Dissertation

The role of microRNA-23a in Acute Myeloid Leukemia

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Assoc.-Prof. Priv.-Doz. Dr. Armin Zebisch

and the co-supervision of

Dr. med. univ. Andreas Reinisch, PhD.

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DECLARATION OF ORIGINALITY

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of “Good Scientific Practice”.

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DANKE EUCH ALLEN.

PUBLICATIONS

This thesis has been published in the following original papers:

Stefan Hatzl,[1] Olivia Geiger,[1] Maja Kim Kuepper,[1] Veronica Caraffini,[1] Till Seime,[2] Tobias Furlan,[2] Erika Nussbaumer,[1] Rotraud Wieser,[3] Martin Pichler,[4,5] Marcel Scheideler,[6,7,8,9] Katarzyna Nowek,[10] Mojca Jongen-Lavrencic,[10] Franz Quehenberger,[11] Albert Wölfler,[1] Jakob Troppmair,[2] Heinz Sill,[1] and Armin Zebisch[1]

Increased Expression of miR-23a Mediates a Loss of Expression in the RAF Kinase Inhibitor Protein RKIP

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[1]Division of Hematology, Medical University of Graz, Graz, Austria; [2]Daniel Swarovski Research Laboratory, Department of Visceral, Transplant and Thoracic Surgery, Innsbruck Medical University, Innsbruck, Austria; [3]Department of Medicine I, Medical University of Vienna, Vienna, Austria and Comprehensive Cancer Center of the Medical University of Vienna, Vienna, Austria; [4]Division of Oncology, Medical University of Graz, Graz, Austria; [5]Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, Texas; [6]Institute for Diabetes and Cancer, Helmholtz Center Munich, Munich, Germany; [7]University Hospital, Heidelberg University, Heidelberg, Germany; [8]German Center for Diabetes Research (DZD), Neuherberg, Germany [9]Technical University of Munich, Munich, Germany; [10]Department of Hematology, Erasmus University Medical Center, Rotterdam, the Netherlands; [11]Institute of Medical Informatics, Statistics and Documentation, Medical University of Graz, Graz, Austria

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Stefan Hatzl,[1] Bianca Perfler,[1] Sonja Wurm,[1] Barbara Uhl,[1] Franz Quehenberger,[2] Susanne Ebner,[3] Jakob Troppmair,[3] Andreas Reinisch,[1] Albert Wölfler,[1] Heinz Sill[1] and Armin Zebisch[1,4]

Increased Expression of micro-RNA-23a Mediates Chemoresistance to Cytarabine in Acute Myeloid Leukemia

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[1]Division of Hematology, Medical University of Graz, Graz, Austria; [2]Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz, Graz, Austria; [3]Daniel Swarovski Research Laboratory, Department of Visceral, Transplant and Thoracic Surgery, Medical University of Innsbruck, Innsbruck, Austria; [4]Otto-Loewi-Research Center for Vascular Biology, Immunology and Inflammation, Division of Pharmacology, Medical University of Graz, Austria

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ABSTRACT

Micro-RNAs are small, evolutionary highly preserved non-coding RNAs, which regulate gene expression on a post-transcriptional level. It has been shown that micro-RNAs are functionally involved in the development of malignant diseases by regulating key proteins of carcinogenesis. In this thesis, we wanted to extend our knowledge about micro-RNAs within the process of myeloid leukemogenesis.

In the first part of the thesis, we aimed to delineate the role of micro-RNAs in loss of the RAF-kinase inhibitor protein (RKIP). RKIP is a central regulator of the RAS/MAPK/ERK signal-transduction pathway and functions as tumor- and metastasis-suppressor. A somatic loss of RKIP has been described previously in different human malignancies and in acute myeloid leukemia (AML). However, the mechanisms behind this RKIP loss remain unclear so far. By analyzing more than 400 primary patient samples with micro-RNA microarrays and qPCR, we could show a significant correlation between increased expression miR-23a and decreased expression of RKIP. Furthermore, we could functionally show that ectopic overexpression of miR-23a decreased the expression of RKIP in hematopoietic cell lines, whereas a knockdown of miR-23a had the opposite effect. Importantly, we could demonstrate that this regulation is mediated via a direct interaction of miR-23a with the RKIP-3`UTR. We additionally could demonstrate that overexpression of miR-23a enforces the proliferation of hematopoietic cells, which pinpoints a relevance for leukemogenesis. Interestingly, co-transfection of miR-23a with an RKIP construct lacking the 3´UTR binding site was able to rescue this phenotype. This suggests that the leukemogenic effects of miR-23a are truly mediated via a miR-23a/RKIP axis. Finally, we could demonstrate the relevance of the miR-23a/RKIP axis in other cancer entities as well. By analyzing 4000 primary patient

samples of different cancers within the cancer and genome atlas, we observed an inverse correlation between miR-23a and RKIP in the majority of cancer subtypes. In conclusion we have identified miR-23a as negative regulator of RKIP in acute myeloid leukemia (AML), and its relevance for cancer research in general.

Secondly, we studied the role of miR-23a in AML drug resistance, which is one of the central obstacles in this disease. This is based on the fact that dysregulation of miR-23a has been linked to myeloid leukemogenesis and drug resistance previously. Therefore, we analyzed the role of miR-23a in mediating therapeutic resistance to cytarabine (AraC), which forms the backbone of AML chemotherapy. We could demonstrate that miR-23a overexpressing hematopoietic cell lines showed decreased sensitivity to AraC, whereas knockdown of miR-23a mediates increased sensitivity. Furthermore, we could show that increased expression of miR-23a significantly correlates with relapsed/refractory AML, the leukemic stem cell compartment as well as with inferior treatment outcomes in standard chemotherapy-treated AML patients. Additionally, we could mechanistically show that miR-23a mediates AraC resistance by direct downregulation of topoisomerase 2β (TOP2B). In agreement with these findings, we could show that decreased TOP2B expression correlates with relapsed/refractory AML and inferior AML treatment outcomes as well. In conclusion, we could demonstrate that miR-23a mediates resistance to AraC in AML via modulation of TOP2B. Moreover, we could show that increased expression of miR-23a and decreased expression of TOP2B correlate with inferior treatment outcomes.

ZUSAMMENFASSUNG

Micro-RNAs sind kurze, evolutionär hochkonservierte, nicht kodierende RNA-Moleküle, welche die Genexpression post-transkriptionell regeln können. In vorausgegangenen Studien konnte gezeigt werden, dass micro-RNAs eine zentrale Rolle in der Tumorentstehung spielen indem sie Schlüsselproteine des Zellwachstums und des Zelltodes modifizieren und regulieren. Mit dieser Dissertation möchten wir das aktuelle Wissen von micro-RNAs in der Pathogenese der akuten myeloischen Leukämie (AML) erweitern.

Im ersten Teil der Arbeit konzentrierten wir uns auf den Einfluss von micro-RNAs auf RAF-Kinase Inhibitor Protein (RKIP) Verlust in der AML. RKIP ist ein zentraler Regulator des RAS/MAPK/ERK Signaltransduktionsweges und hemmt Tumor- und Metastasenwachstum. Der Verlust von RKIP Expression wurde bereits in einer Vielzahl von Krebsformen und speziell auch in der AML beschrieben. Trotz der vielen Daten über RKIP Verlust und dessen Auswirkung auf die Zellbiologie ist der Mechanismus, der den RKIP Verlust verursacht bis dato unklar.

Indem wir über 400 primäre AML-PatientInnen Proben mittels micro-RNA Micro-Arrays und quantitativer PCR analysierten, konnten wir zeigen, dass eine erhöhte Expression von miR-23a mit einer erniedrigten Expression von RKIP signifikant korreliert. In einem nächsten Schritt konnten wir außerdem funktionell zeigen, dass eine artifizielle Überexpression von miR-23a zu einem Verlust von RKIP in hämatopoetischen Zelllinien führt. Zusätzlich führte ein artifizieller knock-down von miR-23a in diesen Zelllinien zu einer Überexpression von RKIP. Ergänzend zu diesen Daten konnten wir mittels Luciferase Assay zeigen, dass die miR-23a/RKIP Interaktion durch die direkte Bindung von miR-23a an die 3´UTR von RKIP passiert. Dann untersuchten wir den Einfluss von miR-23a Überexpression auf das

Zellwachstum. Hier konnten wir zeigen, dass miR-23a Überexpression zu vermehrter Proliferation und Zellteilung führt. Interessanterweise konnten wir diesen Phänotyp durch die Ko-Transfektion von miR-23a mit einem RKIP Konstrukt ohne 3´UTR rückgängig machen. Diese Ergebnisse bestätigen, dass die Effekte von miR-23a auf Proliferation und Zellwachstum tatsächlich durch RKIP vermittelt werden. Schlussendlich konnten wir die Relevanz der miR-23a/RKIP Achse in einer Vielzahl von malignen Tumorentitäten zeigen indem wir über 4000 primäre Patientenproben aus dem „The Cancer and Genome Atlas“ extrahierten und analysierten. Zusammenfassend identifizierten wir miR-23a als negativen Regulator von RKIP in AML und die Relevanz der miR-23a/RKIP für die Krebsforschung insgesamt.

Im zweiten Teil der Dissertation untersuchten wir die Rolle von miR-23a in der therapeutischen Resistenz der AML, welche eines der zentralen Hindernisse in der Heilung dieser Erkrankung darstellt. Sowohl eine Dysregulation von miR-23a in AML als auch ein Konnex von miR-23a und therapeutischer Resistenz in anderen Malignomen, konnte bereits durch uns und andere gezeigt werden. Aus diesem Grund untersuchten wir ob miR-23a einen Einfluss auf Cytarabinresistenz in AML hat. Cytarabin ist ein zentrales Medikament der AML Therapie und stellt das Rückgrat von fast jedem AML Chemotherapie-Regimen dar. In einem ersten Schritt konnten wir zeigen, dass miR-23a überexprimierende Zellen eine verringerte Sensitivität gegenüber Cytarabin haben und dass miR-23a knock-down Zellen eine erhöhte Sensitivität gegenüber Cytarabin haben. Zusätzlich konnten wir zeigen, dass erhöhte Expression von miR-23a mit relapsierter/refraktärer AML, dem leukämischen Stammzell-Kompartiment und auch mit schlechtem Überleben in Standard-Chemotherapie behandelten AML PatientInnen assoziiert ist.

Durch verschiedene *in vitro* Experimente konnten wir mechanistisch zeigen, dass miR-23a Cytarabinresistenz durch die direkte Regulation von Topoisomerase 2B (TOP2B) vermittelt wird. Interessanterweise zeigten wir auch, dass erniedrigte Expression von TOP2B ebenfalls mit relapsierter/refraktärer AML als auch schlechtem Überleben korreliert.

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ABBREVATIONS

3´UTR	3´ untranslated region
5-FU	5-flurouracil
7-AAD	7-aminoactinomycin-D
ABCF1	ATP-binding cassette sub-family F member 1
ABL	Abelson Murine Leukemia Viral Oncogene Homolog
AGO	Argonaut protein
AML	acute myeloid leukemia
AMP	Adenosine monophosphate
ALL	acute lymphoblastic leukemia
APL	acute promyelocytic leukemia
ASXL1	additional sex combs-like 1
ATP	adenosine triphosphate
Allo-HSCT	allogenic hematopoietic stem cell transplantation
AraC	cytarabine
B2M	ß-2-microglobulin
BCR	breakpoint cluster region
BLAST	basic local alignment search tool
BM	bone marrow
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C.elegans	Caenorhabditis elegans
cDNA	complementary deoxyribonucleic acid
CEBPA	CCAAT/enhancer-binding protein alpha
ChIP	chromatin immunoprecipitation
CLL	chronic lymphatic leukemia
CML	chronic myeloid leukemia
CNS	central nervous system
CSC	cancer stem cell
CR	complete remission
Ct	Threshold cycle
DMSO	dimethyl sulfoxide
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
eIF	eukaryotic initiation factor
ELN	European leukemia network
EFS	event free survival
EMA	European Medical Association
ERK	extracellular signal regulated kinase
EZH2	enhancer of zeste homolog 2
FAB	French American British guidelines
FBS	fetal bovine serum
FISH	fluorescence in-situ hybridization
FLT3	FMS-like tyrosine-kinase 3
G6PDH	glucose 6 phosphate dehydrogenase
G-CSF	granulocyte colony stimulating factor
GEB2	GTP-exchange complex growth factor receptor bound-2
GO	gemtuzumab-ozogamicin
GTP	guanosine triphosphate
GUSB	ß-Glucuronidase

HiDAC	high dose cytarabine
HKG	housekeeping gene
HMA	hypomethylating agent
IDAC	intermediate dose cytarabine
IDH	Isocitrate dehydrogenase
IκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
i.v.	intravenously
LSC	leukemic stem cell
LIC	leukemia initiating cell
MAPK	mitogen activated protein kinase
MDS	myelodysplastic syndrome
MEK-1	mitogen associated/ extracellular regulated kinase -1
MiRISC	micro RNA-induced silencing complex
MiRNA	micro RNA
MLL	myeloid/lymphoid mixed linage leukemia
mRNA	messenger RNA
MS	myeloid sarcoma
MST2	mammalian sterile 20-like kinase
MUG	Medical University of Graz
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor κB
NPM1	nucleophosmin
NSCLC	non-small cell lung cancer
nt	nucleotide
OS	overall survival
PB	peripheral blood
PBS	phosphate-buffered saline
PEBP1	phosphatidylethanolamine binding protein 1
PML	promyelocytic leukemia gene
Pre-miRNA	precursor miRNA
Pri-miRNA	primary mircoRNA
PTEN	phosphate and tensin homolog
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
RAF	rapidly accelerated fibrosarcoma (RAF)
RARA	retinoic acid receptor alfa
RFP	red fluorescent protein
RIPA	radioimmunoprecipitation assay buffer
RKIP	RAF-kinase inhibitor protein
RNA	ribonucleotide acid
RNU6	RNA-U6-small nuclear
RT	reverse transcription
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SHC	Src homology (SH) 2 domain-containing protein
SNORD44	small nucleolar RNA 44
SOS	son of sevenless
t-AML	therapy related acute myeloid leukemia
TET2	Tet methylcytosine dioxygenase 2

TG	target gene
TOP1	topoisomerase 1
TOP2	topoisomerase 2
TOP2B	topoisomerase 2 beta
VNTR	variable number of tandem repeats
VOD	veno-occlusive disease
WHO	world health organization
WT1	Wilms tumor

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1 INTRODUCTION

1.1 Acute myeloid leukemia (AML)

Acute myeloid leukemia is a heterogeneous group of malignancies of blood cells that arises from the malignant transformation of hematopoietic progenitor or stem cells. The malignant population of cells in AML is called blasts and displays uncontrolled proliferation and a block of differentiation at a premature stage of the myeloid linage. In most cases, leukemic blasts are detected in the peripheral blood and bone marrow, but involvement or infiltration of any organ system may occur. According to the World Health Organization (WHO) criteria, a blast count of at least 20% in the bone marrow and/or peripheral blood, or the presence of blasts in biopsies of extramedullary tissues is necessary to establish the diagnosis of AML. (Dohner, Weisdorf et al. 2015, Sill, Olipitz et al. 2011, Zebisch, Cerroni et al. 2003, Arber, Orazi et al. 2016)

1.1.1 Epidemiology

AML is the most common acute leukemia in adults and accounts for approximately 80% of cases in this adult population.(Yamamoto, Goodman 2008)
With an annual overall incidence of 3 to 4 per 100.000 in Europe, AML seems to be a rare disease. However, in the older population over 70 years the annual incidence rises to 15 to 20 per 100.000. (Juliusson, Lazarevic et al. 2012, Visser, Trama et al. 2012) Overall the incidence of AML is rising due to general ageing of the population.

1.1.2 Clinical presentation and symptoms

AML patients typically present with symptoms related to pancytopenia, which is caused by the consecutive displacement of normal bone marrow by leukemic blasts. Anemia causes weakness and fatigue (Meyers, Albitar et al. 2005), leukopenia causes infections of variable severity, and thrombocytopenia causes hemorrhagic findings, such as gingival bleeding, ecchymoses, epistaxis, or menorrhagia. (Nebgen, Rhodes et al. 2016)

Leukocytosis can be observed in 60% of adult and 30% of childhood AML patients. In rare cases peripheral leukocyte counts exceed 100.000/µl (Ginsberg, Leeds 1995) and cause a hyperleukocytosis-syndrome with high complication rates like intracranial hemorrhage, pulmonary distress, acute renal failure and signs of acute myocardial infarction.

A special clinical presentation of AML is called myeloid sarcoma (MS) or chloroma. In this situation, myeloid blasts invade extrahematopoietic tissues and grow like a solid tumor therein. MS may present as an isolated manifestation or simultaneously with bone marrow involvement. Importantly, even in the case of isolated MS, all these cases will develop a bone marrow involvement sooner or later when untreated. The frequency of extramedullary involvement at AML diagnosis is estimated to be around 9%, however, data from positron emission tomography studies suggest much higher rates of up to 22-40%. (Stolzel, Rollig et al. 2011, Stolzel, Luer et al. 2019) Skin involvement is more frequent in AML with monocytic or myelomonocytic phenotype. This involvement of the skin is called leukemia cutis and presents with nodular and violaceous/gray-blue colored skin lesions. (Ratnam, Khor et al. 1994) Contrary to acute lymphoblastic leukemia (ALL) central nervous system (CNS) involvement is very rare in AML. Precise data of CNS involvement is not available due to the lack of lumbar puncture and cerebrospinal fluid diagnosis in asymptomatic patients. (Castagnola, Nozza et al. 1997) Lymph node enlargement is also very rare in AML compared with lymphatic disease. Hepato-splenomegaly is only present in about 10% of AML cases and frequently associated with preexisting myeloproliferative disorder or chronic myelogenous leukemia (CML). (Byrd, Mrozek et al. 2002)

1.1.3 Diagnosis

The diagnosis of AML requires the morphologic, cytochemical, immunophenotypic, cytogenetic and molecular typing of the leukemic blasts. (Andreesen, Heimpel 2009) Therefore, bone marrow aspiration and potentially even a bone marrow biopsy are performed in every case. Preferred biopsy location is the posterior superior iliac crest and spine due to low complication rates. Morphologic (May-Grünwald-Giemsa) and cytochemical (periodic acid-Schiff, myeloperoxidase, nonspecific esterase) stains of bone marrow aspiration as well as the peripheral blood smears are used for characterization of the leukemia according to the French American British (FAB) classification system. (Bennett, Catovsky et al. 1976) The newer and currently actual WHO classification is a more comprehensive system and additionally includes clinical information, immunophenotyping by flow cytometric analysis (FACS), cytogenetic analyses, as well as molecular information. (Arber et al. 2016)

1.1.4 Risk-stratification and survival

Survival of AML is dependent on patient-related, leukemia-related, and eventually transplant-related factors. Patient-related factors include age, performance status and comorbidities. While young and/or fit patients can be treated with high-dose chemotherapy and hematopoietic stem cell transplantation (HSCT), a low-intensity, palliative approach has to be chosen in older and/or unfit patients. (H. Kantarjian, O'Brien 2010, Dohner et al. 2015, Dohner, Estey et al. 2017) While there is no strict definition when a patient is considered old, clinical trials usually divide young and old patients at around 60-65 years of age. This is in line with the clinical routine. A large observational study using the SEER-database registry showed that only 40% of patients over 65 years with newly diagnosed AML received high-dose chemotherapy. (Medeiros, Satram-Hoang et al. 2015) But even for fit patients receiving high-dose chemotherapy, age is an important factor influencing the survival of newly diagnosed AML patients. In the “younger” group complete remission rates (CR) of 70-80% can be expected. In contrast, CR in older patients who are still fit and receive high-dose chemotherapy only could be achieved in 40-60% from whom over 85% will relapse and decease due to AML. (A. Burnett, Wetzler et al. 2011) Transplant-related factors only apply for transplanted

patients. Among others, they include the transplanted cell number, disparities in HLA-typing, or differences in the blood groups.

Finally, it is important to assess the leukemia specific risk, which is mainly determined by cytogenetic and molecular genetic factors. The European Leukemia Network (ELN) guidelines thereby established a risk stratification model, which is widely used in clinical routine to assess the AML risk status, and to plan the therapeutic approach. This model encompasses both classical cytogenetics and the mutational status of various genes and provides a prediction system for failure of induction chemotherapy as well as AML related mortality by classifying patients into “favorable”, “intermediate” and “adverse” risk groups. (Dohner et al. 2017) The value of this system has been proven in a broad range of countries regardless their economic stages.(Cape, Islam et al. 2017, Estey 2018)

Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low = allelic\ ratio < 0.5}$
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD$^{high = allelic\ ratio > 0.5}$
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype; monosomal karyotype
	Wild-type *NPM1* and *FLT3*-ITD^{high†}
	Mutated *RUNX1*
	Mutated *ASXL1*
	Mutated *TP53*

Table 1: ELN risk-stratification model
Complex karyotype = ≥3 acquired chromosome aberrations in the absence of prognostically favourable aberrations; Low, low allelic ratio (<0.5); high, high allelic ratio (≥0.5); semiquantitative assessment of *FLT3*-ITD allelic ratio (using DNA fragment analysis) is determined as ratio of the area under the curve "*FLT3*-ITD" divided by area under the curve "*FLT3*-wild type. Adapted from (Dohner et al. 2017)

Independent of the ELN risk stratification model, age, performance status and achievement of complete remission (CR) after the induction therapy is a strong predictor of survival in AML.(Kern, Haferlach et al. 2003) CR after induction therapy is defined as complete recovery of neutrophils and thrombocytes, and a bone marrow blast count below 5%. The value of this factor probably relies on the fact that current risk stratification systems show a lack of relevant aberrations, which influence the chemoresistance crucially. (Creutzig, Kaspers 2004)

1.1.5 Treatment and therapeutic approach

As described in the chapter above, the choice of AML treatment strongly depends on performance status, age and treatment goal. The aim of treatment in young and fit patients is usually the cure of AML. Once diagnosis of AML is established, induction therapy is given with the goal of complete blast clearance and restoration of normal bone marrow function. 70- 80% of patients will attain CR after intensive induction therapy. However, almost all these patients will relapse without further cytotoxic treatment within eight months. (Cassileth, Harrington et al. 1988) Post-remission therapy aims to destroy leukemic cells surviving induction therapy but which can´t be detected by conventional studies. There are two options for post-remission therapy, depending on the ELN risk model. Favorable risk patients will get two to four cycles of consolidating chemotherapies. Intermediate and adverse risk patients will undergo allogenic hematopoietic stem cell transplantation (allo-HSCT) (A. K. Burnett, Goldstone et al. 2013, Grimwade, Walker et al. 1998) Patients, who didn't reach complete clearance of blasts after two cycles of induction therapy are classified as refractory disease and must undergo salvage therapy. These protocols are usually a combination of high dose chemotherapy and highly urgent allo-HSCT. (Kaya, Tekgunduz et al. 2018, Krejci, Doubek et al. 2013)

No matter which therapy is chosen for induction therapy, post remission or salvage therapy, AraC (AraC) is the backbone and the most important chemotherapeutic agent in AML. (Dohner et al. 2015, Lowenberg, Pabst et al. 2017) Elderly patients or patients ineligible for high dose therapy are treated according to low-dose regimen. Within these regimens, hypomethylating agents like 5-azacitidine or decitabine and low dose AraC, respectively, are used with the goal of symptom control and improvement of bone marrow function. (Dombret, Seymour et al. 2015, H. M. Kantarjian, Thomas et al. 2012). Although a cure is usually not achieved, particularly hypomethylating agents have the potential to induce a CR. Sometimes, long-lasting CRs can be observed.

1.1.5.1 Induction therapy

The most commonly used induction therapy is the so called "7+3" regimen, which is the combination of seven-day continuous infusion of AraC with additional infusion of an anthracycline on three days. Usually the anthracycline daunorubicin is used. There are different studies, which compared different anthracyclines like idarubicin or mitoxantrone, but all showed equivalent rates of CR, OS and cardiotoxicity, one of the main side effects of anthracyclines. (Rowe, Neuberg et al. 2004, MacCallum, Davis et al. 1993) In the last three decades, there have been efforts to add a third therapeutic agent to the "7+3" regimen. A broad range of substances have been tested (sorafenib, fludarabine, etoposide, topotecan, thioguanine, mitoxantrone, vorinostat, clofarabine) but didn't show any benefit.(Buchner, Schlenk et al. 2012, Ho, Lipp et al. 1988, Feldman, Alberts et al. 1993, Estey, Thall et al. 2001, Russo, Malagola et al. 2005, Bishop, Lowenthal et al. 1990, Hann, Stevens et al. 1997, J. Jin, Wang et al. 2013) This changed recently with Midostaurin, an unspecific multikinase inhibitor. This drug has shown beneficial results in the context of addition to standard chemotherapy. However, the usage of midostaurin is limited to AML with a mutation in the FMS-like tyrosinkinse 3 (FLT3) producing internal transmembrane duplications (FLT3-ITD) or changes in the activating loop of the kinase domain (*FLT3*-TKD). Midostaurin was the first additional drug, which was able to improve median OS (75 versus 26 months) and median event free survival (EFS) (8 versus 3 months). (Stone, Mandrekar et al. 2017) In the past year, a second nearly forgotten substance got value as combination partner of standard "7+3" induction. Gemtuzumab-Ozogamicin (GO), a humanized anti-CD33 antibody linked to the chemotherapeutic substance calicheamicin, has been approved by the United States Food and Drug Administration (FDA) in 2000 for treatment of relapsed AML and showed promising results as first drug-antibody conjugate in clinical use. (Norsworthy, Ko et al. 2018) In the following years, criticism about the drug increased and the substance lost its value in AML treatment due to its potential to induce veno-occlusive disease (VOD). (Wadleigh, Richardson et al. 2003, Neumeister, Eibl et al. 2001) Later on, however, it was shown that a different and reduced dosing regimen did not cause these problems and improved the outcome of 7+3 treated patients, which caused the re-licensing of this drug by the FDA and the European Medical Association (EMA).(Castaigne, Pautas et al. 2012, A. Burnett,

Cavenagh et al. 2016) This could be confirmed in a series of large meta-analyses.(Hills, Castaigne et al. 2014, X. Li, Xu et al. 2014, Kharfan-Dabaja, Hamadani et al. 2013) A series of other targeted drugs has recently reached FDA and/or EMA approval, including gilteritinib (Perl, Martinelli et al. 2019), venetoclax (DiNardo, Pratz et al. 2019), enasidenib (Stein, DiNardo et al. 2017) , ivosidenib (DiNardo 2018, Roboz, DiNardo et al. 2020) and glasdegib (Cortes, Heidel et al. 2019). However, in-depth discussion of these substances would be beyond the scope of this thesis. (DiNardo, Wei 2020)

1.1.5.2 Post remission therapy

The choice of optimal post remission chemotherapy in AML is strongly depended on the ELN risk of each patient. Therefore, cytogenetic and molecular analysis is essential in every newly diagnosed AML. (Bloomfield, Lawrence et al. 1998)
The main question of post-remission treatment of AML is; does the patient profit from allo-HSCT or rather from consolidation therapy with chemotherapy only. Autologous stem cell transplantation was evaluated in the setting of consolidation therapy but failed to show any bene fit compared with intensive chemotherapy alone. (Farag, Ruppert et al. 2005, Vellenga, van Putten et al. 2011)

1.1.5.2.1 Favorable risk AML

In favorable risk AML, consolidation chemotherapy with high dose AraC (HiDAC) or intermediate dose AraC (IDAC) appears to provide the best survival with the least amount of toxicity. Patients who undergo this procedure yield survival rates from 75-80%. (Cassileth, Lynch et al. 1992) Allogenic stem cell transplantation is not performed in first CR in those patients, as the high treatment related mortality after allo-HSCT would be higher than the risk of relapse after chemotherapy consolidation alone. HiDAC and IDAC produces high intracellular drug concentrations, which saturate the metabolic enzyme pathway, leading to increased levels of the cytotoxic active intracellular agent tri-ara-cytidine which is integrated in the DNA double strand. In this way, HiDAC and IDAC can often effectively eliminate residual blasts that survived induction with standard dose AraC containing regimens. The usual dosage of AraC in these regimens is between 1 to 3 mg/m^2 given over 3 hours twice daily over five days and varies between different centers. A prospective randomized trial compared AraC dosage of

1.5mg/m² (IDAC) with 3mg/m² (HiDAC) and showed similar rates of EFS and OS with less AraC associated side effects. In this trial, 4 cycles of HiDAC were used as consolidation therapy. (Miyawaki, Ohtake et al. 2011) These results were confirmed in a meta-analysis of our own group. (Magina, Pregartner et al. 2017)Indeed, IDAC is the preferred consolidation in this AML risk group.

