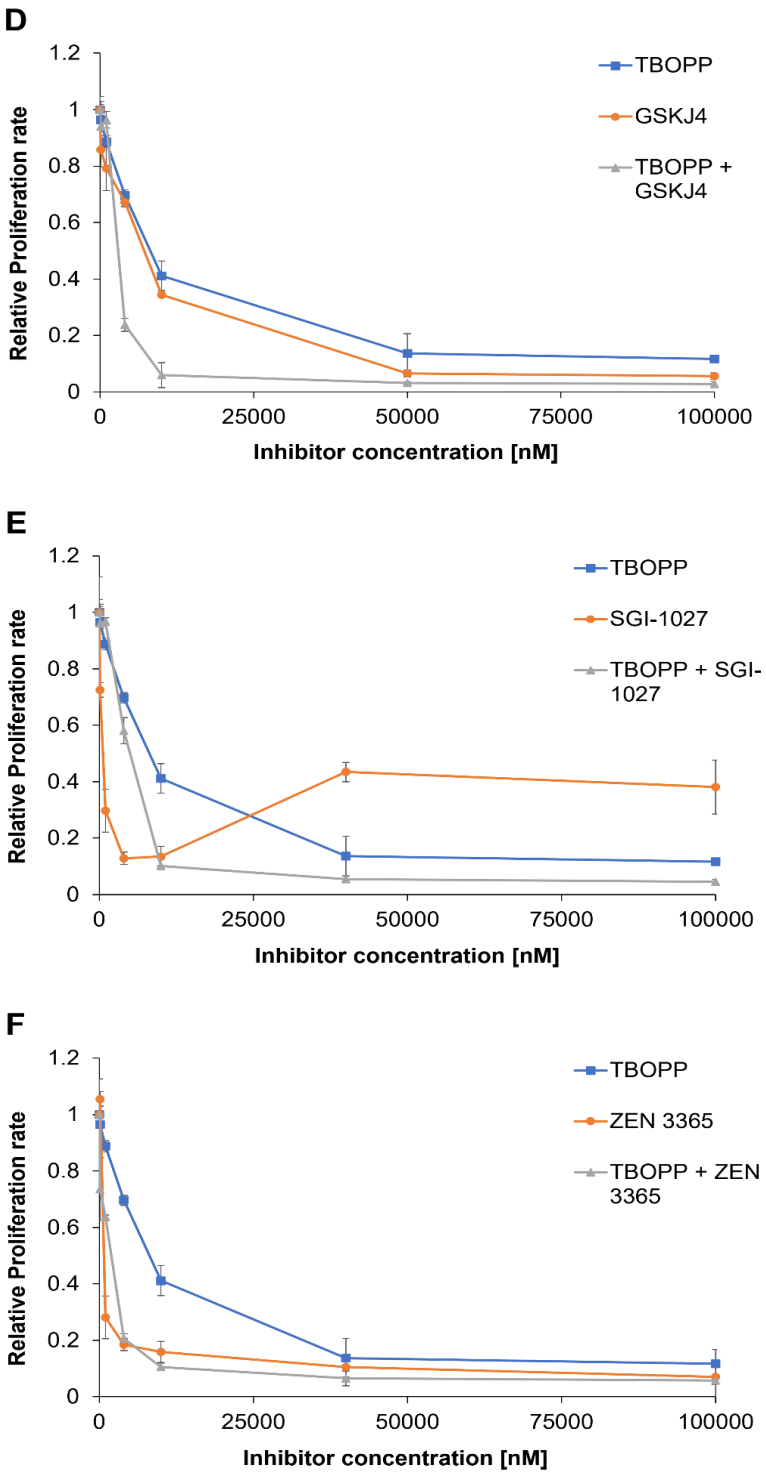


Figure 11. Proliferation data of Molm-13 wild type cells drug combination assays. Small molecule inhibitors CPYPP (**A - C**), TBOPP (**D - F**) and epigenetic inhibitor agents GSKJ4, SGI-1027 and ZEN 3365 were used. Data compares different treatment approaches using monotherapy with a small molecule inhibitor (A - C: CPYPP; D - F: TBOPP), monotherapy with one of three different epigenetic inhibitors and their respective drug combinations**.** For reasons of clarity, CI values are not illustrated decidedly. Corresponding CI values at different effective dose (ED) levels are shown in Table 15. Data represents mean ± SD (n ≥ 3). Viable cell count was conducted after three days.

The Combination of CPYPP + GSKJ4 showed an antagonistic effect at ED_{50} and ED_{95}. CPYPP + SGI-1027 showed a synergistic effect at ED_{50} but was antagonistic at higher doses. Inferior data does not seem to be the reason for this phenomenon since underlying data was coherent without outliers which could falsify results (r value = 0.98). The other drug combinations showed synergistic effects at both effective dose levels. Especially the strong synergisms at ED_{95} in TBOPP combinations are surprising. TBOPP is a specific inhibitor of DOCK1 which is not expressed by wild type Molm-13 cells. Taking the significant antiproliferative effect of TBOPP on Molm-13 cells into account, there is probably a DOCK1- independent or cross-reactive mechanism involved.

