Master thesis

The Role of Micro-RNAs and Transcriptional Repressors in Acute Myeloid Leukaemia with a Loss of the RAF Kinase Inhibitor Protein

submitted by

Maja Kim Küpper, BSc

in order to attain the academic degree

Master of Science

at the

Graz University of Technology

performed in the group ‘Myeloid Cells & Leukemia’

Division of Haematology, Medical University of Graz

under the supervision of

Dr. Armin Zebisch &

Univ.-Prof. Dr. Heinz Sill

Graz, March 2014

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am …… ……..
(Unterschrift)

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Graz, …… ……..
(Signature)

Danksagung

Mein besonderer Dank gilt meinem Betreuer Dr. Armin Zebisch zum einen, dass er mir die Möglichkeit gegeben hat, in seinem Labor an einem spannenden Projekt arbeiten zu können und zum anderen für seine großartige Unterstützung in zahlreichen Diskussionen und Analysen sowie in der Fertigstellung dieser Arbeit. Durch seine Ratschläge und Supervision konnte ich wichtige Dinge lernen, die für das wissenschaftliche Arbeiten erforderlich sind und mir bei meinem weiteren Weg hilfreich sein werden.
Mein weiterer besonderer Dank gilt unserem Gruppenleiter Univ.-Prof. Dr. Heinz Sill dem ich für die Möglichkeit danken möchte, in seiner Forschungseinheit der Klinischen Abteilung für Hämatologie zu arbeiten.

Auch gilt mein Dank der gesamten Arbeitsgruppe “Myeloid Cells & Leukemia” für die herzliche Aufnahme ins Team und die kollegiale Zusammenarbeit. Besonders hervorheben möchte ich dabei Olivia Wolf und Karin Lind, die mir bei technischen Fragen stets mit Rat und Tat zur Seite gestanden haben.

Mein weiterer Dank geht an Martin Pichler und Karin Koller, die mich in die mikro-RNA Analysen eingearbeitet haben.

Zum Schluss aber nicht zu Letzt danke ich meiner Familie, denen ich diese Arbeit widme. Sie geben mir mein ganzes Leben bedingungslosen Rückhalt, unterstützen mich und geben mir Kraft.

Tell me and I forget, teach me and I remember, involve me and I learn

- Benjamin Franklin -

Diese These wurde durch Forschungsgelder der Medizinischen Universität Graz (START) sowie der Leukämiehilfe Steiermark unterstützt.

Zusammenfassung

Die akute myeloische Leukämie (AML) ist eine aggressive Neoplasie, die durch maligne Transformation hämatopoetischer Stamm- und Vorläuferzellen hervorgerufen wird. In einer rezenten Arbeit konnten wir einen somatischen, leukämiespezifischen Verlust des Metastasensupressors RAF Kinase Inhibitor Protein (RKIP) bei ca. 20% aller AML Patienten detektieren. In Folgeuntersuchungen zeigte sich, dass dieser Expressionsverlust von funktioneller Bedeutung für die maligne Transformation hämatopoetischer Zellen ist. Die Mechanismen, die diesen Expressionsverlust verursachen, sind jedoch noch immer unklar. Ziel meiner These war es deshalb, eine mögliche Bedeutung von mikro-RNAs und transkriptionellen Repressoren innerhalb dieses Prozesses zu untersuchen.

Zu Beginn analysierten wir die RKIP Expression in ausgewählten Proben unserer eigenen Leukämie-Biobank mittels Western blot und qPCR auf Protein bzw. mRNA Ebene. Dadurch konnten wir eine Kohorte von 20 AML Patientenproben erstellen, welche eine ausgeglichene Anzahl von Fällen mit und ohne RKIP Verlust enthält. In einem nächsten Schritt haben wir ein Set von zehn mikro-RNAs definiert, welche im Rahmen einer vorangegangenen mikro-RNA ChiP Analyse aberrante Expressionen bei AML Proben mit RKIP Verlust zeigten. Durch qPCR Expressionsanalysen dieser Kandidaten in unserer zuvor definierten AML Kohorte konnten wir zeigen, dass mikro-RNA-15a, mikro-RNA-23a, mikro-RNA-23b, mikro-RNA-24 und mikro-RNA-320a in den Proben die als RKIP Verlust definiert waren, deutlich stärker exprimiert werden. In einem nächsten Schritt haben wir uns gefragt ob die erhöhte mikro-RNA Expression ein Effekt des RKIP Verlustes sein kann und haben dazu ein RKIP knockout Maus Modell herangezogen. Mittels qPCR konnten wir keine Unterschiede in der Expression der oben erwähnen mikro-RNAs zwischen RKIP knockout und wildtyp Mäusen zeigen, was ein Hinweis dafür ist, dass der RKIP Verlust nicht ursächlich für die veränderte mikro-RNA Expression ist. In der letzten Phase haben wir die Expression von sechs transkriptionellen Repressoren, die bereits als RKIP Repressoren beschrieben wurden, mittels qPCR in allen oben angeführten AML Patientenproben analysiert. Dabei konnten wir keinen Unterschied zwischen den Proben mit und ohne RKIP Verlust feststellen, sodass wir ausschließen, dass diese transkriptionellen Repressoren den RKIP Verlust verursachen.

Zusammengefasst haben wir eine gut charakterisierte Kohorte von AML Patientenproben etabliert, die eine Verbindung von molekularen Assoziationen mit RKIP Verlust ermöglicht. Dadurch konnten wir ein spezifisches Set von mikro-RNAs identifizieren, dass eine erhöhte Expression in AML Patientenproben mit RKIP Verlust zeigt. Unter Verwendung eines RKIP knockout Maus Modells waren wir des

Weiteren in der Lage zu zeigen, dass die erhöhte Expression dieser mikro-RNAs kein Effekt des RKIP Verlustes darstellt. Momentan prüfen wir mit Hilfe von *in-vitro* Modellsystemen, ob deren gesteigerte Expression in der Tat die Ursache für den RKIP Verlust ist.

Abstract

Acute myeloid leukaemia is an aggressive neoplasia, which is caused by malignant transformation of haematopoietic stem- and progenitor cells. We recently described a somatic, leukaemia specific loss of the metastasis-suppressor RAF kinase inhibitor protein (RKIP) in approximately 20% of AML specimens. While RKIP loss proved to be of functional importance in malignant transformation of haematopoietic cells, the mechanisms causing its down-regulation are still unclear. Therefore, the aim of my thesis was to delineate a potential implication of micro-RNAs and transcriptional repressors, respectively, within this process.

In a first step, we analysed RKIP expression levels in selected samples of our own leukaemia-biobank, both on the protein and mRNA level by employing Western blot and qPCR, respectively. Thereby, we were able to establish a cohort of 20 AML specimens, containing equal numbers of samples with and without RKIP loss. We then defined a set of ten micro-RNAs, which have been demonstrated to be aberrantly expressed in AML specimens with decreased RKIP expression by the means of micro-RNA ChiP profiling previously. By performing qPCR based expression analysis of these candidates in our predefined AML cohort, we were able to describe increased expression levels of micro-RNA-15a, micro-RNA-23a, micro-RNA-23b, micro-RNA-24 and micro-RNA-320a in samples defined as RKIP loss. In the next part, we asked whether increased micro-RNA expression might display an effect of RKIP down-regulation and therefore employed a murine RKIP knockout model. qPCR based expression analysis revealed no differences in micro-RNA expression levels between RKIP knockout animals and their wildtype littermates, indicating that RKIP loss is not the causative event. In the final phase of this thesis, we analysed the expression of six previously reported RKIP repressors by qPCR in all AML patient samples described above. However, no differences were detected between samples with and without RKIP loss, precluding a role of these transcriptional repressors in the down-regulation of RKIP.

Taken together, we were able to establish a well characterized cohort of AML specimens, which enables the association of molecular associations to RKIP down-regulation. By this means, we were able to identify a specific set of micro-RNAs, showing increased expression levels in AML patient specimens with a loss of RKIP. Using a murine RKIP knockout model, we were further able to demonstrate that overexpression of these micro-RNAs is no effect of RKIP down-regulation. We are currently investigating whether their overexpression is indeed the cause of RKIP loss by employing *in-vitro* model systems.

Index

1. Introduction

1.1 Acute myeloid leukaemia

1.1.1 Definition, classification and epidemiology

Acute myeloid leukaemia (AML) is an aggressive malignant disease of the haematopoietic system, which is caused by malignant transformation of haematopoietic stem- and progenitor cells (HSPCs). The malignant offspring, called leukaemic blasts, is characterized by uncontrolled proliferation and impaired differentiation, respectively (Frohling, Scholl et al. 2005). Typically, leukaemic blasts can be found in the bone marrow and/or peripheral blood, however infiltration of any other tissue may occur as well. A bone marrow biopsy showing a leukaemic blast cell percentage of at least 20% is necessary to establish the diagnosis of AML (Dohner, Estey et al. 2010), however, in some cases extramedullary manifestations might precede the bone marrow affection (Zebisch, Cerroni et al. 2003).

In clinical routine, classification of AML is based on the French-American-British (FAB) and World Health Organization (WHO) criteria, respectively. The FAB classification has already been established in 1976 (Bennett, Catovsky et al. 1976, Bennett, Catovsky et al. 1985) and is based on morphological and cytochemical characteristics of the leukaemic blast cells (Table 1). Due to the shortcomings of the FAB classification in respect to newer diagnostic methods, such as karyotyping and sequence analysis, the WHO classification has been introduced in 2001 (Vardiman et al., 2001). This classification has recently been updated (Vardiman, Thiele et al. 2009) and incorporates clinical, morphological, cytochemical, immunological, cytogenetic and molecular characteristics of the disease (Table 2).

AML is the most frequent acute leukaemia in adults with a reported overall incidence of 3,67 newly diagnosed cases per 100.000 inhabitants each year. Importantly, AML may arise at every age, however older people are affected more frequently. Whereas incidence rates are below 2/100.000/year in individuals younger than 45 years old, they show an increase in the elderly peaking up to 23/100.000/year in people aged 80 years or older (data from SEER Cancer Statistics, www.seer.cancer.gov, downloaded January 2014).

Table 1. The FAB classification of acute myeloid leukaemia (Bennett, Catovsky et al. 1985), which is based on morphological and cytochemical characteristics of leukaemic blasts.

AML type	Name
M0	Acute myeloblastic leukaemia with minimal differentiation
M1	Acute myeloblastic leukaemia without maturation
M2	Acute myeloblastic leukaemia with maturation
M3	Acute promyelocytic leukaemia
M4	Acute myelomonocytic leukaemia
M4eo	Acute myelomonocytic leukaemia with abnormal eosinophils
M5a	Acute monoblastic leukaemia
M5b	Acute monocytic leukaemia
M6a	Erythroleukaemia
M6b	Pure erythroid leukaemia
M7	Acute megakaryocytic leukaemia

Table 2. The 2008 World Health Organization (WHO) classification of acute myeloid leukaemia (Vardiman, Thiele et al. 2009), based on clinical, morphological cytochemical, immunological, cytogenetic and molecular characteristics.

Acute myeloid leukaemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
*APL with t(15;17)(q22;q12); PML-RARA**
AML with t(9;11)(p22;q23); MLLT3-MLL†
AML with t(6;9)(p23;q34); DEK-NUP214
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPα
Acute myeloid leukaemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukaemia, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukaemia
Acute monoblastic/monocytic leukaemia

Acute erythroid leukaemia
Pure erythroid leukaemia
Erythroleukaemia, erythroid/myeloid
Acute megakaryoblastic leukaemia
Acute basophilic leukaemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukaemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukaemias of ambiguous lineage
Acute undifferentiated leukaemia
Mixed phenotype acute leukaemia with t(9;22)(q34;q11.2); BCR-ABL1
Mixed phenotype acute leukaemia with t(v;11q23); MLL rearranged
Mixed phenotype acute leukaemia, B/myeloid, NOS
Mixed phenotype acute leukaemia, T/myeloid, NOS
Provisional entity: Natural killer (NK)–cell lymphoblastic leukaemia/lymphoma

1.1.2 Clinical symptoms and laboratory findings

Proliferation and expansion of the leukaemic clone in the bone marrow results in suppression of the remaining normal haematopoiesis, a situation referred to as bone marrow insufficiency. As a result, cytopenias of peripheral blood cells are often observed in patients with AML. Anaemia, defined by a decrease in haemoglobin, causes fatigue, dyspnoea and sometimes even ischaemic complications, such as myocardial infarction or stroke. Thrombocytopenia on the other hand, results in impaired coagulation. These patients often present with severe bleeding problems. Leukocyte levels can be both decreased and increased, which is referred to as aleukaemic and leukaemic variants of AML, respectively. In any case, leukocytes do not fulfil their physiologic functions in the process of immune response, resulting in the acquisition of severe infections. About 10% of patients exhibit white blood cell (WBC) counts >100.000/µL with few of them even reaching WBC counts of up to 500.000/µL, thereby raising the risk of leukostasis and tumour lysis syndrome. Tumour lysis, which is caused by the high turnover of leukaemic blasts, might result in renal failure, coagulation problems and severe electrolyte imbalances. Increased levels of serum uric acid and lactate dehydrogenase (LDH) are typical laboratory findings in this situation.

1.1.3 Pathogenetics of AML

As outlined above, AML is caused by malignant transformation of HSPC, a process which is caused by the accumulation of (epi)genetic alterations. Broadly, these alterations can be classified into class-I and class-II aberrations (Zebisch, Czernilofsky et al. 2007). Class-I alterations mainly affect signal transduction pathways, thereby causing inhibition of apoptosis and increased proliferation, respectively. Among others, the receptor tyrosine kinase *FMS-like tyrosine kinase 3* (FLT3; 20-30% of cases), *Neuroblastoma RAS viral oncogene homolog* and *Kirsten rat sarcoma viral oncogene homolog* (NRAS and KRAS; 10-20% of cases) as well as *v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog* (c-kit; 10% of cases) are the genes affected most frequently. Class-II alterations are typically detected in transcription factors and cause an arrest in myeloid differentiation. *CCAAT/enhancer-binding protein alpha* (CEBPα; 10% of cases) and *Nucleophosmin* (NPM1; 30-40% of cases) thereby are the most important examples (http://cancer.sanger.ac.uk/cancergenome/projects/ cosmic). Importantly, the simultaneous acquisition of class-I and -II alterations is necessary to cause full AML. Indeed, animal models with isolated class-I alterations developed myeloproliferative disorders, however, frank AML was only seen, when additional class-II alterations were introduced as well (Zebisch, Czernilofsky et al. 2007).

Recently, this model has been challenged as additional mutations acting neither as class-I nor class-II aberrations, have been detected in a substantial subset of AML patients. These include tumour-suppressors, such as *Tumour protein p53 (*TP53; 10% of cases) and *Wilms tumour gene 1* (WT1; 10% of cases), as well as epigenetic modifiers, such as *DNA methyltransferase 3A* (DNMT3A; 20% of cases), *Isocitrate dehydrogenase 1/2* (IDH1/2; 10-15% of cases), *Tet methylcytosine dioxygenase 2* (Tet2; 10-15% of cases) and *Additional sex comb-like protein 1* (ASXL1; 10% of cases) (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).