1.1.5.2.2 Intermediate risk AML

Allo-HSCT showed significant better OS rates in intermediate risk AML patients. Allogeneic HSCT targets cancer cells both by direct destruction of leukemic blast cells via myeloablative or reduced-intensity conditioning therapy, and by employing an immunological active graft-versus-leukemia effect. Unfortunately, however allo-HSCT is related with high treatment related mortality and morbidity due to graft versus host disease (GvHD). Nevertheless, anti-leukemic effect of allo-HSCT exceeds the side effect and leads to improved OS and EFS results in this risk group. (Schmid, Labopin et al. 2015) HiDAC and IDAC is also a valuable opportunity for intermediate risk patients with an impaired performance status especially after induction therapy. Rates of four-year disease-free survival among patients with intermediate-risk disease are approximately 30 percent. (Weick, Kopecky et al. 1996)

1.1.5.2.3 Adverse risk AML

In adverse risk patients, allo-HSCT in first remission or after ablative salvage therapy is the only option for cure. If adverse risk patients are treated with chemotherapy consolidation only, virtually all that die. (Bloomfield et al. 1998, Dohner et al. 2017) Myeloablative or reduced-intensity conditioning regimen combined with allo-HSCT are therefore the best treatment option for these patients (Cornelissen, van Putten et al. 2007) HiDAC and IDAC consolidation is no option for these patients.

1.1.5.3 Chemoresistance

Despite all progress in risk stratification and optimization of the therapeutic approach, a major problem in AML treatment is the development of chemorefractory disease. After initially good response rates to cytotoxic treatment, a significant proportion of patients ultimately relapse and succumb to chemoresistant disease. (Juliusson et al. 2012) As described above the backbone of AML therapy is administration of the nucleoside analogue AraC. Resistance against AraC is mainly based on three different mechanisms. Firstly, resistance can be caused by insufficient intracellular concentration of AraC. This is caused by alterations of membrane bound nucleoside receptors or impaired generation of active and DNA-damaging AraC metabolites. Secondly, by insufficient intercalation and destruction of the DNA strands, which is based on impaired interaction of AraC metabolites with DNA polymerases. Lastly, by inhibition of apoptosis induction.(Galmarini, Mackey et al. 2001, Zebisch, Lal et al. 2016)

Anthracyclines have been employed as second substance class in combination with AraC in AML treatment. Resistance against anthracyclines is also based on three major mechanisms. Impaired cellular uptake by alteration of anthracycline influx pumps which leads to non-toxic intracellular concentrations. The second type of resistance is due to upregulation of the anthracycline metabolizing pathway and key enzymes within this pathway leading to rapid detoxification of the cell. The third mechanism also arises from the inhibition of apoptosis. (Megias-Vericat, Martinez-Cuadron et al. 2018)

Unfortunately, the current knowledge about these molecular aberrations is scarce and cannot currently be used in the clinical routine to improve the response to cytotoxic therapy in AML.

Induction therapy	
16-60 years	**"7+3"** 3 days of anthracycline (daunorubicin 60mg/m², idarubicin 10-12mg/m² mitoxantrone 10-12mg/m²) and 7 days of continuous infusion of AraC (100-200mg/m²)
> 60 years	same induction, lower dosage can be considered - no specific data available
Consolidation therapy	
16-60 years	**IDAC:** favorable risk patients: 2-4 cycles of intermediate dose AraC (1-1,5g/m²) administered every 12 hours over 3 days, in all other patients allo-HSCT should be strongly considered
> 60 years	favorable risk patients: 2-3 cycles of intermediate dose AraC (0,5-1g/m²) administered every 12 hours over 3 days, in unfavorable risk patients no intensive consolidation therapy has been established (allo-HSCT or study)
Salvage therapy	
	IDAC: as described above +/- daunorubicin 45-60mg/m² or mitoxantrone 10-12mg/m² on day 1-3
	MEC: Mitoxantrone 8mg/m² on day 1-5, etoposide 100mg/m² on day 1-5, AraC 1g/m² on day 1-5
16-60 years	**FLAG-Ida:** Fludarabine 30mg/m² on day 1-5. AraC 1,5g/m², idarubicin 12mg/m² on day 3-5, granulocyte colony stimulating factor (G-CSF) from day 6 to white blood cell count > 1000/µl
> 60 years	**FLAG-Ida:** Fludarabine 20mg/m² on day 1-5, AraC 0,5-1g/m², granulocyte colony stimulating factor (G-CSF) from day 6 to white blood cell count > 1000/µl
Low intensity therapy	
	Decitabine 20mg/m² i.v. on day 1-5 four weekly until progression
	5-Azacitidine 75mg/m² s.c. on day 1-7 four weekly until progression
	Low dose AraC 20mg/m² s.c. every 12 hours on day 1-10 four weekly until progression

Table 2: Summary of chemotherapy regimen
(Dohner et al. 2015)

1.1.6 Pathogenesis and molecular pathogenesis

AML is a heterogenous disease, which develops as a result of (epi)genetic changes in a hematopoietic stem or precursor-cell. More than 90% of AML cases show one or more of these aberrations.(Papaemmanuil, Gerstung et al. 2016) Very often, these changes alter the main biologic features of the hematopoietic cell leading to accumulation of an abnormal number of immature myeloid cells in the bone marrow and the peripheral blood. Leukemia is characterized by uncontrolled proliferation of clonal cells that exhibit maturation block on different stages of hematopoietic differentiation. The normal hematopoietic stem cell is multipotent and can differentiate in all blood linages in order to maintain normal hematopoiesis. Normal stem cell function is based on three central functions: maintenance in a non-cycling state, self-renewal, and production of progenitor cells that are committed to a specific linage. Leukemogenesis is based on alterations within this stem cells or the lineage committed precursor cells. (Botnick, Hannon et al. 1979)

The cancer and genome atlas (TCGA) performed a large study including 200 primary AML patient samples. Within this study they tried to unravel the genomic landscape of AML and showed that an average of more than 13 mutations are present at the diagnosis of AML in each patient. Among all mutations, 23 had been proven to be functionally involved in the pathogenesis of AML. The most frequently mutated genes are CCAAT/enhancer-binding protein alpha (CEBPA), FMS-like tyrosine-kinase 3 (FLT3), nucleophosmin 1 (NPM1), isocitrate dehydrogenase 1 and 2 (IDH1/2), DNA-methyltransferase 3A (DNMT3A) rat sarcoma (RAS), splice factor U2AF and enhancer of zeste homolog (EZH2). (Cancer Genome Atlas Research Network, Ley et al. 2013) A second large study of more than 1500 AML patients was able to corroborate these results and showed that more than 90% of patients carries at least one mutation within these currently mutated genes. (Papaemmanuil et al. 2016) The actual model shows that the development of AML follows a specific and order track in which mutations are acquired in order to produce an overt acute myeloid leukemia. Mutations of epigenetic modifiers like Tet methylcytosine dioxygenase 2 (TET2), IDH1/2, additional sex combs-like 1 (ASXL1) and DNMT3A occur early in the development of AML, whereas mutations in signaling genes like RAS or FLT3 develop later. NPM1 could be shown as second

hit as well, and is frequently linked with NRAS, DNMT3A or IDH1 mutations. (Papaemmanuil et al. 2016)

1.1.6.1 Clonal evolution and the leukemic stem cell

The evolution of AML is based on the accumulation of multiple different (epi-)genetic aberrations. Initially, hematopoietic precursor cells acquire mutations and/or other molecular aberrations that transfer an increased fitness on the mutated cell, but which are insufficient to cause full-blown leukemia. This situation is called clonal hematopoiesis of indeterminate potential (CHIP). In 2014, three large sequencing studies have been published, which demonstrated that CHIP occurs in 4% of the general population and shows an increasing incidence by age. Therefore, 18% of humans over 90 years have clonal hematopoiesis. The most common mutations within CHIP are DNMT3A, TET2 and ASXL1. These are mutations commonly found in myelodysplastic associated AML and AML of elderly people. Absolute risk of progression into leukemia is small, however clonal hematopoiesis was associated with an increased risk of hematologic diseases.(Genovese, Kahler et al. 2014, Jaiswal, Fontanillas et al. 2014, Steensma, Bejar et al. 2015) Indeed, the clonal cells have been shown to increase over time, which is often caused by the ability of these mutations to increase the potential for self-renewal. Over the time, the clone acquires more and more mutations and branches into many different subclones. At a certain time, it might be that enough mutations co-exist, which finally causes the development of frank AML. Due to the branching, bulk AML does not consist of only one clone, but rather of many different subclones.(Jan, Majeti 2013)

The leukemic stem cell (LSC) or leukemia initiating cell (LIC) pool describes the cell compartment within the AML bulk, which is able to generate leukemia on its own. This has mainly been shown by xenotransplantation experiments, where transplantation of LSC/LIC into immunocompromised mice causes the engraftment of AML and to transfer AML in serial transplantation experiments. This is not the case for bulk leukemia cells and clones that do not contain LSCs. The compartment of this LSC is cytometrically well described as CD34+/CD38-/HLA-DR-, and a multitude of novel LSC markers are continuously described.(Reinisch, Thomas et al. 2016, Gaksch, Kashofer et al. 2018) LSC are more immature than the vast

majority of bulk leukemic cells and exhibits self-renewal capacity as well as stem cell like properties. (Bonnet, Dick 1997) Importantly, the LSC compartment often remains in a dormant state, which often relates to the G0 cell cycle state.
Therefore, and also because of additional molecular aberrations in these cells, LSC are often refractory to chemotherapy. Although the bulk leukemia is often successfully eradicated by chemotherapy, the small but surviving number of LSCs gives then rise to a relapse. Often, the molecular architecture of the relapse then resembles the LSC compartment at diagnosis. (Kuster, Grausenburger et al. 2011, Yang, Bhojwani et al. 2008, Tremblay, Saw et al. 2018)

1.1.6.2 Causes of genetic damage

Different environmental factors and hereditary conditions can increase AML incidence.
Development of AML following chemotherapy for a broad range of human malignancies (e.g. breast cancer, germ cell cancer or Hodgkin's lymphoma) is an unfortunate complication. This therapy-related AML (t-AML) typically develops five to seven years following therapy with alkylating agents. Alkylating agents induced AML frequently show complex karyotypes with alteration of chromosome 5 and 7, which leads to dismal prognosis. Substances targeting topoisomerase II (TOP2), like anthracyclines and epipodophyllotoxins, frequently cause t-AML with a rearranged myeloid/lymphoid mixed linage leukemia (MLL) gene. These cases develop with a very short latency under 12 months. (Sill, Olipitz et al. 2011)
Ionizing radiation shares the ability of alkylating agents to introduce double strand breaks in the DNA of hematopoietic precursor or stem cells that may cause mutation and translocation required for malignant transformation. (Little 1993)
As best example for the leukemogenic properties of ionizing radiation, epidemiologic studies of atomic bomb survivors can be used. Incidence of AML increased directly proportional with exposure to the dosage of radiation after atomic bomb explosions. (Ishimaru, Otake et al. 1979)
Exposure to chemical substances like benzene or formaldehyde, which are used in the petroleum industry can also reinforce the risk of developing AML. This substance frequently leads to AML with presence of RAS mutations. (Taylor, Sandler et al. 1992)

New techniques like next generation sequencing have fostered rapid analysis of large regions of the human genome leading to the discovery of an increasing number of mutations causing hereditary AML or familial leukemia predisposition syndromes. Inherited forms of AML can occur as an increase of isolated AML in the pedigree of a family or with AML as part of a complex medical syndrome. (University of Chicago Hematopoietic Malignancies Cancer Risk Team 2016)
Despite these underlying pathophysiologic concepts provide an explanation for development of a small number of AML cases the reason of myeloid leukemogenesis remains unclear in the vast majority of patients.

1.1.6.3 RAS/MAPK/ERK pathway

As described above, mutations and other non-mutational molecular aberrations are detected in almost all AML patients. Many of those are relevant for leukemogenesis. In a significant proportion of AML cases, these (epi-) genetic aberrations affect the RAS mitogen activated protein kinase / extracellular signal regulated kinase pathway (RAS-MAPK/ERK). The RAS-MAPK/ERK is an essential intracellular signal transduction cascade, that mediates proliferative and anti-apoptotic signals from the cell surface to the intracellular effector proteins. Activation of this pathway therefore also occurs in the physiologic setting, however, directly, after signal transduction, the pathway is again inactivated. A series of mutations (like mutations in RAS or a multitude of receptor tyrosine kinases) cause a permanent and pathologic activation of this cascade. This "constitutive" activation is an important step during malignant transformation.
The signaling via this pathway works as follows: membrane bound generic growth factor receptors become activated after receptor ligation via association of the C-terminus of the receptor and SHC, a Src homology (SH) 2 domain-containing protein. SHC itself recruits the GTP-exchange complex factor receptor bound-2 (Grb2)/son of sevenless (SOS) exchange proteins (GRB2/SOS) leading to guanosine triphosphate (GTP) loading of RAS. Rapidly accelerated fibrosarcoma (RAF) activation by RAS/GTP is the next step in the signal-transduction pathway. RAF is a multigene family, which consist of RAF1 (also termed CRAF), ARAF and BRAF. RAF in turn activates mitogen associated/ extracellular regulated kinase -1 and -2 (MEK1/2) by phosphorylation. MEK can phosphorylate extracellular-signaling

regulated kinase (ERK 1/2). ERK is the downstream part of this signaling pathway and regulates more than 150 effector proteins. For example, ERK can translocate into the nucleus of the cell and directly target transcription factors like FOS or ELK1. Those are central proteins of normal cell function and proliferation. (Steelman, Abrams et al. 2008)

Activating alterations of the RAS-MAPK/ERK pathway are known in different human malignancies and play a seminal role in tumorigenesis. Activating mutations of at least one RAS (NRAS, KRAS, HRAS) isoform could be found in approximately 20% of human tumors.(Prior, Lewis et al. 2012, Downward 2003) Also BRAF mutations are frequently detected in human tumors, including malignant melanoma and thyroid cancer. In hematologic malignancies, BRAF mutations are detected in almost all cases with hairy cell leukemia.(Falini, Martelli et al. 2016) (Steelman, Bertrand et al. 2004). Indeed, constitutive activation of the RAS/MAPK/ERK pathway by mutations is relevant for the pathogenesis of these tumors. It mediates cell proliferation, growth and inhibition of apoptosis thereby reinforcing tumor and cell survival. (Platanias 2003, Zebisch, Czernilofsky et al. 2007)

This signaling cascade also plays a role in the development of AML. More than 50% of cases show a constitutive activation. (Platanias 2003, Zebisch, Staber et al. 2006)Activating and oncogenic mutations are frequently observed in the pathway itself. Particularly, the so-called RASopathy mutations (NRAS, KRAS, NF1, CBL and PTPN11) can be frequently detected in AML with NRAS being the most frequent of these alterations. (Steelman et al. 2008, Mullally, Ebert 2010, Liu, Ye et al. 2019) Besides, mutations in upstream activators are very frequent events in AML and include mutations in FLT3, c-kit, G-CSF-R and other receptor tyrosine kinases.(Zebisch et al. 2007)

1.1.6.4 RAF-kinase inhibitor protein (RKIP)

As shown above, the RAS-MAPK/ERK pathway is a pivotal signaling cascade for malignant transformation and leukemogenesis. Therefore, the proper function of this important signaling cascade is protected by countless regulators, which fine-tune the signaling and which avoid overactivation of the pathway. (Kolch 2005)

One of these regulators is RAF-kinase inhibitor protein (RKIP), also known as PEBP1 (phosphatidylethanolamine binding protein 1). RKIP is a negative regulator of the RAS/MAPK/ERK pathway, which interacts with RAF1. Due to this interaction, RKIP blocks the RAF1-driven phosphorylation of MEK and is able to inhibit the propagation of signaling in the cascade. (K. Yeung, Seitz et al. 1999) RKIP additionally mediates effects independently of the RAS/MAPK/ERK pathway by regulating other pathways, including the nuclear factor κB signaling cascade. (Wu, Bonavida 2009) A complete or partial loss of RKIP expression can be observed in a broad range of human malignancies, including carcinomas of the breast, colon and prostate. (Al-Mulla, Bitar et al. 2013) Indeed, a lot of functional studies demonstrated, that RKIP plays a role in the development of these tumors. In particular, RKIP was established as metastasis-suppressor gene. Our own group could highlight the importance of RKIP loss in the development of AML. We could show, that RKIP loss occurs in 20% of AML cases and is functionally involved in myeloid leukemogenesis. (Zebisch, Wolfler et al. 2012) Additionally, we could demonstrate, that RKIP loss correlates with myeloid sarcoma (MS). As described above, MS is the presentation of AML in form of a solid tumor exhibiting similar properties as solid cancer. As leukemic cells have to leave the circulation and have to invade extra-hematopoietic tissues, this process resembles the formation of metastases. In line with the role as metastasis suppressor, RKIP loss is functionally involved in MS development and tissue invasion of leukemic cells. (Caraffini, Perfler et al. 2018)

Much is known about the consequences of RKIP loss in cancer and its gatekeeping function in cellular signaling. However, the reasons causing this RKIP loss remain unclear so far.

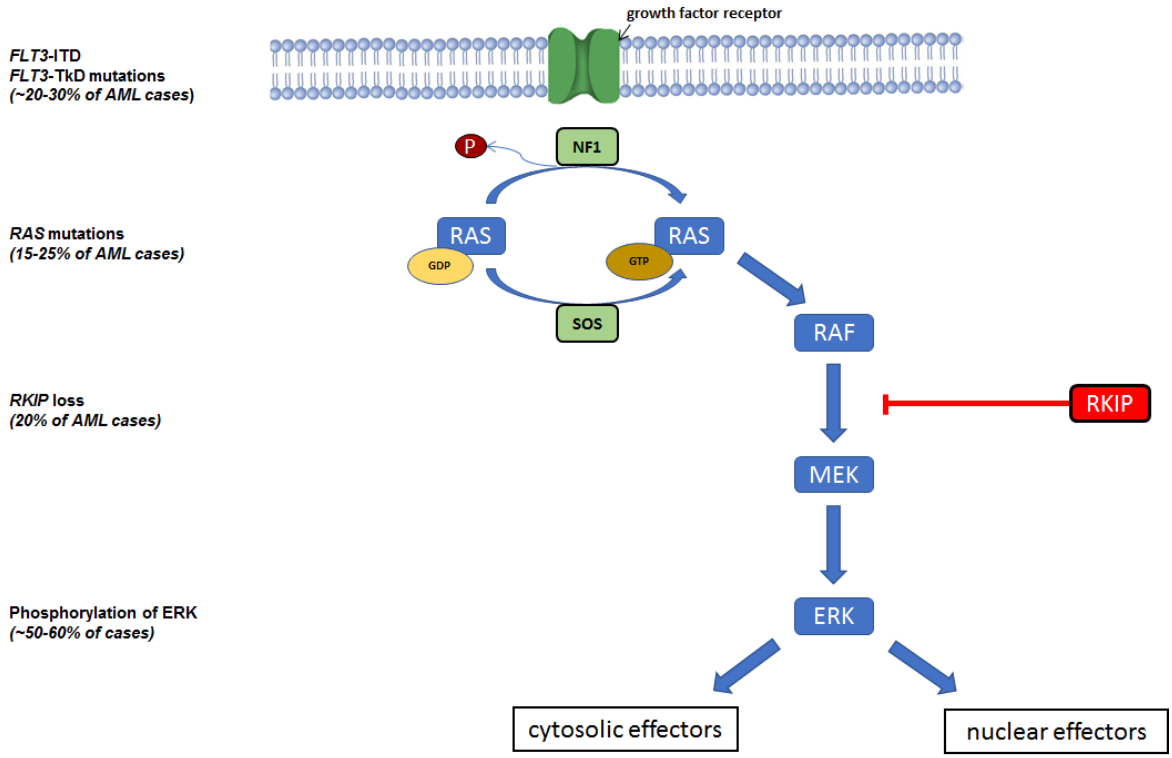

Figure 1: RAS/MAPK/ERK pathway alterations in AML
MAPK pathway is activated in AML by receptor tyrosine kinases like FLT-3. Mutations of the tyrosine kinase domain or internal tandem duplications are present in approximately 30% of AML cases which leads to consecutive downstream activation of the pathway. Next step is activation of RAS by guanine nucleotide-exchange factors (*e.g.*, SOS). Approximately 20% of AML cases harbour an activating mutation of RAS, which leads to activation of RAS/MAPK pathway independent of activation factors. Activated RAS than promotes the formation of RAF dimers which in turn activate the MEK-ERK cascade through phosphorylation. Phosphorylation of ERK is present in 50-60% of AML cases. RKIP is a negative regulator of the RAS/MAPK/ERK pathway and in frequently lost or suppressed in AML.

1.2 MicroRNA´s

1.2.1 Gene regulation by microRNAs

MicroRNAs (miRNAs) are short, evolutionary highly conserved noncoding ribonucleotide acids (RNA), which play a seminal role in gene regulatory networks and gene silencing. MiRNAs are transcribed from genomic sequences that are not involved in protein synthesis and comprise between 21 and 23 nucleotides. The first miRNA was described in 1993 in the nematode Caenorhabditis elegans (C. elegans) and was named lin-4. The lin-4 RNA was able to regulate the expression of the lin-14 gene by posttranscriptional binding to the 3 prime untranslated region (3´UTR) of *lin-14* messenger RNA (mRNA) and inhibiting its translation into the lin-14 protein. (Lee, Feinbaum et al. 1993)

Biogenesis of miRNAs starts with an intronic genome sequence in the nucleus of a cell by transcription due to RNA polymerase II and III which produces the 500 to 3000 nt long primary transcript called primary microRNA (pri-miRNA). In the next step of miRNA synthesis, the microprocessor complex consisting of RNAse III (called Drosha) and the dsRNA (double strand RNA) binding protein DGCR8 (called Pasha) processes the pri-miRNA to a 70-80 nt long precursor miRNA (pre-miRNA) with its typical hairpin structure. After cytosolic export, a specific RNAse III called Dicer cuts away the loop joining the 5´and 3´ part and produces an imperfect complex consisting of two complementary miRNAs (miRNA-5p: miRNA-3p) In most cases only 5´ terminus form of the miRNA complex is functional active and is called guide or mature miRNA (miRNA-5p). The complementary 3´terminus miRNA is called miRNA-3p and plays inferior roles in most cases of gene regulation. (Kim, Nam 2006)

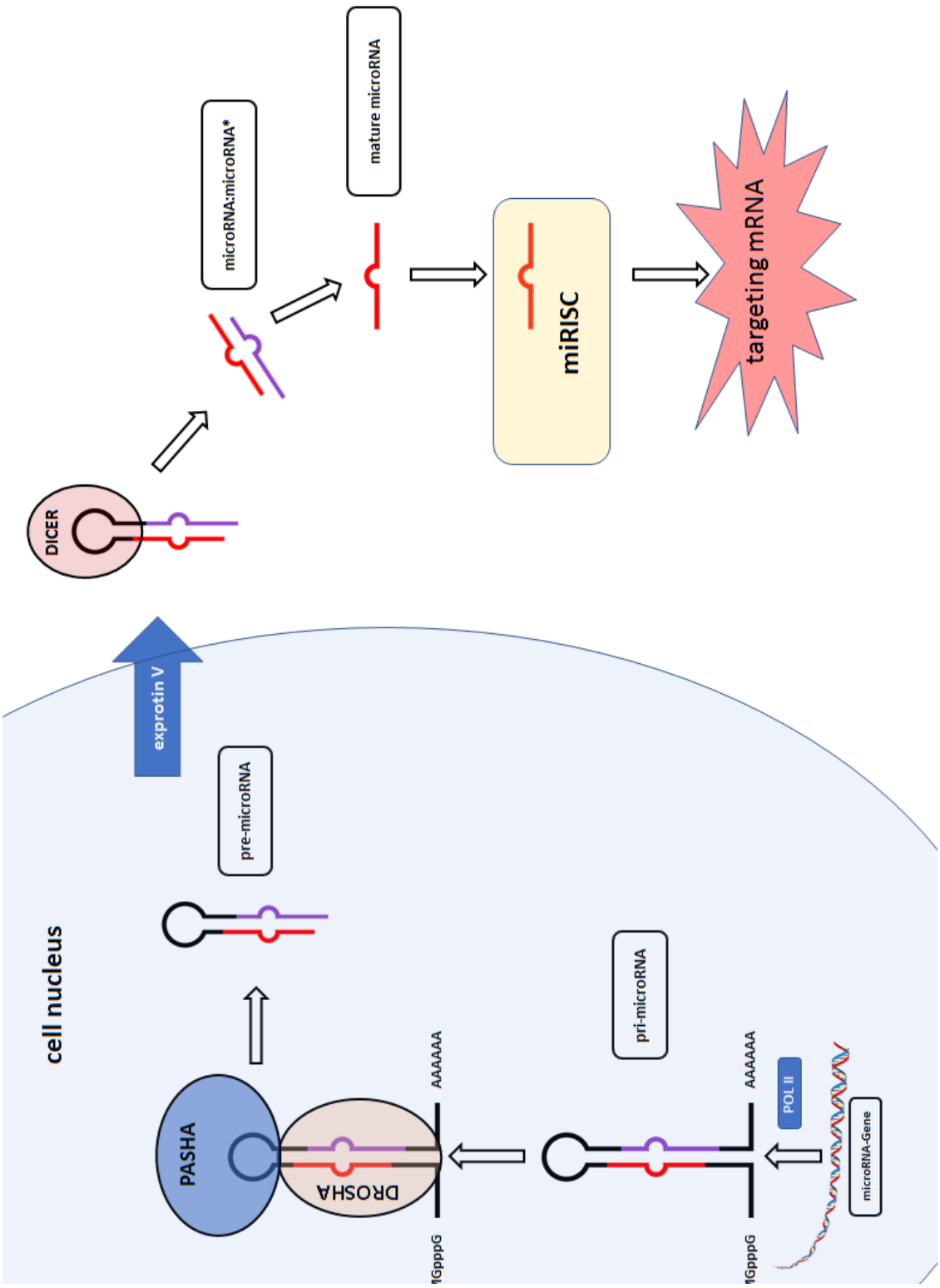

Figure 2: Maturation and action of microRNAs

MicroRNAs are essential in the regulation of cellular gene expression. Computer based algorithms predicted a multitude of genes and cell signaling networks, which are influenced by miRNAs highlighting their importance in healthy and diseased cells. (Lewis, Burge et al. 2005, Xie, Lu et al. 2005)

In mammalian cells most miRNAs bind to the 3´UTR of a target messenger RNA and function as adaptor for miRNA induced silencing complex (miRISC). A key feature of miRNA binding within the 3´UTR involves the Watson-Crick base pairing from the second to eighth nucleotide of miRNA, displaying the seed region. Despite the fact of perfect base pairing in the seed region, animal miRNA binding shows mismatches and bulges. In contrast to animal miRNA binding, plant miRNAs show near perfect complementarity to the whole coding region of their target mRNA, representing an evolutionary difference. Complementarity of miRNA to target 3´UTR determines the mechanism of regulation. Therefore, a high degree of complementarity enables Argonaut (Ago) catalyzed cleavage of messenger RNA, while low degree of complementarity exclude cleavage of Ago and leads to translational block. Argonaut proteins are the key components of RISC, thus involved in the RNA interference pathway influencing post transcriptional gene regulation either by miRNA or small interfering RNAs (siRNA). However, distinction between miRNA and siRNA is part of ongoing debates in RNA science and is still not solved. (Carthew, Sontheimer 2009). The precise mechanisms of miRISC induced inhibition of mRNA translation is a major point of ongoing debates and remains unclear.