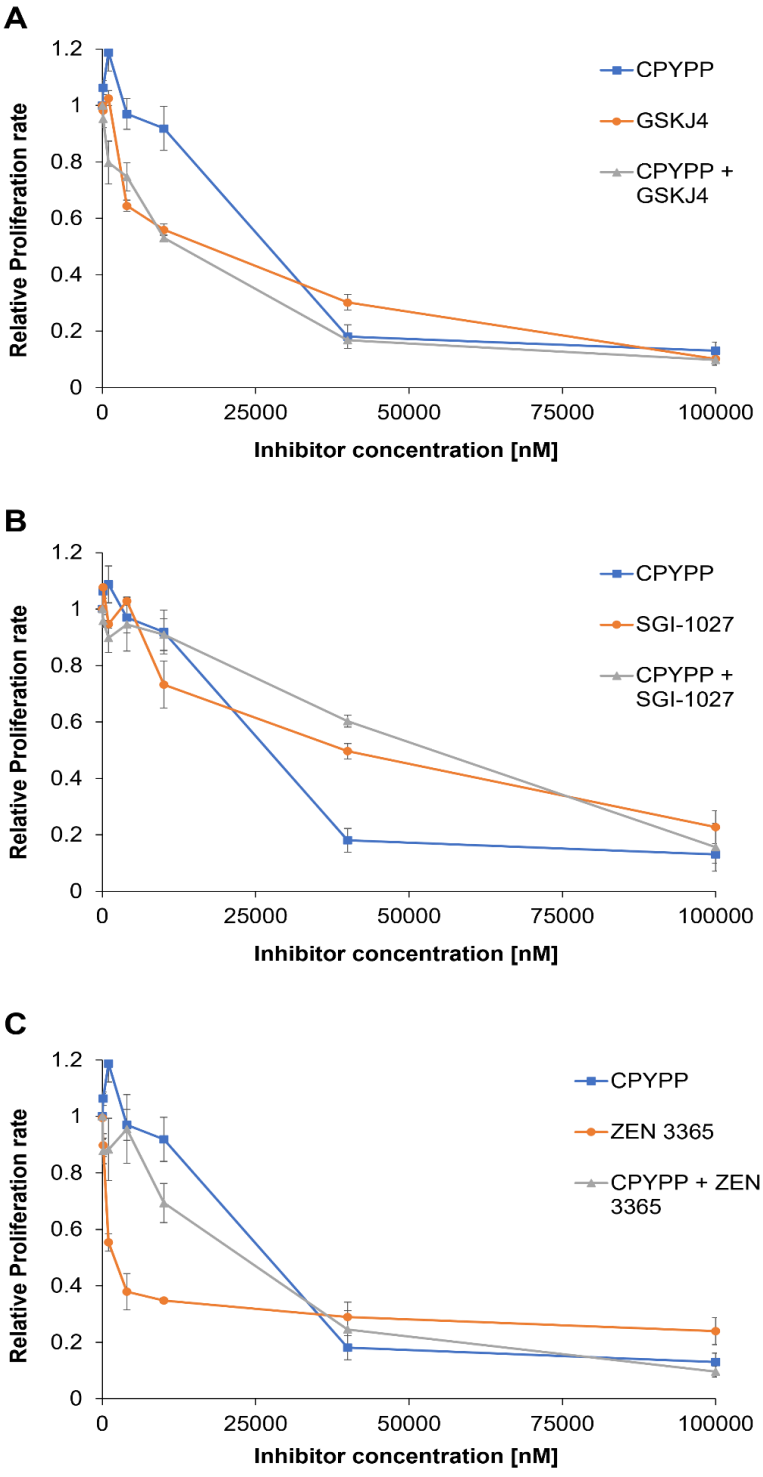
UKE-1 cell line

Combined treatment of UKE-1 wild type cells exhibited strong synergistic effects on proliferation at both effective doses. Combinations of CPYPP + GSKJ4, CPYPP + ZEN 3365 and TBOPP + SGI-1027 displayed particularly strong synergistic effects with CI values < 0.3 at ED_{95}. Table 16 gives an overview of drug combinations and resulting CI values. Figure 12 illustrates proliferation data from respective drug mono- and combination treatment.

Table 16. Combination index (CI) values for UKE-1 wild type cells at ED_{50} and ED_{95}.

Drug combination	Drug ratio	CI at ED_{50}	CI at ED_{95}
CPYPP + GSKJ4	5 : 1	0.11	0.29
CPYPP + SGI-1027	3 : 1	0.72	0.74
CPYPP + ZEN 3365	10 : 1	0.38	0.27
TBOPP + GSKJ4	8 :1	0.57	0.82
TBOPP + SGI-1027	5 : 1	0.47	0.24
TBOPP + ZEN 3365	1.6 : 1	0.22	0.53

AML cells were treated with ascending concentrations of inhibitor while adhering to the specific and fixed drug concentration ratio. CI values are indicated for the respective 50 % effective dose (ED_{50}) and the 95 % effective dose (ED_{95}). Underlying data represents the mean of at least three independent experiments performed in triplicates. CI < 1: synergism; CI = 1: additive effect; CI > 1: antagonism.


A
Relative Proliferation rate
1.2
1
0.8
0.6
0.4
0.2
0
0
25000
50000
75000
100000
Inhibitor concentration [nM]
CPYPP
GSKJ4
CPYPP + GSKJ4
B
Relative Proliferation rate
Inhibitor concentration [nM]
CPYPP
SGI-1027
CPYPP + SGI-1027
C
Relative Proliferation rate
Inhibitor concentration [nM]
CPYPP
ZEN 3365
CPYPP + ZEN 3365

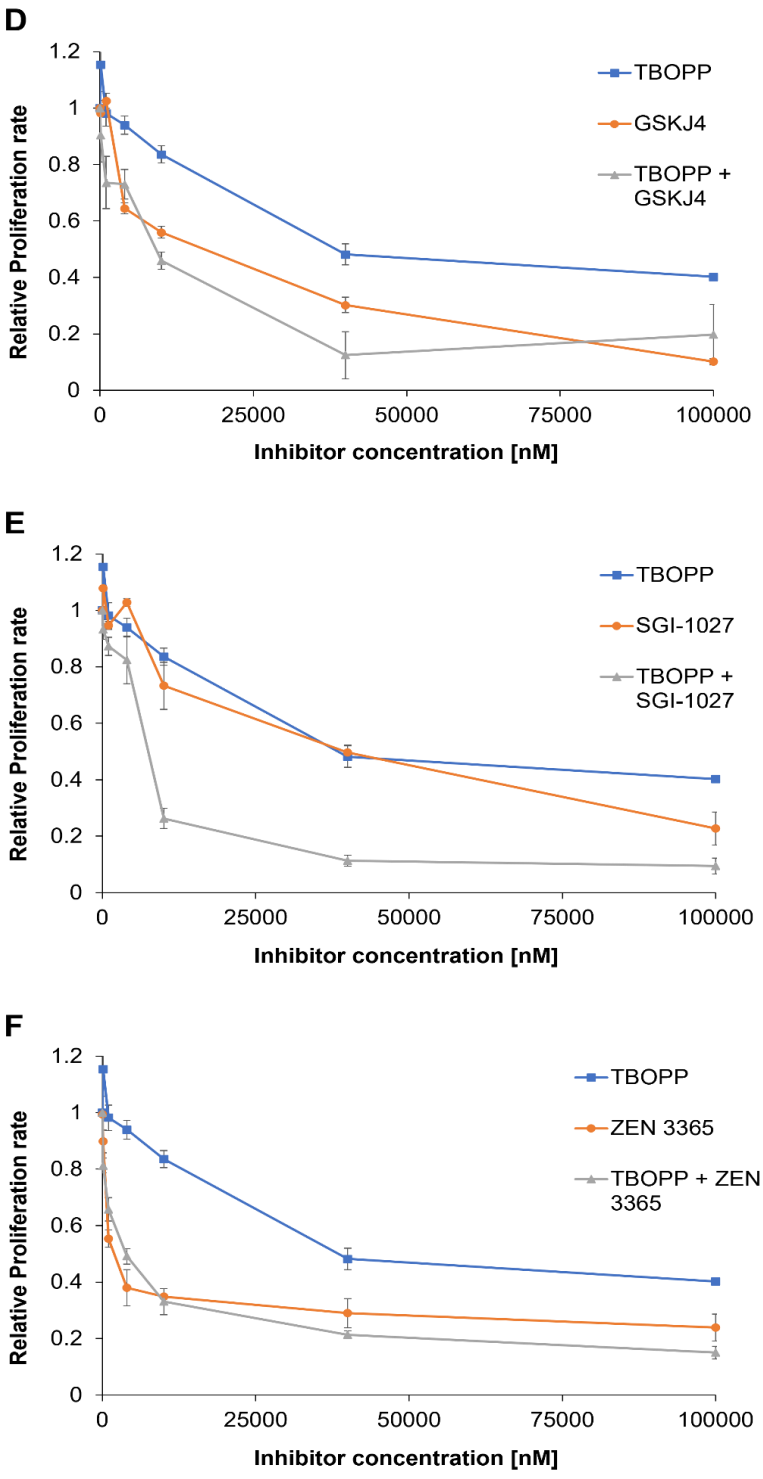


Figure 12. Proliferation data of UKE-1 wild type cells drug combination assays. Small molecule inhibitors CPYPP (**A - C**), TBOPP **(D - F**) and epigenetic inhibitor agents GSKJ4, SGI-1027 and ZEN 3365 were used. Data compares different treatment approaches using monotherapy with a small molecule inhibitor (A - C: CPYPP; D - F: TBOPP), monotherapy with one of three different epigenetic inhibitors and their respective drug combinations**.** For reasons of clarity, CI values are not illustrated decidedly. Corresponding CI values at different effective dose (ED) levels are shown in Table 3. Data represents mean ± SD (n ≥ 3). Viable cell count was conducted after three days.