1.1.4 Prognosis, risk stratification and therapy

In a first place, the prognosis of patients diagnosed with AML is depending on patient specific risk factors, such as age, performance status and the amount of co-morbidities, respectively. Whereas younger patients in good clinical condition may be treated with a curative approach, older and frail patients have to be managed within a palliative setting. For the curative approach, high-dose chemotherapy regimens

including haematopoietic stem cell transplantations are chosen. Although complete remission (CR) can be achieved in more than 60% of cases, many patients succumb to a chemoresistant relapse. Furthermore, a substantial subset of patients die due to the side effects of leukaemia treatment, a situation referred to as treatment related mortality (TRM). Out of these reasons, it is essential to identify leukaemia specific risk factors that help to separate patients needing a more aggressive therapeutic approach from those, who might benefit from a reduction of the dosage. Beside classical factors, such as a high WBC count and increased LDH serum levels (Dohner, Estey et al. 2010), cytogenetic and molecular genetics have been established as the gold-standard for prognostic risk stratification. The prognostic significance of recurrent somatic chromosomal aberrations has already been shown in 1998 by David Grimwade and co-workers (Grimwade, Walker et al. 1998), who analysed the cytogenetic information of almost 2000 AML patients and were able to clearly discriminate three prognostic groups. Meanwhile, this model could be corroborated by many other studies, thereby establishing the leukaemic karyotype as a vital prognostic marker. Through the introduction of molecular techniques, such as DNA sequencing, this model could be further refined. Considering a myriad of publications, the European LeukemiaNet (ELN) recently proposed a prognostic model that is able to define four prognostic groups and that incorporates both, the cytogenetic and molecular information of AML patients. Up to this date, mutations in CEBPα, NPM1 and FLT3 have been included in this score (Dohner, Estey et al. 2010). While CEBPα and NPM1 are associated to a favourable clinical course, mutations in FLT3 are correlated to an adverse risk profile of affected patients (Figure 1).

A

Genetic group	Subsets
Favourable	*t(8;21)(q22;q22); RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD (normal karyotype)
	Mutated CEBPA (normal karyotype)
Intermediate I	*Mutated NPM1 and FLT3-ITD (normal karyotype)*
	Wild-type NPM1 and FLT3-ITD (normal karyotype)
	Wild-type NPM1 without FLT3-ITD (normal karyotype)
Intermediate II	*t(9;11)(p22;q23); MLLT3-MLL*
	Cytogenetic abnormalities not classified as favourable or adverse
Adverse	*inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1*
	t(6;9)(p23;q34); DEK-NUP214
	t(v;11)(v;q23); MLL rearranged
	-5 or del(5q)
	-7
	abnl(17p)
	Complex karyotype (three or more chromosome abnormalities)

B

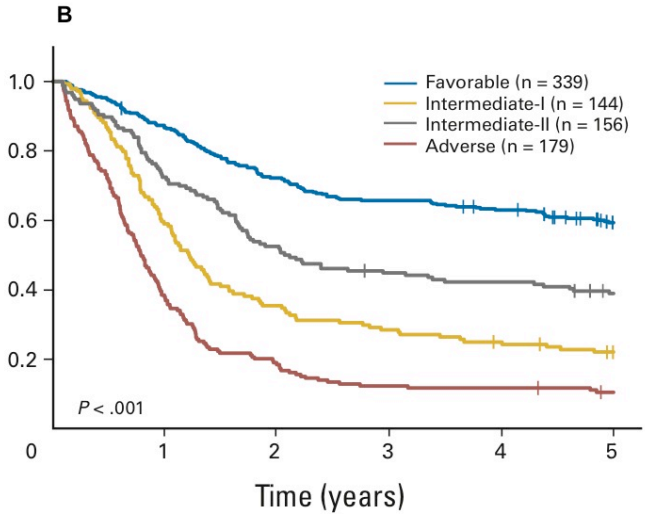

Figure 1. The ELN prognostic risk groups for acute myeloid leukaemia. (A) Classification of the risk groups. (B) 5-year overall survival for the prognostic groups (Dohner, Estey et al. 2010, Mrozek, Marcucci et al. 2012).

1.2 The RAF kinase inhibitor protein: RKIP

1.2.1 The RAS-MAPK/ERK pathway

The Rat sarcoma - mitogen-activated protein kinase/extracellular signal-regulated kinase (RAS-MAPK/ERK) signalling pathway is a highly conserved signalling cascade that transfers extracellular stimuli to the nucleus and cytoplasm (Figure 2). This pathway is important for the regulation of many fundamental cellular processes including proliferation, differentiation and cell cycle progression (Zebisch, Czernilofsky et al. 2007).

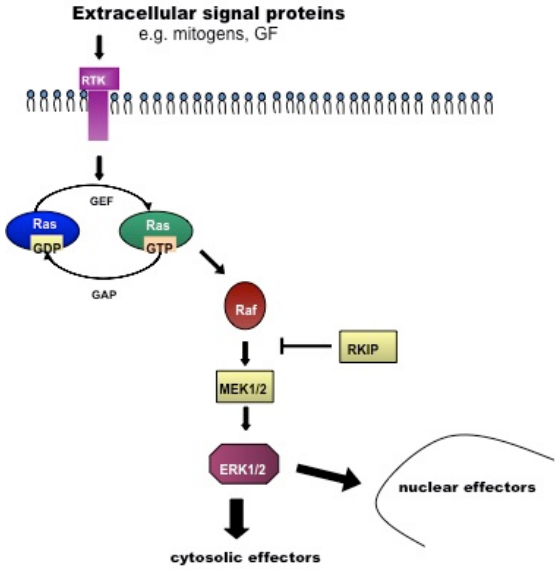

Figure 2. The schematic diagram of the RAS-MAPK/ERK signal transduction cascade.

In the physiologic setting, the RAS-MAPK/ERK pathway in haematopoietic cells is activated by the binding of growth factors and/or mitogens to cellular receptor-tyrosine-kinases, such as FLT3 or vascular endothelial growth factors (VEGF). This binding results in activation of the receptor, which in turn facilitates the transfer of the small GTPase protein RAS to the cellular membrane, where its activation occurs. In the next step, activated RAS binds and activates the family of *Rapidly accelerated fibrosarcoma* (RAF) serine/threonine kinases, consisting of A-RAF, B-RAF and C-RAF (or RAF1). Active RAF then phosphorylates and activates the dual specifity kinase MEK1/2, which in turn phosphorylates and activates the *Extracellular-signal-regulated kinases 1/2* (ERK1/2). ERK1/2 displays the most downstream part of this pathway and regulates more than 160 effector proteins, including transcriptions factors and cytoskeletal proteins (Yoon and Seger 2006).

Importantly, the function of this signalling cascade is safeguarded by a myriad of regulator proteins. These so called scaffolds fine-tune the activity of the pathway and also ensure its proper inactivation after a signal has been mediated to the intracellular effectors.

This pathway is of seminal importance in tumorigenesis. Genetic aberrations within its upstream activators, its regulators or within its member proteins might cause its permanent activation, which ultimately results in malignant transformation. This situation is referred to as constitutive activation and is detected in a broad range of human malignancies. Deregulation of this pathway seems to be of particular importance for the pathogenesis of AML, as outlined by the fact that more than 50% of AML cases exhibit a constitutively activated ERK1/2 protein. Indeed, mutations affecting the signalling in this cascade are among the most frequent genetic aberrations in this disease.

1.2.2 RKIP

RKIP is a small, evolutionary conserved protein that was first discovered as a *Phosphatidylethanolamine binding protein* (PEBP) in bovine brain (Yeung, Seitz et al. 1999) and belongs to the PEBP superfamily. The protein is located in the cytoplasm and expressed in almost all mammalian tissues (Bollengier and Mahler 1988, Frayne, Ingram et al. 1999). RKIP is involved in the regulation of many intracellular signalling cascades, controlling cell growth and apoptosis (Lorenz, Lohse et al. 2003, Hagan, Garcia et al. 2006). Most importantly, RKIP negatively regulates the RAS-MAPK/ERK

pathway. It specifically binds to C-RAF and thereby inhibits the interaction between C-RAF and MEK1/2 (Yeung, Janosch et al. 2000). As a result, phosphorylation and thus activation of MEK1/2 is inhibited. It is this specific function that was first described in 1999 by Yeung and co-workers that resulted in the name RKIP (Yeung, Seitz et al. 1999). Since then, involvement of RKIP in the regulation of many other signalling modules, including the nuclear factor-κB (Yeung, Rose et al. 2001), G-protein coupled receptor kinase-2 (Lorenz, Lohse et al. 2003) and glycogen synthase kinase 3β pathways (Al-Mulla, Bitar et al. 2011) has been described.

The expression of RKIP is lost in a wide range of human carcinomas, including breast, prostate and colon carcinoma cells (Al-Mulla et al., 2006; Fu et al., 2003; Hagan et al., 2005; Minoo et al., 2007). Functional *in-vitro* and *in-vivo* studies demonstrated, that this loss of expression results in constitutive activation of the RAS-MAPK/ERK pathway. Interestingly, while loss of RKIP did have no obvious effects on the growth of the primary tumour, the formation of metastases was greatly affected. Indeed, subsequent studies incorporating primary patient samples and functional *in-vitro*/*in-vivo* models were able to establish RKIP as a bona fide metastasis-suppressor gene (Granovsky and Rosner 2008, Dangi-Garimella, Yun et al. 2009). Additionally, effects on apoptotic cell death and resistance to chemotherapy could be shown in selected studies (Odabaei, Chatterjee et al. 2004, Al-Mulla, Bitar et al. 2012).

1.2.3 RKIP in AML

Our group previously demonstrated a loss of RKIP as a somatic and leukaemia-specific event in patients with therapy-related AML and C-RAF germline mutations (Zebisch, Staber et al. 2006, Zebisch, Haller et al. 2009). Most importantly, the oncogenic potential of mutant C-RAF could be inhibited by the overexpression and increased by the knockdown of RKIP in functional *in-vitro* studies. We therefore concluded that loss of RKIP acts as a second genetic hit facilitating leukaemogenesis in these patients. In a follow up study comprising almost 400 patient samples with all different subtypes of AML, we detected a loss of RKIP in more than 20% of cases (Zebisch, Wolfler et al. 2012). Most interestingly, decreased expression of RKIP thereby demonstrated a highly significant association to monocytic AML phenotypes. In functional *in-vitro* assays, RKIP overexpression – as achieved by transfection of a FLAG-tagged RKIP expression construct – inhibited the growth and proliferation of

AML cell lines. Additionally, it also inhibited their oncogenic potential, as measured by colony formation in soft-agar. Next, we detected a correlation between RKIP loss and mutations in RAS. Subsequent *in-vitro* assays further demonstrated a functional synergism in respect to malignant transformation. RKIP overexpression was able to abrogate the RAS-induced formation of NIH3T3 foci. Taken together, these data indicate a tumour-suppressor role of RKIP in AML, which is an addition to its metastasis-suppressor function described in solid tumours.

Despite these interesting data about the effects of RKIP loss, the mechanisms causing its down-regulation remain to be delineated. Protein instability can be excluded, which is due to the fact that low RKIP protein levels correlated with low mRNA levels in both, AML cell lines and patient samples (Zebisch, Haller et al. 2009, Zebisch, Wolfler et al. 2012). Furthermore, our group screened for methylation of the RKIP promoter, for inactivating mutations within the coding sequence and for DNA copy number alterations, but did not find any abnormalities (Zebisch, Haller et al. 2009, Zebisch, Wolfler et al. 2012).

1.3 Gene silencing through micro-RNAs

As outlined above, gene deletions, inactivating mutations as well as promoter methylation could be excluded as reasons for RKIP loss in AML. Micro-RNAs are naturally occurring, small non-coding RNA molecules with a length of about 22 nucleotides and display an additional possibility of gene silencing. They function at a post-transcriptional level and decrease the levels of their target mRNAs via translational repression or target degradation, respectively.

Micro-RNAs are transcribed from specific genetic micro-RNA loci, resulting in large pri-micro-RNA transcripts. These pri-micro-RNAs are then further processed and shortened within the nucleus to precursor micro-RNAs (pre-micro-RNAs), displaying a length of approximately 70 nucleotides. In a next step, the molecule is exported to the cytoplasm where it is refined to a mature micro-RNA through specific DICER enzyme complexes. These mature micro-RNAs now attach to a multiprotein RNA-induced silencing complex (RISC), which enables its' binding to complementary mRNA sequences and consequently genetic silencing. The binding occurs within the 3' untranslated region (UTR) of the target gene mRNA, downstream of the protein-coding region and upstream of the poly-adenylation site. Importantly, a single micro-

RNA is able to target several different mRNAs. Even more, every gene mRNA may be the target of different micro-RNAs as well (Krek, Grun et al. 2005).

Micro-RNAs play a seminal role in the regulation of many cellular processes, including cell growth, proliferation and differentiation (Hwang and Mendell 2006). In addition, a myriad of micro-RNAs have been shown to be associated with malignant transformation and cancerogenesis (Meltzer 2005, Croce 2008), partly by down-regulation of tumour- and metastasis-suppressor genes (Zhu, Si et al. 2007, Kota, Chivukula et al. 2009, Ru, Steele et al. 2012). Interestingly, they also play a role in both, physiologic haematopoiesis (Chen, Li et al. 2004) and leukaemogenesis (Kluiver, Kroesen et al. 2006). Most importantly, deregulated micro-RNA expression profiles were described in AML patients previously (Garzon, Volinia et al. 2008, Jongen-Lavrencic, Sun et al. 2008, Marcucci, Radmacher et al. 2008) and proved to be valuable prognostic markers (Diaz-Beya, Brunet et al. 2013).

In a recent publication, Rommer and co-workers performed a micro-RNA ChiP expression analysis, using a microarray technology, including 636 different micro-RNAs in leukaemic specimens of patients diagnosed with AML (Rommer, Steinleitner et al. 2013). This study included 33 samples of our groups leukaemia-biobank. Importantly, all of them have been characterized in respect to RKIP protein expression previously (Zebisch, Wolfler et al. 2012). Bioinformatic analysis thereby identified a set of eight micro-RNAs (hsa-miR-10a, hsa-miR-15a, hsa-miR-23a, hsa-miR-23b, hsa-miR-24, hsa-miR-518b, hsa-miR-519b, hsa-miR-320a), showing differential expression levels between patients with and without RKIP loss. It has to be mentioned though, that micro-RNA ChiP expression analysis is a screening approach, which might produces both false-positive and false-negative results in a substantial subset of cases (Cole, Galic et al. 2003). Furthermore, only a restricted number of six patients defined as RKIP loss could be evaluated in these analyses, which additionally limits the results obtained. Unfortunately, validation of these preliminary data in a well characterized cohort of AML patients with equal numbers of specimens with and without RKIP loss by the means of qPCR has not yet been performed.

1.4 Decreased gene expression as a result of transcriptional repression

Another possibility of genetic silencing is the process of transcriptional repression. Transcriptional repression is mediated via so called transcriptional repressor proteins and plays a key role in the regulation and fine-tuning of gene expression profiles and consequently regulates a myriad of fundamental cellular processes. From a mechanistic point of view, these repressor proteins bind to the promotor region of specific effector genes, resulting in an inhibition of transcription and consequently in a decrease of gene expression and protein levels.