Normal mRNA translation starts with the recognition of the 5´mRNA terminus by eukaryotic initiation factors 4e (eIF4e). In the next step, a complex consisting of different eIF develops, leading to the recruitment of ribosomal 40S subunit by eIF3. 40S subunit itself joins 60S ribosomal subunit at the AUG codon to prepare elongation. eIF4G has the ability to bind the poly adenylated 3´end as well as 5´end via interaction with eIF4e allowing the mRNA strand to circularize. Circularization massively enhances the translation efficacy.

On the one hand there is evidence that miRISC can inhibit translation at the initiation stage by blocking of initiation factor binding to the 5´mRNA cap (Humphreys, Westman et al. 2005) or blocking the recruitment of 60S subunit of ribosomes. (Pillai, Bhattacharyya et al. 2005) On the other hand miRISC can repress translation at post-initiation stage by inducing premature ribosomal drop

of. (Petersen, Bordeleau et al. 2006) Alternatively, it could be shown that miRISC can deadenylate the 3´end of target mRNA leading to impossibility of mRNA circularization and mRNA decay. (He, Hannon 2004)
All different mechanisms of miRNA action could not be reconciled under one big unifying theory up to now and it is a big issue of basic RNA research.

1.2.2 MicroRNAs in malignant transformation and leukemogenesis

For a long time, it has been thought that malignant transformation of cells is caused by somatic events leading to alteration in protein coding genes, which are classified as tumor suppressor genes or oncogenes. One of the best examples for this malignant transformation is displayed in CML. This disease is caused by somatic t (9;22) translocation of chromosome 9 and 22 which leads to BCR-ABL (breakpoint cluster region - Abelson murine leukemia viral oncogene homolog) with dysregulation of the ABL oncogene. (Rowley 1973) It is now also known, that tumor development and malignant transformation can be caused by epigenetic alteration of tumor suppressor genes or oncogenes, such as methylation of cytosine and guanosine rich (CpC) islands within their promotors.
MiRNAs as potential cause of malignant transformation were firstly investigated in chronic lymphocytic leukemia (CLL) with a loss or translocation of the long arm of chromosome 13 (13q14). It could be shown that the precursor-genes of miR-15a and miR-16 are located within 13q14 and that loss of this microRNAs are frequent and early events in the development of CLL. (Calin, Dumitru et al. 2002) In CLL, miR-15a and miR-16 function as tumor suppressors. However, microRNAs can also function as oncogenes. For example, miR-155 has been shown to be overexpressed in different tumor entities, including AML and Burkitt's lymphoma. (Metzler, Wilda et al. 2004, Garzon, Volinia et al. 2008). Furthermore, it was proven that miR-155 is directly involved in lymphatic leukemogenesis by transgenic mouse models exhibiting consecutive overexpression of miR-155. Mice with overexpression of miR-155 developed high-grade lymphoma or acute lymphoblastic leukemia (ALL). (Costinean, Zanesi et al. 2006) These findings of oncogenic potential could be corroborated in serval other microRNAs. Another mechanism of microRNAs in oncogenic transformation is the dysregulation of transcription factor or DNA

methyltransferases. On the other hand, microRNAs are itself regulated by transcription factors or underlie epigenetic silencing by methylation of their promotors. (Garzon, Liu et al. 2009) Interestingly, one microRNA can function as tumor suppressor in one specific tissue and enforce proliferation and malignant transformation in other tissue, therefore classification of general microRNA function should be avoided. MiR-222 for example inhibits proliferation and forces differentiation in pure erythroid leukemia by targeting the oncogenic stem cell receptor KIT, on the other hand miR-222 leads to oncogenic transformation in several solid carcinomas via downregulation of the tumor suppressor phosphate and tensin homolog (PTEN).

MicroRNAs are part of complex gene regulatory networks and play seminal roles in malignant transformation of human malignancies, but the assessment of their specific function is complex and strongly depends on the analyzed tumor. (Croce 2009, Croce 2008)

1.2.3 MicroRNAs and therapeutic resistance

Although high rates of complete remission (CR) after the first induction therapy can be achieved, the vast majority of AML patients develop relapse and succumb to chemoresistant disease. The same problem occurs in old and/or frail patients, who get refractory to hypomethylating agents or relapse after good response. Despite all progress, which has been made in risk stratification and optimizing of AML treatment, development of therapeutic resistance over the courses of chemotherapy is still a major problem. In the past time, more and more studies investigated the role of microRNAs in the development of therapeutic resistance. Our group recently published a review regarding this topic: In young patients treated with intensive chemotherapeutic regimens, two groups of microRNAs could be distinguished. The one group comprises all microRNAs where high expression lead to therapeutic resistance, and the other group where high expression to a high sensitivity to chemotherapy. (See Table 4) For example miR-181 is induced by N-terminal mutations of CCAAT/enhancer-binding protein alpha (CEBPA), which itself produces a truncated CEBPA-30p isoform protein. MiR-181 then directly binds the 3´UTR of B-cell lymphoma 2 (BCL2) which leads to its downregulation. Downregulation of BCL2 in turn induces sensitivity against AraC

and daunorubicin. The CEBPA/miR-181/BCL2 axis is a well-studied example of how microRNAs can alter chemotherapeutic properties of AML. MicroRNAs are also able to induce resistance against hypomethylating agents (HMA) and influence prognosis and survival of old and frail patients suffering from AML. The best studied microRNA in context of HMA resistance is miR-29b. Functional experiments showed that gain of function mutations in the stem cell receptor KIT, which are quite frequent in AML, increase KIT activity. KIT in turn decreases miR-29b via downregulation of the oncogene MYC. MiR-29b directly binds the 3´UTR of DNA (cytosine-5)-methyltransferase 3A (DNMT3A). Therefore, KIT induced downregulation of miR-29b increases DNMT3A levels. High DNMT3A expression in turn leads to resistance against the HMA decitabine.

microRNA associated with sensitivity		microRNA associated with resistance	
let-7a	miR-27	(miR-20)	miR-191
let-7f	miR-96	miR-32	miR-196b
miR-9*	(miR-128)	miR-125b	miR-199a
miR-10	miR-135	miR-126	mir-210
miR-19	miR-181a	(miR-128)	miR-644
(miR-20)	miR-331	mir-155	mir3151
	miR-409		

Table 3: miRNAs linked to therapeutic resistance against high dose chemotherapy
This tables shoes the published miRNA with association to resistance or sensitivity against high dose treatment of AML. For (miR-20a) and (miR-128) there is date for resistance and sensitivity available. This table comprises clinical as well as experimental data.

1.2.4 miRNAs in AML – previous data of our group

Our group previously studied the reason for RKIP loss in AML.

In this respect, it is important to know that the reasons for RKIP loss in AML were unknown so far. We and other groups failed to identify mutations or deletions within the RKIP gene leading to decay of RKIP protein. (Al-Mulla et al. 2013, Zebisch et al. 2012, Zebisch, Haller et al. 2009) Epigenetic methylation of CpG rich island of the RKIP promotor could also be excluded.(D. X. Li, Cai et al. 2014, Zebisch et al. 2009, Zebisch et al. 2012) Maja Kim Küpper analyzed the involvement of transcriptional repressors as part of her master thesis in our group. Therefore, expression of six transcriptional repressors (EZH2, SNAI1, BACH1, NFE2L2, YY1, SLUG) was analyzed by quantitative polymerase chain reaction (qPCR). In a next step, expression levels of the six transcriptional repressors were compared between 10 patients with normal RKIP and 10 exhibiting a loss of RKIP. Interestingly no difference in transcriptional repressor expression could be overserved among the two groups suggesting that transcriptional repressors do not influence RKIP expression in AML. After exclusion of common reasons of protein loss, microRNAs are a potential remaining reason for RKIP loss. Indeed, Maja then analyzed the results of a microRNA chromatin immunoprecipitation (ChIP) array in 33 primary AML patient samples, which were characterized for RKIP expression as well. These analyses included over 600 different microRNAs. (Rommer, Steinleitner et al. 2013) When the samples with normal RKIP expression and RKIP loss were compared, she found a set of 8 microRNA with a different expression between these RKIP groups. Six microRNAs (miR-15a, miR-23a, miR-23b, miR-24, miR-320a and miR-519d) showed higher expression in patients with RKIP loss. Two microRNAs (miR-10a, miR-518b) showed lower expression in patients with RKIP loss. As second part of her master thesis Maja Kim Küpper evaluated these candidate microRNAs in an independent cohort of 10 AML patient specimens exhibiting RKIP loss and 10 patients with normal RKIP expression by qPCR. This was to confirm the array results, because of the high false positivity rate that can occur in microRNA ChiP arrays.

Indeed, four microRNAs could be validated (miR15a, miR-23a, miR-23b, miR-24 and miR-320a) and showed higher expression in patients harboring a loss of RKIP. This means that they could be a potential reason behind loss of RKIP in AML. To further clarify this possibility, it was initially important to exclude that the

increased expression of this microRNAs in AML cases with RKIP loss is not a result of RKIP downregulation. This was done by studying the role of these microRNA expressions in a transgenic mouse model with *RKIP* deletion. Indeed, microRNA expression didn't differ between RKIP wildtype mice and those animals with *RKIP* deletion, which proves that the increased expression of these microRNAs is not a result of RKIP loss. Taken together, these data suggest that indeed one of these microRNAs might be the reason for the downregulation of RKIP in AML. However, no functional experiments were performed to clarify this issue.

1.3 Hypothesis and aims of the study

In my thesis, we aimed to extend our knowledge about microRNAs in AML.

In a first part of the thesis, we aimed to further clarify the role of microRNAs in the development of RKIP loss in AML. Based on the preliminary data presented above, we thereby hypothesized that loss of RKIP in AML is caused by the increased expression of one or more miRNAs. To test this hypothesis, we decided to test the functional role of the candidate miRNA described above on regulation of RKIP expression, and consequently, on leukemogenesis.

The second part of the thesis has been performed and designed as follow-up part of the section above. At this time, we already knew that miR-23a is a functional regulator of RKIP and demonstrates increased expression in AML. As miR-23a has been shown to play a central role in the development of therapeutic resistance in solid cancers, we thereby hypothesized that miR-23a confers therapeutic resistance to conventional 7+3 chemotherapy regimens in AML as well. We decided to use a series of in-vitro assays to delineate the effects of RKIP on therapeutic sensitivity in AML. In case miR-23a causes therapeutic resistance, we further decided to delineate potential mechanisms and target genes, which mediate these effects.

2 MATERIAL AND METHODS

The experiments in this study were performed as described in the following section and are also illustrated in Hatzl et al. (Hatzl, Geiger et al. 2016) and (Hatzl et al. 2020)

2.1 Patient samples and cell lines

The study was approved by the institutional review board of the Medical University of Graz (MUG), Graz, Austria. (ethical vote 24-036 ex 11/12) Written informed consent was obtained from every individual involved in this study.

AML patient samples for microRNA and *RKIP* qPCR as well as RKIP immunoblot were collected at the Division of Hematology at the MUG (cohort I). For *RKIP* and microRNA array analysis, an independent Dutch-Belgian cohort (cohort II) consisting of 214 AML patients was collected by cooperation partners at the Erasmus University of Rotterdam, the Netherlands. (Jongen-Lavrencic, Sun et al. 2008)

Twenty-four paired AML patient samples for microRNA as well as *topoisomerase 2 beta* (*TOP2B*) qPCR were obtained at diagnosis and relapse at the Division of Hematology at the MUG.

All patient samples used within this study were purified for mononucleated cells to yield blast count > 80% using Ficoll-Hypaque (Nygaard, Oslo, Norway) centrifugation. Patient samples were derived from bone marrow (BM) aspiration and peripheral blood (PB) samples, which were then stored in liquid nitrogen (-195.5°C) in 0.8 mL (80%) RPMI-1640, with 0.1 mL (10%) dimethyl sulfoxide (DMSO) and 0.1 mL (10%) fetal calf serum (FCS).

AML was classified according to the French American British (FAB) (Bennett et al. 1976) and WHO (Arber et al. 2016) guidelines. Risk stratification was performed according to the ELN guidelines (Dohner et al. 2017) For use in this project cryopreserved patient samples have been thawed up in a water bath at 37°C. In order to remove residual cytotoxic DMSO, cell pellets were washed in 40ml phosphate-buffered saline (PBS) (8000/min, 5min, 4°C), resuspended in 500µl PBS and immediately used for further experiments.

AML cell lines (THP-1, NB-4, U937, HL-60) were obtained in 2007 from the German National Resource Centre for Biological Material *(DSMZ, Braunschweig, Germany)*.

HEK-293 cells were obtained from the Centre for Biomedical Research at MUG in 2015.

The identity of stable cell lines was confirmed by variable number tandem repeat (VNTR) DNA profiling using the AmpF/STR Profiler Plus Kit and ABI PRISM 310 Genetic Analyzer (both by Applied Biosystems, respectively) according to the manufacturer's protocols and as described previously.(Zebisch et al. 2012)

Suspension cell lines were cultivated for no longer than six months in fully supplemented RPMI-1640 (Sigma-Aldrich) with 10% HyClone foetal bovine serum (FBS) (Thermo Scientific) and 1% 1x Antibiotic-Antimycotic (Life technologies) in a humidified chamber at 37 °C and 5% CO^2. Adherent HEK-293 cells were cultivated in fully supplemented DMEM (Sigma-Aldrich) with 10% HyClone FBS (Thermo Scientific) and 1% 1x Antibiotic-Antimycotic (Thermo Fisher Scientific; comprising 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B) in a humidified chamber at 37 °C and 5% CO^2.

Cell line	Cell type	Doubling	Medium
U937	monocytic /histiocytic sarcoma	30-40h	RPMI-1640
THP-1	acute monocytic leukemia	35-50h	RPMI-1640
NB-4	acute promyelocytic leukemia	35-45h	RPMI-1640
HL-60	acute undifferentiated myeloid leukemia	~40h	RPMI-1640
HEK-293	embryonal kidney cells	24-30h	DMEM

Table 4: Cell lines and their origin

2.2 qPCR expression analysis

2.2.1 RNA isolation and cDNA preparation

Whole RNA was extracted from patient samples and cell lines using TRIzol® (Invitrogen) according to the manufacturer's protocol. TRIzol® reagent is a monophasic solution of phenol and guanidine isothiocyanate, which was established for single step liquid-liquid phase extraction. This technique is based on different solubility of RNA and DNA. RNA is separated from DNA by acidic guanidinium thiocyanate, sodium acetate, phenol and chloroform solution followed by centrifugation. Total RNA remains in the acidic aqueous phase while DNA is dissolved in the organic phase. The original protocol of this technique was established in 1987 and is now widely used within different ready-to-use kits. (Chomczynski, Sacchi 1987, Chomczynski, Sacchi 2006)

RNA quantity and quality were evaluated using a spectrophotometric method. Therefore, total RNA was diluted 1:50 in RNAse free water after TRIzol® extraction and isopropanol precipitation. Optical density was assessed at a wavelength of 260nm using the NanoDrop Microvolume Spectrophotometer (Thermo Scientific). Quality of RNA was assessed using absorbance ratio 260:280. These methods are based on the principal that nucleic acids absorb ultraviolet light in a specific manner. The more light absorbed in the sample the higher the nucleotide concentration. (Glasel 1995)

Due to instability of RNA, reverse transcription (RT) of total RNA was performed in order to produce complementary DNA (cDNA). cDNA of samples was stored at -20°C until usage in experiments. cDNA was synthesized from 1µg RNA using the Taq Man® Reverse Transcription Reagents (Applied Biosystems) for mRNA and miScript II Reverse Transcriptase Kit (Qiagen) for miRNA, respectively. Random hexamers were used as RT primer for mRNA. Random hexamers are six randomly composited nucleotides that are able to bind anywhere on mRNA and induce reverse transcription.(Gubler, Hoffman 1983) Stem-loop RT primers were used for miRNA reverse transcription. (M. F. Kramer 2011)

2.2.2 Expression analysis using qPCR

Real-time quantitative PCR (qPCR) for miRNA expression analysis was performed on a LightCycler 480 Instrument II (Roche Life Sciences) using the miScript SYBR Green PCR Kit (Qiagen). qPCR for mRNA expression analysis was performed on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) using the SYBR Green method (Invitrogen). Both modalities use the SYBR-green methodology. SYBR green is an asymmetric cyanine fluorescent dye, which binds ds-DNA and allows its quantification. SYBR green and ds-DNA produces a complex, which absorbs blue light at a wavelength of 494nm and emits green light at a wavelength of 521nm. SYBR-green change its fluorescence emission properties when ds-DNA bound compared to free state.

During temperature increase in PCR, both complementary DNA strands become separated and fluorescence goes down. In the next step of PCR, primers bind the single stranded DNA and DNA-polymerase synthesizes new DNA molecules leading to an increase of ds-DNA. With rising concentrations of ds-DNA, fluorescence increases due to increasing SYBR-green which intercalates the ds-DNA.(Zipper, Brunner et al. 2004)

In order to guarantee reproducibility of qPCR data, every sample and the negative control were performed in triplicates. The median was used for further calculations. Negative controls failed to produce fluorescence during the qPCR analysis (Ct > 34).

Cycle of threshold (Ct) is an arbitrary set value in exponential amplification phase of qPCR and must be the same for all samples in each PCR plate.

Expression level of mRNA as well as miRNA were evaluated using the ΔΔCt method which is a calculation model for quantification of genes relative to ubiquitously expressed non regulated genes, so called housekeeping genes (HKG), in qPCR.(Livak, Schmittgen 2001)

ß-2-microglobulin (B2M) and ß-glucuronidase (GUSB) served as housekeeping genes for mRNA expression analysis. Both genes were shown to be stably expressed in normal and leukemic patient samples and are suitable for qPCR analysis. Additionally, both genes were stably expressed over the course of chemotherapy and expression didn't differ in diagnostic and relapse samples. (Beillard, Pallisgaard et al. 2003) RNA-U6-small nuclear (RNU6) and small nucleolar RNA 44

(SNORD44) were used as housekeeping genes for microRNA qPCR. (Morata-Tarifa, Picon-Ruiz et al. 2017)

In the first step of ΔΔCt method geometric mean of the two HKG is built.

$$Ct_{GM}(HKG) = \prod_{i=1}^{2}(Ct(HKG)_i)^{\frac{1}{2}}$$

The use of two HKG and calculation of their geometric mean provides more accuracy and cross-laboratory data comparability than use of only one HKG. (Vermeulen, Pattyn et al. 2009)
In the next step median of HKG and target gene (TG) triplicates is built in order to provide higher precision and stability against outliers

$$\widetilde{Ct_{GM}}(HKG) = \left\lfloor \frac{n+1}{2} \right\rfloor$$

$$\widetilde{Ct}(TG) = \left\lfloor \frac{n+1}{2} \right\rfloor$$

Then, the TG median is normalized with the median of HKG triplicates, which promotes compensation for differences in sample RNA content.

$$\Delta Ct = \widetilde{Ct}(TG) - \widetilde{Ct_{GM}}(HKG))$$

In the last step, expression of a sample is compared with a given calibrator.
$\Delta Ct_{calibrator}$ is calculated in the same manner than ΔCt_{TG}.
NB-4 AML cells were included on each qPCR plate and were used as calibrator for miRNA and mRNA qPCR of patient samples as well as cells transfected with the empty vector/scrambled control were used as calibrator for all other experiments.

$$\Delta\Delta Ct = \Delta Ct - \Delta Ct_{calibrator}$$

The ΔΔCt value is expressed as $2^{-\Delta\Delta Ct}$ and multiplied with 100 in order to calculate the expression of the target gene as a percentage of the calibrator, which thereby demonstrates a value of 100%.

All primers used in this study for mRNA amplification were selected form “Harvard Primer Bank” (*https://pga.mgh.harvard.edu/primerbank/*) and tested with National Centre for Biotechnology Information (NCBI) Primer – Basic Local Alignment Search Tool
(BLAST) (*https://www.ncbi.nlm.nih.gov/tools/primer-blast/*) to validate their specificity, confirm that they comprise all relevant transcript variants, and to rule out similarities between primer sequences leading to self-annealing or dimerization. Furthermore, all primers (reverse and forward) were designed to bridge exon-exon junction to beware of genomic DNA amplification. Primers for mRNA amplification were purchased from MWG Eurofins. (Eurofins MWG Operon, Ebersberg, Germany)

Gene	Forward	Reverse
TOP2B	AGCCATTGACGCAGTTCATGT	CCTGGCACAAAGGTAACCTCC
RKIP	CAGACAGGAAGTAGCAGCTCCT	CTGGTCATGTTGATGAAGGTGCT
B2M	CGCTCCGAGATGCATGTG	TTGGCTGGCAGTCCTTTAGG
GUSB	CCTGAAGGTGGCTGTGAAGATG	GCTCCCAGAAGGTTGACGATG
SNORD44	Qiagen, Cat# MS00007518	
RNU6	Qiagen, Cat# MS00003740	
miR-15a	Qiagen, Cat# MS00003178	
miR-23a	Qiagen, Cat# MS00031633	
miR-24	Qiagen, Cat# MS00006552	

Table 5: Primer sequences
Primers used for mRNA expression analysis were designed like described in the chapter above via Primer bank. Primers for expression analysis of short RNA sequences used as control genes for miRNA expression as well miRNA primers were order using this specific ordering information.

2.3 Transfection and transduction of cell lines

All stably transfected cell lines were generated by lentiviral transduction.
In a first step the virus is amplified in packaging cells, therefore 293T (a derivate of HEK-293 cell) were cultured as described in the chapter above and transfected by a lentivirus. The used lentivirus consists of two empty backbone constructs (w262, w263) (Addgene) and the gene of interest DNA. For packaging and amplification of the virus, 6µg of each backbone vector as well as 2µg of the gene of interest DNA were mixed. In order to enhance DNA uptake into the cells the nucleic acid is presented to the 293T cells as co-precipitated of calcium phosphate and DNA. This insoluble complex attaches to the surface of the cells and is incorporated by them by endocytosis. (Sambrook, Russell 2006) This step is performed using the CalPhos ™ Mammalian Transfection Kit (Clonetech/Takara) according the manufactures instructions. However, most of the input DNA is degraded before it reaches the nucleus of the cells, where gene expression and DNA replication take place. Much of the degradation apparently occurs in the lysosomes. To further improve virus uptake, 293T cell were treated with 6µl of 100µM chloroquine (Sigma-Aldrich). Chloroquine inhibits the lysosomal degradation and decreases plasmid concentration within the cell nucleus. (Luthman, Magnusson 1983) In the next step, hematopoietic cells were transduced by 293T produced virus. To optimize viral intake into the hematopoietic suspension cells, 24-well plates are coated with RetroNectin® - Recombinant Human Fibronectin Fragment (Clontech/Takara). It could be shown that recombinant fibronectin fragment was an efficient tool for enhancing gene transfer into hematopoietic cells using retro/lenti-viral systems. RetroNectin® which contains three functional domains, *i.e.* the cell-binding domain (C-domain), heparin-binding domain (H-domain), and CS-1 sequence, enhances retroviral mediated gene transduction by merging target cells and viral particle on the RetroNectin® molecule. The C-domain and CS-1 sequence interact with target cells through integrin receptors, and viral particles can be localized upon the H-domain composed. (Chono, Yoshioka et al. 2001) After RetroNectin® coating of not TC treated 24-well Suspension Culture Plate (Greiner Bio-one Cellstar) $5x10^4$ haematopoietic cells were added to each well and incubated for 72h. Addition of 1000µl of viral supernatant produced by transfected 293T was repeated every 24h

to improve transduction efficacy. After successful transduction, stable selection was performed using 2.5 µg/mL puromycin (Sigma-Aldrich)

Stable U-937 RKIP overexpressing cell lines and empty control vector cells were generated previously either by transfecting $1x10^6$ U-937 cell with *pMSCV-FLAG-hRKIP* or empty control vector.(Zebisch et al. 2012)

For stable RKIP knockdown U-937 were lentivirally transduced with either *RKIP shRNA* or empty control with psi-LVRU6GP (Genecopeia) as described above.

MiR-23a stably overexpressing U-937 as well as THP-1 cells were generated by the lentiviral transduction procedure as described above. Therefore, a miR-23a stem-loop overexpression vector and a scrambled control vector, both pEZX-MR03 (Genecopeia) were used.

For transient transfection of cells, optimized lipofection protocols were used. Lipofection uses the phenomenon that eukaryotic cells can take up exogenous DNA under appropriate conditions, and a portion of this DNA becomes localized in the nucleus. However, due in part to the size and charge of DNA the spontaneous entry of intact DNA into the cell and its subsequent expression in the nucleus is a very inefficient process. All commercially available lipofection reagents are cationic, bilayer-forming lipids that give rise to physically stable liposomes. DNA interacts spontaneously with the lipofection solutions in order to form lipid-DNA complexes. This complex formation presumably is due to ionic interactions between the positively charged group on the lipofection reagent molecule and the negatively charged phosphate groups on the DNA. In the next step the lipid-DNA complexes fuse with the cell membrane and allow transient transfer of DNA into the cell.