5. Discussion

Relapse is common in the treatment of AML with relapse rates ranging up to 80 %, especially in older patients with adverse risk factors (de Lima et al., 2021). Patients with a relapsed or refractory disease have a dismal prognosis with standard chemotherapy (Perl et al., 2019). Better understanding of the pathophysiology and molecular biology of AML led to the introduction of several new therapeutic approaches. However, the mechanisms leading to relapse and chemotherapy-resilient AML cells are still not fully understood, yet. One of the main drivers of relapse is thought to be the existence of leukemic stem cells (LSCs). LSCs display mutations, epigenetic modifications and a specific metabolism which usually make them resistant to conventional chemotherapy (Marchand and Pinho, 2021). A better understanding of the physiology and molecular biology of LSCs and their microenvironment has the potential of substantially enhancing outcomes by defining new therapeutic approaches and targets.

Proteins of the DOCK family act as GEFs and are key elements in processes of cell physiology, such as proliferation, migration and adhesion. DOCK2 regulates the exchange between GDP and GTP to activate Rac (Guo and Chen, 2017). DOCK2 is specifically expressed in hematopoietic cells which potentially makes it an attractive target in the treatment of leukemia. Previous studies showed that DOCK2 interacts with FLT3, the most commonly mutated gene in AML, which is associated with a poor prognosis (Wu et al., 2017). FLT3 inhibitors have already found their way into treatment algorithms for AML but resistance to monotherapy with these drugs has shown to be a limiting factor in treatment efficacy (Qiao et al., 2021). This makes the combination of FLT3-inhibition through interconnected pathways with inhibition on different molecular levels a potentially effective treatment strategy.

Further studies have shown that epigenetic mutations in AML patients, which are associated with a poor prognosis correlate with a higher activity of DOCK5 (Biswas et al., 2019). Previous in-vitro experiments by our working group (not published) showed that DOCK5 is expressed by 5 out of 11 investigated AML cell lines. In the aggregate, this leads to the hypothesis that single as well as combined epigenetic inhibition of DOCK5 could have significant antileukemic effects

In the present study, the role of DOCK2 and DOCK5 in the pathophysiology of AML was investigated by directly inhibiting these proteins, using a lentiviral knockdown as well as small molecule inhibitors. The functional consequences of an altered expression of the DOCK proteins were investigated using in vitro assays. Furthermore, the effect of combined pharmacological inhibition of multiple DOCK-associated regulator proteins on the epigenetic level with DOCK proteins was investigated in vitro.

5.1. Functional effects of establishing a DOCK2 and DOCK5 knockdown in AML cell lines

In the present study, an shRNA-based lentiviral knockdown was used to downregulate DOCK2 and DOCK5 in AML cell lines Kasumi-1, Molm-13, UKE-1 and TF-1. Two different shRNAs were used for knockdown of DOCK2 and DOCK5 in each cell line respectively. Repeated validation of knockdown capacity through RT-qPCR showed a stable and distinct knockdown of DOCK2 and DOCK5 with at least one shRNA in each cell line. Moreover, it showed a reproduceable disparity in knockdown capacity between different shRNAs (for DOCK2 and DOCK5, respectively). This observation can be assigned to inherent differences between the shRNA's knockdown capacities and is therefore not related to methodological weaknesses. After successfully transducing the cells, functional in vitro assays were performed. The results of proliferation assays were rather indifferent. Downregulation of DOCK2 resulted in measurably lower proliferation rates across all cell lines but failed to reach statistical significance in 2 out 4 cell lines. Molm-13 cells carrying a DOCK2 knockdown showed the most consistent antiproliferative effects in proliferation assays. DOCK5 knockdown did not show statistically significant inhibitory effects on leukemic proliferation.

The results of colony formation assays with DOCK2 and DOCK5 knockdown cells were mostly coherent with findings from the proliferation assays. DOCK2 knockdown in Molm-13 cells resulted in a significant reduction in colony size, density, and dissemination. Kasumi-1 and TF-1 showed a statistically significant reduction in colony numbers for one (out of two) DOCK2 knockdown approaches. However, the lack of reproducibility with the second DOCK2 knockdown approach and lacking impact on the other result dimensions (dissemination, density) limit the results' significance. These findings must be evaluated with caution since the high standard deviation as well as the inherently semiquantitative/-objective nature of colony formation assays reduce their validity. DOCK5 knockdown surprisingly led to an increase of colonies formed in 5 out of 6 experimental approaches. A high internal variability was observed here as well.

The results support the hypothesis that DOCK2 signaling, and functionality are directly connected to the activity of FLT3. Through mass spectroscopy and Co-Immunoprecipitation Wu et al. previously showed that DOCK2 interacts with FLT3 (Wu et al., 2017). This is consistent with our findings since out of the AML cell lines which were used within this study, only Molm-13 cells carry a FLT3-ITD mutation (Quentmeier et al., 2003). However, contrary to previous findings about the relevance of DOCK2 in AML, we could not reproduce antileukemic effects with the same clarity and statistical

significance as described in the literature. To exclude the possibility of dwindling knockdown strength, repeated validation of knockdown capacity was performed throughout the experimental course. The reasons for the discrepancies between our results and previous studies cannot be addressed with certainty. However, one of the reasons could include alternate signaling and cross reactivity between different proteins of the DOCK family and/or other GEFs. In fact, DOCK2 belongs to the subfamily of DOCK A proteins, together with DOCK1 and DOCK5. Each of these proteins interact with ELMO1 to form an activation complex for Rac1 further downstream (Chang et al., 2020). This is consistent with the findings of one of my fellow lab group members, Dr. Frauke Fuchs, who investigated antileukemic effects resulting from pharmacological and molecular biological inhibition of DOCK1 in AML. Part of her concluding hypothesis is that related members of the DOCK subfamilies compensate for each other, in case of downregulation (Fuchs, 2020). Therefore, a viable approach could be a double or even triple inhibition of DOCK A proteins DOCK1, DOCK2 and DOCK5. Limitations to this strategy could prospectively manifest in an unfavorable side-effect profile through increased toxicity when transferred to in-vivo or clinical settings. DOCK proteins act as GEFs for the ubiquitous Rho-GTPases which are substantially involved in cellular homeostasis. Since the single knockdown of DOCK5 did not show inhibitory effects on proliferation and migration/colony formation in vitro, it seems like specifically targeted DOCK5 downregulation does not have a substantial antileukemic effect. Especially Biswas et al. elucidated the role of DOCK5 in AML before and subsequently concluded that DOCK5 overexpression correlates with increased proliferative and migratory abilities in AML cells (Biswas et al., 2019). However, these findings are put into context with epigenetic dysregulation – specifically loss of the chromatin remodelers MBD3/NuRD and the KDM6A pathway. Hence, the causalities between epigenetic regulation, DOCK5 signaling and functional antileukemic effects are still rather opaque what led us to further investigate the interactions between epigenetic regulators and DOCK proteins through drug combination experiments. Previous studies suggest that DOCK5 downregulation does not induce compensatory DOCK1 and/or DOCK2 overexpression which further supports our hypothesis of an extensive epigenetic-DOCK5-signaling-network (Vives et al., 2011). In retrospect, it would have been helpful to determine the expression rate of the respective other DOCK paralogue in the transduced DOCK2/DOCK5 knockdown cells e.g., via Real-Time qualitative PCR.