Importantly, aberrant expression of transcriptonal repressors might result in the pathologic down-regulation of important tumour-suppressor genes, thereby facilitating malignant transformation. Indeed, deregulated transcriptional repressors have already been connected to human cancers (De Craene, Gilbert et al. 2005, O'Neil and Look 2007, Ben-Batalla, Seoane et al. 2010). Altered expression of transcriptional repressors induce changes in cell proliferation, apoptosis, invasion and tumoural growth or aberrant activation of epithelial mesenchymal transition (EMT), leading to drastic morphological changes in cancer cells. In the haematopoietic system, transcriptional repressors, in particular Zink-finger E-box-binding (Zeb2), PU.1 and CEBPα were shown to play an important role in differentiation and linage commitment of stem and progenitor cells (Rosmarin, Yang et al. 2005). Zeb2 has an essential role for normal differentiation of haematopoietic stem and pregenitor cells, whereas deletions lead to reduced mature haematopietic cells (Goossens, Janzen et al. 2011). PU.1 and CEBPα are crucial for myeloid development and abberational expression contribute to pathogenesis of AML (Rosmarin, Yang et al. 2005). Furthermore, Somech and co-workers detected a significant overexpression of the transcriptional repressor Lamina-associated polypeptide 2 beta (LAP2) in both, acute lymphoid and myeloid leukaemia and further reported a correlation to higher proliferation rates (Somech, Gal-Yam et al. 2007).

Importantly, a variety of transcriptional repressors have been linked to RKIP down-regulation previously. These include EZH2, SNAI1, SLUG, BACH1, NFE2L2 and YY1. However, their role in the pathogenetics of RKIP down-regulation in AML is currently unclear.

1.5 Aim of the thesis

Loss of RKIP is a frequent event in AML and contributes to leukaemogenesis as shown by our group previously (Zebisch, Haller et al. 2009, Zebisch, Wolfler et al. 2012). Although a lot of knowledge about the effects of RKIP loss in AML could already be gained, the mechanisms causing its down-regulation are still unclear. Preliminary data obtained with micro-RNA ChiP profiling now pinpoint to the overexpression of specific micro-RNAs in patients with RKIP loss, however systematic validation of these experiments has not yet been performed.

The primary aim of my thesis is to test the expression of a predefined set of micro-RNAs in a well-defined cohort of AML patients including an equal number of specimens with and without loss of RKIP using qPCR. This set includes the micro-RNAs identified by a micro-RNA ChiP analysis, which has been outlined in more detail above. Additionally, it comprises micro-RNAs, which have been connected to RKIP in the literature previously. In case differentially expressed micro-RNAs are detected, we further aim for a functional characterization, delineating whether they are cause or effect of RKIP down-regulation.

Another aim of my thesis is to delineate whether RKIP loss in AML correlates to the overexpression of specific transcriptional repressor genes. For this purpose, a set of six candidates, that have been shown to affect RKIP previously, were selected in the literature. Again, their expression will be tested by the means of qPCR and functionally characterized in case differences between patients with and without RKIP loss are observed.

2. Material and Methods

2.1 AML patient samples, cell lines and mouse specimens

2.1.1 Reagents

- RPMI-1640 Medium (Cat.No. R8758, Sigma-Aldirch, Steinheim, Germany)
- Antifreeze solution dimethyl sulfoxide, DMSO (Cat.No. WAK-DMSO-10, WAK-Chemie Medical GmbH, Steinbach, Germany)
- Foetal Bovine Serum (FCS), heat inactivated (Cat.No. A15-104, PAA Laboratories GmbH, Cambridge, UK)
- Phosphate buffered saline (PBS), 1000ml (Pharmacy LKH Universitätsklinikum Graz, Graz, Austria) ph 7.2-7.3, containing 1.5129g Na_2HPO_4*2 H_2O, 0.2041g KH_2PO_4 and 8.50g KH_2PO_4, sterilized at 120°C; 30'
- BD Pharm Lyse™, Lysing Buffer 10X concentrate (Cat.No. 555899, BD Biosciences, San Jose, CA, USA)
- DNeasy® Blood & Tissue Kit (Cat.No. 69506, Qiagen, Hilden, Germany)
- HotStarTaq DNA Polymerase (Cat.No. 203203, Qiagen)
- Biozym ME Agarose (Cat.No. 840010, Biozyme Diagnostics GmbH, Hessisch Oldendorf, Germany)
- 1x TAE buffer (Pharmacy LKH Universitätsklinikum Graz)
- GeneRuler Plus 100 bp DNA Ladder (Cat.No. SM0323, Thermo Fisher Scientific, Rochester, NY, USA)

2.1.2 AML patient samples

Twenty AML patient samples, derived from leukaemic bone marrow or peripheral blood, were included in this study. All of these patients were treated at the Division of Haematology, Medical University of Graz, Austria. These leukaemia specimens contain a purified AML blast cell population of more than 80% and have been stored in liquid nitrogen within the course of previous research projects in 0.8 mL RPMI-1640, with 0.1 mL antifreeze solution dimethyl sulfoxide (DMSO) and 0.1 mL foetal calf serum (FCS). Importantly, all samples have been characterized in respect to RKIP protein expression by the means of Western blot by our own group previously (Zebisch, Wolfler et al. 2012). One half (n=10) thereby exhibited a normal expression whereas the rest (n=10) harboured a leukaemia-specific, somatic loss of the RKIP

protein. All patients handed written informed consent at the time of sample collection, allowing the use of this material for research purposes. In addition, the project has been reviewed and approved by the institutional review board of the Medical University of Graz, Austria.

To use the samples for the analyses performed within this project, they were thawed in a water bath at 37°C. To remove residual DMSO, pellets were washed once (40 mL PBS; 8000 rpm at 4°C for 5 minutes), resuspended in PBS and immediately used for execution of the experiment.

2.1.3 AML cell lines

AML cell lines were obtained from the *Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures* (DSMZ, Braunschweig, Germany) and have been characterized in respect to RKIP protein expression previously (Zebisch, Wolfler et al. 2012). The AML cell line NB-4 (DSMZ No. ACC-207) was selected as a calibrator for qPCR and Western blot experiments which is due to the fact, that it demonstrates a prominent and stable expression of RKIP (Zebisch, Wolfler et al. 2012). It was established from the bone marrow of a woman with acute promyelocytic leukaemia (AML FAB M3) in second relapse (Lanotte, Martin-Thouvenin et al. 1991). The AML cell line ML-2 (DSMZ No. ACC-15) - which has been shown to exhibit a low RKIP expression previously (Zebisch, Wolfler et al. 2012) - was included in all experiments as an additional negative control. ML-2 has been derived from the peripheral blood of a patient with acute myelomonocytic AML (AML FAB M4) (Ohyashiki, Ohyashiki et al. 1989) following T-non-Hodgkin lymphoma and T-ALL (Drexler, Quentmeier et al. 2004).

The human colon carcinoma cell line HCT-116 (DSMZ No. ACC-581) was used as a calibrator in qPCR experiments, analysing the expression of snail family zinc finger 1 (SNAI1) and snail family zinc finger 2 (SNAI2, also known as SLUG), which is due to the fact that these genes did not show detectable expression levels in the cell lines mentioned above. HCT-116 has been established from a colon carcinoma and carries a RAS mutation in codon 13 (Brattain, Fine et al. 1981). Cell lines were stored in DMSO in liquid nitrogen and cell pellets for further experiments were prepared as described for AML patient samples.

2.1.4 Mouse specimens

To investigate the functional effects of RKIP loss on mRNA expression, we employed a murine RKIP knockout model. In this model, a germline knockout of the RKIP gene ($RKIP^{-/-}$) has been established by gene trapping as described in detail previously (Stanford, Cohn et al. 2001). Briefly, the gene trap cassette, consisting of a selectable marker (β-geo), a 3' splicing acceptor site and a transcriptional termination sequence (poly-adenylation site), was inserted into the first intron of the RKIP gene, resulting in a shortened and non-functional protein (De-Zolt, Schnutgen et al. 2006). $RKIP^{-/-}$ mice are viable and show a normal life expectancy (Moffit, Boekelheide et al. 2007, Theroux, Pereira et al. 2007). So far, only minor defects in olfactory functions and spermatogenesis have been reported (Moffit, Boekelheide et al. 2007), although it has to be noted that the majority of tissues have not been examined in detail so far. The model has been obtained from Professor John Sedivy (Brown University, RI, USA) and is currently maintained on a C57/BL6 background in our group. All mouse experiments were carried out according to the EU directive. The institute in Graz holds a permit for mouse experiments, which was successfully extended to include the experiments performed within this study.

To extract haematopoietic tissue for Western blot and qPCR analyses (bone marrow and spleen), ten mice (five $RKIP^{-/-}$ and five wildtype [$RKIP^{+/+}$] animals) had to be sacrificed, which was done by cervical dislocation after anaesthesia with isofluran (4%). Then, sternum, femur and tibiae, which contain the BM, as well as the spleen were carefully removed and transferred to 40 mL PBS on ice. After bones have been crushed, haematopoietic cells were isolated by filtration using 70 µm nylon mesh cell strainer followed by repeated wash steps in PBS. Red blood cells were lysed by incubation in 1 mL lysis buffer for 2 minutes.

2.1.4.1 Genotyping

Importantly, mice were maintained in a heterozygous situation ($RKIP^{+/-}$). As a result, breedings always produced an offspring, which was either $RKIP^{+/-}$, $RKIP^{-/-}$ or $RKIP^{+/+}$. To select homozygous knockout and wildtype animals, genotyping was performed from tail tips achieved from all newborn pups by polymerase chain reaction (PCR) using primer pairs specific for either the wildtype or the knockout allele. Therefore, DNA was purified using the DNeasy® Blood & Tissue Kit according to the manufacturer's protocol. Briefly, the tail tips were lysed in 180 µL of the provided ATL

lysis buffer and 20 µL proteinase K at 56°C until complete lysis of the tissue was achieved. Then, DNA was extracted and purified in a series of wash and precipitation steps using a set of optimised columns and buffers, which were provided with the kit. DNA concentrations were measured with a BioPhotometer (Eppendorf, Hamburg, Germany) and 1:10 dilutions were used for PCR experiments.

In a next step, two separate PCR amplifications were performed for each sample. Both reactions used the same forward primer (RKIP F2). The reverse primer, however, was designed to specifically bind either the full-length (RKIP R4) or the truncated (RKIP R2) version of RKIP, which resulted in generation of either wildtype (978 bp – F2 and R4) or knockout (639 bp F2 and R2) amplicons. In more detail, PCR reactions were carried out using the HotStarTaq DNA Polymerase in a total volume of 50 µL, using 2µL of DNA dilution; 50mM $MgCl_2$; 10mM dNTPs; 10µM forward primer (RKIP F2); 10µM reverse primer (either RKIP R4 or R2); 1,5U HotStarTaq DNA Polymerase and 1x PCR buffer. An initial hot-start activation was required at 94°C for 15 minutes. Then amplification was performed in 40 cycles of denaturing at 94°C for 1 minute, annealing at 62°C for 1 minute and elongation at 72°C for 1 minute and final extension for 10 minutes at 72°C.

PCR products were separated on a 2% agarose gel for 30 minutes at 120 V, prepared with 1x TAE buffer and ethidium bromide (1:10.000), using a 100 bp GeneRuler Plus DNA Ladder to estimate the sizes of the PCR products. A representative gel, demonstrating all possible conditions is displayed in Figure 3.

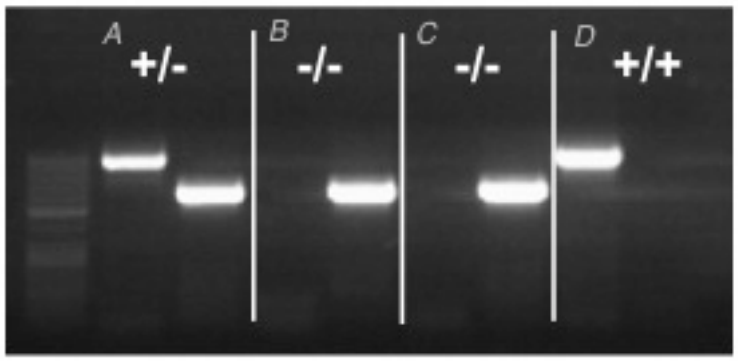

Figure 3. Genotyping of RKIP mice by PCR, demonstrating the (A) RKIP heterozygote ($RKIP^{+/-}$) (B/C) homozygote ($RKIP^{-/-}$) and (D) wild type ($RKIP^{+/+}$) situation.

2.2 Analysis of RKIP expression levels in AML patient samples, cell lines and mice

In this phase of the project we analysed RKIP expression levels in AML patient samples and mouse specimens by the means of qPCR and Western blot. The aim of this project phase was to ensure that the groups selected truly differed in respect to their RKIP expression on both, the mRNA and protein level.

2.2.1 RKIP mRNA expression analysis by qPCR

2.2.1.1 Reagents

- TRIzol® Reagent (Cat.No. 15596-026, Invitrogen / Life Techologies™, Carlsbad, CA, USA)
- Isoproply alcohol (Cat.No. I9516-500mL, Sigma-Aldrich)
- Chloroform, extra pure (Cat.No. 1024311000, Merck KGaA, Darmstadt, Germany)
- Ethanol for analysis (Cat.No. 1.00983.1011, Merck KGaA)
- RNA 6000 Nano LabChip Kit (Cat.No. 5067-1511, Agilent Techologies, Foster City, CA, USA)
- Taq Man® Reverse Transcription Reagents (Cat.No. N808-0234, Applied Biosystems / Life Techologies™)
- Oligonucleotid Primer RKIP, GUSB and B2M (Eurofins MWG Operon, Ebersberg, Germany)
- SYBR® GreenER™ (Cat.No. 11760-500, Applied Biosystems / Life Techologies™) qPCR SuperMix for ABI PRISM®Instrument, a ready-to-use cocktail containing all components except primers and template for real-time quantitative PCR (qPCR)

2.2.1.2 RNA isolation, RNA quality assurance and cDNA preparation

Total RNA was extracted from AML patient samples, AML cell lines and mice using TRIzol® Reagent according to the manufacturer's instructions. This is a single-step liquid-liquid extraction technique based on different solubility's, separating under acidic conditions RNA in an upper aqueous phase and DNA in a lower organic phase (Chomczynski and Sacchi 2006). Precipitating with isopropanol yields total RNA, which was used for further applications.

To increase the RNA yield, cell pellets were additionally disrupted by sonication, using a Sonopuls Ultrasonic Homogenizer HD2070 (Bandelin electronic GmbH & Co. KG, Berlin, Germany). Subsequently, 150 µL chloroform and the suspension was mixed for 15 seconds followed by an incubation period at room temperature for 3 minutes. Thereafter, three phases were separated by centrifugation (2.000 x g at 4°C for 15 minutes): a lower red, organic phase containing proteins and lipids; an interphase comprising DNA and a colourless upper aqueous phase, which contains RNA. This RNA containing fluid was carefully removed and transferred into a new tube.

In a last step, nucleic acids were precipitated with 375 µL isopropanol for 10 minutes at room temperature and spun down with 12.000 x g for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet washed with 750 µL 75% ethanol. Finally, the pellet was air-dried for 10 minutes, re-dissolved in 30 µL RNase-free water and heated for 10 minutes at 56°C.