In this study, miR-23a-3p mimics, hairpin inhibitors, and scrambled controls (Dharmacon and Qiagen) as well as *TOP2b siRNA* (ON-TARGETplus® TOP2B siRNA – Dhramacon) were transfected at a concentration of 20 nmol/L using DharmaFECT2 (Dharmacon) for suspension cell lines or Lipofectamine RNAiMAX (Thermo Fisher Scientific) for adherent cell lines. Transient transfection was carried out in 6-well plates according to the manufacturer protocol.

2.4 Evaluation of chemoresistance

Therapeutic resistance was evaluated by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-based cell viability assays. MTT cytotoxic is based on the ability of living cells to reduce the yellow tetrazolium salts to their intensely violet colored formazan. The intensity of color is assessed by a multi-well scanning spectrophotometer (ELISA reader) and is directly proportional to the number of cells viable. The cells can then additionally be incubated with cytotoxic agents, and the MTT-assay is used to assess the viability after this incubation.

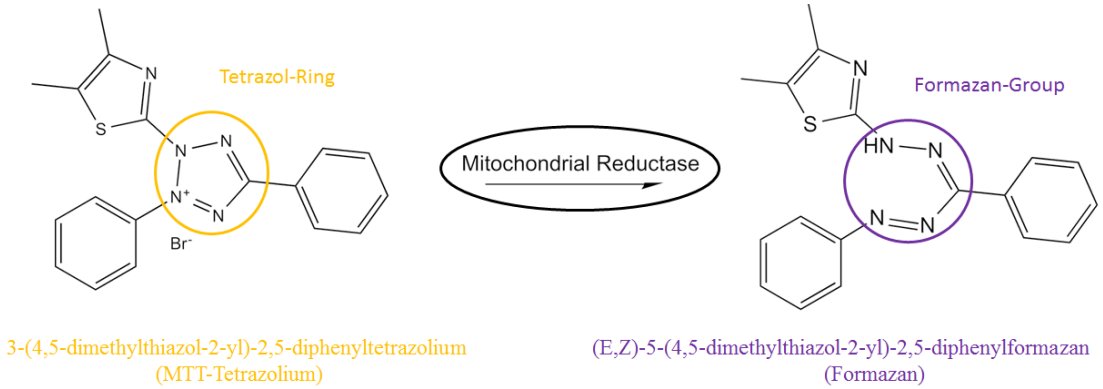

Figure 3: Reduction of tetrazolium to formazan
Tetrazolium salts are chromogenic dyes produced by reduction of Tetrazolium. Tetrazoles consist of a 5-member ring of four nitrogen and on carbon atom. Typical for the MTT-formazan is the deep purple color and its solubility in DMSO. The main part of the reduction of MTT takes place in the mitochondria of cell by mitochondrial reductases. Both, tetrazoles and formazans are artificial molecules which are unknown in nature.

In more detail, transfected/transduced cells in exponential growth phase were harvested and seeded into 96-well culture plates at a density of 3×10^4 cells. Cells were then treated with AraC (Sigma-Aldrich) or Daunorubicin (Sigma-Aldrich) at various concentrations. Chemotherapeutic agents were dissolved in RPMI-1640 supplement with 10%FBS in order to yield final concentration. Cells were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2. Towards the end of the incubation for 48h a CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT-assay) (Promega) was performed according to the manufacturer's protocol.

Therefore, 20 µl MTT assay solution was added into each well. Subsequent to 4 h incubation at 37°C, the 96-well plate was centrifuged at 2500/min for 10 min at room temperature in order to sediment the purple colored precipitate of formazan.

In the last step, the supernatant was discarded, and the precipitate was dissolved in 200µl DMSO. Absorbance was detected at 490 nm wavelength using an automatic multi-well spectrophotometer Infinite®F50 (Tecan)

The dose-response curve was recorded at different concentrations and IC50 values were calculated. Each concentration was analyzed in triplicate. Each experiment was repeated at least 3 times to increase the reliability and reproducibility.

For AraC depending colony forming assays, cells were pre-incubated with 5 µM AraC and empty dissolvent, respectively, for 2 hours in 48-well plates. Soft-agar assays were set up in 6-well plates, each well containing 2 ml of a bottom layer of 0.5% SeaPlaque agarose (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and a top layer (2 ml) of 0.4% agarose containing 7500 cells. The plates were incubated for 8-10 days. To assess colony frequencies, at least 15 pictures were taken per well and large colonies (> 5000 pixels) were quantified with ImageJ (*https://imagej.net*).

2.5 Immunoblotting

Cell lysis was performed using a combination of chemical lysis in special buffer and mechanical disruption of cells by thawing and freezing. Therefore, cell pellets were initially dissolved in 100µl radioimmunoprecipitation assay (RIPA)-buffer (Sigma-Aldrich), containing 5µl of Phosphatase Inhibitor Cocktail 3® (Sigma-Aldrich) and 5µl of HALT® Protease Inhibitor Cocktail (Thermo Fisher) as well as repeated freezing in liquid nitrogen and thawing at 4°C. The combination of both methods achieves higher yields of protein as well as protein quality. (Miskiewicz, MacPhee 2019) After complete homogenization of the cell pellet, the solution was centrifuged (10000/min; 10min; 4°C) and the supernatant, containing the protein fraction was obtained for further steps.

Then, protein concentration was determined by Lowry protein assay (LOWRY, ROSEBROUGH et al. 1951) and spectrophotometric measurement in a SpectraMax Plus® microplate reader (Molecular Devices) at a wavelength of 660nm.

Function of Lowry assay relies on two chemical reactions. In the first reaction divalent copper ion (Cu (II)) forms a complex with peptide bonds in which it is reduced to a monovalent copper ion (Cu (I)). In the second reaction Cu(I) and the radical groups of tyrosine, tryptophan, and cysteine react with Folin-Ciocalteu reagent (mixture of phosphomolybdate and phosophotungstate) to produce an unstable product that becomes reduced to molybdenum-tungsten blue. The absorbance of molybdenum-tungsten blue is measured at a wavelength of 660nm by a photo-spectrometer. For total protein measurement DC-Protein Assay® (BioRad) was used according manufactures protocol. RIPA buffer served as blank, and concentrations of 1,2,4 and 8mg/ml bovine serum albumin (BSA) dissolved in RIPA respectively were used for standard curve generation. (Peterson 1977) Total protein content was calculated by Microsoft® Excel using the specific absorbance values of sample relative to the determined standard curve (measured absorbance vs. known protein concentration). In order to linearize and break three-dimensional structure of proteins as well as render proteins with negative charge for further electrophoresis, lysates were mixed with 4x Laemmli buffer (BioRad) (final concentration 1x) and heated at 95°C for 10min.

Laemmli buffer consists of sodium dodecyl sulphate (SDS), which charges proteins negative as well as bromophenol blue and ß-mercaptoethanol, which break disulphide bridges of proteins. (Laemmli 1970)

For subsequent protein electrophoresis 10µl of lysate mixed with Laemmli-buffer were loaded into each well of Mini-Protean® TGX Precast Gel, which was run for 45min at 120V with 1x Tris/Glycine/SDS (BioRad) running buffer (Running buffer = 100 mL 1xTris/Glycine/SDS plus 900 mL distilled water). In the applied electric field, negatively charged proteins separate depending on the molecular weight. Smaller proteins migrate faster towards the cathode, bigger proteins stay near the anode. Precision Plus Protein® Kaleidoscope (BioRad) was used as standard in order to identify exact size of the analysed proteins. In the next step, proteins were transferred on polyvinylidene difluoride (PVDF) membranes (Bio-Rad) in order to enable detection by specific antibodies. Protein-transfer was performed with the BioRad Trans-Blot® Turbo transfer system using the standard transfer program for any protein size (25V constant, 30min) Subsequent to protein transfer, PVDF membranes were blocked with 5% dry milk blotting grade block buffer for 1 hour at 4°C to prevent unspecific binding of the antibody. For analysis of RKIP, TOP2B and inhibitor kappa B (IκB) expression, the PVDF membrane was incubated with the primary antibody, anti-RKIP anti-TOP2B and anti-IκB, respectively. After three 15-minute wash steps in 1x TBST washing buffer (TBST washing buffer = 100 ml 10x TBS (BioRad), 1ml Tween®20 (Sigma Aldrich) and 900 ml distilled water), the PVDF-membrane was incubated with a secondary antibody, which is directed against the species of the primary antibody and labelled with the enzyme horseradish peroxidase (HRP). HRP oxidates luminol to 3-aminophthalate and produces a chemiluminescent reaction. The reaction is associated by emission of light which could be recorded on a photographic film.

Subsequent to secondary antibody incubation, three washing steps are carried out as described above. Thereafter, the PVDF-membrane was incubated with WesternBright® ECL HRP substrate (Advansta) for 2 minutes according to manufactures instructions, resulting in oxidation of the substrate and leading to a chemiluminescent reaction. The signal produced by this luminescent reaction could be detected by exposing the membrane to a CL-XPosure® (Thermo Fisher) photographic film, where it produces a band, whose intensity correlates to expression of the protein studied. The films were developed in an Agfa Curix 60

(Agfa Health Care) automated developing machine as well as ChemiDoc imaging system (BIO-RAD).

In order to correct for differences in protein loading, RKIP, TOP2B and IκB expression were normalized to the expression of a loading control.
ß-Actin has been chosen as loading control for RKIP and TOP2B because its different size and the fact of equal expression in AML samples. (Dos Santos, McDonald et al. 2013)
Vinculin has been chosen as loading control for IκB due to its optimal molecular weight.
(Schiappacassi, Lovisa et al. 2011) After detection of the protein of interest (RKIP, TOP2B and IκB) membranes were washed three times in TBST washing buffer.
Subsequently, antibody staining as well as protein detection has been done as described above using the corresponding antibodies. Stripping of the membranes between the antibodies has not been performed, because the proteins of interest and their loading controls differ in molecular weight and the specific antibodies were derived from different species. To determine relative protein expression, band intensities were compared using ImageJ for further calculations. (Schneider, Rasband et al. 2012)

	pc/mc	species	blocking	staining	temp	time	weight	company
RKIP	pAB	Rabbit	5% milk	TBST	4°C	1 hour	23 kDa	Millipore
TOP2B	mAB	Mouse	5% milk	5% milk	4°C	over night	180 kDa	Santa Cruz
β-Actin	mAB	Mouse	5% milk	TBST	4°C	over night	42 kDa	Sigma
Vinculin	mAB	Rabbit	5% milk	TBST	4°C	over night	124 kDa	Abcam
GAPDH	mAB	Mouse	5% milk	5% milk	RT	1 hour	37 kDa	Santa Cruz

Table 6: Primary antibodies
pAB = polyclonal antibody, mAB = monoclonal antibody, TBST = Tris-buffered saline with Tween20, RT = room temperature, Da = Dalton

	source	dilution	staining	temp	Time	company
Anti-Rabbit	goat IgG + HRP	1:10.000	TBST	4°C	1 hour	Cell Signaling
Anti-Mouse	horse IgG + HRP	1:10.000	TBST	4°C	1 hour	Dako

Table 7: Secondary Antibodies
TBST = Tris-buffered saline with Tween20, HRP = horse radish peroxidase

2.6 Luciferase reporter assay

Luciferase assays were performed at the Medical University of Innsbruck under the supervision of Prof. Jakob Troppmair.

For luciferase reporter assays HEK-293 cells were transiently transfected with 0.25 ng/µl pCS2-red fluorescent protein (RFP) (Addgene), together with 0.125 ng/µL RKIP-3′-untranslated region (UTR)-pMirTarget (Origene) or TOP-2B – 3′ UTR-pMirTarget (Origene) as well as with miR-23a mimics or scrambled control as described in the chapter above. RFP was co-transfected in order to normalize for differences in transfection efficacy. To alter the binding site of miR-23a within the 3′ UTR of RKIP or TOP2B, site directed mutagenesis has been done using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufactures protocol. Presence of the mutation or deletion was confirmed by direct sequencing.

Site-directed mutagenesis is a method to introduce specific changes into a DNA sequence. This method relies on the use of synthetic oligodeoxynucleotides as highly specific mutagens. A mutagenic oligodeoxynucleotide, typically 15-20 nt long, is annealed to single-stranded template, the primer is extended by DNA polymerisation, and the ends of the nascent strand are ligated. The result is a heteroduplex molecule containing mismatched base pairs at the mutation site. Transformation of a suitable competent cell host strain with this heteroduplex DNA molecule results in both mutant and wild-type progeny. (B. Kramer, Kramer et al. 1984, Zoller, Smith 1983)

DNA gained from site directed mutagenesis and miR-23a mimics were mixed with 4µL jetPrime transfection reagent (Polyplus) in jetPrime buffer (Polyplus) to reach a final volume of 200µL transfection mix per well of a 24 well plate. After incubation for 10 minutes at room temperature, the mix was added dropwise to HEK-293 cells. Twenty-four hours after transfection HEK-293 cells were harvested and lysed in cell culture lysis reagent (Promega) according to the manufacturer's protocol. 20µl of cell lysate was pipeted into each well of a 96-well plate. In a next step, luciferase activity as well as RFP activity were assed using a Plate chameleon reader (Hidex). The reader automatically injected 100µl of luciferase Assay System Reagent (Promega) into each well of a 96-well plate. Luminescence

of Luciferase was measured for 10 seconds after a delay of 2 seconds. Fluorescence of RFP was detected at a wavelength of 544 and 616 nm.

The function of the luciferase assay is based on the oxidative properties of luciferases.

Luciferases are a class of enzymes found in different organisms that enable them to shine which is also called bioluminescence. The most famous luciferase is found in fireflies (*Lamprohiza splendidula)* which enables them to emit light by the oxidation of luciferin to oxyluciferin. In the luciferase reporter assay the regulatory region of the gene of interest must be cloned upstream the luciferase gene. Since the gene of interest is linked to the luciferase gene, the luciferase activity can by directly correlated with the gene of interest. (Barriscale, O'Sullivan et al. 2014)

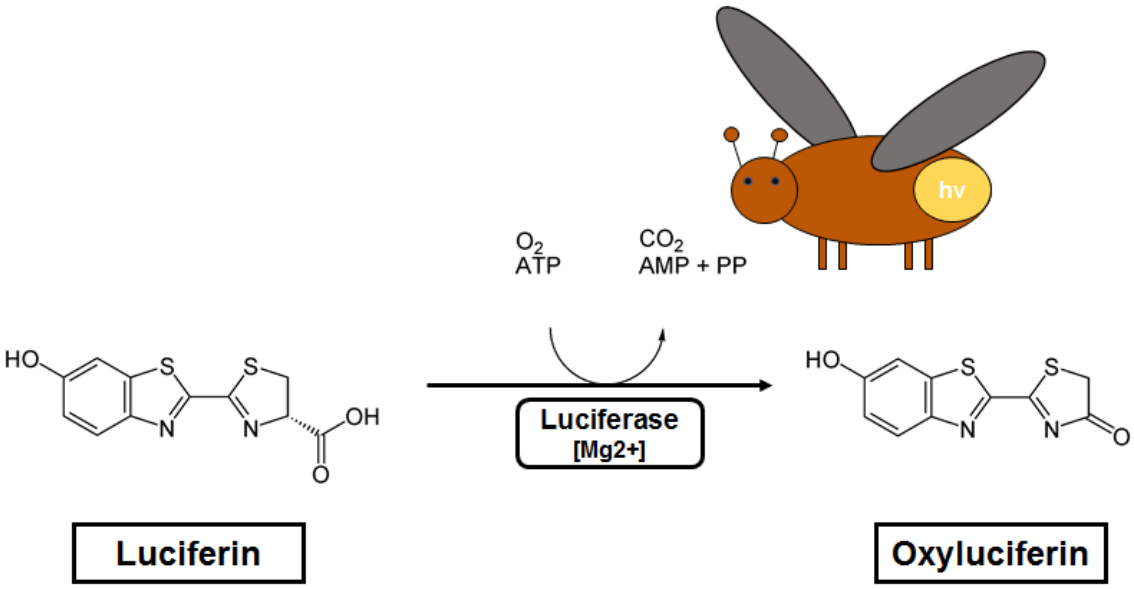

Figure 4: Function of luciferase reporter assay
Oxidation of Luciferin to Oxyluciferin under the consumption of adenosine triphosphate (ATP) and production of a light-quant (hu) as well as adenosine monophosphate (AMP) + 2 phosphate ions (PP) and CO_2.

2.7 Analysis of cell growth and cell viability

For the assessment of growth curves U-937 cells containing different expression constructs were seeded at a density of $1x10^4$ cells/ml and starved at reduced serum condition (5% FCS). The total amount of viable cells was measured by a trypan blue exclusion assay using a Bio-Rad TC20 automated cell counter (Bio-Rad). Trypan blue is an anionic diazo dye which colours dead cells blue and allows distinction between vital and dead cells.

Proliferation was additionally assed by bromodeoxyuridine/ 7-aminoactinomycin-D BrdU/7-AAD staining using the APC BrdU Flow Kit (BD Pharmingen). Therefore, U-937 cell containing different expression constructs were harvested and $1x10^6$ cells were transferred to a 12-well plate. Then cells were grown under puromycin-free normal conditions for twenty-four hours. Subsequently, BrdU at a final concentration of 50µM was added to each well and cells were incubated for one hour under light protected conditions. $1x10^6$ cells were washed in ice cold PBS and centrifuged (4°C, 5min, 8.000/min). In the next step, cells were fixed for 30 min (room temperature), permeabilised for 15 min re-fixed for 5 min (room temperature), treated with DNase and finally stained

with APC anti-BrdU antibody (dilution 1:50; 30 min; room temperature) as well as 7-AAD according the manufactures protocol. Unlabeled native cells were used as negative controls. 10^4 stained cells were measured on a BD-LSRII Flow Cytometer operated with FACSDiva Software (BD Biosciences) with a flow rate of less than 400 cells/s on the same day of staining and analyzed with Kaluza Flow Cytometry Analysis Software v1.2 (Beckman Coulter).

2.8 Database retrieval and statistics

Expression data for *RKIP* and *TOP2B* (obtained by RNA Sequencing V2 RSEM as well as by Affymetrix U133 and Agilent microarrays) and miRNAs (obtained by miRNA arrays and miRNA Sequencing) were downloaded and analyzed from The Cancer Genome Atlas (TCGA, http//:www.cancergenome.nih.gov). In case of availability, data were downloaded and analyzed using the cBioPortal for Cancer Genomics (http//:www.cbioportal.org/public-portal/index.do)(Gao, Aksoy et al. 2013, Cerami, Gao et al. 2012)

RKIP mRNA and miRNA expression values were analyzed in previously published array and qPCR datasets (data available at http://www.ncbi.nlm.nih.gov/geo under accession numbers GSE49665 (Rommer et al. 2013), GSE1159 and GSE6891 (Valk, Verhaak et al. 2004, Jongen-Lavrencic, Sun et al. 2008, Nowek, Sun et al. 2016)) For LSC analyses, previously published miR-microarray expression data were downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession number GSE55916) and re-analyzed for the expression of miR-23a.(Lechman, Gentner et al. 2016)

Wilcoxon signed-rank tests were used for comparison of miRNA expression levels between primary patient samples with and without RKIP loss as well as for comparison of *RKIP* and miR-23a expression between AML patients with and without myelo-monocytic phenotypes.

Wilcoxon signed-rank tests were also employed for comparison of miR-23a as well as *TOP2B* expression levels between primary patient samples at diagnosis and R/R stages, as well as for comparison of miR-23a expression between LSCs and corresponding AML bulk samples.

Spearman-Rho correlation coefficients were calculated in order to test for a correlation between miR-23a and *RKIP* or *TOP2B* expression level.

For survival analyses, miR-23a and *TOP2B* expression levels were evaluated as dichotomous variables (samples with low versus samples with high expression). The optimal cut-off to separate these groups was assessed by employing a maximized Youden's Index within a receiver operating characteristic (ROC) analysis of event free survival (EFS). Overall survival (OS) and EFS within these groups were calculated with a Kaplan-Meier estimator and compared with log-rank tests. Furthermore, associations with OS and EFS, respectively, were calculated in both uni- and multivariable Cox proportional hazards regression models. Beside

miR-23a and *TOP2B*, respectively, these calculations included the well-established AML risk factors age, white blood cell count (WBC), and cytogenetic risk group.
For calculation of IC_{50} values and comparison of dose response curves cytotoxicity experiments ANOVA within R 3.2.2 (http//:www.r-project.org) was used.
For analysis of *in vitro* experiments, Student *t* test was calculated from at least three independent experiments. SPSS 22.0 (SPSS Inc.) and R 3.2.2 (http//:www.r-project.org) were used for analysis. All tests were performed two-sided and a *P* value of <0.050 was considered statistically significant.

3 RESULTS

The following sections are dedicated to describe the results of this thesis and discuss them. These data were also largely published in Hatzl et al. (Hatzl, Geiger et al. 2016) and (Hatzl et al. 2020)

3.1 Increased expression of miR-23a mediates a loss of expression of RKIP

3.1.1 Increased expression of miR-23a in AML correlates with RKIP loss

To identify miRNAs, which correlate with the expression of RKIP in AML, we analyzed 33 primary AML specimens in a first step. In these samples, RKIP protein expression as well as the expression of more than 600 miRNAs had been evaluated by immunoblot and microarray analysis previously. (Zebisch et al. 2012, Rommer et al. 2013) RKIP loss was defined as an expression of <25% of the RKIP expression observed in the calibrator cell line NB-4. (Zebisch et al. 2012) These experiments revealed a set of seven differently expressed miRNAs (miR-23a, miR-23b, miR-24, miR-15a, miR-320a, miR-518b and miR-519d) between patients with either normal (*n* = 27) or loss of (*n* = 6) RKIP.

In the next step we validated these findings in an additional cohort of 20 AML patient specimens (cohort I). Within this cohort 10 patients showed normal RKIP expression, and 10 exhibited RKIP loss. Importantly, only three out of seven miRNAs showed an increased expression in patient samples with a loss of RKIP in these analyses (miR-23a, miR-24, and miR-15a).

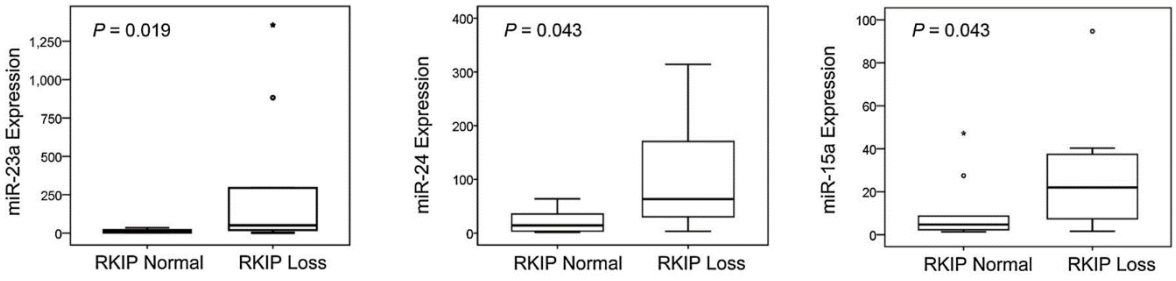

Figure 5: Box plots showing a significant increase of miR-23a, miR-24, and miR-15a expression in AML patient specimens defined as RKIP loss in AML cohort I (*n* = 20).
miRNA expression values were assessed by qPCR and are displayed as *x*-fold expression of the calibrator (NB4 cells). RKIP expression was evaluated by qPCR and immunoblot.
The figure is reproduced from (Hatzl, Geiger et al. 2016) with permission of *Cancer Research*.

We then aimed to further corroborate these results in two additional cohorts. Therefore, we analyzed *RKIP* mRNA expression, as well as the expression of the three miRNAs validated in cohort I (miR-23a, miR-24, and miR-15a) in cohort II (consisted of 214 Dutch-Belgian AML patients and was previously analyzed by mRNA arrays and miRNA qPCRs (30–32)) and cohort III (consisted of 173 specimens and was acquired via TCGA database retrieval)

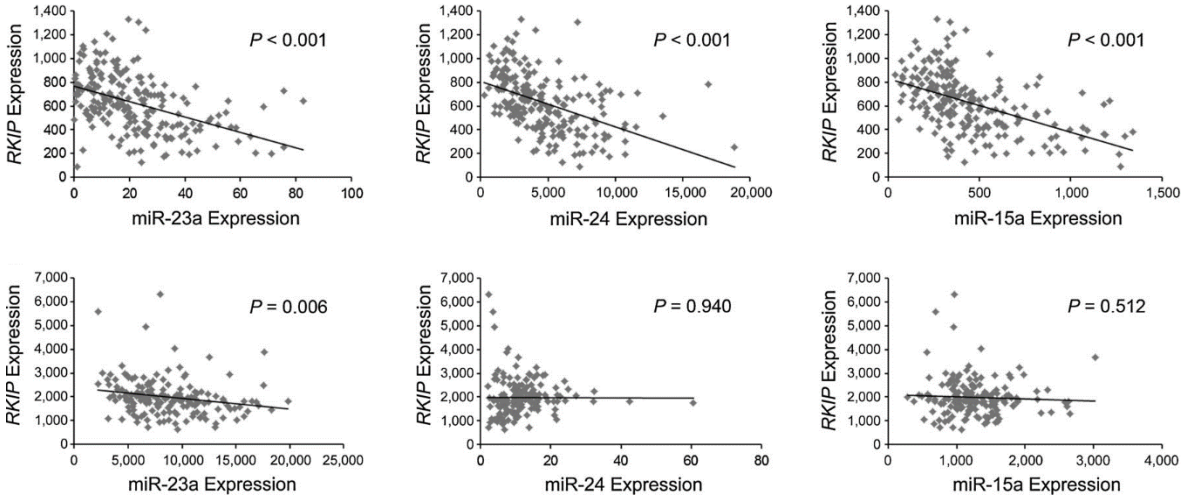

Figure 6: scatter plots showing correlation between the expression of *RKIP* mRNA and microRNAs
(miR-23a, miR-24, and miR-15a) in 214 patient specimens of the Dutch-Belgian AML cohort II (first row) and significant and inverse correlation between the expression of *RKIP* mRNA (displayed as RNA Sequencing V2 RSEM expression values at the *y*-axis) and miR-23a (displayed as miRNA-sequencing expression values at the *x*-axis) in the TCGA AML cohort III (second row)
The figure is reproduced from (Hatzl et al. 2016) with permission of *Cancer Research*.

Interestingly, although different techniques of expression analysis were used across these cohorts, one microRNA (miR-23a) demonstrated a significant and inverse correlation with *RKIP* expression in all of these patient groups. Although increased levels of miR-24 and miR-15a showed correlation with decreased RKIP expression in cohort II as well, no additional correlation with statistical significance could be observed in cohort III.

These data pinpoint a significant correlation between RKIP loss and increased expression of miR-23a in AML. Consequently, we aimed to investigate the role of miR-23a in RKIP modulation in AML in the following experiments.