Overall, the results of the in vitro assays, we carried out using transduced AML cell lines leave some room for uncertainty and the need for further research. We could partly confirm the findings of Wu and colleagues who stated that DOCK2 does in fact play a role in leukemogenesis and that downregulation of DOCK2 influences leukemic cells'

leukemogenic abilities (Wu et al., 2019). However, our results implicate that antileukemic effects of DOCK2 knockdown are directly correlated with and/or dependent on an internal tandem duplication mutation of FLT3. As FLT3 is the most common genetic alteration among a set of recurring mutations in acute myeloid leukemia (Kennedy and Smith, 2020), it certainly adds to the relevance of further research towards translation of these findings into new therapeutic approaches involving DOCK2 inhibition. However, new experimental efforts should be aimed at understanding the exact signaling and the reciprocal connections in DOCK2 signaling to get a holistic understanding of its functioning. Furthermore, a single DOCK5 knockdown did not expose significant alterations of AML cells' abilities to proliferate and migrate. Currently, the data available regarding DOCK5 is relatively limited with Biswas et al. as the only study which decidedly investigated the role of DOCK5 in AML. Biswas as well as other authors postulated the crosstalk in signaling pathways between DOCK5 and epigenetic regulator proteins. E.g., Liu et al. described the pathophysiological connection between alternative splicing events and an oncogenic DOCK5 variant in Human Papilloma Virus-negative head and neck squamous cell carcinoma (Liu et al., 2018). ASE are co-transcriptional processes which lead to the generation of multiple mRNA isoforms and increasing evidence suggests that epigenetic mechanisms are reciprocally involved in the regulation of specific chromatin regions which ultimately lead to co-transcriptional RNA-processing (J. Zhang et al., 2020). The results of combined epigenetic and DOCK2 and DOCK5 inhibition will be discussed in section 5.5.

5.2. Could pharmacological inhibition of DOCK proteins become a paradigm shift in the therapy of AML?

Wild Type AML cell lines Molm-13 and UKE-1 were treated with the small molecule inhibitors CPYPP and TBOPP and subsequently proliferation assays were performed. As CPYPP mainly inhibits DOCK2 and DOCK5 (Watanabe et al., 2014), we sought to compare these effects with the DOCK1-specific inhibitor TBOPP and calculate the respective IC_{50}s from the resulting data. Preliminary expression data from our laboratory as well as data from the *Harmonizome* project (Rouillard et al., 2016) indicate that Molm-13 expresses DOCK2 and DOCK5 whereas UKE-1 expresses DOCK1, DOCK2 and DOCK5.

Treating Molm-13 and UKE-1 cells with CPYPP led to a significant reduction in proliferation rates for both cell lines. These results are in line with the literature (Nishikimi et al., 2012; Wu et al., 2017), earlier findings by our research group (Fuchs, 2020) and the anticipated results, based on the molecular mode of action. The results clearly

support the hypothesis that DOCK2 does play a role in the pathophysiology of AML and that there is an inverse correlation between DOCK2 activity and AML cells' proliferative behavior. However, the underlying molecular mechanisms still need to be elucidated, since CPYPP is not a DOCK2-specific inhibitor and the causalities between cause and effect in DOCK-inhibition remain rather hypothetical. Unexpectedly, the administration of TBOPP exhibited stronger antiproliferative effects for the DOCK1-non-expressing cell line Molm-13 (IC_{50} 11.32 µM; $p < 0.01$) than the DOCK1-expressing cell line UKE-1 (IC_{50} 43.23 µM $p < 0.01$). A possible explanation could be that the functional consequences of DOCK1-inhibition and biochemical traits of the inhibitor drug (e.g., the chemically determined specificity and binding strength) have diverging implications for the pathophysiology of AML cells. Tajiri et al. could show that TBOPP specifically binds to DOCK1 with high affinity (Tajiri et al., 2017). Our suggestion is that this does not necessarily imply a proportional relationship to the functional in vitro or in vivo effects. Overall, we could not confirm a direct relationship between DOCK1-specific pharmacological inhibition and proportionally correlated significant antileukemic effects. We rather suggest an explanation which puts DOCK1 into a more differentiated network of reciprocal interaction with the remaining DOCK A protein family members and other signaling pathways and molecules. Possibly the effectiveness of specific as well as non-specific DOCK-inhibition may be dependent on certain (patho-)physiological prerequisites, such as mutations or combined treatment with e.g., chemotherapy agents. Lately another research group could show that TBOPP enhances the effects of the chemotherapy agent cisplatin in renal cell carcinoma by sensitizing the cells to the treatment and decreasing chemoresistance (W. Zhang et al., 2020). This approach certainly needs further experimental investigation and understanding.

5.3. The effects of pharmacological inhibition on the epigenetic level in AML cell lines

Our previous findings as well as published data (Biswas et al., 2019; Liu et al., 2018; Qiao et al., 2021) indicate reciprocal connections between the epigenetic level and DOCK proteins. This includes molecules which dynamically control different mechanisms of chromatin remodeling which lead to an alteration of the functionality of DOCK proteins and its downstream pathways and effector proteins, such as Rac, ELMO and FLT3. A total of 4 inhibitor compounds was used to treat wild type AML cell lines Molm-13 and UKE-1 to perform proliferation assays. The data was utilized to calculate the respective IC_{50} for each compound and gain further insights on the individual efficacy. The inhibitors targeting KDM6A/KDM6B (GSKJ4), DNMT3A (SGI-1027) and BRD4 (ZEN

3365) all showed a significant antiproliferative effect in vitro in Molm-13 and UKE-1 cells. Compared to small molecule inhibitors (see 5.2.) the respective IC_{50}s for the epigenetic inhibitors were significantly lower (i.e., exposing stronger antiproliferative effect) with ZEN 3365 being the most potent. It is notable that $IC_{50[Molm-13]}$ (713 nM; $p < 0.001$) was significantly lower than the $IC_{50[UKE-1]}$ (2.63 µM; $p < 0.001$). A possible explanatory approach for the efficacy disparities between the two cell lines could be a dual activation of the Hedgehog pathway in Molm-13 cells. Our research group could recently show that, besides the canonical activation of the Hedgehog pathway via Patch (PTCH) and Smoothened (SMO), the Hedgehog pathway can be activated in a non-canonical way in FLT3-mutated AML cells through the RAS-RAF-MEK-ERK pathway as well (Latuske et al., 2017; Pietrobono et al., 2019). Since Molm-13 cells carry a FLT3-ITD mutation, the "basal expression rate" of the Hedgehog pathway is possibly higher, compared to a FLT3-non-carrying AML cell. Current findings show that the BET bromodomain protein, BRD4, regulates GLI transcription downstream of SMO and directly interacts with GLI1 and GLI2 promotors, the final effectors of the Hedgehog/GLI pathway (Pietrobono et al., 2021; Tang et al., 2014; Wellbrock et al., 2021). Thus, the inhibition of BRD4 leads to a higher relative inhibition of the Hedgehog/GLI signaling cascade through targeting of the downstream effector, the GLI transcription factors. It is important to note that BRD4 was shown to transcriptionally regulate multiple proto-oncogenes such as MYC or BCL2 (Latif et al., 2021; Spriano et al., 2020). However, pharmacological and molecular biologic inhibition of BRD4 in vivo and in vitro has shown to be a promising way of targeting disease maintenance in AML (Wellbrock et al., 2021; Zuber et al., 2011). Figure 13 shows a proposed model of the molecular interactions of the Hedgehog pathway and BRD4(-inhibitors).