For evaluation of RNA quantity, the optical density at a wavelength of 260 nm (OD_{260}) was measured using a BioPhotometer (Eppendorf). For these measurements, RNA was diluted 1:50 in RNase-free water. RNA quality was evaluated using RNA 6000 Nano LabChip Kit on a BioAnalyzer BA2100 (Agilent Technologies) according to the manufacturer's protocol. The RNA Integrity Number (RIN) - values obtained were between 6.3 and 9.8, which indicates sufficient RNA quality (Fleige and Pfaffl 2006, Schroeder, Mueller et al. 2006) in the samples analysed (data not shown).

Due to the increased stability of cDNA, reverse transcription (RT) of RNA was performed before samples were stored (at -20°C) or used for experiments. Therefore the Taq Man® Reverse Transcription Reagents were used. cDNA preparation was done from 1 µg of RNA with 1,25 U/µL reverse transcriptase; 0,4 U/µL RNase inhibitor; 1x taqman RT buffer; 2,5 µM random hexamers; 2 mM desoxyribonucleoside triphosphates (dNTP mix; 0,5 mM each) and 5,5 mM $MgCl_2$ solution. The reaction was carried out in a total volume of 100 µL at 25°C for 10 minutes (incubation), 48°C for 30 minutes (reverse transcription) and 95°C for 5 minutes (inactivation of the reverse transcriptase).

2.2.1.3 cDNA expression analysis using qPCR

Relative quantitation of RKIP cDNA expression was done using the SYBR Green methodology. SYBR Green is a cyanine-fluorescent dye, designed for detection and

quantification of PCR products. The fluorescent dye binds to double-stranded DNA and shows an alteration in fluorescence emission when ds-bound instead of the free state (Zipper, Brunner et al. 2004). The principle is based on the fluorescence resonance energy transfer (FRET). For this practice, two oligonucleotides are used, one carrying an acceptor (reporter fluorophor I), the other one containing a donor. Both oligonucleotides bind in striking distance on the template DNA, enabling the donor to support a fluorescence signal from the acceptor. The intensity of fluorescence is measured at the end of elongation of each PCR cycle, increases with each PCR cycle and corresponds to the amount of generated PCR product. As a result, its continuous detection enables a “real-time” measurement of the amount of the amplificate at any time of the reaction, which facilitates measurement of the amount of PCR product at a point in which the reaction is still in the exponential phase (Figure 4).

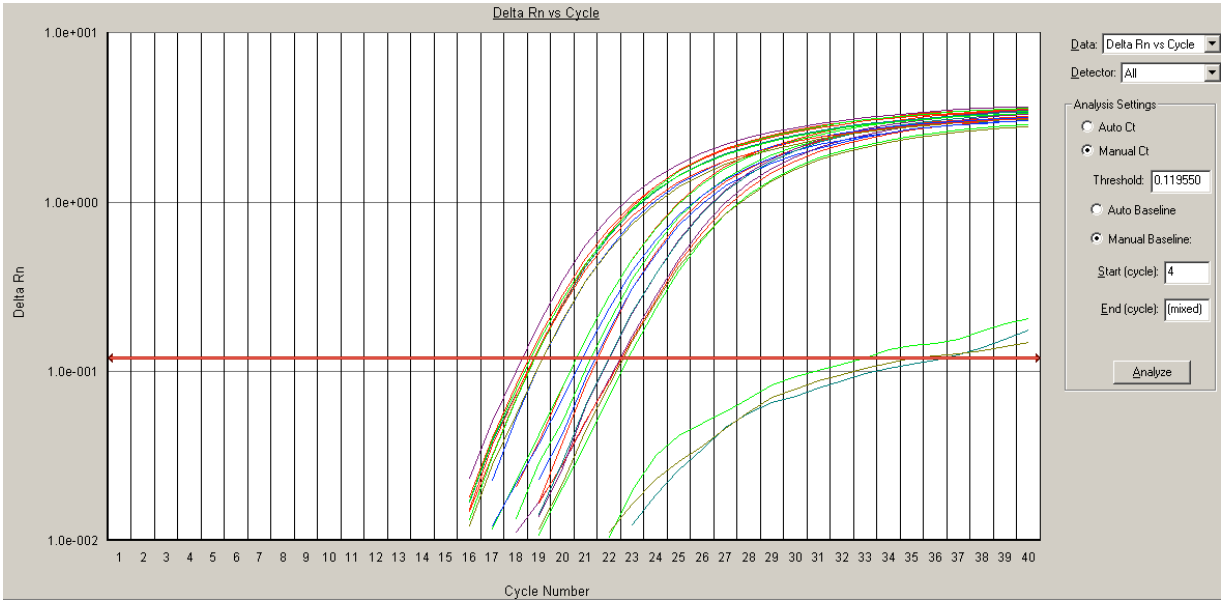

Figure 4. A representative qPCR expression curve with a threshold (red line) located in the exponential amplification phase.

This is one of the greatest advantages compared to a conventional PCR reaction, where an end point quantitation is performed for expression analysis, which bears the risk that the PCR reaction is no longer generating templates at an exponential rate. The PCR cycle number, at which the fluorescence intensity reaches a defined amount is called cycle of threshold (C_T). This C_T cycle – which has to be constant for all samples within each plate – is arbitrarily set within the exponential amplification

phase and subsequently used for further calculations. Lower C_T values therefore indicate higher expression of a specific gene. For the analysis of RKIP gene expression, relative quantitation using the $\Delta\Delta C_T$ method has been performed as previously described (Livak and Schmittgen 2001) and the MIQE guidelines were followed for all qPCR experiments (Bustin, Benes et al. 2009). In a first step, expression of RKIP was normalized to two control genes, which facilitates a compensation for differences in RNA content and integrity between different samples (Pfaffl 2001). To increase the accuracy, the geometric mean of the control genes was used (Vandesompele, De Preter et al. 2002) for further calculations ($\Delta C_T = C_{T\ RKIP} - C_{T\ averaged\ control\ genes}$). In this set of experiments, beta-2-microglobulin (B2M) and beta-glucuronidase (GUSB) were selected as control genes. Both genes are recommended as suitable endogenous controls for qPCR in leukaemia, which is due to the fact that they exhibit stable expression levels in specimens of this malignancy (Beillard, Pallisgaard et al. 2003). In a next step, expression of a given sample was compared to a calibrator ($\Delta C_{T\ sample} - \Delta C_{T\ calibrator}$). As a calibrator we used the AML cell line NB-4, which has been shown to exhibit prominent RKIP expression previously (Zebisch, Wolfler et al. 2012). For expression analysis in $RKIP^{-/-}$ mice, an $RKIP^{+/+}$ littermate was used as calibrator. The $\Delta\Delta C_T$ value was expressed as 2^{-ddC_T} and multiplied with 100 ($2^{-ddC_T} \times 100$) to allow comparison as a percentage of the calibrator, which thereby exhibits a value of 100%. To further increase the accuracy of our experiments, we additionally included ML-2 AML cells (characterized by low RKIP expression (Zebisch, Wolfler et al. 2012)) in each PCR plate. The average expression of RKIP in ML-2 was 13% with a standard deviation of 5.5, which indicates comparable conditions among all PCR runs.

Relative $\Delta\Delta C_T$ method quantitation with SYBR Green qPCR requires well-designed primers. Firstly, it is important to avoid primer dimers, as the fluorescent dye binds to every double-stranded DNA and cannot distinguish between specific and non-specific PCR products. Additionally, the PCR efficiencies for all primers used should be between 90% and 110% to enable representative normalisation of C_T values (Vermeulen, Pattyn et al. 2009). Primers were selected from 'Harvard PrimerBank' (http://pga.mgh.harvard.edu/ primerbank/) and checked with National Center for Biotechnology Information (NCBI) Primer – Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Hence, similarities between the primer sequences, which may lead to self-annealing and primer dimers,

could be excluded. Additionally, their specifity to the target gene could be confirmed. As we were interested in amplification of cDNA, primers were chosen that bridge an exon-exon junction, which excludes the amplification of genomic DNA. All primers were purchased from MWG Eurofins (Appendix, Table 3).

In a next step, we evaluated the PCR efficiency of every single primer pair by the means of standard curve analysis. Therefore cDNA of the calibrator cell line NB-4 was serially diluted over a range from 1:1 to 1:125. The resulting C_T values were plotted against $\log_{10}$ concentration and used in the following equation to calculate the PCR efficiency (Pfaffl 2001):

$$\text{PCR-efficiency [\%]} = ([10^{1/-S}]-1) \times 100; \text{ slope [S]}$$

With a slope between 3.61 and 3.23, corresponding to PCR efficiencies between 90 and 104%, PCR efficiencies were satisfactory for all primer assays. As a representative example, the standard curve of B2M is shown in Figure 5.

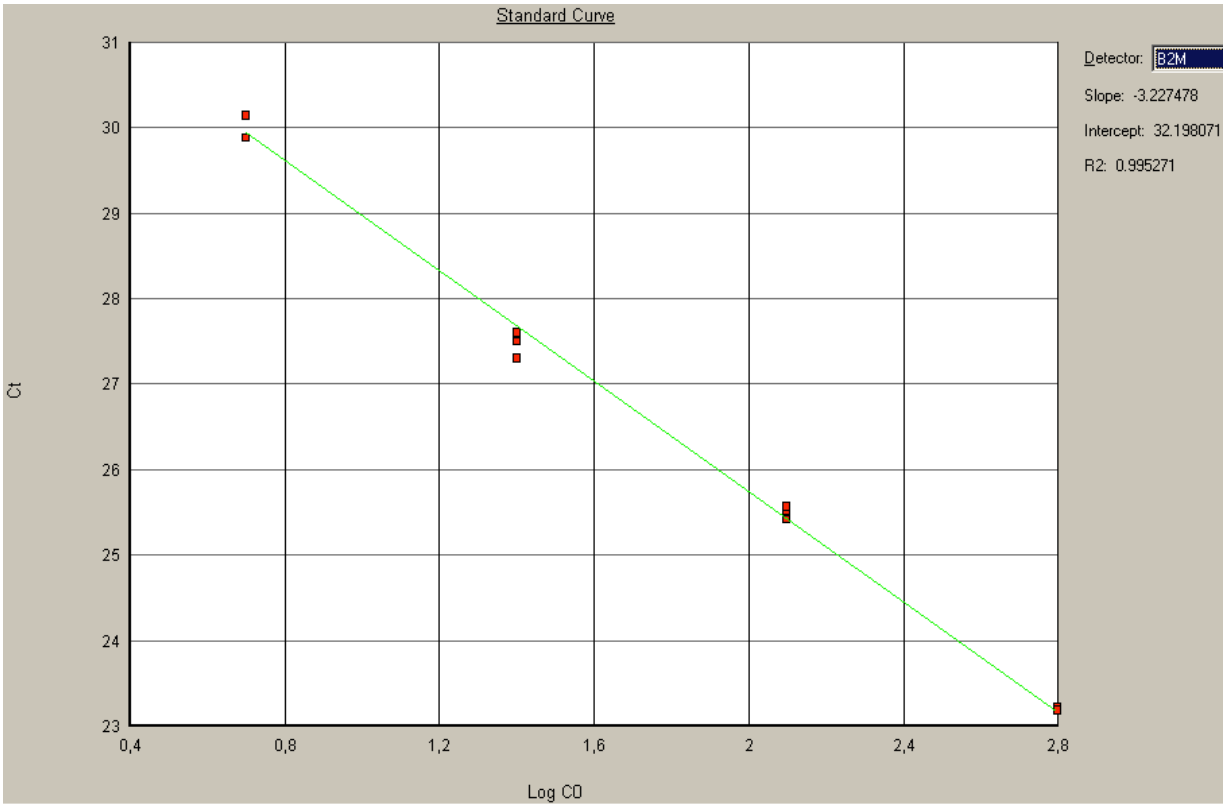

Figure 5. Standard curve determining the PCR amplification efficiency for B2M. A slope of 3.23 indicates a PCR efficiency of 104%.

Negative controls were included as additional quality controls. Firstly, a no template control (NTC), which contains all PCR components but no cDNA to exclude contamination of the master mix reagents. Secondly, a no reverse transcription

control (NRTC), where no multiscribe reverse transcriptase enzyme is added to the RT-PCR procedure. This control excludes contamination of the sample itself, either before or after RT-PCR (Martel, Grundemann et al. 2002).

The reactions were carried out in an ABI Prism 7000 Sequence Detection System (Applied Biosystems), which features a 96-well thermal cycler. The initial hot-start activation is required for the DNA polymerase. During cycling, the DNA is melted at 95°C to enable primer annealing at single-stranded DNA and extension by the polymerase at 60°C. The qPCR program started with initial incubation at 50°C for 2 minutes, followed by an initial hot-start activation at 95°C for 10 minutes. Then amplification was done in 40 cycles of denaturation at 95°C for 15 seconds and annealing plus elongation at 60°C for 1 minute. Reactions were performed in a total volume of 20 µl containing 10 ng cDNA; 1x SYBR Green Master Mix; 0.25 µM forward and reverse primer and RNase-free water.

To ensure high quality and reproducibility of the data, every sample was performed in triplicates and the median was used for further calculations. Negative controls were performed in triplicates as well and failed to produce fluorescence throughout the study (defined as C_T cycles above 35).

2.2.2 RKIP protein expression analysis by Western blot

2.2.2.1 Reagents

- RIPA buffer (Cat.No. R0278-50ML, Sigma-Aldrich)
- Phosphatase Inhibitor Cocktail 3 (Cat.No. P0044-1ML, Sigma-Aldrich) DMSO solution
- Halt Protease Inhibitor Cocktail 100x (Cat.No. 1858566, Thermo Fisher)
- DC™ Protein Assay Reagent A (Cat.No. 500-0113, Bio-Rad, Hercules, CA)
- DC™ Protein Assay Reagent B (Cat.No. 500-0114, Bio-Rad)
- DC™ Protein Assay Reagent S (Cat.No. 500-0115, Bio-Rad)
- 4x Laemmli Sample Buffer (Cat.No. 161-0747, Bio-Rad)
- Mini-Protean® TGX™ Precast Gels (Cat.No. 456-1083, Bio-Rad)
- 10x Tris/Glycine/SDS (Cat.No. #161-0732, Bio-Rad)
- Precision Plus Protein™ Kaleidoscope Standards (Cat.No. #161-0375, Bio-Rad)
- Mini Trans-Blot® Filter paper (Cat.No. 1703932, Bio-Rad)

- Low fluorescence PVDF transfer membrane (Cat.No. L-08001-010, Advansta, Menlo Park, CA, USA)
- Methanol for analysis (Cat.No. 1.06009.1011, Merck KGaA)
- 10x Tris/Glycine Buffer (Cat.No. 161-0734, Bio-Rad)
- Blotting-Grade Blocker (Cat.No. 170-6404, Bio-Rad) Nonfat dry milk
- 10x TBS (Cat.No. 170-6435, Bio-Rad)
- TWEEN® 20 (Cat.No. P2287-500ML, Sigma-Aldrich)
- Polyclonal Anti-RKIP Antibody produced in rabbit (Cat.No. 07-137, Millipore Corporation, Billerica, MA, USA)
- Anti-rabbit IgG, HRP-linked antibody produced in goat (Cat.No 7074S, Cell Signaling Technology, Boson, MA)
- WesternBright ECL HRP substrate (Cat.No. 541004, Advansta)
- CL-XPosure^{TM} Film (Cat.No. 34089, Thermo Fisher Scientific)
- Agfa Curix 60 (Agfa HealthCare, Mortsel, Belgium)
- Monoclonal anti-β-actin, antibody produced in mouse (Cat.No. A5441-2ML, Sigma-Aldrich)
- Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP (Cat.No. P0260, DakoCytomation)

2.2.2.2 Cell lysis and protein extraction

Initially, cell pellets were lysed on ice in 200 µL RIPA buffer, containing protease and phosphatase inhibitors for 5 minutes until the pellet was soluble. After a centrifugation step for 5 minutes at 8000 rpm at 4°C, the supernatant containing the proteins was obtained.