3.1.2 RKIP loss and increased expression of miR-23a correlate with myelomonocytic and monocytic AML phenotypes

In a previous paper, our group could show that low expression levels of AML correlate with myelomonocytic and monocytic differentiation of AML (AML M4/5 according to French American British classification system). (Zebisch et al. 2012) We also could demonstrate that RKIP is indeed involved in the myelomonocytic differentiation process, and that RKIP loss induces the myelomonocytic lineage commitment of HSPCs.(Caraffini, Geiger et al. 2019)

We now aimed to confirm these findings in the cohorts studied within this project. Therefore, we analyzed the twenty primary AML specimens of cohort I (Austrian cohort), and indeed AML M4/5 phenotypes were significantly enriched in those patient samples exhibiting a loss of RKIP.

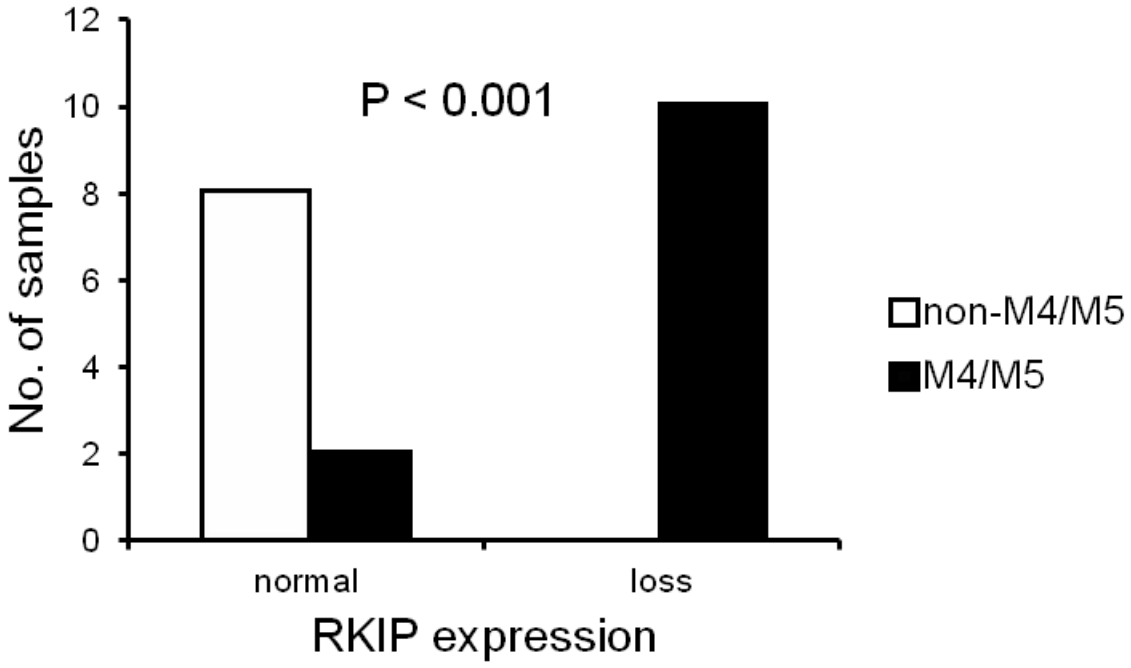

Figure 7: Myelomonocytic and monocytic AML phenotypes are enriched in AML with RKIP loss
Phenotypes of the 20 AML specimens used for qPCR validation of miRNA array data (cohort I). Expression of RKIP has been assessed by qPCR and Immunoblot, RKIP loss was defined as previously defined (1). Distribution of myelomonocytic and monocytic AML phenotypes (M4/M5) as well as all other phenotypes (non-M4/M5) between the two different RKIP expression groups was calculated using Fisher´s exact test.
The figure is reproduced from (Hatzl et al. 2016) with permission of *Cancer Research*.

In agreement with these data, *RKIP* expression levels were also significantly decreased in AML M4/M5 as compared with all other subtypes in cohorts II and III as well. Figure 8 shows the expression data generated in cohort III. Of note *RKIP* expression in myelomonocytic and monocytic subgroups in the Dutch-Belgian AML cohort II has been previously published. (Zebisch et al. 2012)

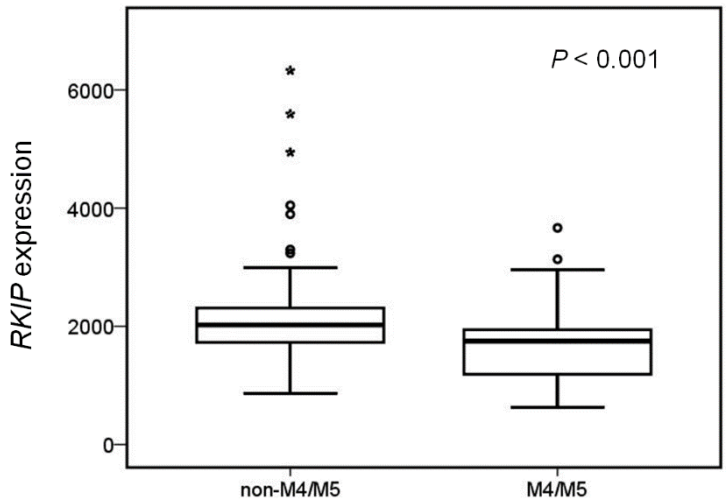

Figure 8: RKIP expression is decreased in AML M4/5 in cohort III (TCGA)
Box plot analysis showing a decrease of *RKIP* mRNA expression (displayed as RNA Sequencing V2 RSEM expression values at the y-axis) in myelomonocytic and monocytic AML phenotypes (M4/M5) as compared to all other phenotypes (non-M4/M5).
The figure is reproduced from (Hatzl et al. 2016) with permission of *Cancer Research*.

We were then interested whether myelomonocytic AML phenotypes do not only correlate with decreased expression of RKIP, but also with increased expression of miR-23a. Indeed, when studying cohort I, miR-23a expression was significantly increased in AML specimens characterized as AML M4/M5.

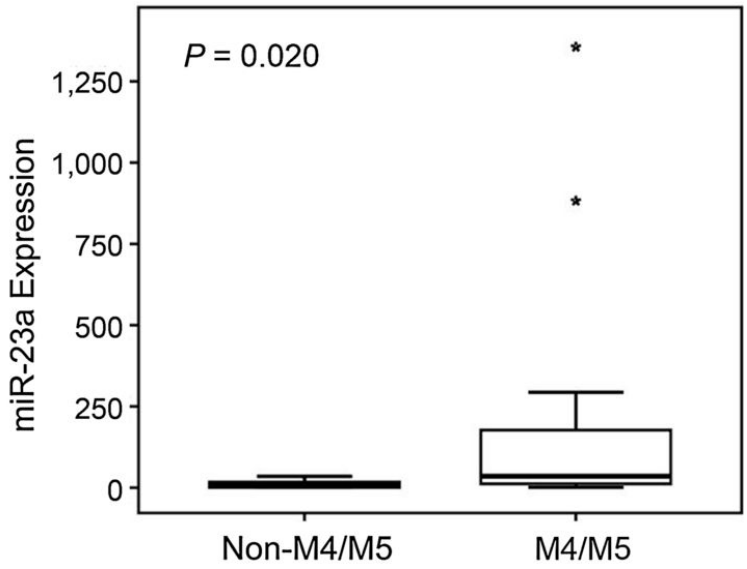

Figure 9: Box plot showing a significant increase of miR-23a expression (cohort I) measured by qPCR and displayed as *x*-fold expression of a calibrator; NB4 cells) in AML phenotypes with myelomonocytic and monocytic phenotypes (M4/M5) in the Austrian AML cohort I (*n* = 20) The figure is reproduced from (Hatzl et al. 2016) with permission of *Cancer Research*.

Importantly, we were also able to corroborate these findings in the two additional AML cohorts comprising almost 400 patients showing that the expression of miR-23a was significantly increased in AML M4/M5 phenotypes.

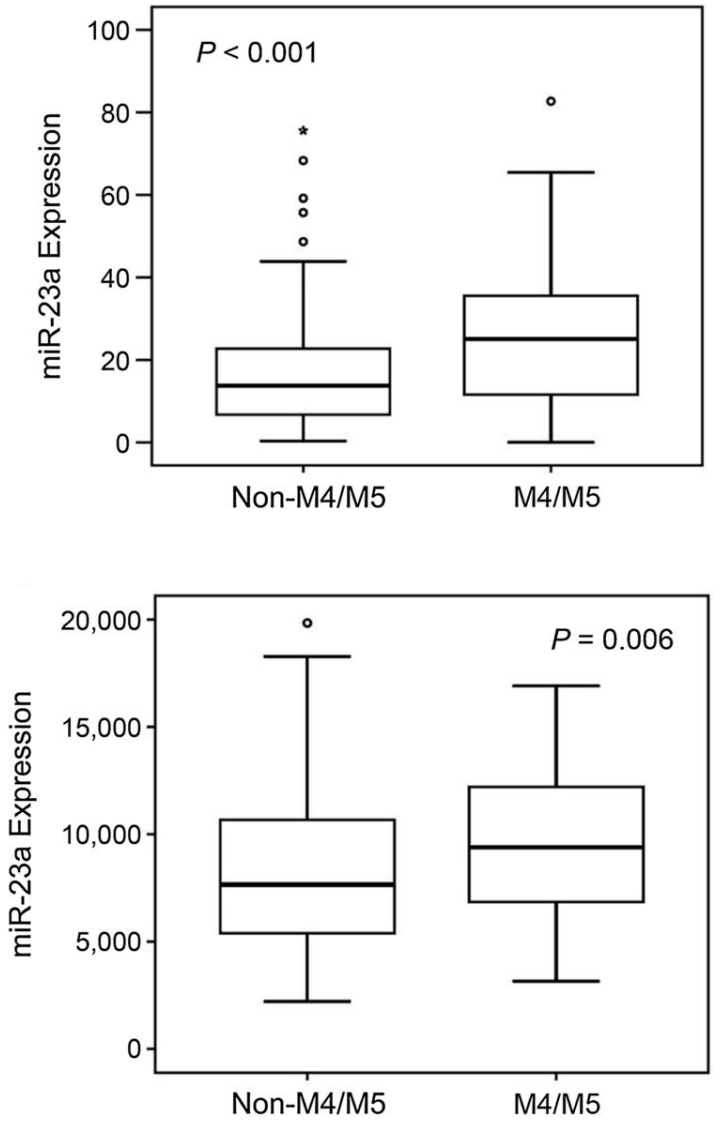

Figure 10: miR-23a was significantly increased in AML M4/5 in almost 400 patients
Above: Dutch-Belgian AML cohort II (*n* = 214; miR-23a expression displayed as miRNA array expression values;)
Below: TCGA cohort III (*n* = 173; miR-23a expression displayed as miRNA-sequencing expression values)
The figure is reproduced from (Hatzl et al. 2016) with permission of *Cancer Research*.

3.1.3 RKIP expression is regulated by miR-23a

Up to this point, we could conclude that RKIP and miR-23a correlate inversely in AML, and that they are both associated with myelomonocytic AML phenotypes.
In a next step, we aimed to delineate whether miR-23a truly regulates RKIP. Therefore, we performed miR-23a modulation in the AML cell lines U937 and THP-1, as well as in the non-hematopoietic cell line HEK293. First, we used these cells to exclude that upregulation of miR-23a is not the consequence of RKIP loss. In such a situation, RKIP loss would be the primary event, which then causes the increased expression of miR-23a. Such a scenario could be shown in recent studies for miR-98 and miR-200.(Chen, Cheng et al. 2013) (Sun, Gomes et al. 2014) Hence, we lentivirally transduced U937 by *RKIP* shRNA to knockdown RKIP. We monitored the expression of miR-23a measured by qPCR in both cell lines (Cntrl, *RKIP*shRNA). No difference in its expression could be observed.

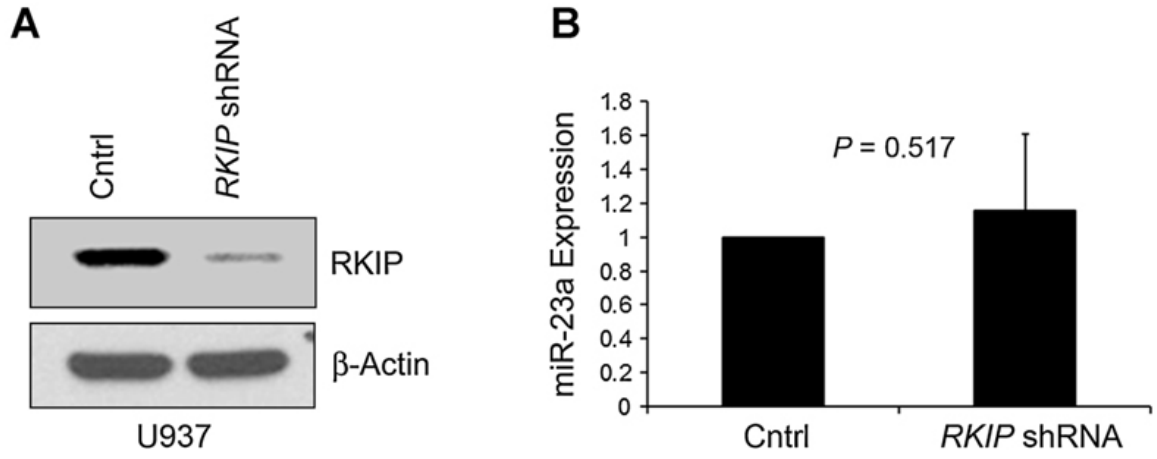

Figure 11: miR-23a overexpression is no effect of RKIP knockdown
A) Knockdown of RKIP was performed by a lentiviral shRNA. Cntrl cells were transduced by an empty vector. Expression of *RKIP* was monitored by immunoblot. ß-Actin was used as loading control
B) miR-23a expression analysis by qPCR revealed no difference between *RKIP* shRNA and Cntrl cells. The figure is reproduced from (Hatzl et al. 2016) with permission of *Cancer Research*.

We then aimed to study this scenario in more detail, as a recent study demonstrated the induction of miR-23a by activated nuclear factor- κB (NF-κB). (Y. C. Zhang, Ye, Zeng, Chin, Huang, and Fu 2015a). In this respect, it is worth mentioning that RKIP is a well-established inhibitor of NF-κB. (K. C. Yeung, Rose et al. 2001) Thus, one might speculate that RKIP loss causes increased activation of NF-κB , and consequently the increased expression of miR-23a. We, therefore, studied the expression of nuclear factor of kappa light polypeptide gene enhancer

in B-cells inhibitor (IκB) within these cells. Previous studies could show that IκB mediates NF-κB inhibition by RKIP in non-hematopoietic cell lines. (K. C. Yeung et al. 2001) Therefore we analyzed IκB in *RKIP* knockdown U937 cell as well as control U937 cells. These cells had been transduced lentivirally by *RKIP* shRNA or empty vector. Importantly, there was no difference in the expression of IκB in the hematopoietic cell line U937. Taken together, these data indicate that the increased expression of miR-23a is not an effect of RKIP loss in AML.

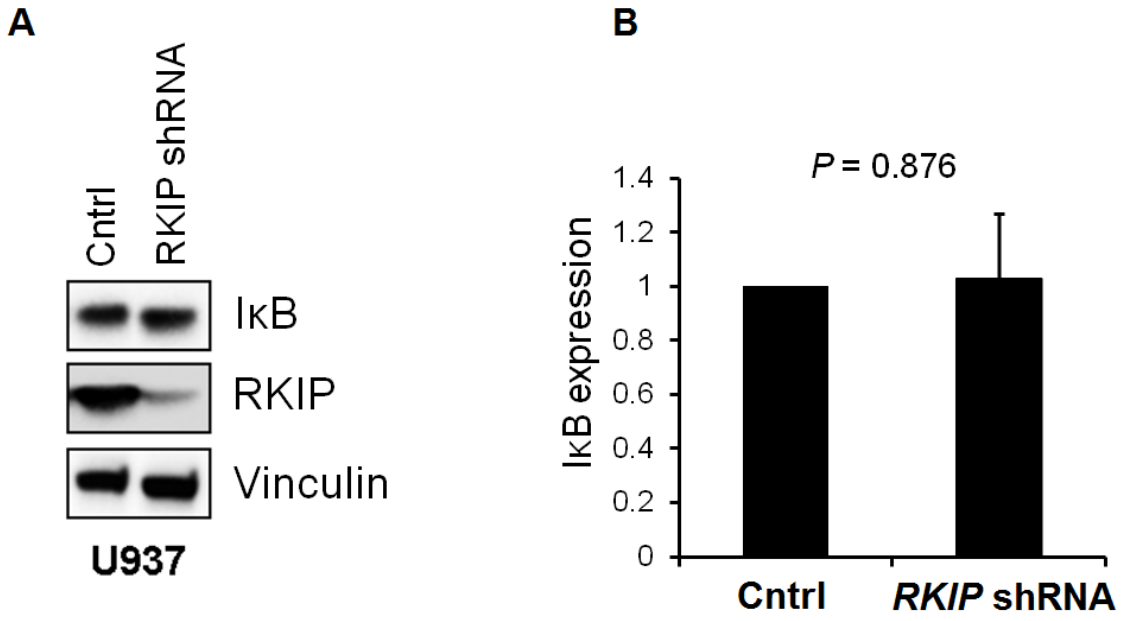

Figure 12: RKIP knockdown in U937 does not affect IκB as surrogate of NF-κB.
A) Successful knockdown of RKIP was confirmed via immunoblot. Vinculin was used as loading control.
B) Densitometric studies on IκB expression in empty vector transduced Ctrl cells as well as RKIP knockdown cells transduced by *RKIP* shRNA
The figure is reproduced from (Hatzl et al. 2016) with permission of *Cancer Research*.

In the next step, we aimed to clarify whether increased expression of miR-23a is the reason behind RKIP loss. Therefore, we transiently transfected one non-hematopoietic cell line (HEK293) and one hematopoietic cell line (U937) with either miR-23a mimics or scrambled controls. Subsequently, we studied the expression of RKIP. Indeed, ectopic overexpression of miR-23a caused significant downregulation of RKIP both on mRNA and protein level in HEK293 and U937 cells. To further corroborate these findings, we aimed to show these effects in the other direction as well. Therefore, we performed miR-23a shRNA-mediated knockdown in AML cells with low endogenous RKIP expression levels (THP-1).(Zebisch et al. 2012) Interestingly, miR-23a hairpin inhibitor transfected cells exhibited

significantly higher expression levels of RKIP both on mRNA and protein level compared with scrambled control hairpin inhibitor transfected cells. These results of RKIP modulation by miR-23a overexpression and downregulation could be confirmed by using mimic and hairpin inhibitors of different companies. Taken together, these functional studies demonstrate that miR-23a regulates RKIP expression in AML.

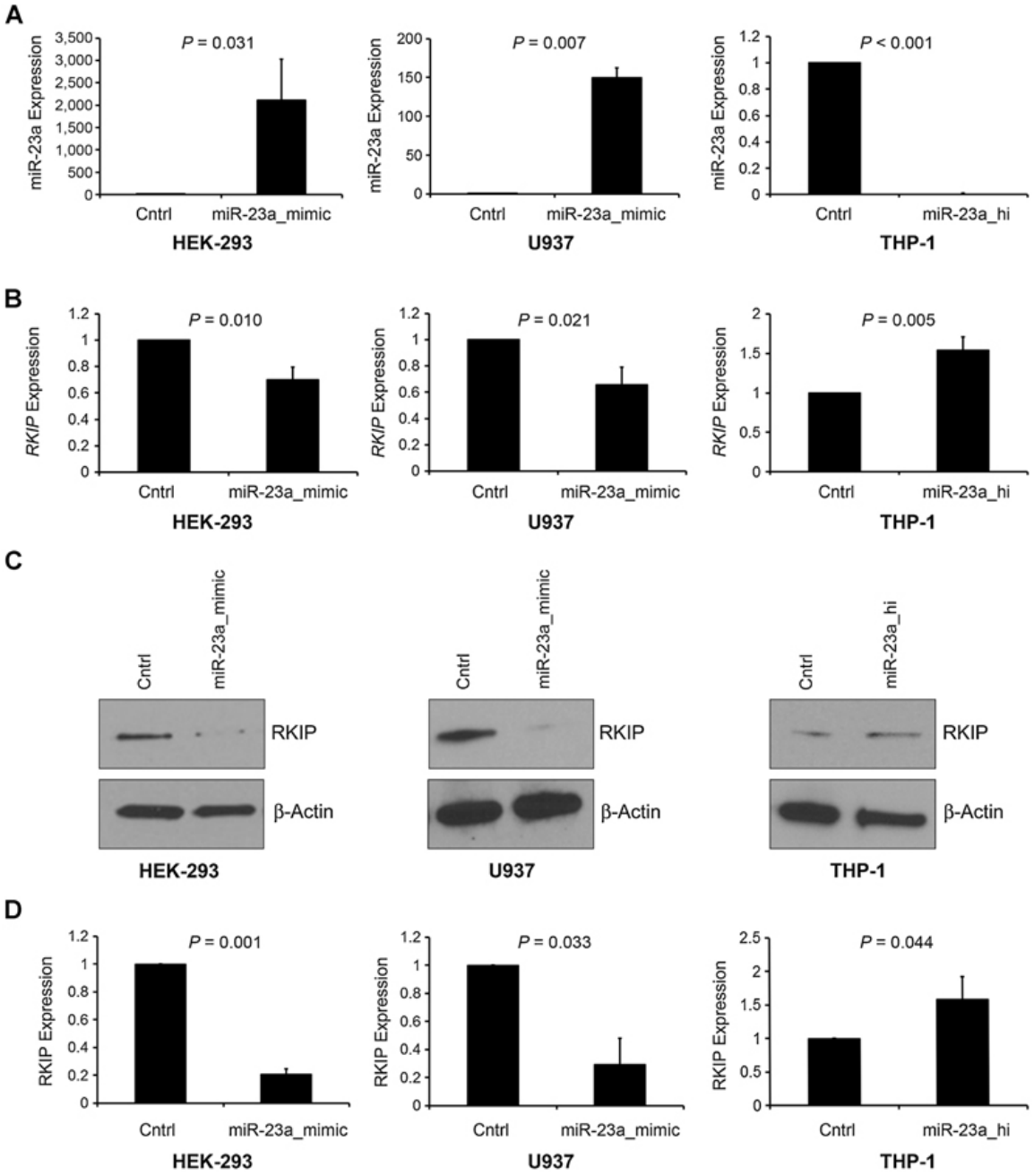

Figure 13: RKIP is regulated by miR-23a
A) Overexpression of miR-23a in HEK293 and U937 cells (both exhibiting high endogenous RKIP expression). Downregulation of miR-23a in THP-1 cells (exhibiting low endogenous RKIP expression) Expression of miR-23a levels were measured by qPCR.
B) *RKIP* mRNA expression analysis by qPCR of the condition mentioned above
C) Immunoblots showing RKIP modulation in the different cell lines
D) Densitometric analysis of immunoblots
All graphs represent the mean of three independent experiments ± standard deviation; expression levels are given as *x*-fold expression of scrambled control.
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3.1.4 miR-23a modulates RKIP expression by direct binding to the RKIP 3'UTR

We now wanted to delineate whether miR-23a regulates RKIP via direct binding. Therefore, we initially performed an in-silico binding analysis, where we employed 10 different target prediction tools and algorithms. Five out of ten revealed *RKIP* 3´UTR as putative target of miR-23a, which suggests that RKIP might indeed be regulated by miR-23a via a direct interaction.

Gene Name	MicroRNA	DIANAmT	miRanda	miRDB	miRWalk	RNAhybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	SUM
RKIP (PEBP1)	hsa-miR-23a	yes	yes	no	yes	no	no	yes	no	no	yes	5

Figure 14: In silico target prediction of miR-23a and RKIP
Target prediction tools marked with green boxes revealed *RKIP* 3´UTR as putative target of miR-23a.
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We then analyzed the 3´UTR of *RKIP* and identified two potential binding sites for miR-23a, with one of them (binding site 1) showing a particularly strong match with the seed region of miR-23a. We then aimed to evaluate whether miR-23a truly binds to this binding site and therefore attached the 3´UTR of *RKIP* to the coding region of a luciferase plasmid. Interestingly, co-transfection of this construct with miR-23a mimics resulted in a significant downregulation of its luciferase activity, which indicates that the miR-23a mimic interacted with the RKIP 3´UTR in these assays. To further evaluate if this effect is indeed mediated by direct interaction of miR-23a and binding site 1 of the *RKIP* 3´UTR, we altered this binding site by either introducing a mutation or a deletion. Indeed, both approaches prevented the miR-23a mediated downregulation of luciferase activity, which indicates that miR-23a regulates RKIP expression via direct binding to binding site 1 of its 3´UTR.