GSKJ4 targets histone lysine demethylases (KDM's). Recently, it has been shown that this leads to a downregulation of cAMP response element-binding protein (CREB) which plays a key role in the multistep process of leukemogenesis (Illiano et al., 2020). Our experimental results support the hypothesis that GSKJ4 could play a role in new approaches for AML therapy.

SGI-1027 is an inhibitor of DNMT3A – an epigenetic regulator which catalyzes DNA methylation to regulate essential processes such as embryonic development and cell differentiation (Zwergel et al., 2019). Consistent with recent findings by other groups we conclude from our data that inhibition of DNMT3A is a promising approach for novel AML therapies. Again, the antiproliferative effect was distinctively stronger in Molm-13 than in UKE-1 cells. This further supports our hypothesis that inhibition of commonly aberrated

epi-/genetic molecules in AML in the presence of another mutation – in the case of Molm-13 it is FLT3-ITD – leads to an amplification of antileukemic effects.

The pharmacological inhibition of EZH2 through GSK126 was the least effective out of the four used compounds. However, GSK126 did show antiproliferative effects in vitro which surpassed the threshold of statistical significance. Recent investigations by other authors stress the relevance of EZH2 as a new molecular target in AML therapy with reference to the physiological functions of EZH2 as a lysine methyltransferase which regulates the methylation of lysine 27 on histone H3. The subsequent decondensation of the chromatin increases accessibility to the respective DNA region and thus, enhances the damage resulting from chemotherapy application (Porazzi et al., 2022). Due to the lower efficacy and the need for prioritization of the broader experimental approach, we decided not to include GSK126 in further experiments. With that being said, the combination of currently used chemotherapy agents with GSK126 could be a viable approach for further research, nevertheless.

In conclusion, it should be noted that our results certainly have limits to their validity and significance since we used only a single experimental method. In doing so, our aim was to iterate our approach towards a combination of epigenetic inhibition and DOCK inhibition by deductively prioritizing the most viable options among an exhaustive field of different inhibitor compounds.

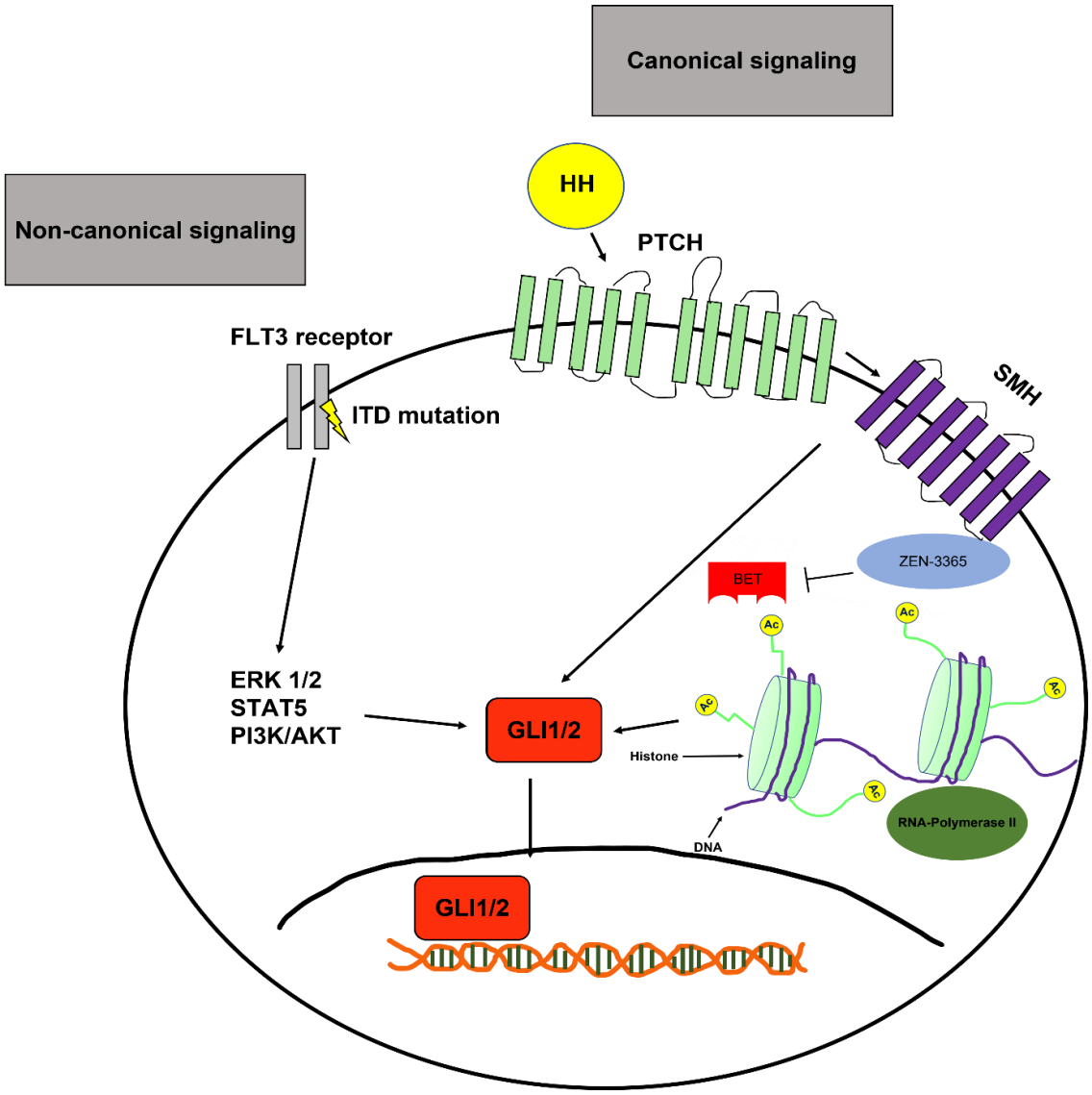


Figure 13. Proposed model of the signaling of GLI1/2 and the amplified effects of BET inhibition by the BRD4 inhibitor ZEN-3365 due to the presence of a FLT3-ITD mutation. Arrows symbolize activation/amplification. T-bar indicates inhibition. Abbreviations: FLT3, FMS like tyrosine kinase 3; ITD, internal tandem duplication; HH, Hedgehog; PTCH, Patched; SMH, Smoothened; BET, Bromo- and Extra-Terminal domain; Ac, Acetyllysine; ERK, extracellular signal-regulated kinases; STAT5, Signal transducer and activator of transcription 5; PI3K, Phosphoinositide 3-kinase; AKT, RAC-alpha serine/threonine-protein kinase, GLI, glioma-associated oncogene. Activation of the transcription factor GLI works through Hedgehog and the canonical way and the non-canonical way through FLT3-ITD. The presence of both signaling pathway leads to a higher basal expression/activation rate of GLI which potentially gives inhibition of GLI more “leverage” in relative reduction of GLI expression and its cellular effects. BRD4 is a BET and epigenetically regulates the transcription rate of GLI by binding acetylated lysine residues in histones. By blocking BRD4 it is possible to amplify the inhibition of GLI expression and subsequent leukemic proliferation since BRD4 acts independently from non-/canonical activation of GLI and therefore makes ZEN-3365 a promising candidate for (combined) treatment in FLT3-ITD mutated AML.