For quantification of protein concentration, a Lowry Protein Assay was performed in 96-well plates and analysed in a SpectraMax Plus^{384} Microplate Reader (Molecular Devices, Sunyvale, CA, USA). The master mix was set up with 1 mL reagent A and 20 µL solution S. Out of that, 25 µL were transferred in each well and mixed with 5 µL protein solution. Finally, 200 µL reagent B were added in each well and incubated for 15 minutes at room temperature. The measurement was done in triplicates for each sample. RIPA lysis buffer served as blank and concentrations of 1, 2, 4 and 8 mg/mL BSA RIPA, respectively, were used for definition of the standard curve.

2.2.2.3 Western blot analysis

In a first step, lysates were mixed with 4x Laemmli buffer (final concentration 1x) and heated for 5 minutes at 95°C. This buffer contains denaturing SDS, which renders proteins with a negative charge, as well as bromophenol blue and β-mercaptoethanol, the latter breaking the disulphide bounds, which makes the proteins linear. Subsequently, 20 µg of protein for each sample was loaded on a Mini-Protean® TGX™ Precast Gel, which was run for 1 hour at 100 V with 1x Tris/Glycine/SDS running buffer (100 mL 10x Tris/Glycine/SDS plus 900 mL aqua dest.). In this electric field, the negatively charged and linearized proteins migrate to the positive pole, which results in their separation according to size (molecular weight) with smaller proteins migrating faster. To identify the size of target proteins, the Precision Plus Protein™ Kaleidoscope Standard from Bio-Rad (Figure 6) was additionally loaded on each gel. This marker contains a mixture of 10 multicolour proteins, identifying predefined standard sizes ranging from 10 to 250 kDa.

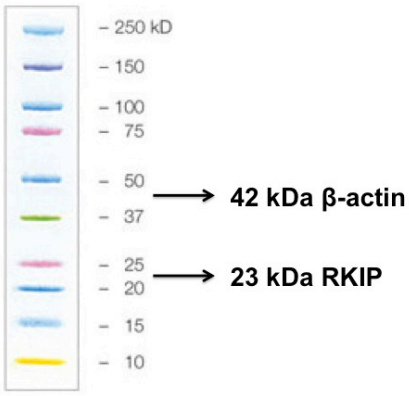

Figure 6. Precision Plus Protein™ Kaleidoscope Standard (Bio-Rad) which was used for Western blotting. The sizes of proteins studied in this project (β-actin and RKIP) are illustrated within this figure.

In a next step, proteins were transferred on a polyvinylidene fluoride (PVDF) membrane. This step is necessary to fix the proteins in a stable membrane and to enable their detection by specific antibodies. Therefore, pre-cooled transfer buffer, consisting of 100 mL 10x Tris/Glycine, 200 mL Methanol and 700 mL aqua dest. was used. As PVDF membranes are hydrophobic, they were rinsed in 100% methanol (MeOH), prior to their equilibration in the transfer buffer. The transfer was carried out at 400 mA for 75 minutes at 4°C. Subsequently the membrane was blocked in 50 mL 5%-blotting grade blocker for 1 hour at 4°C to prevent unspecific binding of the

antibodies. Thereafter, membranes were washed 3 times for 10 minutes with 1x TBST (100 mL 10x TBS, 1 mL Tween20 and 900 mL aqua dest.).

For analysis of RKIP expression, the membrane was incubated with the primary antibody, anti-RKIP (rabbit; polyclonal; 1:1000; 45 minutes at 4°C). After three 10 minute wash steps in 1x TBST, the 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), in the case of RKIP, HRP-linked anti-rabbit IgG (goat; 1:10 000; 1 hour at 4°C).

Next, membranes were washed three times in 1x TBST to remove all the HRP, which is not bound to the RKIP/primary antibody/secondary antibody complex. Thereafter, the membrane was incubated with freshly prepared WesternBright ECL HRP substrate for 2 minutes, resulting in oxidation of HRP, which in turn produces a chemiluminescent signal. This signal can be detected by exposing the membrane to a photographic film, where it produces a band, whose intensity correlates to expression of the protein studied. The intensity of the bands has been measured densitometrically by ImageJ (Schneider, Rasband et al. 2012) and was used for further calculations.

To correct for differences in protein loading, RKIP expression was normalized to the expression of a loading control. Therefore, β-actin has been chosen, which is due to the fact that it has been demonstrated to be equally expressed among various human tissues and diseases, including AML (Dos Santos, McDonald et al. 2013) and mouse experiments (Kim, Ives et al. 2012) previously. Briefly, membranes used for the detection of RKIP were washed in 1x TBST once more and subsequently blocked for 1 hour in 5%-blotting grade blocker. Antibody staining and development has been done as described above using the following conditions: anti-β-actin (mouse; monoclonal; 1:10 000; 1 hour at 4°C), anti-mouse-IgG-HRP (rabbit; polyclonal; 1:10 000; 1 hour at 4°C). Due to the fact that RKIP and β-actin exhibit different protein sizes and that the specific antibodies are obtained from different species (rabbit for RKIP and mouse for β-actin, respectively), stripping of the membrane between the antibodies has not been performed.

To correct for different efficiencies in transfer and antibody-binding among membranes, all samples were normalized to a calibrator, which was included on every blot. As in the qPCR experiments, the NB-4 AML cell line, characterized by prominent RKIP expression (Zebisch, Wolfler et al. 2012), was chosen.

2.3 Expression analysis of transcriptional repressors in AML patient samples

The aim of this part was to investigate the role of transcriptional repressors in RKIP down-regulation in AML. Therefore, the expression of transcriptional repressors, that have been shown to bind the RKIP promoter previously (EZH2 (Ren, Baritaki et al. 2012), SNAI1 (Beach, Tang et al. 2008), SLUG (Al-Mulla, Bitar et al. 2011), BACH1 (Yun, Frankenberger et al. 2011), NFE2L2 (Al-Mulla, Bitar et al. 2012), Yin yang 1 (Baritaki, Katsman et al. 2007)), has been analysed by the means of qPCR and compared between patients with and without loss of RKIP expression. Therefore, the two cohorts of AML patients described above, as well as the NB-4 and ML-2 cell lines were used.

2.3.1 Reagents

- MicroAmp® Optical 96-Well Reaction Plate (Cat.No. N8010560, Applied Biosystems)
- SYBR® GreenER™ (Cat.No. 11760-500, Applied Biosystems / Life Techologies™) qPCR SuperMix for ABI PRISM®Instrument, a ready-to-use cocktail containing all components except primers and template for qPCR
- Oligonucleotide primers for EZH2, SNAI1, SLUG, BACH1, NFE2L2, YY1, B2M and GUSB (Eurofins MWG Operon)

2.3.2 RNA-extraction and cDNA synthesis

RNA extraction using TRIzol® Reagent and cDNA synthesis using Taq Man® Reverse Transcription Reagents were performed as described in part 2.2.1.2.

2.3.3 Expression analysis by qPCR

Primers used for the amplification of transcriptional repressors were selected from ‘Harvard PrimerBank’ and tested in an NCBI-BLAST search to validate their specifity, ensuring that they cover all transcription variants and precluding their self-annealing. Forward and/or reverse primer(s) were chosen to span an exon-exon boundary to beware genomic DNA contamination. All primers were purchased at MWG Eurofins - the sequences are displayed in the appendix, Table 3.

In a next step, according to the MIQE guidelines (Bustin, Benes et al. 2009), we evaluated the PCR efficiencies of every single primer pair by the means of standard curve analysis (see part 2.2.1.3). Achieving slopes between 3.10 and 3.57

corresponding to PCR efficiencies between 110% and 91%, all primer pairs were ascertained to be satisfactory for our experiments.

The AML cell line NB-4 served as calibrator, except for the analysis of SNAI1 and SLUG, which did not show detectable expression values in this cell line. HCT-116 colon carcinoma cells served as calibrator for these genes, which is due to the fact that we could detect prominent expression of both, SNAI1 and SLUG in these cells in preliminary experiments (data not shown). As outlined for RKIP expression analysis, B2M and GUSB were used as control genes for these experiments as well. Expression of these control genes demonstrated a high correlation (correlation coefficient 0.52 – 0.90) in all experiments. To increase the accuracy of our experiments even further, the geometric mean of both genes was calculated and used for further calculations.

The ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with a 96-well thermal cycler and the SYBR Green Master Mix was used for quantitative real-time PCR as specified above (see part 2.2.1.3). As additional quality controls, the following negative controls were included: a no template control (NTC), to exclude contamination of the master mix reagents and a no reverse transcription control (NRTC), without adding multiscribe reverse transcriptase enzyme to the RT-PCR procedure.

2.4 Expression analysis of micro-RNAs in AML patient samples

Genes cannot only be down-regulated by overexpression of transcriptional repressors, but also by the overexpression of specific micro-RNAs (Introduction, part 1.3). As outlined above, data from micro-RNA ChiP profiling, assessing the expression status of more than 600 micro-RNAs in patients with AML (Rommer, Steinleitner et al. 2013), pinpoint to a differential expression of eight micro-RNAs between samples with and without RKIP expression. However, as the amount of samples with defined RKIP loss according to Zebisch and colleagues within this cohort was small (ranging from two to six) (Zebisch, Wolfler et al. 2012) and as ChiP profiling may produce a significant rate of false positive and unspecific results (Cole, Galic et al. 2003, Stec, Wang et al. 2005, Konishi 2006), we aimed at validating these data in the cohort of AML patients described above. The following micro-RNAs were analysed within this project: micro-RNA-10a, -15a, -23a, -23b, -24, -320a, -518b, -519d (Introduction, part 1.3). Additionally, two micro-RNAs, that have been shown to

be linked to RKIP in solid tumours previously, were included (micro-RNA-101 and -224).

2.4.1 Reagents

- miScript® II RT Kit (Cat.No. 218161, Qiagen) for reverse transcription of total RNA containing micro-RNAs
- miScript® SYBR® Green PCR Kit (Cat.No. 218075, Qiagen) for miRNA detection and quantification by real-time PCR
- Hs_miR-23a_2 miScript Primer Assay (Cat.No. MS00031633, Qiagen) miRNA-specific primer
- Hs_miR-23b_2 miScript Primer Assay (Cat.No MS00031647, Qiagen) miRNA-specific primer
- Hs_miR-24_1 miScript Primer Assay (Cat.No. MS00006552, Qiagen) miRNA-specific primer
- Hs_miR-320a_1 miScript Primer Assay (Cat.No. MS00014707, Qiagen) miRNA-specific primer
- Hs_miR-10a_2 miScript Primer Assay (Cat.No. MS00031262, Qiagen) miRNA-specific primer
- Hs_miR-518b_1 miScript Primer Assay (Cat.No. MS00004466, Qiagen) miRNA-specific primer
- Hs_miR-519d_1 miScript Primer Assay (Cat.No. MS00004508, Qiagen) miRNA-specific primer
- Hs_miR-224_1 miScript Primer Assay (Cat.No. MS00003878, Qiagen) miRNA-specific primer
- Hs_miR-101_3 miScript Primer Assay (Cat.No. MS00008372, Qiagen) miRNA-specific primer
- Hs_miR-15a_1 miScript Primer Assay (Cat.No. MS00003178, Qiagen) miRNA-specific primer
- Hs_RNU6-2_11 miScript Primer Assay (Cat.No. MS00033740, Qiagen) miRNA-specific primer
- Hs_SNORD48_11 miScript Primer Assay (Cat.No. MS00007511, Qiagen) miRNA-specific primer
- LightCycler® 480 Multiwell Plates 96 (Cat.No. 04729692001, Roche Diagnostics GmbH, Mannheim, Germany)

- LightCycler® 480 Sealing Foil (Cat.No. 04729757001, Roche Diagnostics GmbH)

2.4.2 Micro-RNA extraction and cDNA synthesis

TRIzol has been shown to successfully extract cellular micro-RNAs previously (Rio, Ares et al. 2010, Eikmans, Rekers et al. 2013). Hence, RNAs used for the qPCR experiments outlined in chapters 2.2.1.2 have been used for the analysis of micro-RNA expression as well.

Reverse transcription was performed using the miScript II RT Kit according to the manufacturer's protocol. 1 µg RNA (including micro-RNAs) of each sample was transcribed into cDNA with 1x miScript HiSpec Buffer; 1x miScript Nucleics Mix and 2µl miScript Reverse Transcriptase Mix. HiSPec buffer was used, as all primers that were used were specific to amplicons of 21 nucleotides. The reaction was performed in a total volume of 20 µL, incubating for 60 minutes at 37°C followed by inactivating the reverse transcriptase mix for 5 minutes at 95°C. The resulting cDNA was diluted to a final concentration of 1 ng/µl, adding 1 µL to each qPCR reaction and stored at -20°C.

2.4.3 Analysis of micro-RNA expression by qPCR

Detection and quantification of PCR products was performed using the SYBR Green methodology, as described earlier within this work.

In this specific setting, however, the miScript SYBR Green PCR Kit was used as master mix, as it is optimised for the amplification of cDNA, transcribed from micro-RNAs. Pre-designed micro-RNA specific primer assays were applied, containing specific forward primers only. For reverse primers, one universal primer mix was used, which was included in the master mix. Reactions were performed in a total volume of 25 µL containing 1 ng template cDNA; 1x QuantiTect SYBR Green PCR Master Mix; 1x miScript Universal Primer and 1x miScript Primer Assay. An initial activation of the HotStarTaq® DNA Polymerase of 15 minutes at 95°C was followed by 45 amplification cycles (15 seconds denaturation at 94°C; 30 seconds annealing at 55°C; 30 seconds extension at 70°C). The real-time PCR instrument LightCycler® 480 (Roche Diagnostics GmbH) with 96-well plates has been used for these experiments.

As outlined above, the $\Delta\Delta C_T$ method, with the AML cell line NB-4 serving as calibrator, was applied to analyse the measured expression data. However, in this

specific setting, U6 small nuclear 2 RNA (RNU6) and the small nuclear RNA - C/D box 48 (SNORD48) were used as control genes.

SNORD 48 is a non-coding RNA usually expressed in the nucleus, showing consistent expression in AML blasts (Jongen-Lavrencic, Sun et al. 2008). RNU6 is part of the spliceosome, located in the nucleus and a commonly used endogenous control in micro-RNA expression experiments. As SNORD48, its expression has been shown to be stable in samples of AML (Rucker, Russ et al. 2013). The expression of both control genes demonstrated a high correlation (correlation coefficient 0.5 to 0.9) in all experiments. To increase the accuracy even further, the geometric mean of both genes was calculated and used for further calculations. To further increase reproducibility, all experiments were performed in triplicates and the median was used for further calculations.