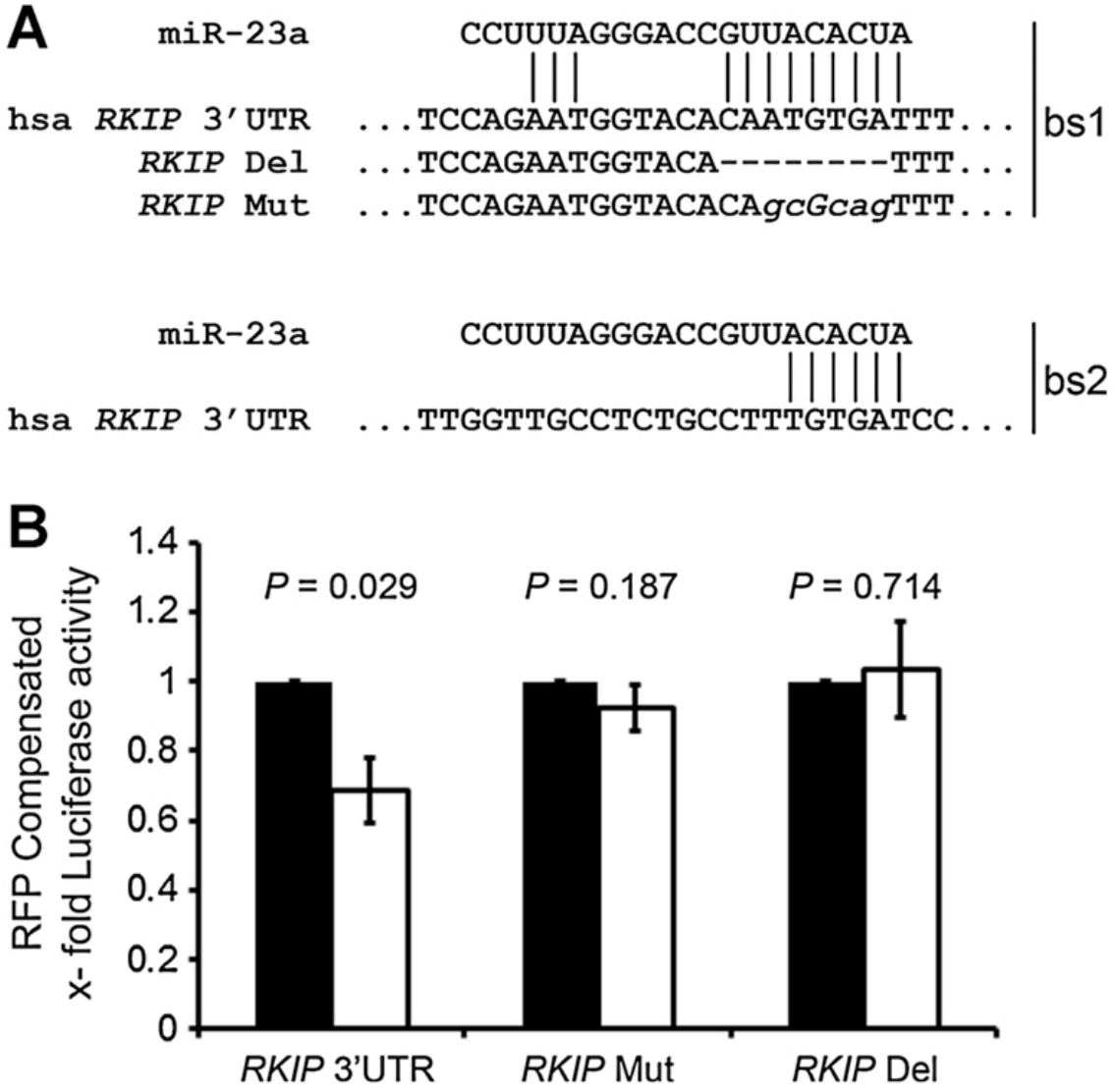

Figure 15: miR-23a binds the 3´UTR of RKIP and modulates its expression
A) Genomic sequence of human 3´UTR of RKIP with both binding sites. Only binding site 1 (bs1) was altered for luciferase experiments. *RKIP* Del denotes an eight-base deletion of bs1. *RKIP* Mut denotes a five-base exchange of bs1
B) HEK-293 cells were transfected with miR-23a mimics (white bars) or with scrambled control mimics (black bars) together with a luciferase reporter vectors containing wild-type *RKIP* 3′UTR (*RKIP* 3′UTR), *RKIP* Del, or *RKIP* Mut. pCS2-RFP was co-transfected in each experiment to compensate for different transfection efficacy.
Graphs display the mean three independent experiments ± SD compensated with RFP expression; values are given as *x*-fold expression of scrambled control-transfected cells
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3.1.5 miR-23a increases the proliferation of hematopoietic cells via downregulation of RKIP

Our group could previously demonstrate that stable overexpression of RKIP by a FLAG-tagged RKIP overexpression vector causes a decrease in cellular growth. This decrease in cellular growth was mediated by a decreased proliferation rate. (Zebisch et al. 2012) As we have now shown that miR-23a regulates the expression of RKIP, we wanted to delineate the effects of miR-23a overexpression on cellular proliferation in hematopoietic cells. Therefore, miR-23a mimics and scrambled controls, respectively, were transfected into parental U937 cells to yield miR-23a overexpression. Transfection with miR-23a mimics thereby resulted in higher proliferation rates, as assessed by a 7-AAD/BrdU/PI cell-cycle assay. It also increased the cellular growth compared with scrambled control transfected cells. To delineate if this biologic effect is caused by miR-23a mediated downregulation of RKIP, we continued to work with FLAG-RKIP overexpressing U937 cells as outlined above. FLAG-RKIP doesn't harbour a 3´UTR and is resistant against miR-23a mediated decay. Consequently, it should be able to rescue the increase of proliferation caused by miR-23a. Indeed, transfection of miR-23a mimics in U937 cells containing a FLAG-RKIP vector failed to alter proliferation rates and was not able to induce a specific biologic phenotype. To further validate these findings, we performed *shRNA*-based knockdown of *RKIP* in U937 cells. This caused the same phenotype as miR-23a overexpression in the 7-AAD/BrdU/PI cell-cycle assay (increase in proliferation and cellular growth).

Taken together, we can conclude that increased expression of miR-23a causes a decrease of RKIP expression, which in turn induces the proliferation and cellular growth of hematopoietic cells.

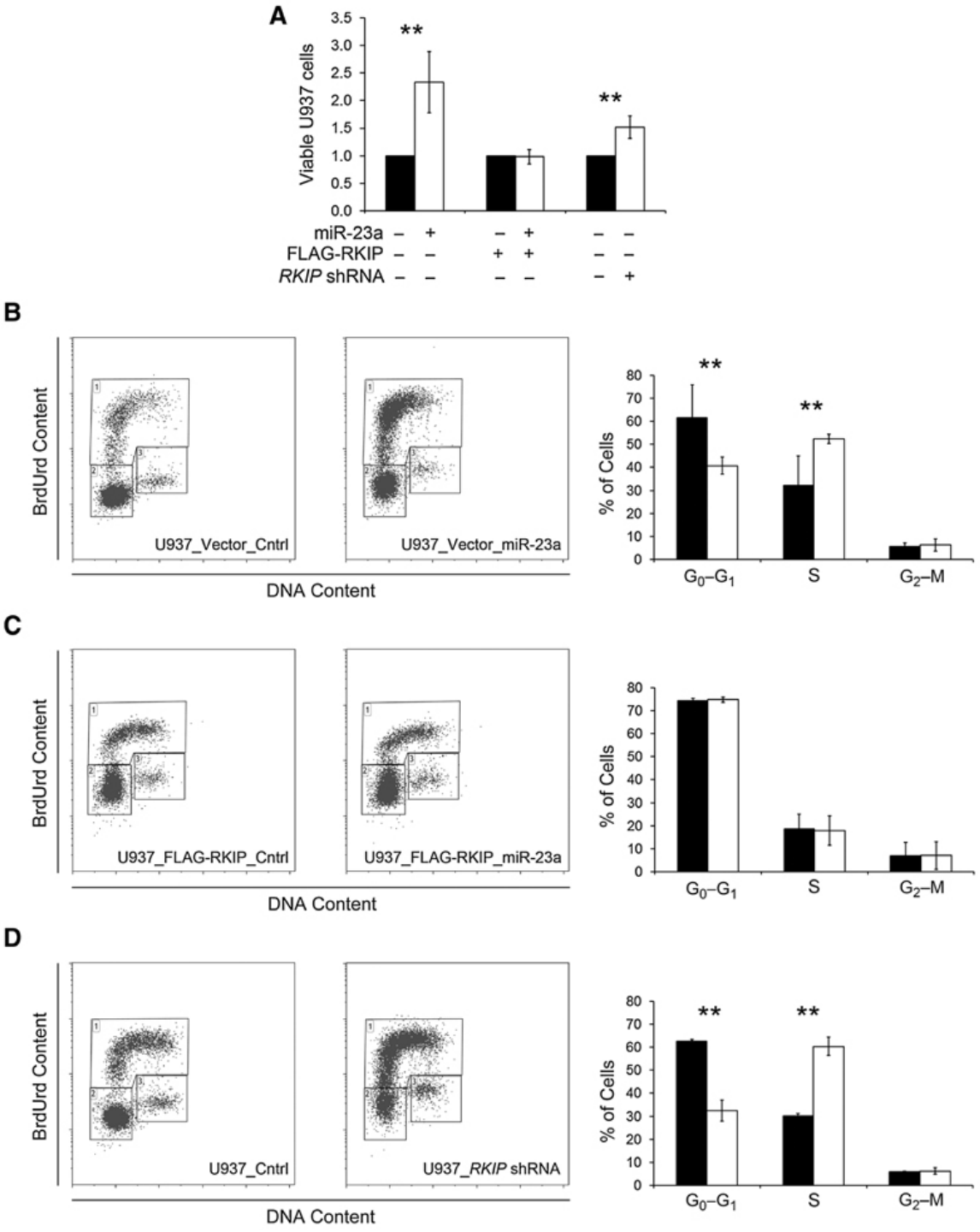


Figure 16: miR-23a induces the proliferation of hematopoietic cells via downregulation of RKIP
A) U937 cells transfected or transduced as outlined above seeded and maintained as described in Materials and Methods and viable cells were counted after 5 days.
B–D) 7-AAD/BrdU cell-cycle /proliferation assays were (settings described above)
S-phase (top gate), G_0–G_1-phase (left bottom gate), and G_2–M-phase (right bottom gate). Control (black bars); miR-23a/*RKIP* shRNA (white bars)
The figure is reproduced from (Hatzl et al. 2016) with permission of *Cancer Research*.

3.1.6 The miR-23a/27a/24-2/RKIP axis is of general relevance for other cancer entities as well

In the last step of this study, we were interested if miR-23a-mediated RKIP modulation is of interest for other tumor entities than AML as well. Out of these reasons, we were interested if a member of the miR-23a/27a/24-2 cluster is linked to RKIP knockdown in other human malignancies. Therefore, we performed a database retrieval via TCGA. We analyzed malignancies in which loss of RKIP has been reported previously in the literature, and where data for RKIP expression as well as for miR-23a/27a/24-2 expression were available. Overall, we were able to analyze 14 additional cancer entities comprising 4,342 primary patient specimens. We could show that at least one member of the miR-23a/27a/24-2 cluster correlates significantly and inverse with *RKIP* expression in 10 out of 14 cancer entities. We could further confirm that miR-23a is the predominant cluster member with significant inverse correlation in 9 out of 10 malignancies.

Cancer entity (n = 14)	n = 4342	miR-23a		miR-24-2		miR-27a	
		Coeff.	*P*	Coeff.	*P*	Coeff.	*P*
Lung squamous cell carcinoma (LUSC)	104	−0.131	0.186	0.176	0.074	−0.291	**0.003**
Lung adenocarcinoma (LUAD)	447	−0.201	**<0.001**	−0.021	0.661	0.172	**<0.001**
Prostate adenocarcinoma (PRAD)	493	−0.190	**<0.001**	−0.174	**<0.001**	−0.095	0.060
Kidney renal clear cell carcinoma (KIRC)	162	−0.280	**<0.001**	−0.224	**0.004**	−0.272	**<0.001**
Bladder urothelial carcinoma (BLCA)	405	−0.306	**<0.001**	−0.187	**<0.001**	−0.354	**<0.001**
Invasive breast carcinoma (BRCA)	299	−0.188	**0.001**	−0.141	**0.015**	−0.158	**0.006**
Ovarian serous cystadenocarcinoma (OV)	513	−0.137	**0.002**	−0.084	0.058	−0.051	0.253
Uterine corpus endometrial carcinoma (UCEC)	174	−0.089	0.242	0.025	0.747	−0.098	0.196
Pancreatic adenocarcinoma (PAAD)	179	−0.492	**<0.001**	−0.368	**<0.001**	−0.297	**<0.001**
Liver hepatocellular carcinoma (LIHC)	369	−0.425	**<0.001**	−0.277	**<0.001**	−0.407	**<0.001**
Colon and rectal adenocarcinoma (COAD)	78	0.1	0.382	0.001	0.997	0.073	0.526
Head and neck squamous cell carcinoma (HNSC)	476	−0.104	**0.023**	−0.126	**0.006**	−0.07	0.087
Skin cutaneous melanoma (SKCM)	447	0.044	0.354	0.039	0.412	0.069	0.145
Glioblastoma multiforme (GBM)	196	0.048	0.506	0.002	0.975	0.044	0.542

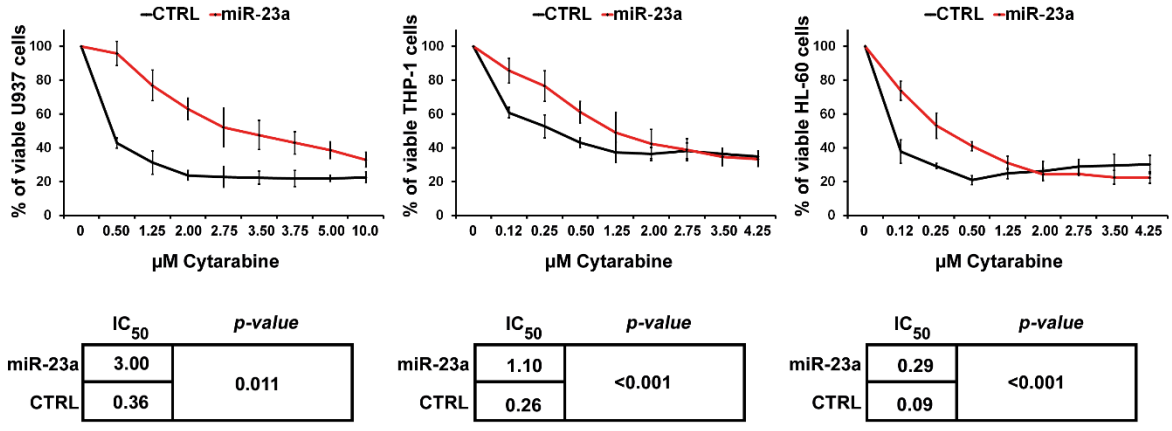
Table 8: Correlation between *RKIP* and miRNA expression in human cancer
Expression values of *RKIP* mRNA (as obtained by RNA Sequencing V2 RSEM, Affymetrix U133, or Agilent microarrays) and miRNAs (as obtained by miRNA arrays) were correlated using Spearman-Rho.
Coeff. denotes Spearman-Rho. correlation coefficient
The table is reproduced from (Hatzl et al. 2016) with permission of *Cancer Research*.

3.2 Increased expression of miR-23a mediates chemoresistance to AraC in acute myeloid leukemia

3.2.1 miR-23a mediates resistance to AraC

In the first step of this second part of the thesis, we sought to delineate if miR-23a is involved in therapeutic resistance to AraC in AML. AraC is the backbone of AML chemotherapy in every line of treatment. (Dohner et al. 2017) Therefore we performed overexpression of miR-23a in three different AML cell lines. In more detail, we stably overexpressed miR-23a by lentiviral transduction in U937 and THP-1 cells, and transiently overexpressed miR-23a by miR-23a mimics in HL-60 cells. Initially, we verified that miR-23a is indeed overexpressed in the cells transfected with miR-23a overexpression constructs by employing qPCR.

Subsequently, the cells were exposed to increasing concentrations of AraC and cell viability was measured by MTT assays. Indeed, miR-23a overexpressing cells were significantly less sensitive to AraC-induced cell death with IC_{50} values increasing from 0.36 µM to 3.0 µM in U937, from 0.26 µM to 1.1 µM in THP-1, and from 0.09 µM to 0.29 µM in HL-60 cells.



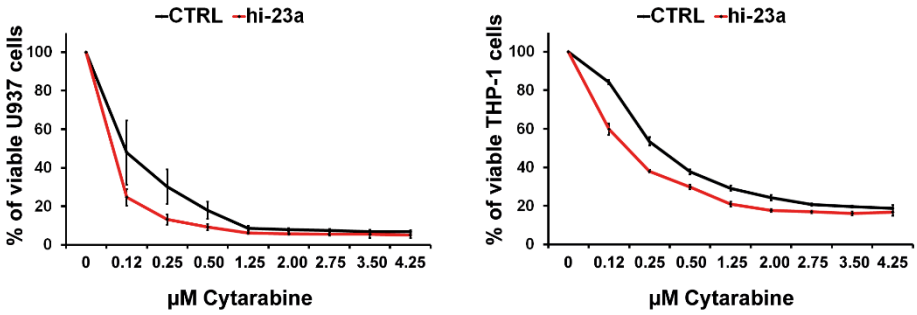
	IC_{50}	*p-value*
miR-23a	3.00	0.011
CTRL	0.36	

	IC_{50}	*p-value*
miR-23a	1.10	<0.001
CTRL	0.26	

	IC_{50}	*p-value*
miR-23a	0.29	<0.001
CTRL	0.09	

Figure 17: Ectopic expression of miR-23a reduces sensitivity to AraC
MTT cytotoxicity assays in AML cell lines after incubation with AraC. miR-23a denotes transfection/transduction with a miR-23a overexpression construct; CTRL denotes transfection/transduction with an empty control vector. The figure is reproduced from (Hatzl, Perfler et al. 2020) with permission of *Cancers*.

To further validate this effect, we performed hairpin-inhibitor based transient knockdown of miR-23a in U937 and THP-1 cells. In agreement with the overexpression studies, miR-23a knockdown reduced the sensitivity to AraC in all cell lines tested with IC_{50} values decreasing from 0.11 µM to 0.06 µM in U937, and from 0.31 µM to 0.17 µM in THP-1 cells.



	IC_{50}	*p-value*
hi-23a	0.06	<0.001
CTRL	0.11	

	IC_{50}	*p-value*
miR-23a	0.17	<0.001
CTRL	0.31	

Figure 18: Knockdown of miR-23a reinforces sensitivity to AraC
MTT cytotoxicity assays in AML cell lines after incubation with AraC. hi-23a denotes transfection/ with a miR-23a hairpin inhibitors; CTRL denotes transfection with scrambled control hairpin inhibitors
The figure is reproduced from (Hatzl et al. 2020) with permission of *Cancers*.

AraC forms the standard cytotoxic therapy of AML together with anthracyclines. Daunorubicin is the most common anthracycline used in AML therapy. Therefore, we were interested if miR-23a alters sensitivity to this substance as well. Interestingly, miR-23a failed to alter sensitivity to daunorubicin in MTT assays.

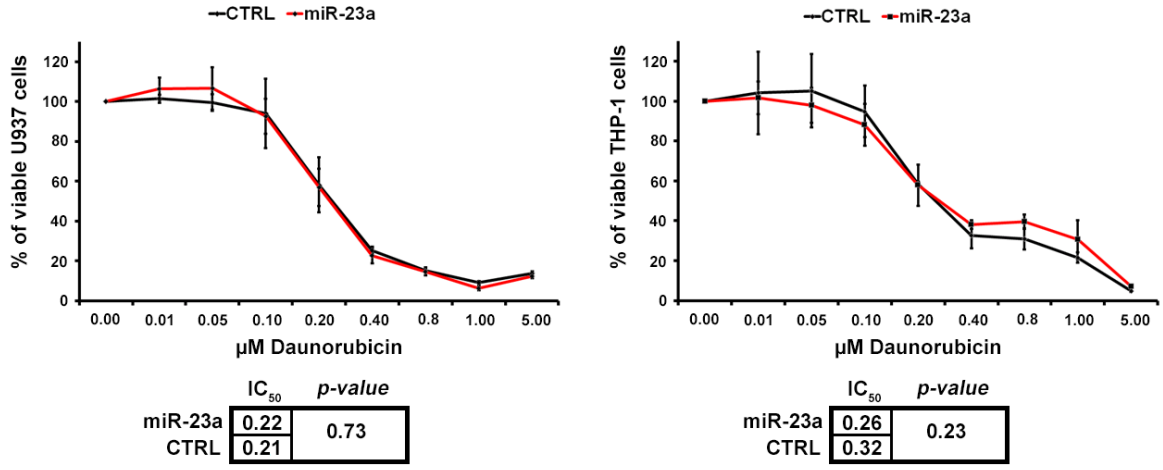


Figure 19: Ectopic expression of miR-23a doesn't alter sensitivity to daunorubicin
MTT cytotoxicity assays in U937 and THP-1 AML cells after incubation with daunorubicin. miR-23a denotes transduction with a miR-23a overexpression construct, CTRL denotes transduction with an empty control vector.
The figure is reproduced from (Hatzl et al. 2020) with permission of *Cancers*.

We then aimed to confirm this data in an assay, which assesses the cytotoxic properties of cells in a more extended time period. Hence, we employed U937 cells and performed colony formation assays in semi-solid media. In these assays, U937 cells were pre-incubated with 5µM AraC and seeded onto soft agar plates. Of note U937 cell were the only cell line which show sufficient formation of colonies, so we focused on them in these experiments. In agreement with the data generated in the MTT assays, U937 cells with stable overexpression of miR-23a showed a significantly increased formation of colonies after AraC pre-incubation when compared with empty control vector-transfected U937 cells. Taken together, ectopic expression of miR-23a mediates resistance to AraC in short term MTT assays, as well as in long term colony forming assays.

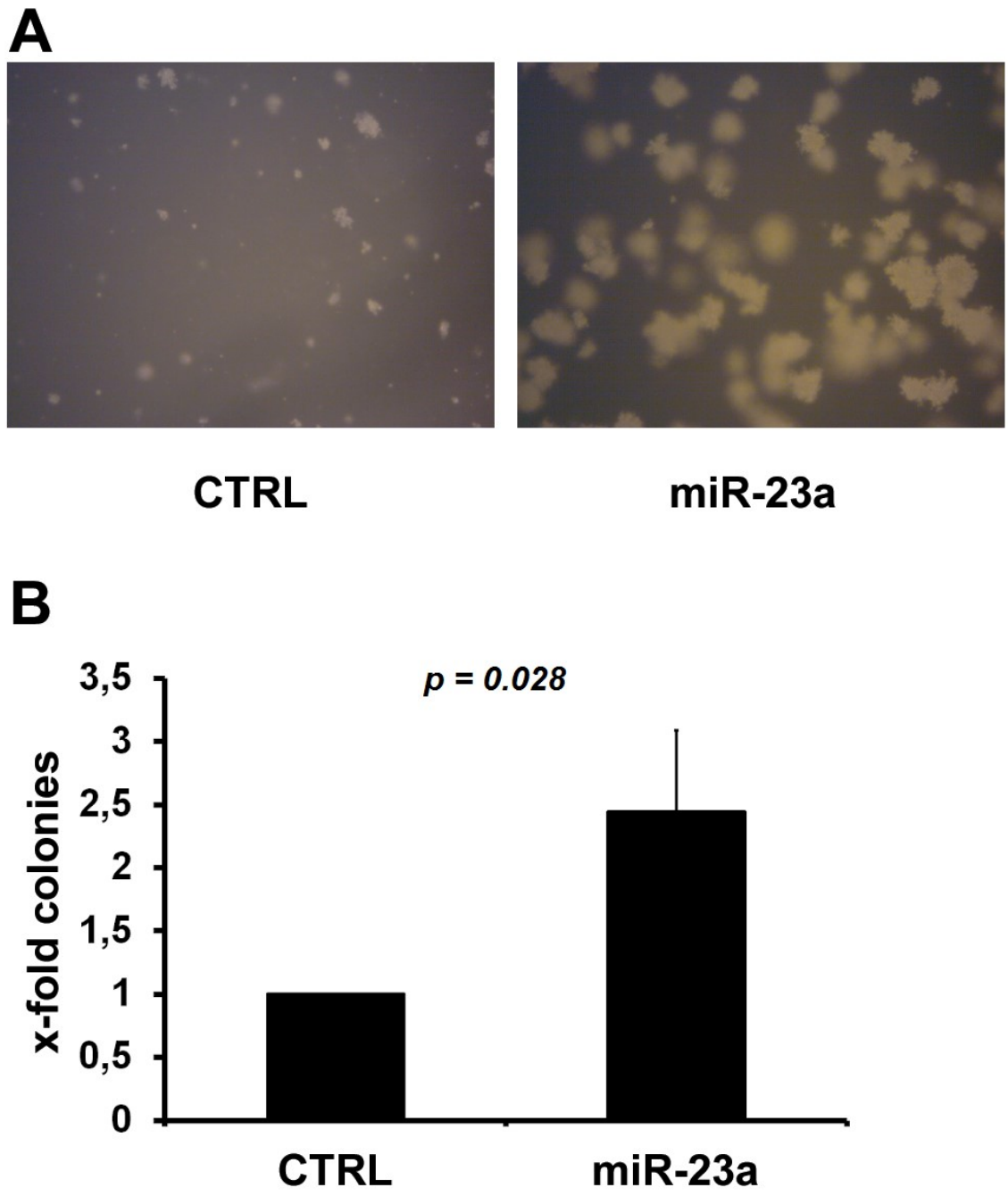


Figure 20: Clonogenic assays after miR-23a overexpression and AraC-treatment
For soft agar colony formation assays, cells carrying a miR-23a overexpression construct (miR-23a) and an empty vector control (CTRL), were incubated with 5 µM AraC. Subsequently, cells were seeded in soft agar and colonies were counted after 8-10 days.
A) Representative pictures of colonies after incubation
B) The graphs summarize the results of at three independent experiments. Data are expressed as mean ±SD
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3.2.2 Increased expression of miR-23a correlates with relapsed/refractory AML, with the leukemic stem cell pool and with shorter survival in AraC-treated patients

In the next step, we sought to identify the clinical relevance of this finding. Therefore, we initially measured miR-23a expression by qPCR in 24 paired patient samples at the time point of diagnosis and at chemorefractory or resistant (R/R) stages. Interestingly we could show that miR-23a is significantly increased in R/R samples as compared with diagnostic samples of the same patients. All 24 patients were treated we 7+3 standard induction chemotherapy and received consolidation therapy containing high dose AraC or allogenic stem cell transplantation. (see table 9: Patients characteristics on next page) These data indicate that increased expression of miR-23a correlates with AraC resistant disease stages in AML.