5.4. In vitro effects of the combination of lentiviral shRNA based DOCK2/DOCK5 knockdown with epigenetic inhibitor compounds

To further elucidate the interplay of epigenetics and DOCK inhibition, we used transduced Molm-13 and UKE-1 DOCK2 and DOCK5 knockdown cells to perform proliferation assays. The cells were treated with three different epigenetic inhibitor compounds from the previous experiment. Selection of the compounds used in this experiment was based on their individual level of efficacy in vitro (see section 4.5.). The following compounds were included in the experiments for both transgenic cell lines (target molecule in brackets): GSKJ4 (KDM), SGI-1027 (DNMT3A), ZEN-3365 (BRD4).

In transgenic Molm-13 cells, neither DOCK2 nor DOCK5 knockdown could show a significant antiproliferative effect, when combined with any of the three different epigenetic inhibitor compounds. These results are counterintuitive, given the data, we had collected about the effects of singular inhibition of DOCK and on the epigenetic level, so far. We anticipated effects which would at least match the results of single treatment with the epigenetic inhibitor compounds. Surprisingly, the IC_{50}s (for the epigenetic inhibitors) for Molm-13 scrambled cells diverged from the respective wild type IC_{50}s which could be an indicator for experimental failure since scrambled is meant to act as a control for the knockdown cells, thus behaving analogous to the respective wild type cell. To exclude the possibility of methodological and statistical weaknesses as well as experimental errors in transduction, repeated experiment approaches were performed while using RT-qPCR to validate the knockdowns as well as scrambled. Furthermore, another batch of freshly transduced cells was used to repeat the experiment under the same conditions stated above. However, these measures did not lead to clarification of these conspicuities. Although, we could exclude some evident sources of experimental errors which could possibly lead to the unexpected results described, we are not able to give a definite evaluation regarding the validity of this experiment.

The results for DOCK2 and DOCK5 UKE-1 knockdown cells were analogous to our findings in Molm-13 cells. In none of the knockdown cells, a combined treatment with epigenetic inhibitor drugs led to significant antileukemic effects in vitro (with respect to scrambled acting as the control). With regards to the IC_{50} of UKE-1 scrambled cells we also observed slight discrepancies compared to UKE-1 wild type cells.

Collectively, the results leave room for incongruity. We anticipated antiproliferative effects (when comparing scrambled to the respective knockdown cells) which would have a baseline effect, similar to mono-inhibited cells from the previous experiments (either from shRNA-based knockdown, pharmacological DOCK, or epigenetic inhibition). However, one continuum we observed here was that, in different experimental settings,

the effect of pharmacological DOCK inhibition through small molecule inhibitors exceeded effects of DOCK knockdowns. Again, a possible explanation for these findings could be extensive cross-talk between different DOCK proteins. This results in weaker antileukemic effects when specifically targeting DOCK2 or DOCK5 by knockdown, compared to pharmacological inhibition. This emphasizes our incomplete knowledge of the dynamics of DOCK signaling and the molecular nodal points in which DOCK proteins are embedded. What we observed is, that even though a certain chemical specificity is attributed to inhibitor compounds, it is not always clear which neighboring signal cascades are affected and which functional effects to expect.

5.5. The synergistic effect of combined pharmacological DOCK2/DOCK5 and epigenetic inhibition

In the light of our previously obtained results, we sought to further investigate the interactions and functional outcomes with combined epigenetic and DOCK2/DOCK5 inhibition. After using a knockdown-pharmacological-inhibition before, we conducted another experiment with dual pharmacological inhibition of DOCK proteins and epigenetic molecules, using the same three compounds as in the previous experiment. We performed proliferation assays, using Molm-13 and UKE-1 wild type cells and the acquired data was utilized to calculate combination indexes (for detailed information see 3.1.5.3.).

For Molm-13 cells, the drug combination of CPYPP and GSKJ4 led to an antagonistic effect at both effective dose levels ($CI_{[ED50]}$ = 4.9, $CI_{[ED95]}$ = 1.6). These results do not support the findings of other groups regarding the interactions of DOCK proteins and KDM6A. While Biswas et al. could show that DOCK5 levels are positively correlated with KDM6A levels and poor AML survival, our data implies that this does not equal a causality between the two signal cascades. KDM6A as well as DOCK2 and DOCK5 do apparently play a role in the pathophysiology of AML. At the same time (based on the available data), we are not able to definitely reject the concept of DOCK proteins having pathophysiological interconnections to KDM6A. CPYPP is a non-specific DOCK inhibitor which likely affects the activity of all DOCK A proteins (Nishikimi et al., 2012) which impedes an evaluation of cause and effect in this case. In addition, combination of the DOCK1-specific inhibitor TBOPP with GSKJ4 led to strong synergistic effects ($CI_{[ED50]}$ = 0.9, $CI_{[ED95]}$ = 0.1). On the on hand, this is surprising since Molm-13 wild type cells do not express DOCK1, but on the other hand, it further supports our hypothesis that DOCK-inhibition through small molecule inhibitors seems to have DOCK-independent effects in AML cells. Based on the literature, we know that KDM6A is somatically mutated in

2 - 15 % of AMLs (depending on the patient series), but its exact role in driving leukemogenesis remains unclear, yet (Tian et al., 2021). Generally, we observed a “trend” for Molm-13 cells to display stronger synergism when combining TBOPP with another compound which indicates adverse effects with the relatively unselectively inhibition of three DOCK proteins (CPYPP) when combining this with further epigenetic inhibition. The possibly contrary effects in combined DOCK and epigenetic inhibition are not described in the literature so far since most studies including DOCK inhibition are limited to the single inhibition of DOCK and/or examining correlations between DOCK expression and other molecules (Biswas et al., 2019; Wu et al., 2017). To illuminate the underlying molecular mechanisms, supplementary experiments like Rac1 pull-down activation assays and genetic reporter cell lines are needed to determine activation levels of downstream effector pathways. In Molm-13, the combination of SGI-1027 (DNMT3A inhibitor) and CPYPP/TBOPP proved to be very effective, overall (CPYPP: $CI_{[ED50]}$ = 0.8, $CI_{[ED95]}$ = 1.6; TBOPP: $CI_{[ED50]}$ = 0.8, $CI_{[ED95]}$ = 0.2). However, one limitation of CPYPP combination and SGI-1027 (as in the combination with GSKJ4) is the antagonistic effect of the drugs at higher ED_{95} (CI = 1.6). As in GSKJ4, the DOCK-unspecific inhibition at higher effective dose levels seems to impair signaling pathways in an undesirable way. Given the fact that anticancer drugs have the highest CI values at high effective dose levels (Chou, 2010), it is coherent to see these effects more explicitly at the higher dose levels. As for the relevance of DNMT3A in AML, other groups have suggested that DNMT3A mutations in the presence of FLT3-ITD is a poor prognostic factor (Ardestani et al., 2018; Tang et al., 2017). Our results support this hypothesis since the FLT3-ITD-carrying Molm-13 showed significant synergistical effects –although, there is a substantial need for further experimental clarification of underlying mechanisms and explanation as to why TBOPP shows significantly higher levels of synergy in the DOCK1-non-carrying Molm-13.