Two negative controls were included as additional quality control. Firstly, a no template control (NTC), which contains all PCR components but no cDNA to exclude contamination of the master mix reagents. Secondly, a no reverse transcription control (NRTC), where no multiscribe reverse transcriptase enzyme is added to the RT-PCR procedure. This control excludes contamination of the sample itself, either before or after RT-PCR (Martel, Grundemann et al. 2002).

2.5 Analysis of micro-RNA expression in mice

In this part we investigated, whether altered micro-RNA expression is an effect of RKIP down-regulation. Therefore, we used an $RKIP^{-/-}$ mouse-model (see part 2.1.4) compared with sex- and age-matched $RKIP^{+/+}$ mice and analysed micro-RNA expression in haematopoietic tissue, including bone marrow (BM) and spleen (SP) by qPCR. The knockout of RKIP was verified on mRNA level by qPCR and on protein level through Western blot (Results, part 3.1, Figure 8).

2.5.1 Reagents Mice

- Hs_RNU1A_11 miScript Primer Assay (Cat.No. MS00013986, Qiagen) miRNA-specific primer
- Mm_miR-23a_2 miScript Primer Assay (Cat.No. MS00032599, Qiagen) miRNA-specific primer
- Mm_miR-23b_2 miScript Primer Assay (Cat.No. MS00032606, Qiagen) miRNA-specific primer

- Mm_miR-24_1 miScript Primer Assay (Cat.No. MS00005922, Qiagen) miRNA-specific primer
- Mm_miR-320_3 miScript Primer Assay (Cat.No. MS00011767, Qiagen) miRNA-specific primer
- Mm_miR-15a_1 miScript Primer Assay (Cat.No. MS00001281, Qiagen) miRNA-specific primer

2.5.2 RNA extraction and cDNA synthesis

Cell pellets from both, bone marrow and spleen of five $RKIP^{-/-}$ and five $RKIP^{+/+}$ mice, respectively, were used for RNA extraction. We therefore employed the TRIzol® method as described in detail above. Again, we performed cDNA synthesis using the miScript II RT Kit (see part 2.4.2). However, in this specific situation, we used 1x miScript HiFlex Buffer instead of 1x miScript HiSpec Buffer, to account for differing amplicon sizes of the control primers used.

2.5.3 Analysis of micro-RNA expression in mice by qPCR

As outlined for the qPCR experiments mentioned above in detail, the SYBR Green methodology, combined with $\Delta\Delta C_T$ data analysis, was used for micro-RNA expression analysis in mice as well. However, considering the fact that all experiments described above were performed in human material, the following mouse-specific modifications were employed:

In a first step, the SNORD48 control was replaced by U1A small nuclear 4 RNA (RNU1A), which is due to the fact that the primers used for SNORD48 were available for the human species only. We therefore chose RNU1A. RNU1A is a component of the spliceosome, responsible for pre-mRNA processing. It was used for normalization of micro-RNA expression in dog serum and showed stable expression (Steudemann, Bauersachs et al. 2013). Primers used for RNU6, which was commonly used as endogenous control for normalization of micro-RNA expression analyses in mouse experiments previously (Kishore, Verma et al. 2013) remained unchanged, while mouse-specific primer assays were purchased for the micro-RNAs analysed. The expression of both control genes demonstrated a high correlation in all experiments (correlation coefficient 0.54 to 0.98) corroborating their role as appropriate reference genes. To increase the accuracy even further, the geometric mean of both genes was calculated and used for further calculations.

As a final modification, we employed $RKIP^{+/+}$ mice as calibrators within this part of the project. The human AML cell lines NB-4 and ML-2 were not included in these experiments, as the primer settings used did not allow a comparison between different species.

2.6 Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics 20.0 software package (SPSS Inc., Chicago, IL, USA). As human AML patient samples present a heterogeneous group, normal distribution of qPCR results within this sample set cannot be assumed. Therefore, we employed the Mann-Whitney U test to compare the expression of genes and micro-RNAs, respectively, between specimens with and without RKIP loss. This test ranks the results within a group in an ordinal scale and thereby enables the comparison of independent sample sets with non-normal distribution (Mann, Whitney 1947). The mouse specimens, on the other hand are genetically identical. The presence or absence of the RKIP knockout is the only difference between the two groups analysed, suggesting that expression levels obtained within every group are normally distributed. Therefore, we employed the Student's t-test for comparison of micro-RNA expressions within these specimens (Rivas-Ruiz, Moreno-Palacios et al. 2013). All tests were performed two-sided and *P*-values below 0.05 were considered as statistically significant.

3. Results

3.1 Analysis of RKIP expression levels in AML patient samples and mice

As outlined above in more detail, this study is based on the comparison between AML specimens with and without loss of RKIP. Therefore, we selected a group of 20 AML patients, that have been characterized in respect to RKIP protein expression by the means of Western blot by our own group previously (Zebisch, Wolfler et al. 2012). One half (n=10) thereby exhibited a normal expression whereas the rest (n=10) harboured a leukaemia-specific, somatic loss of the RKIP protein. In this part of the study, we initially aimed at corroborating these results by analysing RKIP expression levels by the means of Western blot. Therefore, the RKIP expression of a given sample was related to the NB-4 AML cell line, which served as a calibrator and was included on every blot. As published previously, samples showing a relative expression of less than 25% were classified as RKIP loss. Importantly, our results clearly confirmed the RKIP expression status of the selected patients (Figure 7), although it has to be mentioned that four samples could not be measured (2951, 1848, 5536, 4803), due to limitations in sample quality resulting in protein degradation. In a next step, we extended our analyses by analysing the RKIP mRNA expression of these samples by the means of qPCR. Therefore, we employed the $\Delta\Delta C_T$ method using the SYBR green methodology. As for the protein analysis, NB-4 served as a calibrator and was arbitrarily set to an expression value of 100%. Expression values of samples analysed were displayed as x-fold expression of the calibrator.

The qPCR results clearly demonstrated, that the two groups of AML patient samples not only differed in respect to RKIP protein, but also in respect to RKIP mRNA expression. Statistical evaluation by the means of Mann-Whitney U test (Figure 7) revealed the difference to be highly significant ($P < 0.0001$**).

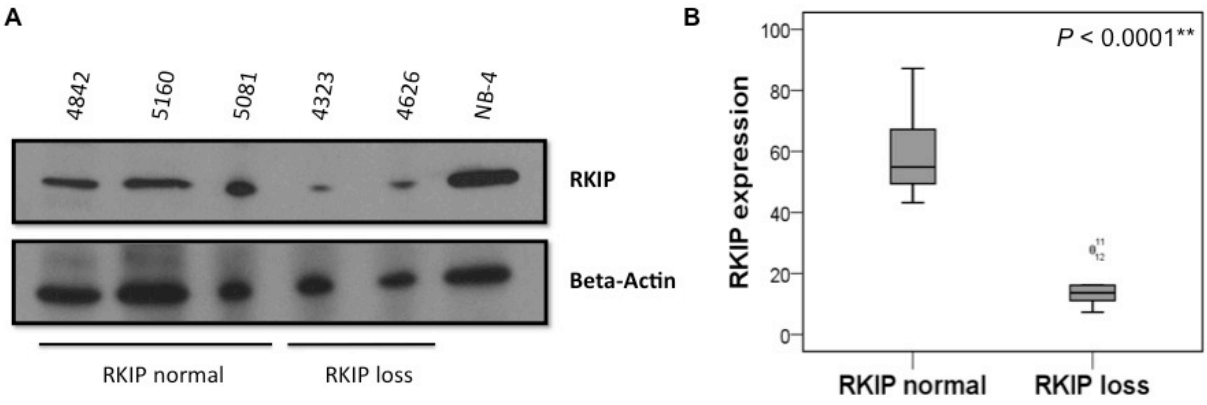

Figure 7. RKIP expression levels in AML patients. (A) Representative Western blot of AML patient samples showing a loss of RKIP protein expression in samples 4323 and 4626. (B) Box plot of RKIP mRNA expression, as analysed by qPCR, in AML patient samples with and without loss of RKIP protein (referred to as "RKIP normal" and "RKIP loss"). RKIP expression is displayed in percent of the calibrator NB-4, which was set to an expression value of 100%.

Beside the AML patient samples, we also employed an RKIP mouse model within this study. In more detail, haematopoietic tissues (bone marrow and spleen) of five mice with a complete RKIP knockout were compared to five wildtype littermates. Therefore, we analysed the RKIP protein and mRNA expression levels as described above. Again, RKIP expression differed statistically significant between $RKIP^{-/-}$ and $^{+/+}$ animals confirming the successful knockdown of RKIP (Figure 8). Thereby the *P*-values are 0.006** for bone marrow and 0.0002** for spleen. Interestingly, only a homozygous knockout resulted in a complete abrogation of RKIP expression, whereas the heterozygous situation did not differ from the wildtype animals.

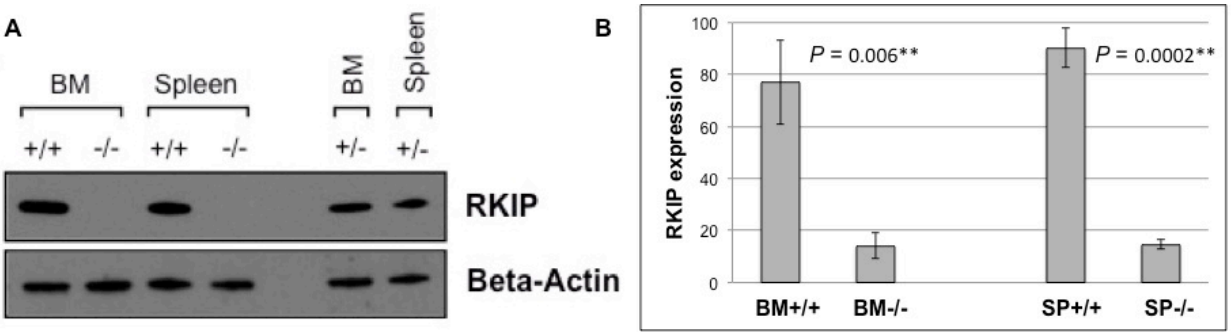

Figure 8. RKIP expression levels in mice. (A) Western blot RKIP protein analysis in haematopoietic tissues (BM and SP) of mouse specimens demonstrating prominent expression in $RKIP^{+/+}$ and $RKIP^{+/-}$ animals but a complete abrogation in homozygous knockouts ($RKIP^{-/-}$) (B) RKIP mRNA expression levels in $RKIP^{+/+}$ and $RKIP^{-/-}$ mice, corroborating Western blot results.

3.2 Expression analysis of transcriptional repressors in AML patient samples

As shown in the previous chapter, we were clearly able to distinguish two groups of AML patients differing in both, RKIP mRNA and protein expression, respectively. In this part, we aimed at delineating, whether the overexpression of transcriptional repressors might be associated to AML samples exhibiting a loss of RKIP and consequently might serve as an explanation for its down-regulation. Therefore, we analysed the mRNA expression levels of transcriptional repressors that have been linked to RKIP previously, in all AML patient samples by the means of qPCR and screened for differences between samples with and without loss of RKIP. Our analyses included EZH2, SNAI1, SLUG, BACH1, NFE2L2 and YY1. Again, the AML cell line NB-4 was used as a calibrator, included in every PCR run and set to a value of 100%, thereby facilitating the relative quantitation as outlined above. It has to be noted, that the colon carcinoma cell line HCT-116 served as a calibrator for the analysis of SNAI1 and SLUG, which is due to the fact that we did not detect any measurable expression of these genes in NB-4.

Our analyses demonstrated prominent expression levels of EZH2, SNAI1, BACH1, NFE2L2 and YY1 in AML patient samples. However, statistical evaluation using the Mann-Whitney U test revealed that their expression levels did not differ between samples with and without RKIP loss. The *P*-value for EZH2 thereby was 0.353, for SNAI1 0.436, for BACH1 0.912, for NFE2L2 0.123 and for YY1 0.436 (Figure 9). Of note, we did not detect measurable expression levels in the case of SLUG in any of the samples analysed.

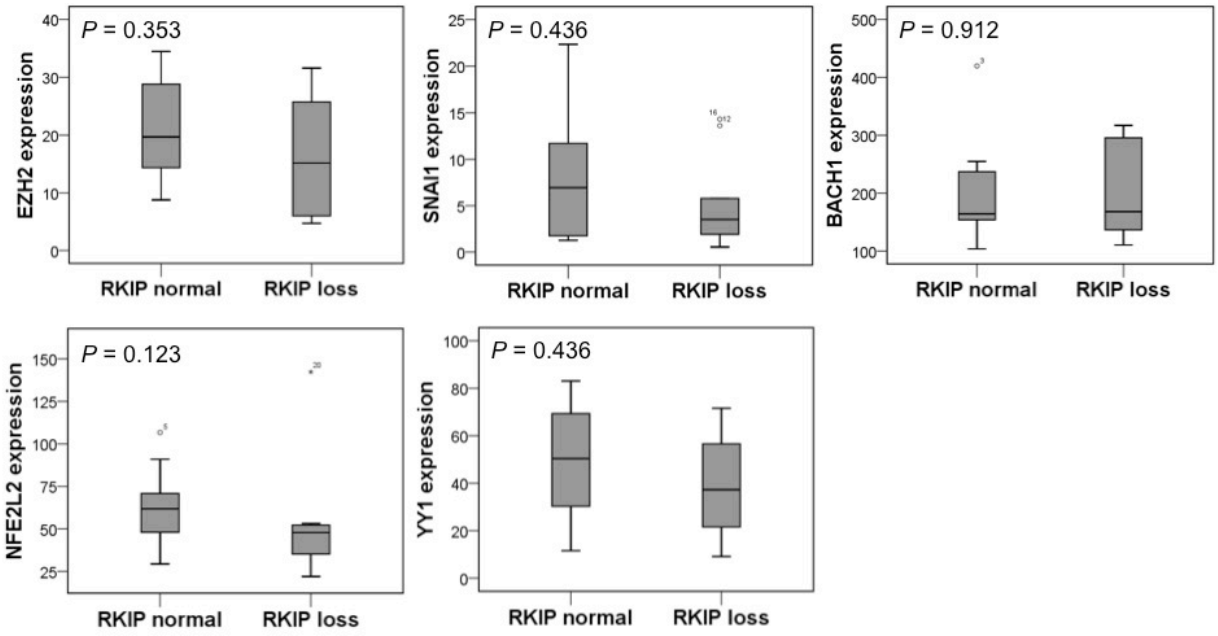

Figure 9. Box plot analysis demonstrating the mRNA expression of transcriptional repressors, linked to RKIP previously, in AML patient samples. mRNA expression levels were measured by qPCR and compared between AML patients with and without RKIP loss.