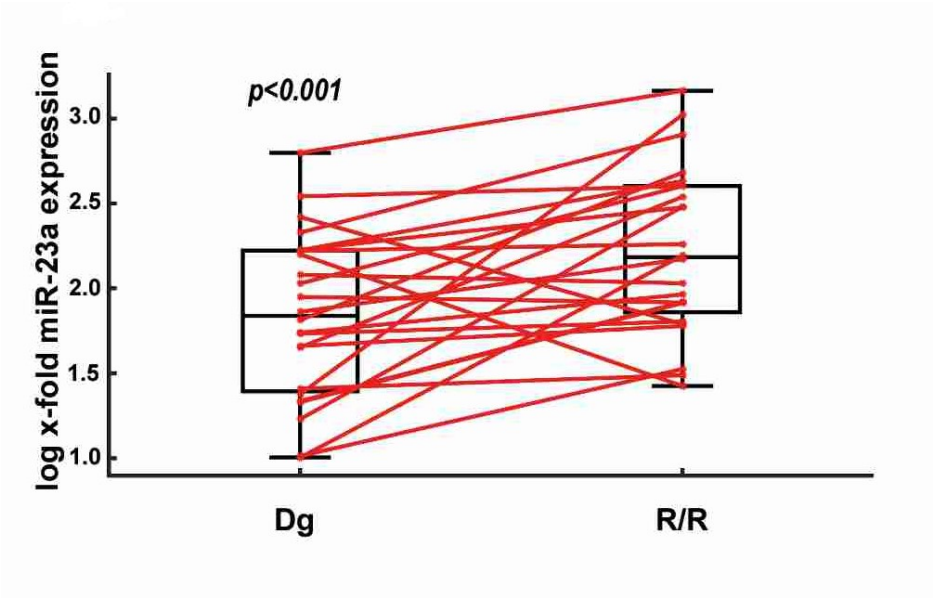

Figure 21: Box plots of miR-23a expression levels in 24 paired AML patient specimens
Dg denotes stage of diagnosis and R/R denotes relapsed/refractory disease. miR-23a expression levels were analyzed by qPCR and are shown as the log-transformed x-fold expression of the calibrator (NB4 cells). The *p*-value has been calculated using the Wilcoxon signed-rank test
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Pat	Age	Gender	WBC [G/L]	Marrow Blasts [%]	LDH [U/L]	Karyotype	ELN	FAB	Therapy
A1	59	f	110	95	910	45,XX,t(2;3)(p23;q26),-7	ad	M4	"7+3" + HD-AraC
A2	58	f	143	95	301	46,XX	fav	M1	"7+3" + HD-AraC
A3	47	f	44.95	90	379	46,XX	fav	M5	"7+3" + HD-AraC
A4	38	f	2.379	80	443	46,XX	int	M2	"7+3" + HD-AraC
A5	38	f	186.12	95	2002	46,XX	int	M4	"7+3" + HD-AraC+HSCT
A6	44	m	6.34	40	2101	46,XY	int	M5	"7+3" + HD-AraC+HSCT
A7	21	m	0.78	50	5774	46,XY,t(6;11)(q27;q23)	int	M5	"7+3" + HD-AraC+HSCT
A8	49	f	107	90	559	47,XX	int	M0	"7+3"
A9	48	m	178	100	886	46,XY	int	M2	"7+3" + HD-AraC
A10	54	m	49.24	80	1650	44~45,XY,-5,-7,-10,+2mar	ad	M2	"7+3"
A11	54	m	24.78	70	313	46,XY	int	M2	"7+3" + HD-AraC+HSCT
A12	49	m	2.01	80	203	46,XY	ad	M1	"7+3"
A13	41	f	91	90	813	46,XX	fav	M4	"7+3"
A14	65	f	40.73	90	393	46.XX	fav	M4	"7+3" + HD-AraC
A15	68	m	30.85	95	786	45~47,XY,der(7)t(7;11)(p13;q13)del(7)(q31)	ad	M0	"7+3" + HD-AraC
A16	58	f	60.53	95	622	49,XX,+6,+8,+22	ad	M4	"7+3" + HD-AraC
A17	52	m	6,1	80	360	46.XY	int	M4	"7+3" + HD-AraC+HSCT
A18	44	m	36.97	95	2041	46~48,XY,+8,ins(10;11)(p12;q23),+19	ad	M0	"7+3" + HD-AraC
A19	55	f	234	95	883	46,XX	int	M4	"7+3" + HD-AraC
A20	59	m	10.85	90	419	46~50,XY,+8, t(10;11)+13,+14,+19	ad	M5	"7+3" + HD-AraC+HSCT
A21	46	f	64.87	95	498	46,XX	int	M4	"7+3" + HD-AraC+HSCT
A22	66	f	3.85	90	288	46,XX	fav	M1	"7+3" + HD-AraC
A23	67	m	12	85	213	46,XX, del(16)	fav	M1	"7+3" + HD-AraC
A24	34	f	5.87	80	209	46,XX	int	M2	"7+3" + HD-AraC+HSCT

Table 9: Patient characteristics of the 24 paired AML specimens (Graz cohort)
Pat., patient-number; WBC, white blood cells; f, female; m, male; G/L, giga per liter; LDH, lactate dehydrogenase; U/L, units per litre; ELN, European Leukemia Net risk stratification; FAB, French-American.British classification; HD, high-dose; AraC, AraC; HSCT, hematopoietic stem cell transplantation, fav, favorable; int, intermediate; ad, adverse;
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In a next step we want to delineate if miR-23a actually correlates with the LSC compartment in AML. In this respect, it is important to notice that AML relapse often develops from an intrinsically resistant LSC pool. (Bonnet, Dick 1997, Shallis, Boddu et al. 2020) Intensive chemotherapy is able to destroy the bulk leukemia but a small fraction of LSCs remains and form chemoresistant relapse. (Yang et al. 2008, Stavropoulou, Kaspar et al. 2016) Interestingly, chemorefractory or resistant (R/R) leukemic cells often show molecular properties, which are similar to those observed in LSCs.(Tremblay et al. 2018) Our data of increased miR-23a expression at R/R stages pinpoint such a scenario, hence we were interested whether miR-23a expression is indeed increased in the LSC pool. Therefore, we analyzed a recently published miRNA array dataset from Lechman ER et al by using the Gene Expression Omnibus (Series GSE55916).(Lechman et al. 2016) The authors of the paper sorted AML specimens according to their CD34/CD38 expression status into four subsets (CD34+/CD38-; CD34+/CD38+; CD34-/CD38+; CD34-/CD38-) and tested the leukemic engraftment potential of each subset in NOD/SCID/gamma-null (NSG) mice. They performed miRNA array analysis in each subset and calculated an LSC miRNA expression score. Compartments were considered to contain LSCs, when they successfully engrafted in NSG mice. For our purpose, we compared the miR-23a expression of the bulk leukemia with the miR-23a expression in the corresponding engrafting LSC compartments. Indeed, miR-23a expression was significantly higher in engrafting subsets (=LSC rich subsets) compared with corresponding bulk leukemia.

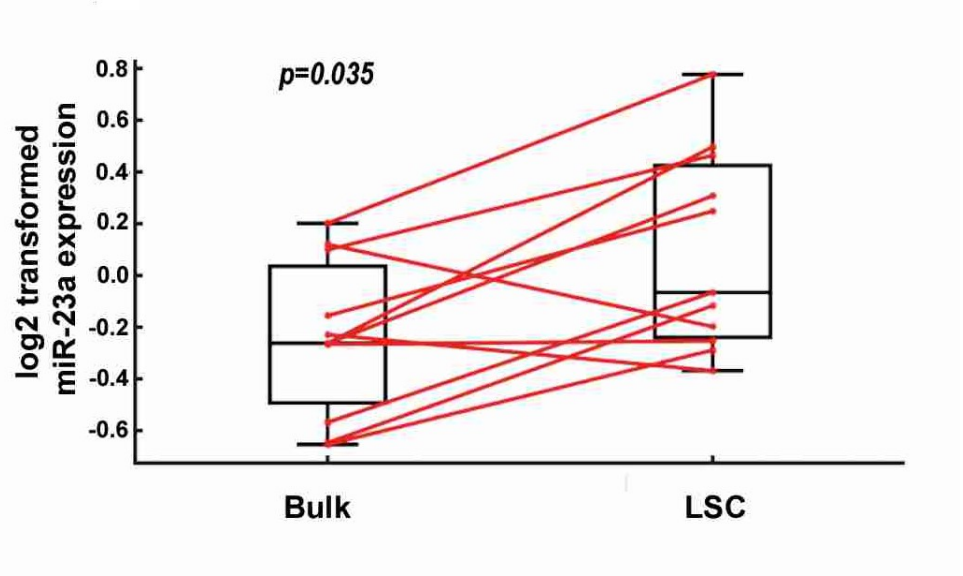

Figure 22: Box plots of miR-23a expression within the Lechman dataset
Bulk denotes eleven AML bulk specimens and LSC the corresponding leukemic stem cell compartment. Expression data were downloaded from the Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE55916; and re-analyzed for the expression of miR-23a. The *p*-value has been calculated using the Wilcoxon signed-rank test. The y-axis displays normalized and log2- transformed miR-microarray expression data (Lechman et al. 2016)
The figure is reproduced from (Hatzl et al. 2020) with permission of *Cancers*.

In the last step, we aimed to corroborate these findings in an independent AML cohort and aimed to evaluate the potential of miR-23a as predictive biomarker for AraC-based therapy. We, therefore, analyzed the AML dataset of TCGA, where data about treatment regimen as well as OS and EFS are available. We analyzed miR-23a expression in all patients who received high dose AraC (n=146). Interestingly, we observed a significantly shorter OS as well as EFS in patients with high expression of miR-23a. This finding could be confirmed in a multivariable Cox regression model by including the established AML risk factors age at diagnosis, WBC and cytogenetic risk.(Cancer Genome Atlas Research Network et al. 2013)

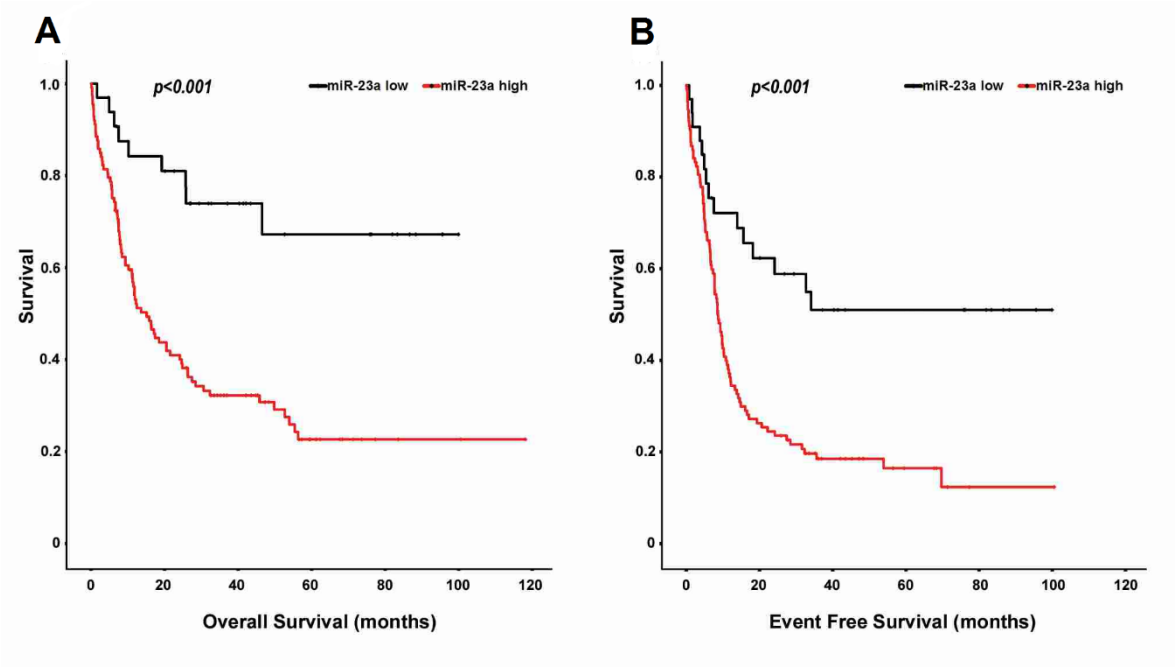

Figure 23: OS and EFS in 146 patients of TCGA AML correlated with miR-23a
A) Overall survival (OS) and B) Event free survival (EFS) in TCGA-AML cohort treated with AraC-containing high-dose regimens (n=146) according to the miR-23a expression status.
Data were downloaded from the TCGA-AML dataset and re-analyzed for the expression of miR-23a. Statistical significance was calculated with a Kaplan-Meier estimator and compared with log-rank tests.(Cancer Genome Atlas Research Network et al. 2013)
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miR-23a				
Parameter	***Variable***	***Hazard ratio***	***95% CI***	***p-value***
OS	**miR-23a high**	2.862	1.385-5.913	**0.005**
	WBC, G/L	1.000	1.000-1.001	0.637
	Age at diagnosis	1.019	1.002-1.035	**0.024**
	Cytogenetics	1.418	1.013-1.984	**0.042**
EFS	**miR-23a high**	1.854	1.023-3.363	**0.042**
	WBC, G/L	1.000	1.000-1.001	0.279
	Age at diagnosis	1.011	0.996-1.025	0.144
	Cytogenetics	1.549	1.130-2.110	**0.006**

Table 10: Multivariable Cox proportional hazard model for miR23a
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3.2.3 *TOP2B* is regulated by miR-23a and affects AraC-sensitivity as well

Up to this point, we can conclude that miR-23a is involved in AraC chemoresistance in AML. However, we wanted to unravel the mechanism behind this effect. To identify potential targets of miR-23a which affect chemosensitivity or resistance properties of AML, we performed an *in-silico* analysis by using the miR-walk 2.0 algorithm.(Dweep, Gretz 2015) The best fifty hits were screened in the literature for involvement in AML chemoresistance. Only *TOP2B*, *ATP-binding cassette transporter 1* (*ABCA1*), and *monocyte-specific enhancer factor 2C* (*MEF2C*) met all of these criteria and were further analyzed. (see Table 11 on next page) Therefore, we re-analyzed the expression of each gene by using the TCGA-AML cohort. Interestingly, only *TOP2B* showed a significant negative correlation with miR-23a, which pinpoints a regulation by miR-23a. (Cancer Genome Atlas Research Network et al. 2013)

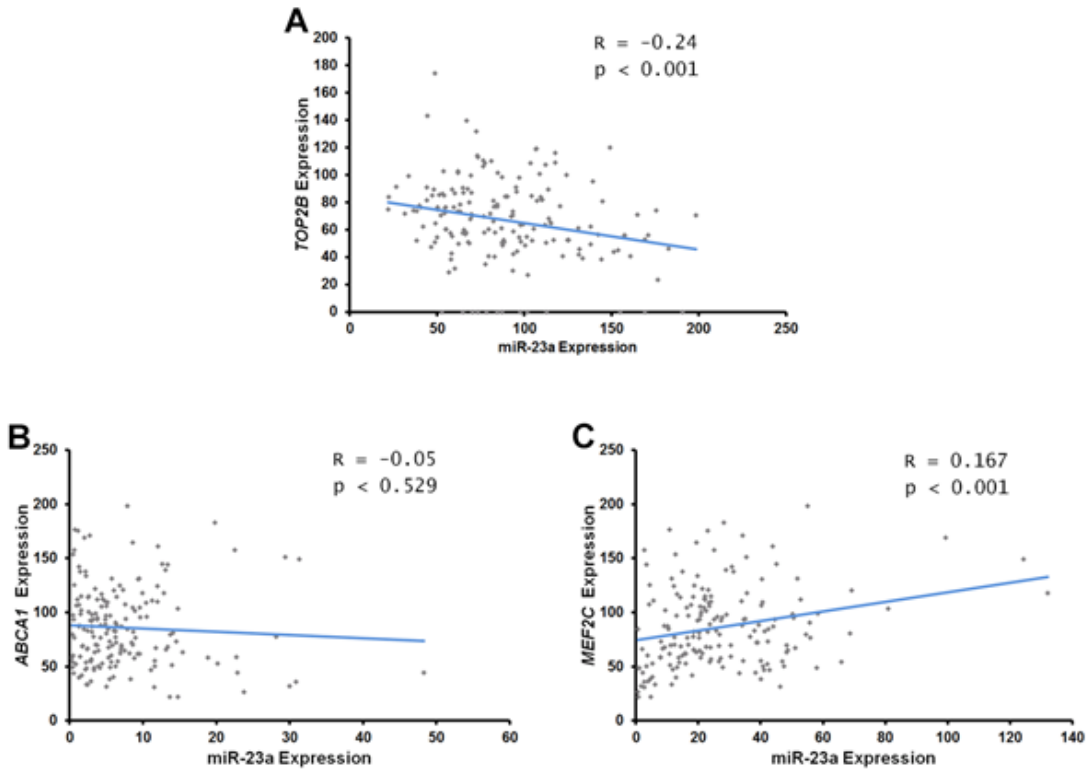

Figure 24: Scatter plots of miR-23a correlation with *TOP2B*, *ABCA1* and *MEF2C within TCGA-AML cohort (n=173)*
Only A) *TOP2B* showed a significant negative correlation with miR-23a; B) *ABCA1* was not significant and C) *MEF2C* showed a significant positive correlation
Expression values are displayed as RNA Sequencing V2 RSEM expression values for mRNAs (depicted at the *y*-axes), and as miRNA-sequencing expression values for miR-23a (depicted at the *x*-axes).
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Gene	Prediction	PMID	Gene	Prediction	PMID	Gene	Prediction	PMID
CCDC6	7/7	x	*WNK3*	7/7	x	*DICER1*	7/7	x
TENM1	7/7	x	*PTCH1*	7/7	x	*FNTA*	7/7	x
METAP1	7/7	x	*VGLL2*	7/7	x	*SUCO*	7/7	x
CAPN6	7/7	x	*TERF2*	7/7	x	*LDHB*	7/7	x
TENM4	7/7	x	*DNAJC6*	7/7	x	*SLC39A10*	7/7	x
FOXA1	7/7	x	*SEC24A*	7/7	x	*NAP1L1*	7/7	x
MSL2	7/7	x	*ESYT1*	7/7	x	*KLHL15*	7/7	x
MRC1	7/7	x	*DLX1*	7/7	x	*RXRG*	7/7	x
RBM25	7/7	x	*BBS9*	7/7	x	*LIN9*	7/7	x
PPP1CB	7/7	x	*ITGB8*	7/7	x	*KDM6A*	7/7	x
RNF38	7/7	x	*MYNN*	7/7	x	*LRPPRC*	7/7	x
TADA2A	7/7	x	*MYH2*	7/7	x	*UHRF1BP1L*	7/7	x
CUL3	7/7	x	*VCPIP1*	7/7	x	*CACNB2*	7/7	x
SEC23A	7/7	x	*RAD23B*	7/7	x	*SMPX*	7/7	x
NEDD4L	7/7	x	*CNOT6L*	7/7	x	*GALNT1*	7/7	x
RUNX2	7/7	x	*KLF10*	7/7	x	*BRWD1*	7/7	x
FOXP1	7/7	x	*ABI1*	7/7	x	*MCM6*	7/7	x
HOXC11	7/7	x	*ZHX1*	7/7	X	*NLGN4X*	7/7	x
LGR4	7/7	x	*ABCA1*	7/7	26463638	*NTS*	7/7	x
MYH1	7/7	x	*MED13L*	7/7	x	*ZIC4*	7/7	x
RBM26	7/7	x	*EGR2*	7/7	x	*SLC20A1*	7/7	x
PPP6C	7/7	x	*HOOK2*	7/7	x	*SPOPL*	7/7	x
RDH10	7/7	x	*IPO5*	7/7	x	*VSNL1*	7/7	x
TCF20	7/7	x	*MYO5C*	7/7	x	*ZMYM2*	7/7	x
SLC4A4	7/7	x	*MYH4*	7/7	x	*GPR64*	7/7	x
CELF2	7/7	x	*NAA15*	7/7	x	*POGZ*	7/7	x
WDR7	7/7	x	*RPS6KA3*	7/7	x	*CALCR*	7/7	x
CLK3	7/7	x	*MAGI3*	7/7	x	*ZNF521*	7/7	x
ARFIP1	7/7	x	*TOP2B*	7/7	22627319	*GAP43*	7/7	x
HOXD10	7/7	x	*COL4A3BP*	7/7	x	*RC3H2*	7/7	x
AMBRA1	7/7	x	*ZBTB1*	7/7	x	*MEF2C*	7/7	29431698
MYH2	7/7	x	*ADH5*	7/7	x	*ARRDC3*	7/7	x
SPTBN1	7/7	x	*TTC7B*	7/7	x	*POU4F2*	7/7	x

Table 11: TOP fifty gene hits of the miR-walk 2.0 algorithm for miR-23a
The column "Prediction" demonstrates the results of the seven target prediction tools included in this algorithm (miRWalk; Microt4, *http://diana.imis.athena-innovation.gr/DianaTools*; miRanda, *http://*microrna.*org/*; miRMap, *https://mirmap.ezlab.org/*; miRNAMap, *http://mirnamap.mbc.nctu.edu.tw/*; RNAhybrid, *https://bio.tools/rnahybrid*; Targetscan, *http://www.targetscan.org/vert_72/*. 7/7 indicates that a specific gene has been identified as a potential miR-23a target in all seven target prediction machines. The column "PMID" presents the results of a PubMed-based literature search. The respective gene was entered along with "AML" or "Acute myeloid leukemia", and "therapeutic resistance" or "chemoresistance". Matches are displayed with the respective PubMed ID and highlighted in red, no match is indicated as x.
The table is reproduced from (Hatzl et al. 2020) with permission of *Cancers*.

To further validate this assumption, we analyzed *TOP2B* expression both on mRNA as well as on protein level after miR-23a modulation in two different hematopoietic cell lines.

We performed stable miR-23a overexpression by lentiviral transduction in U937 and THP-1 cells and monitored *TOP2B mRNA* expression by qPCR as well as TOP2B protein expression by immunoblot. Indeed, ectopic overexpression of miR-23a caused a significant downregulation of TOP2B both on mRNA as well as on protein level. To further corroborate these findings, we additionally transiently transfected miR-23a hairpin inhibitor into THP-1 AML cells. Interestingly, miR-23a knockdown caused a significant overexpression of TOP2B both on mRNA and protein level.

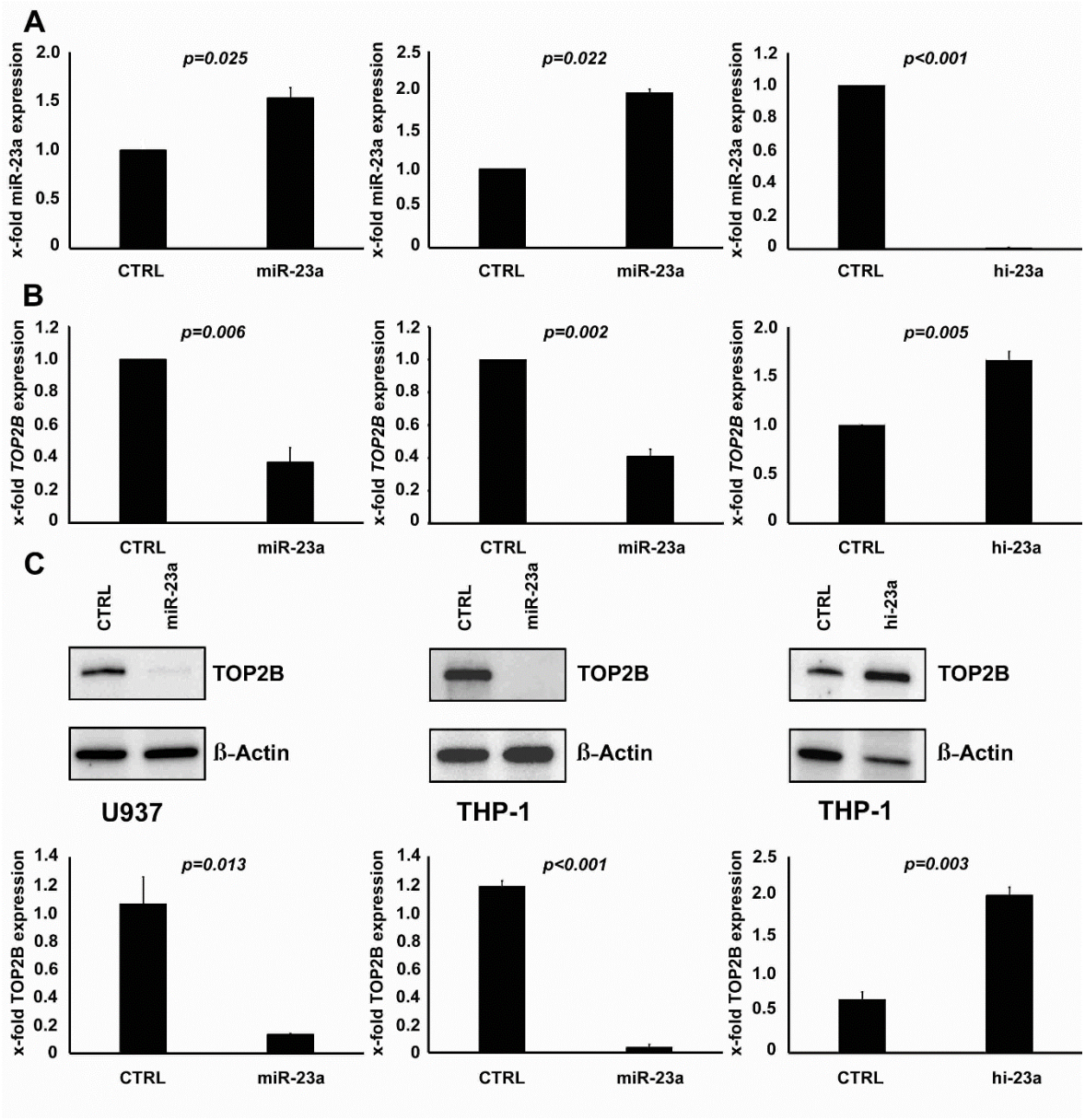

Figure 25: TOP2B expression after miR-23a modulation in hematopoietic cell lines
A) Overexpression of miR-23a in U937 and THP-1 cells Downregulation of miR-23a in THP-1 cells Expression of miR-23a levels were measured by qPCR.
B) *TOP2B* mRNA expression analysis by qPCR of the condition mentioned above
C) Immunoblots showing *TOP2B* modulation in the different cell lines and densitometric analysis of immunoblots
All graphs represent the mean of three independent experiments ± standard deviation; expression levels are given as *x*-fold expression of scrambled control.
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Up to this point, we can conclude that TOP2B is regulated by miR-23a. In the next step, we were interested if decreased expression of TOP2B mediates resistance to AraC in the same way as overexpression of miR-23a did induce this chemoresistance. Therefore, we performed a siRNA based knockdown of TOP2B in U937 and THP-1 cells and repeated the MTT cytotoxicity assays. Indeed, TOP2B knock down cells showed a significantly lower sensitivity against AraC.

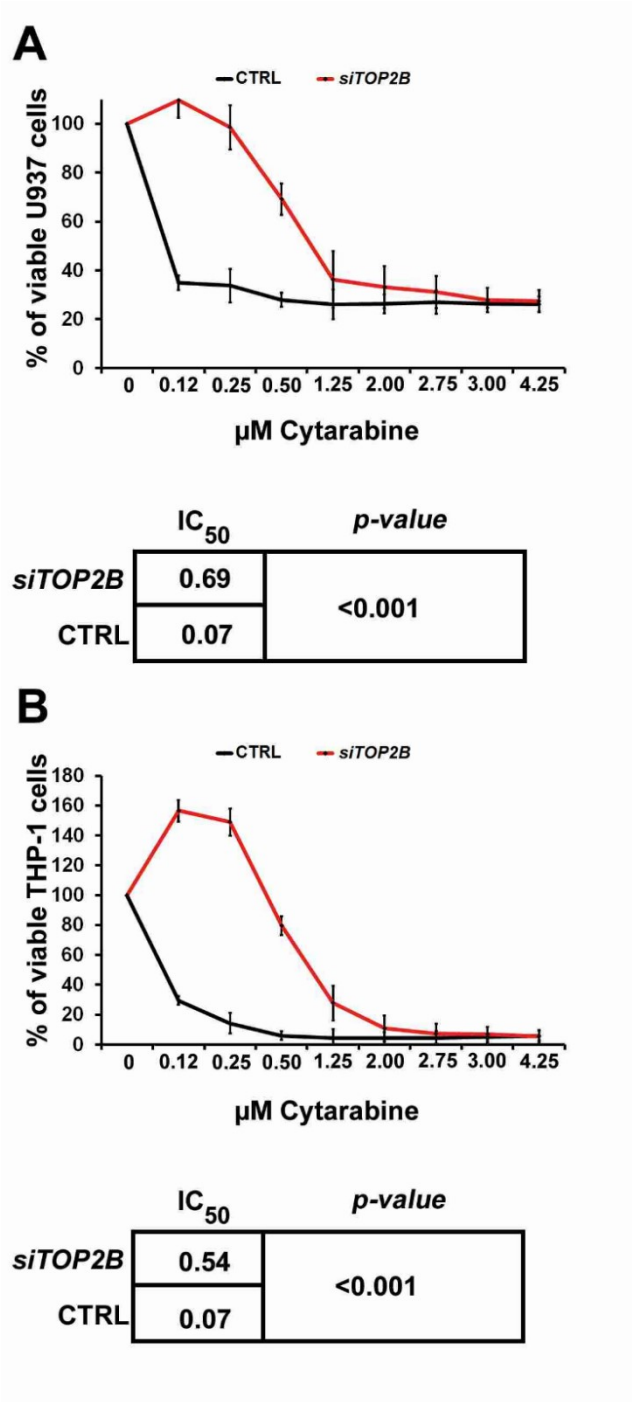

Figure 26: Knockdown of TOP2B reduces sensitivity to AraC
MTT cytotoxicity assays in A) U937cells and B) THP-1 cells after incubation with AraC. *SiTOP2B* denotes transfection/transduction with a *TOP2B* silencing construct; CTRL denotes transfection with an empty scrambled control
The figure is reproduced from (Hatzl et al. 2020) with permission of *Cancers*.