The combination treatment with the BRD4 inhibitor ZEN-3365 was the most effective in Molm-13 across both DOCK-inhibitors (CPYPP: $CI_{[ED50]}$ = 0.6, $CI_{[ED95]}$ = 0.88; TBOPP: $CI_{[ED50]}$ = 0.1, $CI_{[ED95]}$ = 0.3), consistent with our previous results from single pharmacological inhibition (see 4.5.). ZEN-3365 is the only epigenetic inhibitor that did not show antagonistic effects in combination with CPYPP at ED_{95}. As previously described, we hypothesize that one of the reasons for ZEN-3365’s superior antiproliferative effects is based on its amplified inhibition of protooncogenic GLI transcription factor signaling which seems to be enhanced by the presence of FLT3-ITD. Based on our results and recent findings from other research groups, we deduce that a viable approach for new therapeutic approaches in AML could include the combinatory inhibition of FLT3 + BRD4 + DOCK A protein family members. E.g., Qiao et al. could

recently show that a combination therapy of CUDC-907, a dual inhibitor of PI3K and histone deacetylases, plus Gilteritinib, an FDA-approved 2nd generation FLT3-inhibitor, synergistically induces apoptosis in FLT3-ITD AML cell lines and indicates therapeutical superiority, as compared to Gilteritinib monotherapy (Qiao et al., 2021). The extension of this approach by additionally targeting DOCK A proteins encompasses the possibility of potentiating the results seen so far, since DOCK proteins heavily interact with FLT3.

The results for synergy assays with UKE-1 and Molm-13 wild type cells were mostly consistent. Most combinations exhibited strong synergistic effects. Particularly to mention, is the fact that, despite the inherent differences in expressed DOCK A protein isoforms in UKE-1 and Molm-13 we did not see significant discrepancies among TBOPP and CPYPP. This strengthens our previous conclusions that the effects of small molecule DOCK inhibitors do not solely rely on the specific inhibition of their respective DOCK isoform but heavily includes mechanisms of signaling crosstalk. Furthermore, we did not observe antagonistic effects in any UKE-1 drug combination, as opposed to Molm-13, where we repeatedly detected antagonism at ED_{95}. This possibly implies that combined DOCK-inhibition does not follow the pattern of “the more the better” but rather requires a “tailor-made” approach based on the existing mutational pattern. As discussed before, the FLT3-ITD mutation in Molm-13 showed to be rather beneficial for the combination with DOCK1-specific inhibitor TBOPP whereas the less specific CPYPP led to adverse effects (i.e., antagonism) in some cases. As UKE-1 does not bear a mutated FLT3, the risk of “over-inhibition” with resulting adverse effects is less distinct when combining these cells with DOCK-inhibitors.

Overall, the drug synergy assays provided us with new insights and strengthened some of our previously formulated hypotheses. This especially includes the potential of combining inhibition on different molecular levels – including epigenetics, DOCK-inhibition and leveraging on existing FLT3-ITD mutations. Based on our data, targeting BRD4 via the specific inhibitor ZEN-3365 is particularly promising. This is coherent with recent findings by our research group who identified ZEN-3365 to be a potential new therapy in AML by modulating Hedgehog and GLI (Wellbrock et al., 2021). At the same time, the present data should be interpreted with caution since the scale and the scope of our results are not exhaustive and need further experimental confirmation.

6. Conclusion

The present thesis sought to investigate the role of DOCK2/DOCK5 in AML and place these findings in its context of epigenetic regulation and dysregulation. The pathophysiological mechanisms of leukemogenesis, the idiosyncrasies of its tumor microenvironment and the leukemic stem cell are drivers of therapy resistance, relapse and poor survival.

We could show that DOCK2 and DOCK5 play a role in leukemogenesis, and that leukemic cells' proliferative abilities are mitigated through inhibition of DOCK proteins. DOCK2 knockdown demonstrated to be effective in decreasing proliferation and colony formation in FLT3-ITD mutated AML cells. DOCK5 knockdown did not display a significant impact in vitro. However, the present results negate an immediate/predictable causality between inhibition of a specific DOCK protein and an immediate functional response. This emphasizes the need for more profound understanding and decoding of signaling pathways in which DOCK proteins are embedded – beyond our current understanding of their role as GEFs for Rho-GTPases. The results from pharmacological inhibition of DOCK proteins through small molecule inhibitors further support our hypothesis that the scope of complexity for DOCK proteins and their molecular interactions exceed the limited insights we receive by selectively inhibiting a single DOCK isoform. Although, the inhibitors showed significant antileukemic effects in vitro, it was not possible to align these functional effects with the underlying molecular biology.

Lastly, we demonstrated that in vitro AML cells are susceptive to different kinds of compounds who target commonly mutated epigenetic molecules in AML. Furthermore, the combination of pharmacological inhibition of DOCK2 and DOCK5 with epigenetic inhibition displayed significant synergistic effects, in vitro. Contrarily, an shRNA based DOCK2/DOCK5 knockdown is not as effective and showed inconsistent results. A particularly effective epigenetic inhibitor was the BRD4 inhibitor ZEN-3365. Across all AML cell lines, in mono- and in combination therapy, ZEN-3365 demonstrated a significant reduction in AML cell proliferation and showed strong synergy when combined with pharmacological DOCK2/DOCK5 inhibition. FLT3-ITD mutated cells exhibited the strongest effects when treated with ZEN-3365, either in mono- or DOCK-combinatory-inhibition. The effects of BRD4 inhibition can partly be attributed to its effect on GLI which indicates an important role of the GLI transcription factors and its up- and downstream effectors. Next experimental steps could consist of further in vitro approaches to determine the expression levels of different effectors of DOCK proteins and epigenetic molecules while conducting functional assays. This should be followed by in vivo experiments to transfer acquired knowledge into a more complex biological setting.

7. Abstract

7.1. English

Introduction: Clinical outcomes in AML have improved dramatically during the last decades. However, the molecular and clinical complexity of the disease require further advances in personalized therapies. DOCK proteins are guanine nucleotide exchange factors which interact with Rac and are closely related to mechanisms of cellular homeostasis. Recent findings show that genomic alterations on the epigenetic level occur frequently in AML.

Objectives: The present study sought to investigate the role of DOCK 2/DOCK5 and their interactions with epigenetic dysregulation in AML.