3.3 Expression analysis of micro-RNAs in AML patient samples

As outlined in more detail above, preliminary results based on micro-RNA expression arrays (Rommer, Steinleitner et al. 2013) suggested a differential expression of eight micro-RNAs between AML specimens with and without RKIP loss. This set of micro-RNAs included micro-RNA-15a, -23a, -23b, -24, -320a, and -519d, showing a higher expression in samples with RKIP loss and micro-RNA-10a and -518b, demonstrating higher expression values in samples with normal RKIP expression. However, as explained in the introduction in more detail, micro-RNA ChiP profiling produces a substantial rate of false-positive results. Therefore, we aimed at delineating, whether RKIP loss in AML patient samples truly correlated to the overexpression of specific micro-RNAs by using qPCR as described above. Although not differentially expressed in the ChiP analyses, we included the analysis of micro-RNA-101 and -224 into our experiments, which is based on the fact that they have been shown to suppress RKIP in solid cancer cell lines previously. Again, NB-4 served as a calibrator and was set to a value of 100%, except for micro-RNA-10a, which was not expressed within this cell line. In this case, ML-2 was used as a calibrator. Six of ten micro-RNAs studied (micro-RNA-10a, -15a, -23a, -23b, -24, -320a) exhibited expression levels detectable by the means of qPCR. Expression of micro-RNA-101,

-224, -518b and -519d was below the detection threshold, which precluded their further analysis. Three of the six micro-RNAs expressed in AML demonstrated statistically significant higher expression levels in AML specimens defined as RKIP loss as compared to samples with normal RKIP expression. These include micro-RNA-15a with a *P*-value of 0.043*, micro-RNA-23a with 0.019* and micro-RNA-24 with 0.043* (Figure 10). Importantly, micro-RNA-23b (*P* = 0.063) and micro-RNA-320a (*P* = 0.052) show a trend to higher expression in AML samples with RKIP loss as well, although statistical significance was not reached (Figure 10). Micro-RNA-10a failed to demonstrate a differential expression (*P* = 0.123) between the two groups of AML patients (Figure 10).

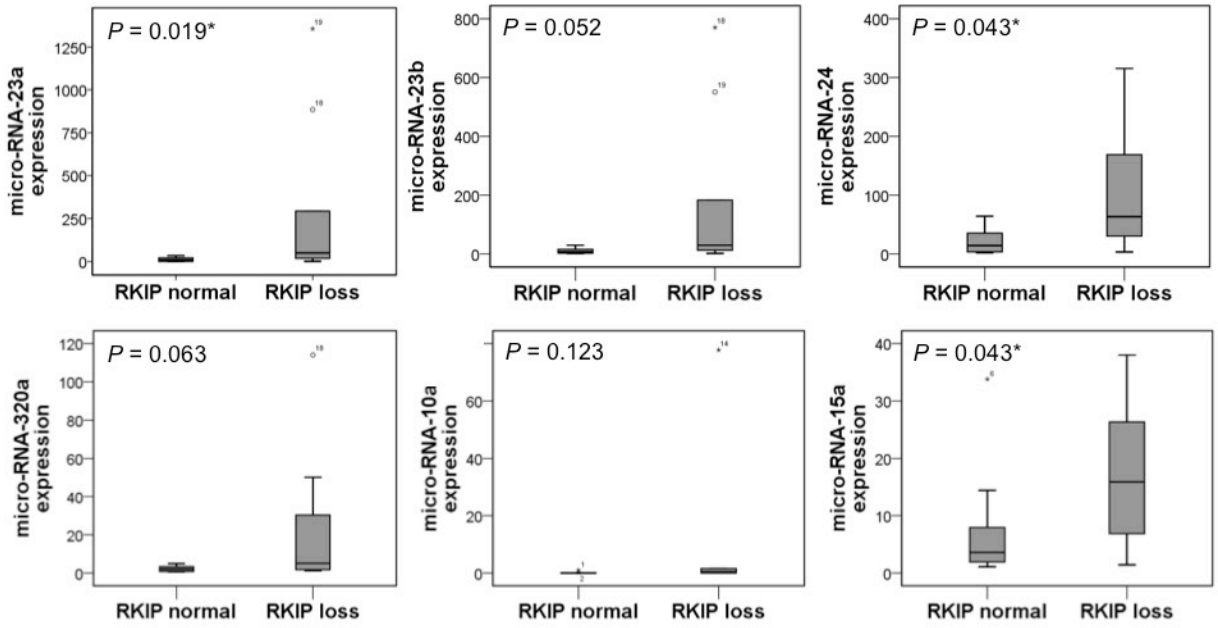

Figure 10. Box plot analysis demonstrating the expression of micro-RNAs in AML patient samples. Micro-RNA expression levels were measured by qPCR and compared between AML patients with and without RKIP loss.

3.4 Analysis of micro-RNA expression in mice

As depicted in the previous chapter, we were able to show that RKIP loss in AML is correlated to the overexpression of five micro-RNAs, including micro-RNA-15a, -23a, -23b, -24 and -320a. To further characterize a potential functional relation between these aberrations, we investigated, whether micro-RNA up-regulation in AML patient samples with RKIP loss (see part 3.3) is an effect of the decreased RKIP expression.

Therefore, we employed a murine RKIP knockout model, which is characterized by a complete loss of RKIP within the haematopoietic system (Figure 8). We performed a qPCR based expression analysis of these micro-RNAs in both, bone marrow and spleen in five animals and compared the results to five wildtype littermates. As described above in more detail, we used a relative quantitation, where samples are normalized to a calibrator sample, which is arbitrarily set to a value of 100%. As the human NB-4 cell line could not be used due to sequence variations between humans and mice, specimens of a wildtype mouse were employed as calibrator for these experiments. As shown in Figure 11, we could not detect any differences in the expression of micro-RNAs between RKIP knockout and wildtype animals. The *P*-values are 0.600 for micro-RNA-15a for BM and 0.753 for SP, 0.753 for micro-RNA-23a for BM and 0.600 for SP, 0.600 for micro-RNA-23b for BM and 0.917 for SP, 0.346 for micro-RNA-24 for BM and 0.917 for SP and 0.465 for micro-RNA-320a for BM and SP.

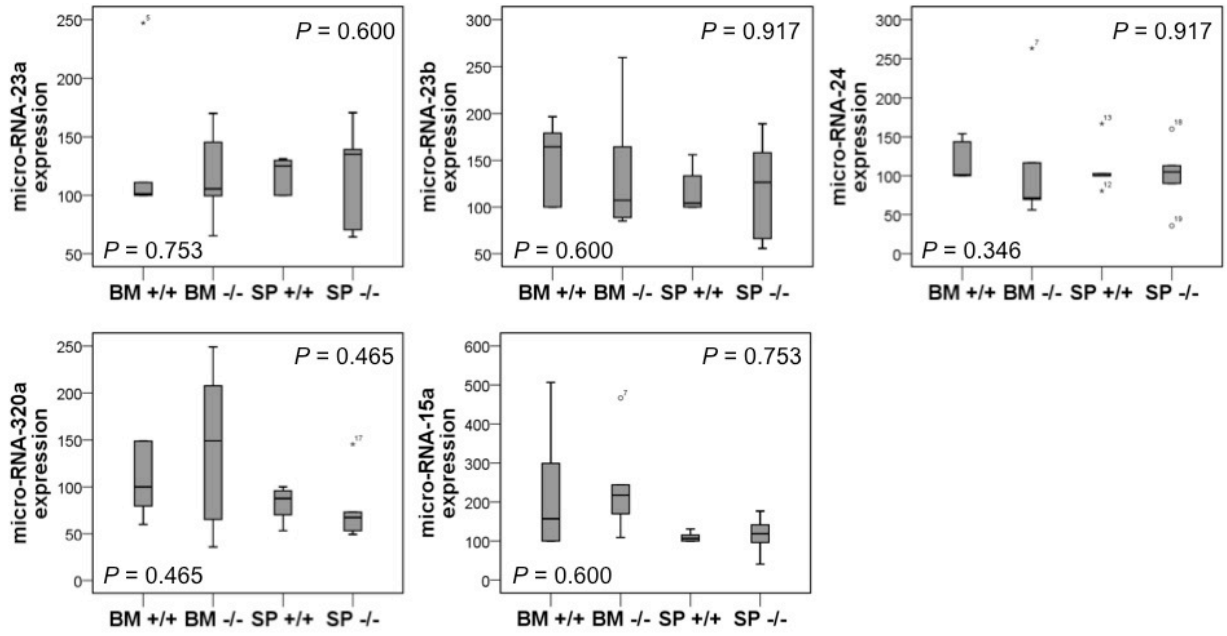

Figure 11. Box plot analysis demonstrating the expression of micro-RNAs in RKIP wildtype and knockout mice. Micro-RNA expression levels were measured by qPCR and compared between $BM^{+/+}$ and $BM^{-/-}$ on the one hand and between $SP^{+/+}$ and $SP^{-/-}$ on the other hand.

4. Discussion

AML is an aggressive neoplasm, which is caused by the accumulation of (epi)genetic alterations in haematopoietic stem- and progenitor cells. The progenies of these malignantly transformed cells, which are called leukaemic blasts, are characterised by uncontrolled proliferation on the one hand and impaired differentiation on the other hand (Frohling, Scholl et al. 2005, Zebisch, Czernilofsky et al. 2007). The RAS-MAPK/ERK pathway, which regulates many fundamental cellular processes including proliferation, differentiation and cell cycle progression (Chang and Karin 2001, Zebisch, Staber et al. 2006), plays a key role in the pathogenesis of this disorder. Its functional involvement in the malignant transformation of haematopoietic stem cells has been shown in various *in-vitro* and *in-vivo* studies and indeed, its constitutive activation, caused by genetic alterations within its members and regulators, has been observed in more than 50% of AML cases. RKIP is an ubiquitously expressed negative regulator protein of the RAS-MAPK/ERK pathway and has been shown to play a seminal role as metastasis-suppressor in solid tumours within the last decade. In a recent publication of our own group we could demonstrate, that RKIP expression is lost in a substantial subset of patients with AML. RKIP loss correlated to the development of monocytic AML phenotypes and to mutations in RAS on the one hand and proved to be a valuable prognostic marker on the other hand. Importantly, RKIP decreased the proliferation and colony formation of AML cell lines in functional *in-vitro* assays, which indicates a tumour-suppressor function of RKIP within the haematopoietic system. Therefore, these data describe a novel role of RKIP, which is a substantial addition to its sole metastasis-suppressor role in solid tumours (Zebisch, Wolfler et al. 2012).

Although we could gain a lot of insight into the effects of RKIP loss in AML (Zebisch, Haller et al. 2009, Fried, Wolfler et al. 2012, Zebisch, Wolfler et al. 2012), the mechanisms causing this important genetic aberration are still unknown. We previously observed that a loss of the RKIP protein correlated to a significant decrease in its mRNA expression in both AML cell lines and patient samples (Zebisch et al., 2012). These data preclude protein instability as the reason behind RKIP loss (Zebisch, Haller et al. 2009, Zebisch, Wolfler et al. 2012). We then screened for hypermethylation of the RKIP promoter by methylation specific PCR, for

inactivating mutations within the RKIP gene by sequence analysis and for gene deletions by qPCR (Zebisch, Haller et al. 2009, Zebisch, Wolfler et al. 2012). However, we did not detect any abnormalities in the AML patients defined as RKIP loss, suggesting other, hitherto unkown (epi-) genetic mechanisms behind the development of RKIP down-regulation. In this thesis, we now aimed at analysing micro-RNAs and transcriptional repressor proteins, which describe two additional possibilities of genetic silencing.

Micro-RNAs are small non-coding RNA molecules that function in post-transcriptional gene silencing, either by translational repression or by degradation of the target mRNA. Pathologic overexpression of a myriad of micro-RNAs has been observed in human malignancies and was functionally related to the down-regulation of important tumour-suppressor genes. In a recent publication, Rommer and co-workers analysed the role of micro-RNAs in the development of AML and therefore used a micro-RNA ChiP technology, which allowed them to study the expression of 636 micro-RNAs per specimen (Rommer, Steinleitner et al. 2013). Importantly, this study included 33 AML specimens from our leukaemia-biobank. We thereafter performed a Western blot protein expression analysis of RKIP in these samples and correlated the results to the micro-RNA ChiP data. Interestingly, we were able to define a set of eight micro-RNAs (hsa-miR-10a, hsa-miR-15a, hsa-miR-23a, hsa-miR-23b, hsa-miR-24, hsa-miR-518b, hsa-miR-519b, hsa-miR-320a), showing differential expression between AML samples with and without RKIP loss. However, as the number of patients with RKIP loss within this cohort was small (n=6), and as ChiP based expression analyses are prone to false-positive and false-negative results (Cole, Galic et al. 2003), we decided to validate the ChiP data by the means of qPCR within this thesis. Additionally, micro-RNA-101 and micro-RNA-224, which have been linked to RKIP down-regulation in solid tumors previously (Huang, Dai et al. 2012, Ren, Baritaki et al. 2012), were added to our analyses.

In a first step of my thesis, we used our leukaemia-biobank, which contains AML samples of patients treated at the Division of Haematology, Medical University of Graz, to define a well characterized cohort of 20 AML specimens. We selected the samples by expression analysis of the RKIP protein by Western blot and of RKIP mRNA by qPCR, respectively. Thereby, we were able to generate a cohort of well

characterized AML patient samples, containing equal numbers of specimens with and without RKIP loss. These specimens are the prerequisite for a representative comparison of genetic events associated to RKIP down-regulation. They have been used for the subsequent analyses of my thesis on the one hand and will provide the basis for potential additional research projects pertaining to the role of RKIP in AML on the other hand.

In a next step, we used this cohort to analyse the expression of the ten micro-RNAs listed above by the means of qPCR. Importantly, we were able to vaildate a statistically significant increase of micro-RNA-15a (P = 0.043*), -23a (P = 0.019*) and -24 (P = 0.043*) in specimens defined as RKIP loss. We further detected a trend to higher expression levels in samples defined as RKIP loss for micro-RNA-23b (P = 0.063) and -320a (P = 0.052), although statistic significance was just not reached. The P-value indicates overexpression of these two micro-RNAs close to significance (defined as a P-value <0.05). Therefore, it would be interesting to analyse a bigger cohort, as this would enable us increase the statistical power of these analyses. Currently, generation of such a sample set is underway within our laboratory which will enable these analyses within the near future.

The most interesting candidates among the five micro-RNAs with increased expression in AML specimens defined by RKIP loss were -23a (P = 0.019*), -23b (P = 0.063) and -24 (P = 0.043*). They are all part of a common micro-RNA cluster, the so called miR-24-23-27 cluster. It describes a highly conserved module containing the micro-RNAs mentioned above and micro-RNA-27. Altered expression of this cluster was shown in various human diseases and malignancies (Chhabra, Dubey et al. 2010). Most interestingly, however, Mi and colleagues studied its role in acute leukaemias and observed its significant up-regulation in specimens of AML (Mi, Lu et al. 2007). In agreement with this publication, the overexpression of the micro-RNAs pertaining to this cluster in AML could be corroborated in subsequent studies. Zaidi and colleagues were able to demonstrate micro-RNA-24 overexpression in AML when compared to haematopoietic stem cells and observed an additional correlation to cases carrying a translocation of chromosomes eight and 21 (t[8;21]) (Zaidi, Dowdy et al. 2009). In another study, overexpression of micro-RNA-23a could be correlated to AML cases with a normal karyotype and mutant NPM1 (Havelange, Stauffer et al. 2011). Importantly, the data obtained in my thesis also confirm a role of miR-24-23-27 overexpression in AML. However, they additionally suggest a

connection of three members of this cluster to the development of RKIP loss. This association is further supported by bioinformatic prediction analyses, carried out by Chhabra and colleagues previously (Chhabra, Dubey et al. 2010). By using two different databases (PANTHER and GeneCodis), they delineated that micro-RNAs-24 and -23, respectively, target both the Wnt and RAS-MAPK/ERK signalling pathways. Both pathways are tightly regulated and inhibited by RKIP (Yeung, Seitz et al. 1999, Yeung, Janosch et al. 2000, Kim, Rath et al. 2007). Most interestingly, however, inhibition of micro-RNA-23b expression, by the means of antagomir-23b reduced the activity of the RAS-MAPK/ERK pathway in endothelial cells, which suggests the regulation of a hitherto unknown RAS-MAPK/ERK regulator by this micro-RNA (He, Li et al. 2012). In agreement with these data, we now associate the overexpression of micro-RNA-23a, -23b and -24 to the down-regulation of the RAS-MAPK/ERK inhibitor RKIP. Whether these micro-RNAs truly inhibit RAS-MAPK/ERK signalling via a down-regulation of RKIP expression will be a follow-up study of my thesis and will be performed in our lab using functional *in-vitro* studies.