3.2.4 *TOP2B* is a direct target of miR-23a

In the next step, we sought to clarify if *TOP2B* is a direct target of miR-23a. By, analyzing the 3´UTR of *TOP2B,* we were able to identify a putative miR-23a binding site. Next, we attached the 3´UTR of *TOP2B* to a luciferase expression construct. Importantly, co-transfection of this construct together with miR-23a mimics into HEK-293 cells resulted in a significant decrease of luciferase activity. To verify if this regulation is caused via direct interaction to the putative binding site within the 3´UTR we altered the binding site either by deletion, or by two different mutations. Indeed, both approaches - either deletion or mutation - failed to influence luciferase activity.

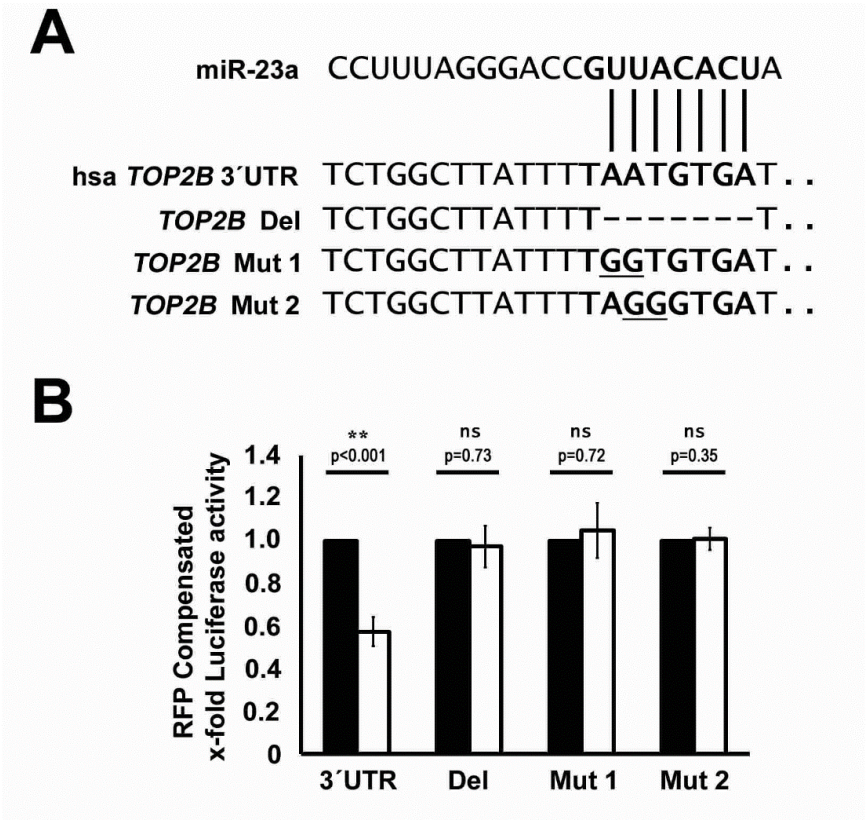

Figure 27: miR-23a binds the 3´UTR of *TOP2B* and modulates its expression
A) Genomic sequence of human 3´UTR of *TOP2B* with both binding sites. *TOP2B* Del denotes an seven-base deletion of the binding site. *TOP2B* Mut1 and 2 denotes a two-base exchange of the binding site
B) HEK-293 cells were transfected with miR-23a mimics (white bars) or with scrambled control mimics (black bars) together with a luciferase reporter vectors containing wild-type *TOP2B* 3'UTR (*TOP2B* 3'UTR), *TOP2B* Del, or *TOP2B* Mut1 and 2. pCS2-RFP was co-transfected in each experiment to compensate for different transfection efficacy. Graphs display the mean three independent experiments ± SD compensated with RFP expression; values are given as *x*-fold expression of scrambled control-transfected cells
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3.2.5 Decreased expression of TOP2B correlates with R/R AML and with shorter survival in AraC-treated patients

In the last step, we aimed to identify the clinical role of TOP2B expression. Therefore, we analyzed the *TOP2B* expression by qPCR the same way as outlined above for miR-23a in 24 paired patient samples at time point of diagnosis and at the R/R stage. Interestingly, TOP2B was significantly decreased in R/R samples as compared with diagnostic samples of the same patients. This observation is contrary to that we have seen for miR-23a and highlights the role of a miR-23a/TOP2B axis in the development of AraC resistance in AML.

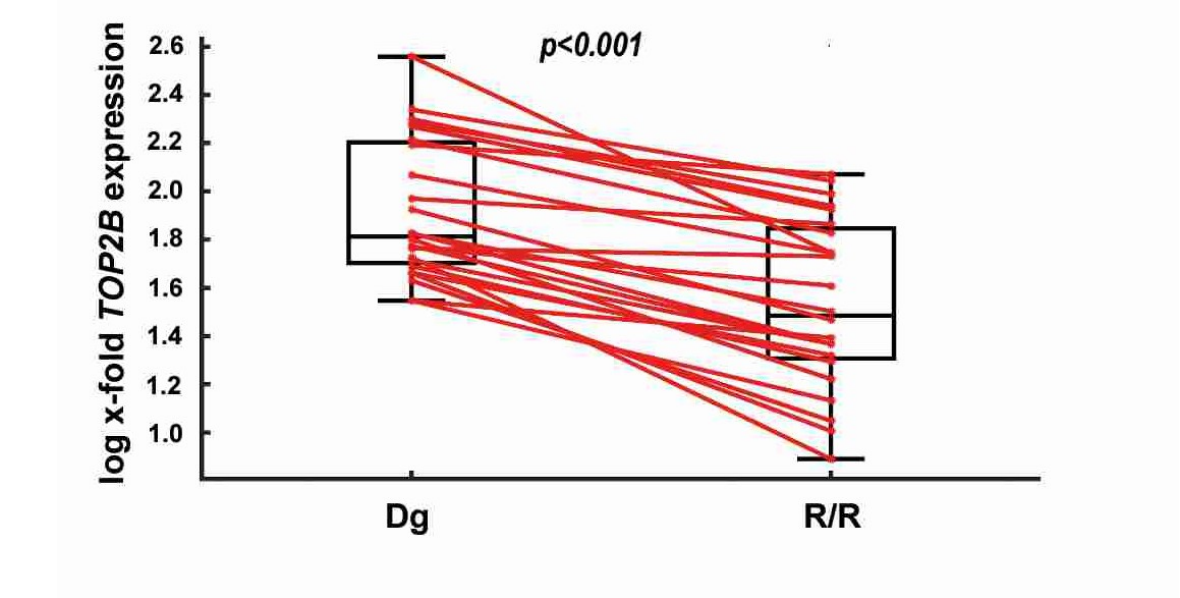

Figure 28: Box plots of *TOP2B* expression levels in 24 paired AML patient specimens
Dg denotes stage of diagnosis and R/R denotes relapsed/refractory disease. *TOP2B* expression levels were analyzed by qPCR and are shown as the log-transformed x-fold expression of the calibrator (NB4 cells). The *p*-value has been calculated using the Wilcoxon signed-rank test
The figure is reproduced from (Hatzl et al. 2020) with permission of *Cancers*.

Finally, we sought to validate these findings in an independent cohort of TCGA. Therefore, we re-analyzed the above-described cohort for TOP2B expression and correlated the results with OS and EFS in patients treated with high-dose AraC (n=154)

Contrary to the results for miR-23a expression, we observed a significantly longer OS as well as EFS in patients with high expression of *TOP2B*. This finding could be confirmed in a multivariable Cox regression model by including the established AML risk factors age at diagnosis, WBC and cytogenetic risk. (Cancer Genome Atlas Research Network et al. 2013)

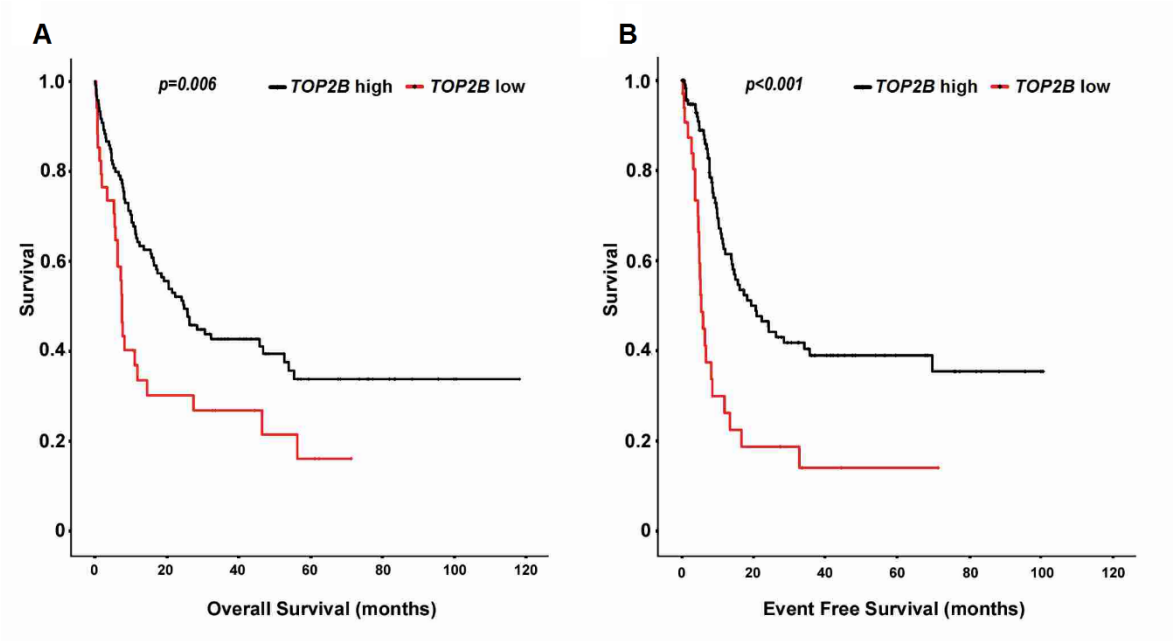

Figure 29: OS and EFS in 154 patients of TCGA AML correlated with *TOP2B*
A) Overall survival (OS) and B) Event free survival (EFS) in TCGA-AML cohort treated with AraC-containing high-dose regimens (n=154) according to the *TOP2B* expression status.
Data were downloaded from the TCGA-AML dataset and re-analyzed for the expression of *TOP2B*. Statistical significance was calculated with a Kaplan-Meier estimator and compared with log-rank tests.
The figure is reproduced from (Hatzl et al. 2020) with permission of *Cancers*.

TOP2B				
Parameter	***Variable***	***Hazard ratio***	***95% CI***	***p-value***
OS	***TOP2B* high**	0.581	0.367–0.922	**0.021**
	WBC, G/L	1.000	1.000–1.001	0.092
	Age at diagnosis	1.031	1.015–1.047	**<0.001**
	Cytogenetics	1.844	1.328–2.561	**<0.001**
EFS	***TOP2B* high**	0.399	0.245–0.650	**<0.001**
	WBC, G/L	1.000	1.000–1.001	0.067
	Age at diagnosis	1.009	0.994–1.025	0.232
	Cytogenetics	1.513	1.056–2.168	**0.024**

Table 12: Multivariable Cox proportional hazard model for *TOP2B*
The table is reproduced from (Hatzl et al. 2020) with permission of *Cancers*.

4 DISCUSSION

4.1 Increased expression of miR-23a mediates a loss of expression of RKIP

Loss of RKIP expression is a frequent event in acute myeloid leukemia and has been observed in approximately 20% of cases. In addition, its role in myeloid leukemogenesis has been well established in previous projects of our group. (Zebisch et al. 2012, Zebisch et al. 2009, Caraffini et al. 2018, Caraffini et al. 2019)

In this part of my thesis, we aimed to unravel the mechanism behind RKIP loss in AML and focused on micro-RNAs as potential reason. In preliminary screening experiments, we correlated RKIP expression levels with miR-microarray expression profiling data and thereby identified an inverse correlation between the expression of RKIP and miR-23a.We could then show a strong association between high expression levels of miR-23a and low expression of RKIP in more than 400 primary AML patient samples, which suggests a role of this miR in the development of RKIP loss. Indeed, we could functionally show that miR-23a overexpression causes RKIP knockdown in different in vitro cell line studies. Interestingly, this effect was mediated via direct interaction of miR-23a with the 3´UTR of RKIP. In agreement with our results, Mi and coworkers could show high expression levels of miR-23a in AML as well. They also observed that increased expression of miR-23a is part of a unique micro-RNA signature of AML.(Mi, Lu et al. 2007) Zhang et al. recently showed *in vivo* the functional involvement of increased miR-23a expression in myeloid leukemogenesis. (Y. C. Zhang, Ye, Zeng, Chin, Huang, and Fu 2015) In agreement with these data, we could show that overexpression of miR-23a, which induces the same phenotype as RKIP knockdown, enforces cell proliferation in hematopoietic cell lines. Interestingly, we were able to rescue this proliferative phenotype by an artificial concomitant overexpression of RKIP in these cells. This indicates that at least some parts of the miR-23a-mediated leukemogenic effects are caused by RKIP functioning as effector protein. Interestingly, our group could show that loss of RKIP is associated with favorable prognosis in AML in a previous study.(Zebisch et al. 2012) Surprisingly, increased expression of miR-23a failed to influence survival of AML patients in this work.

Though, the mechanisms and reasons behind this differential influence on outcome parameters remain unclear so far. We can hypothesize that miR-23a orchestrates a broad network of different effector proteins, in which RKIP only mediates a small part of the whole effect. One can further speculate that miR-23a targets enzymes, which are related to drug resistance in an RKIP-independent manner. This topic is investigated in detail in the second part of my thesis and will be discussed separately within this section.

Another interesting finding of my thesis was the association of miR-23a expression with myelomonocytic AML phenotypes. In detail, we could demonstrate that AML with myelomonocytic differentiation shows increased expression of miR-23a and decreased expression of RKIP compared with all other AML phenotypes, which suggests that the miR-23a/RKIP axis could act as potential regulator of differentiation in AML. These data are also supported by other groups. Chhabra et al could demonstrate that increased expression of miR-23a exists exclusively in myeloid-differentiated leukemias.(Chhabra, Dubey et al. 2010) Another group postulated that miR-23a is part of a myeloid micro-RNA differentiation signature, which can distinguish between AML and acute lymphoblastic leukemia.(Mi et al. 2007) These findings are further supported by functional in-vitro and in-vivo data of our group. Caraffini et al., demonstrated that HSPCs with a deletion of RKIP have a differentiation bias into the myelomonocytic lineage. Indeed myeloproliferative diseases in mice with RKIP deletion had a more pronounced myelomonocytic phenotype as compared to mice without RKIP deletion. (Caraffini et al. 2019)

MiR-23a is part of a micro-RNA cluster consisting of three different but still closely related micro-RNAs. In detail, miR-23a works in a network with miR-27a and miR-24-2, the so-called miR23a/27a/24-2 cluster. Within this network, all micro-RNAs execute similar functions and target related mRNAs. Interestingly, the importance of miR-23a/27a/24-2 cluster dysregulation as well as the dysregulation of singular micro-RNAs of this cluster for malignant transformation has been described in different human malignancies previously.(Chhabra et al. 2010) Recently, Li and coworkers could show that miR-27a mediates cisplatin resistance in lung cancer cells via downregulation of RKIP, which shows a relationship of this micro-RNA cluster to RKIP. However, although the cluster-members share multiple common target messenger RNAs, each single micro-RNA within this cluster underlies

different regulation-mechanisms and responds to different cellular stimuli. In line with this complex regulation of this cluster, only increased miR-23a expression correlated with decreased expression of RKIP in AML, whereas miR-27a and miR-24-2 failed to do so. This finding underscores the functional involvement of miR-23a in the mechanisms of RKIP loss, suggesting miR-23a as causative factor within this process. This conclusion is further strengthened by data provided from Zhang et al., who showed the pro-leukemogenic effect of miR-23a overexpression in a murine *in vivo* model.(Y. C. Zhang et al. 2015)

Having shown the importance of the miR-23a/RKIP axis in AML, we than aimed to identify if a potential correlation between RKIP and micro-RNAs of the miR-23a/27a/24-2 cluster exists in other human cancer entities as well. Therefore, we performed a database retrieval within the TCGA and searched for correlations between the single members of the cluster and RKIP. Overall, we were able to analyze 14 additional cancer entities comprising almost 4300 primary patient samples. Interestingly, we could observe a significant and inverse correlation between a member of the miR-23a/27a/24-2 cluster and RKIP in the vast majority of tumor entities (10/14) suggesting a general role of the miR-23a/27a/24-2/RKIP-axis in human carcinogenesis. Of note, miR-23a and RKIP showed a significant inverse correlation in all significantly associated cancer entities with exception of lung squamous cell carcinoma, in which only miR-27a showed this correlation, suggesting miR-23a as the predominant cluster member. These results are further supported by the fact that dysregulation of miR-23a has been described in the most of these cancer entities previously.(N. Wang, Tan et al. 2018) Even more, functional relevance for carcinogenesis as well as influence on drug resistance has been suggested by different groups for miR-23a previously.(Chhabra et al. 2010, X. W. Zhang, Liu et al. 2015, Qu, Liu et al. 2015, A. H. Jin, Wei 2015, Bao, Zhao et al. 2014)

In conclusion, we have found that increased expression of miR-23a negatively correlates with decreased expression of RKIP in more than 400 primary AML patient specimens. Furthermore, we have identified increased expression of miR-23a as well as decreased expression of RKIP associated with myelomonocytic differentiation of AML. We could further functionally show that miR-23a modulates RKIP expression via direct interaction with its 3´UTR. Finally, we could show that the miR-23a/27a/24-2/RKIP axis is of relevance for a broad range of

human cancer entities highlighting its importance for basic and clinical research in the field of hematology and oncology.

4.2 Increased expression of miR-23a mediates chemoresistance to AraC in acute myeloid leukemia

Chemoresistance in AML describes the failure of cytostatic substances to kill leukemic cells. It displays a major problem in AML therapy and is one of the major reasons for the poor prognosis of AML patients. In the past decades it has been a key challenge in hematological research to understand the molecular mechanisms behind chemoresistance, and to design strategies to might overcome this phenomenon. Many mechanisms how leukemic cells promote their survival and avoid cell death in the presence of therapeutic substances could be unraveled. Recently, miRNAs could be shown as novel players which potentially pull the strings within a network of deregulated signaling pathways leading to ultimately chemoresistant AML. (Zebisch, Hatzl et al. 2016) As part of my thesis, we investigated the role of miR-23a in chemoresistance to AraC and Daunorubicin, which form the backbone of AML chemotherapy. We could demonstrate that increased expression of miR-23a reduces the sensitivity of different human leukemic cell lines against AraC. Furthermore, we could show that increased expression of miR-23a correlates with adverse outcomes in AraC-treated AML patients. Over the past years others and our own group could prove the functional significance of miR-23a within the process of myeloid leukemogenesis. (Hatzl et al. 2016, Y. C. Zhang et al. 2015, Zhao, Wang et al. 2019) However, its role in AraC resistance in AML is novel. This finding matches with previous data from solid tumors. Different groups could demonstrate that overexpression of miR-23a mediates resistance to the antimetabolite 5-flurouracil (5-FU) and the intercalating drug cisplatin.(X. Li, Li et al. 2015, Peng, Zhang et al. 2015, Yu, Zhong et al. 2010, Komatsu, Ichikawa et al. 2016) One central finding of our study was that miR-23a was significantly higher expressed in patient samples at R/R stage compared with diagnostic samples. This observation allows two possible hypotheses. In the first hypothesis, we can assume that leukemic blasts with an increased expression of miR-23a form one of the major clones of the bulk leukemia. Those cells with increased miR-23a expression are primary refractory to chemotherapy and form the relapse or refractory disease. The second hypothesis is based on increased expression of miR-23a in the small fraction of LSCs. (Saito, Kitamura et al. 2010) It its well-known that a resistant LSC compartment can persist after chemotherapy and give rise to a relapse, even if

the bulk leukemia is cleared by conventional cytotoxic drugs. (Kreso, Dick 2014, Lechman et al. 2016, Thomas, Majeti 2017, Reinisch, Chan et al. 2015) These relapse cells often exhibit the same molecular phenotype as their paternal LSCs with the same chemoresistant properties. Lechman et al. previously published a microRNA expression profiling dataset of validated LSCs and their corresponding bulk leukemia. (Lechman et al. 2016) By reanalyzing this dataset, we could show that miR-23a expression is significantly higher in the LSC compartment as compared with bulk leukemia. This suggests that miR-23a could be a potential LSC marker. Furthermore, we can hypothesize that miR-23a helps LSCs to overcome AraC-induced cell death and apoptosis. Other groups could generate similar results in solid human cancers and could highlight the importance of miR-23a within the cancer stem cell (CSC). Jahid et al. could functionally show that miR-23a promotes invasion and migration of CSCs of colorectal carcinoma and that miR-23a is one of the highest expressed microRNAs amongst colorectal CSC. (Jahid, Sun et al. 2012) Other groups suggested the involvement of miR-23a and miR-23a/miR-27a cluster within drug-resistance of colorectal carcinoma and metastases formation. (Z. Wang, Wei et al. 2012) Han et al. find similar expression results of miR-23a within the CSC pool of non-small cell lung cancer (NSCLC). Furthermore, they could show that isolated NSCLC-CSC were resistant to erlotinib which inhibits the epidermal growth factor receptor signaling and plays a major role in the treatment of NSCLC. Interestingly, they could restore erlotinib-sensitivity by miR-23a knockdown via miR-23a hairpin inhibitors. (Han, Zhou et al. 2017) These results are interesting because targeted therapies like tyrosine kinase inhibitors can only cure cancer patients if they are possible to eradicate and destroy the cancer or LSC pool. In this work, the authors demonstrated that concomitant treatment of targeted therapies with antisense RNAs is able to target the CSC. If miR-23a provides a potential target in order to sensitize LSCs against cytotoxic drugs remains unclear at the moment and requires special studies which focus on this issue in the future. Within our study we could further show that high expression of miR-23a is an independent prognostic marker of adverse outcome in standard AraC-treated AML patients. This finding highlights the potential of miR-23a as attractive biomarker. In more detail, it could help to distinguish between patients who will benefit from high dose AraC treatment and those who will not. Especially, older patients with comorbidities would possibly benefit from such knowledge. For example, those

patients with high expression of miR-23a will profit from treatment with low-intensity treatment regimens with hypomethylating agents like 5-azacitidine or decitabine. (Dohner et al. 2017, Pleyer, Dohner et al. 2017, Dombret, Itzykson 2017, Glasel 1995, Pleyer, Burgstaller et al. 2016) Of note, microRNAs and especially miR-23a have been used as biomarker previously in other malignancies. (Komatsu, Ichikawa et al. 2016, Gordon, Wong et al. 2013) The usage of microRNAs as biomarkers provides several advantages compared with conventional messenger RNA analysis. In detail, the small size of microRNAs makes them highly stable during tissue processing thereby ensuring stability and reproducibility of the expression analysis. Previous studies have shown that microRNA expression analysis is possible from different hematological tissues like buffy coat, formalin-fixed paraffin embedded tissues and decalcified bone marrow biopsies. (Gordon et al. 2013)

Despite all advantages of miR-23a as potential decision-support biomarker for the best therapeutic regimen (high-dose AraC vs. non-AraC containing regimens like hypomethylating agents), the role of miR-23a within hypomethylating agent resistance is still unclear. In order to clarify this question, further laboratory studies and clinical trials will be needed.

Within this study, we were also interested in the mechanisms behind the miR-23a-mediated AraC resistance. Therefore, we searched for potential miR-23a targets. By a combination of in-silico target prediction tools and literature review focusing on targets which were associated with chemoresistance previously, we were able to identify TOP2B as miR-23a target. Furthermore, we could show in functional studies that TOP2B-3´UTR is a direct target of mir-23a, and that miR-23a regulates TOP2B expression.

DNA-topoisomerases are ubiquitously expressed enzymes that are crucial for unlinking and cleaving DNA as well as DNA replication and transcription. (Delgado, Hsieh et al. 2018) TOP2B is responsible for introducing single and double strand breaks as well as warranting the optimal position of the DNA in order to secure its re-cleavage, which is catalyzed by other topoisomerases.(Austin, Lee et al. 2018) Anthracyclines and other topoisomerase inhibitors block topoisomerases after the introduction of double and/or single strand breaks of DNA thereby inhibiting the re-ligation process. This double-strand breaks of the DNA in turn induces apoptosis and cell death. Previous studies have shown that reduced expression of

topoisomerases is able to mediate resistance to topoisomerase inhibitors like anthracyclines. (Austin et al. 2018, Delgado et al. 2018)

Surprisingly, our functional in vitro cytotoxicity experiments revealed that knockdown of TOP2B reduces the sensitivity to the antimetabolic active nucleoside analog AraC. TOP2B knockdown exhibits the same AraC resistant phenotype than miR-23a overexpression; therefore, we can hypothesize that TOP2B is the executor-protein within the process of miR-23a mediated AraC resistance in AML.

Although these observations that TOP2B mediates resistance to an antimetabolite were unexpected, Pourquier et al. published a model of AraC-resistance, where AraC-resistance was caused by decreased expression levels of a topoisomerase previously. They could show that AraC alters the conformational structure of the DNA and that the antimetabolic incorporation at the +1 position (immediately 3') relative to a unique TOP1 (topoisomerase 1) cleavage site reinforces TOP1-induced single strand breaks. This group could further mechanistically show that the incorporation of AraC into the DNA prohibits the TOP1-dependent re-ligation of the DNA strands, which suggest that topoisomerases and their interaction with the DNA double strand is of fundamental relevance for the cytotoxic effects of AraC. In agreement with our study, Pourquier and co-workers showed that cells which lack TOP1 were resistant to AraC. (Pourquier, Takebayashi et al. 2000)

To put the findings in a more specific context of AML, our study is also in agreement with previously published data from Song et al. They could show that high expression levels of TOP2B correlate with prolonged survival in a cohort of "7+3" treated AML patients.

In conclusion, we demonstrated that increased expression of miR-23a in AML mediates resistance to AraC and correlates with an inferior outcome in AraC-treated AML patients. We also show that miR-23a expression is particularly high at the stage of primary chemoresistant disease and/or chemoresistant relapse and that it is linked to the intrinsically resistant LSC pool. Mechanistically, we demonstrate that miR-23a causes the downregulation of *TOP2B*, which is likely to mediate its effects on AraC sensitivity.

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