Methods: The antileukemic effects of DOCK inhibition were investigated in vitro, in AML cell lines. Pharmacological DOCK inhibition was conducted by using small molecule inhibitors CPYPP and TBOPP. Molecular biological inhibition of DOCK proteins was achieved by using a lentiviral shRNA based knockdown (KD). AML wild type (wt) cells were treated with epigenetic inhibitors GSK126, GSKJ4, SGI-1023 and ZEN-3365. In vitro synergy assays were performed by (a) combining DOCK KD cells with epigenetic inhibitors GSKJ4, SGI-1027 and ZEN-3365; (b) treating AML wild type cells with a combination of CPYPP/TBOPP + GSKJ4, SGI-1027, ZEN-3365. Subsequently, IC_{50}s and combination indexes (CI) were calculated.

Results: Treatment with CPYPP led to a significant decrease in proliferation in Molm-13 and UKE-1 wt cells ($p < 0.05$). DOCK1 inhibition through TBOPP was more effective in Molm-13 than in UKE-1 wt cells ($p < 0.01$). Monotreatment with GSKJ4 and SGI-1027 led to significant reductions in leukemic proliferation ($p < 0.01$). The strongest mono-inhibitor in both wt cell lines was ZEN-3365, reducing the proliferation rate by ~ 70 % ($p < 0.001$). Combination treatment of AML wt cells with small molecule inhibitors + epigenetic inhibitors was significantly effective along multiple concentrations/combinations. Combinations including ZEN-3365 were particularly effective with CI values < 0.1 for both cell lines at multiple median effect doses (r value 0.9 - 0.98).

Conclusion: Pharmacological combination of DOCK- and epigenetic inhibition is a promising therapeutic approach. BRD4 inhibition through ZEN-3365 was particularly effective. Further experiments to illuminate the underlying molecular signaling and in vivo studies are necessary in future research.

7.2. German

Einleitung: Das Outcome bei der AML hat sich in den letzten Jahrzehnten dramatisch verbessert. Aufgrund der molekularen und klinischen Komplexität der Erkrankung sind jedoch weitere Fortschritte bei personalisierten Therapien erforderlich. DOCK-Proteine sind *Guanine Nucleotide Exchange Factors*, die mit Rac interagieren und essenziell für die zelluläre Homöostase sind. Neuere Erkenntnisse zeigen zudem, dass in der AML regelmäßig Mutationen auf epigenetischer Ebene auftreten.

Zielsetzung: Ziel der vorliegenden Studie war es, die Rolle von DOCK 2/DOCK5 und ihre Wechselwirkungen mit der epigenetischen Dysregulation bei der AML zu untersuchen.

Methoden: Die antileukämischen Effekte einer DOCK-Hemmung wurden in vitro an AML-Zelllinien untersucht. Pharmakologische DOCK-Inhibition wurde durch die niedermolekularen Inhibitoren CPYPP und TBOPP erreicht. Molekularbiologische Hemmung von DOCK-Proteinen wurde durch einen lentiviralen shRNA-basierten Knockdown (KD) erreicht. AML-Wildtypzellen (wt) wurden mit den epigenetischen Inhibitoren GSK126, GSKJ4, SGI-1023 und ZEN-3365 behandelt. Synergieassays wurden durchgeführt, indem (a) DOCK-KD-Zellen mit den epigenetischen Inhibitoren GSKJ4, SGI-1027, ZEN-3365 kombiniert wurden; (b) AML-Wildtyp-Zellen mit einer Kombination aus CPYPP/TBOPP + GSKJ4, SGI-1027, ZEN-3365 behandelt wurden. Anschließend wurden die IC_{50}-Werte und *Combination Indexes* (CI) berechnet.

Ergebnisse: Die Behandlung mit CPYPP führte zu einer signifikanter Hemmung der Proliferation in Molm-13- und UKE-1 wt-Zellen ($p < 0,05$). Die Hemmung von DOCK1 durch TBOPP war bei Molm-13-Zellen wirksamer als bei UKE-1 wt-Zellen ($p < 0,01$). Die Monobehandlung mit GSKJ4 und SGI-1027 führte zu einer signifikanten Verringerung der leukämischen Proliferation ($p < 0,01$). Der stärkste Mono-Inhibitor in beiden wt-Zelllinien war ZEN-3365, der die Proliferationsraten um ~ 70 % reduzierte ($p < 0,001$). Die Kombination von AML wt-Zellen mit CPYPP/TBOPP + epigenetischen Inhibitoren war signifikant wirksam. In beiden Zelllinien sowie verschiedenen effektiven Dosislevels waren Kombinationen mit ZEN-3365 besonders wirksam (CI < 0,1; r-Wert 0,9 - 0,98).

Fazit: Die Kombination von pharmakologischer DOCK- und epigenetischer Inhibition ist ein vielversprechender therapeutischer Ansatz. Die Hemmung von BRD4 durch ZEN-3365 zeigte sich als besonders effektiv. Weitere Experimente zur Klärung der zugrundeliegenden Signaltransduktion sowie in-vivo-Versuche sind zukünftig erforderlich.

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List of Abbreviations

AML	Acute myeloid leukemia
aSCT	Allogenic stem cell transplantation
ASE	Alternative splicing event
ASXL1	Additional Sex Combs-Like 1
BET	Bromodomain and extra terminal domain
BRD4	Bromodomain-containing protein 4
CAR cells	CXCL12-abundant reticular cells
CD	Cluster of differentiation
cDNA	Commentary DNA
CEBPA	CCAAT/enhancer-binding protein alpha
CI	Combination index
CR	Complete remission
CREB	cAMP response element-binding protein
CXCL12	CX chemokine ligand 12
CXCR4	C-X-C chemokine receptor type
DEPC	Deionized diethylpyrocarbonate
DHR-1/2	DOCK homology region-1/2
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
DOCK	Dedicator of cytokinesis
eGFP	enhanced Green Fluorescent Protein
ELMO1	Engulfment and cell motility factor 1
FAB	French-American-British Cooperative Group
FBS	Fetal bovine serum
FLT3	FMS-like tyrosine kinase 3
FSC	Forward scatter
g	Unit for gravity of earth (9.81 m/s^2)
GAP	GTPase-activating proteins
GAPDH	Glycerinaldehyde-3-phosphate-dehydrogenase

GDI	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
GTPase	Small guanosine triphosphatases
HBS buffer	HEPES-buffered saline buffer
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IDH1	Isocitrate dehydrogenase 1
ITD	Internal tandem duplication
KDM	Histone lysine demethylases
KDM6A	Histone 3 lysine 27 demethylase Lysine-specific demethylase 6A
LeGO	lentiviral gene ontology
LSC	Leukemic stem cell
MBD3	Methyl-CpG-binding domain protein 3
MDS	Myelodysplastic syndrome
MRD	Minimal residual disease
mRNA	messenger RNA
MSC	Mesenchymal stem cell
NMP1	Nucleophosmin
PA	Phosphatidic acid
PAA	Polyamino acid
PBS	Phosphate buffered saline
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PTCH	Patch
RNA	Ribonucleic acid
SCID	Severe combined immunodeficient
SD	Standard deviation
SH3	Src-homology 3
shRNA	short hairpin RNA
SMO	Smoothened
SSC	Side scatter

TET2	Tet methylcytosine dioxygenase 2
TKD	Tyrosine kinase domain
TRM	Treatment-related mortality
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
WHO	World Health Organization

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