The next micro-RNA with increased expression in patients with RKIP loss was micro-RNA-320a ($P = 0.052$). Using a micro-RNA ChiP based analysis, its expression could be included into a specific micro-RNA signature previously, which was subsequently used to successfully predict the clinical outcome of patients with AML and normal cytogenetics. This model even proved to be of prognostic relevance in multivariate analyses including established AML risk factors, such as mutations in FLT3-ITD and NPM1, respectively, as well as WBC counts (Marcucci, Radmacher et al. 2008). Micro-RNA-320a was additionally connected to monocytic differentiation, as its up-regulation was observed during the TPA induced monocytic maturation of immature HL-60 AML cells (Schaar, Medina et al. 2009). This is of particular interest, as we could previously show that RKP loss is associated to a monocytic AML phenotype (Zebisch, Wolfler et al. 2012). In subsequent *in-vitro* analyses, we were able to demonstrate that RKIP is down-regulated during the TPA induced monocytic maturation of HL-60 cells and causally involved in the process of monocytic differentiation (unpublished data). It will be of enormous interest to delineate, whether the increased expression of micro-RNA-320a indeed causes the RKIP loss in AML and thereby drives the development of a monocytic AML phenotype. Functional *in-vitro* studies, to test this hypothesis are currently underway within our laboratory.

The last micro-RNA showing increased expression levels in patients with RKIP loss was micro-RNA-15a (*P* = 0.043*). This micro-RNA has been shown to regulate lymphocytic leukaemia cell proliferation via down-regulation of the Wilms' Tumor 1 (WT1) oncogene (Gao, Xing et al. 2011). Accordingly, it has been shown to be down-regulated in specimens of chronic lymphocytic leukaemia (CLL) carrying a chromosomal deletion at 13q14 (Calin, Dumitru et al. 2002) and to be involved in CLL development in mice (Klein, Lia et al. 2010). Importantly, our data reporting micro-RNA-15a overexpression in patients with RKIP loss are the first report of a potential role of micro-RNA-15a in the pathogenesis of AML.

As a summary of this part of my thesis, we were able to establish a well-defined cohort of AML specimens, containing equal numbers of patients with and without RKIP loss. By qPCR based expression analyses within these samples, we were able to identify a set of five micro-RNAs, including -15a, -23a, -23b, -24 and -320a, respectively, which demonstrate increased expression levels in specimens defined as RKIP loss (Results, Figure 10).

In the next part of this study, we were interested whether up-regulation of these micro-RNAs might display an effect of RKIP down-regulation and therefore employed a functional *in-vivo* model. In more detail, we used RKIP knockout mice, which are currently kept within our laboratory, and which are characterized by a complete knockout of the RKIP protein. Initially, we identified a cohort of ten animals, containing equal numbers of RKIP knockouts and wildtype littermates. By qPCR and Western blot analysis, we were able to confirm the successful RKIP knockdown both at the mRNA and protein level. In a next step, we isolated haematopoietic cells from the bone marrow and the spleen and analysed the expression of the micro-RNAs mentioned above by the means of qPCR. Then, expression values of RKIP knockouts were compared to the wildtype controls. Importantly, no differences were observed between mice with and without RKIP loss (Results, Figure 11), indicating that the micro-RNA up-regulation observed in AML patients with RKIP loss is no effect of RKIP down-regulation. As already mentioned above, there is evidence within the literature, that overexpression of these micro-RNAs might be the reason for RKIP loss in AML, which is further supported by these data. Functional *in-vitro* experiments to test this hypothesis are currently underway within our laboratory.

In the final part of my thesis, we were interested in the role of transcriptional repressors in the process of RKIP down-regulation. Transcriptional repressors regulate gene expression by binding to the promoter region of specific effector genes. Aberrational expression of transcriptional repressors might result in pathologic down-regulation of important tumour-suppressor genes, thus allowing malignant transformation. To screen for a potential association of RKIP loss to transcriptional repressors, we initially performed a research of the available literature and thereby selected a set of six candidate repressors. These repressors were chosen due to the fact that they have been connected to RKIP down-regulation in solid tumours previously and included EZH2, SNAI1, SLUG, BACH1, NFE2L2 and YY1, respectively. The most promising candidates thereby seemed to be EZH2 and SNAI1. EZH2 has been linked to deregulation of the RAS-MAPK/ERK pathway (Fujii, Tokita et al. 2011), particularly to the transcriptional repression of RKIP in breast and prostate cancer (Ren, Baritaki et al. 2012). SNAI1 on the other hand was linked to down-regulation of RKIP in metastatic prostate cancer cell lines previously (Beach, Tang et al. 2008). Most importantly, it has been shown to repress the transcription of RKIP by targeting four different binding sites within the RKIP promoter (Beach, Tang et al. 2008).
In the next step, we analysed the expression of these transriptional repressors within the same cohort of AML specimens that have been used for the analysis of micro-RNAs. However, we failed to observe differential expression levels for any of the transcriptional repressors between AML specimens with and without RKIP loss. Therefore, our data preclude a role of these transcriptional repressors in the pathogenesis of RKIP loss in AML.

In conclusion, we were able to establish a well characterized cohort of AML specimens, which enables the association of molecular associations to the loss of RKIP. By this means, we were able to identify a specific set of micro-RNAs (including -15a, -23a, -23b, -24, -320a), which shows increased expression levels in AML patient specimens and cell lines defined as RKIP loss. Using a murine RKIP knockout model, we were further able to demonstrate that overexpression of these micro-RNAs is no effect of RKIP down-regulation. We are currently investigating whether their overexpression is indeed the cause of RKIP loss by employing *in-vitro* model systems.

5. References

Al-Mulla, F., M. S. Bitar, M. Al-Maghrebi, A. I. Behbehani, W. Al-Ali, O. Rath, B. Doyle, K. Y. Tan, A. Pitt and W. Kolch (2011). "Raf kinase inhibitor protein RKIP enhances signaling by glycogen synthase kinase-3beta." Cancer Res **71**(4): 1334-1343.

Al-Mulla, F., M. S. Bitar, J. Feng, S. Park and K. C. Yeung (2012). "A new model for raf kinase inhibitory protein induced chemotherapeutic resistance." PLoS One **7**(1): e29532.

Baritaki, S., A. Katsman, D. Chatterjee, K. C. Yeung, D. A. Spandidos and B. Bonavida (2007). "Regulation of tumor cell sensitivity to TRAIL-induced apoptosis by the metastatic suppressor Raf kinase inhibitor protein via Yin Yang 1 inhibition and death receptor 5 up-regulation." J Immunol **179**(8): 5441-5453.

Beach, S., H. Tang, S. Park, A. S. Dhillon, E. T. Keller, W. Kolch and K. C. Yeung (2008). "Snail is a repressor of RKIP transcription in metastatic prostate cancer cells." Oncogene **27**(15): 2243-2248.

Beillard, E., N. Pallisgaard, V. H. van der Velden, W. Bi, R. Dee, E. van der Schoot, E. Delabesse, E. Macintyre, E. Gottardi, G. Saglio, F. Watzinger, T. Lion, J. J. van Dongen, P. Hokland and J. Gabert. (2003, Dec). "Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program." Leukemia Retrieved 12, 17, from http://www.ncbi.nlm.nih.gov/pubmed/14562124.

Ben-Batalla, I., S. Seoane, T. Garcia-Caballero, R. Gallego, M. Macia, L. O. Gonzalez, F. Vizoso and R. Perez-Fernandez (2010). "Deregulation of the Pit-1 transcription factor in human breast cancer cells promotes tumor growth and metastasis." J Clin Invest **120**(12): 4289-4302.

Bennett, J. M., D. Catovsky, M. T. Daniel, G. Flandrin, D. A. Galton, H. R. Gralnick and C. Sultan (1976). "Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group." Br J Haematol **33**(4): 451-458.

Bennett, J. M., D. Catovsky, M. T. Daniel, G. Flandrin, D. A. Galton, H. R. Gralnick and C. Sultan (1985). "Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group." Ann Intern Med **103**(4): 620-625.

Bollengier, F. and A. Mahler (1988). "Localization of the novel neuropolypeptide h3 in subsets of tissues from different species." J Neurochem **50**(4): 1210-1214.

Brattain, M. G., W. D. Fine, F. M. Khaled, J. Thompson and D. E. Brattain (1981). "Heterogeneity of malignant cells from a human colonic carcinoma." Cancer Res **41**(5): 1751-1756.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele and C. T. Wittwer (2009). "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments." Clin Chem **55**(4): 611-622.

Calin, G. A., C. D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich and C. M. Croce (2002). "Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia." Proc Natl Acad Sci U S A **99**(24): 15524-15529.

Chang, L. and M. Karin (2001). "Mammalian MAP kinase signalling cascades." Nature **410**(6824): 37-40.

Chen, C. Z., L. Li, H. F. Lodish and D. P. Bartel (2004). "MicroRNAs modulate hematopoietic lineage differentiation." Science **303**(5654): 83-86.

Chhabra, R., R. Dubey and N. Saini (2010). "Cooperative and individualistic functions of the microRNAs in the miR-23a~27a~24-2 cluster and its implication in human diseases." Mol Cancer **9**: 232.

Chomczynski, P. and N. Sacchi (2006). "The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on." Nat Protoc **1**(2): 581-585.

Cole, S. W., Z. Galic and J. A. Zack (2003). "Controlling false-negative errors in microarray differential expression analysis: a PRIM approach." Bioinformatics **19**(14): 1808-1816.

Croce, C. M. (2008). "Oncogenes and cancer." N Engl J Med **358**(5): 502-511.

Dangi-Garimella, S., J. Yun, E. M. Eves, M. Newman, S. J. Erkeland, S. M. Hammond, A. J. Minn and M. R. Rosner (2009). "Raf kinase inhibitory protein suppresses a metastasis signalling cascade involving LIN28 and let-7." EMBO J **28**(4): 347-358.

De Craene, B., B. Gilbert, C. Stove, E. Bruyneel, F. van Roy and G. Berx (2005). "The transcription factor snail induces tumor cell invasion through modulation of the epithelial cell differentiation program." Cancer Res **65**(14): 6237-6244.

De-Zolt, S., F. Schnutgen, C. Seisenberger, J. Hansen, M. Hollatz, T. Floss, P. Ruiz, W. Wurst and H. von Melchner (2006). "High-throughput trapping of secretory pathway genes in mouse embryonic stem cells." Nucleic Acids Res **34**(3): e25.

Diaz-Beya, M., S. Brunet, J. Nomdedeu, R. Tejero, T. Diaz, M. Pratcorona, M. Tormo, J. M. Ribera, L. Escoda, R. Duarte, D. Gallardo, I. Heras, M. P. Queipo de Llano, J. Bargay, M. Monzo, J. Sierra, A. Navarro and J. Esteve (2013). "MicroRNA expression at diagnosis adds relevant prognostic information to molecular categorization in patients with intermediate-risk cytogenetic acute myeloid leukemia." Leukemia.

Dohner, H., E. H. Estey, S. Amadori, F. R. Appelbaum, T. Buchner, A. K. Burnett, H. Dombret, P. Fenaux, D. Grimwade, R. A. Larson, F. Lo-Coco, T. Naoe, D. Niederwieser, G. J. Ossenkoppele, M. A. Sanz, J. Sierra, M. S. Tallman, B. Lowenberg, C. D. Bloomfield and L. European (2010). "Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet." Blood **115**(3): 453-474.

Dos Santos, C., T. McDonald, Y. W. Ho, H. Liu, A. Lin, S. J. Forman, Y. H. Kuo and R. Bhatia (2013). "The Src and c-Kit kinase inhibitor dasatinib enhances p53-mediated targeting of human acute myeloid leukemia stem cell by chemotherapeutic agents." Blood.

Drexler, H. G., H. Quentmeier and R. A. MacLeod (2004). "Malignant hematopoietic cell lines: in vitro models for the study of MLL gene alterations." Leukemia **18**(2): 227-232.

Eikmans, M., N. V. Rekers, J. D. Anholts, S. Heidt and F. H. Claas (2013). "Blood cell mRNAs and microRNAs: optimized protocols for extraction and preservation." Blood **121**(11): e81-89.

Fleige, S. and M. W. Pfaffl (2006). "RNA integrity and the effect on the real-time qRT-PCR performance." Mol Aspects Med **27**(2-3): 126-139.

Frayne, J., C. Ingram, S. Love and L. Hall (1999). "Localisation of phosphatidylethanolamine-binding protein in the brain and other tissues of the rat." Cell Tissue Res **298**(3): 415-423.

Fried, I., A. Wolfler, F. Quehenberger, G. Hoefler, H. Sill and A. Zebisch (2012). "Mutations inDNMT3A and loss of RKIP are independent events in acute monocytic leukemia." Haematologica **97**(12): 1936-1937.

Frohling, S., C. Scholl, D. G. Gilliland and R. L. Levine (2005). "Genetics of myeloid malignancies: pathogenetic and clinical implications." J Clin Oncol **23**(26): 6285-6295.

Fujii, S., K. Tokita, N. Wada, K. Ito, C. Yamauchi, Y. Ito and A. Ochiai (2011). "MEK-ERK pathway regulates EZH2 overexpression in association with aggressive breast cancer subtypes." Oncogene **30**(39): 4118-4128.

Gao, S. M., C. Y. Xing, C. Q. Chen, S. S. Lin, P. H. Dong and F. J. Yu (2011). "miR-15a and miR-16-1 inhibit the proliferation of leukemic cells by down-regulating WT1 protein level." J Exp Clin Cancer Res **30**: 110.

Garzon, R., S. Volinia, C. G. Liu, C. Fernandez-Cymering, T. Palumbo, F. Pichiorri, M. Fabbri, K. Coombes, H. Alder, T. Nakamura, N. Flomenberg, G. Marcucci, G. A. Calin, S. M. Kornblau, H. Kantarjian, C. D. Bloomfield, M. Andreeff and C. M. Croce (2008). "MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia." Blood **111**(6): 3183-3189.

Goossens, S., V. Janzen, S. Bartunkova, T. Yokomizo, B. Drogat, M. Crisan, K. Haigh, E. Seuntjens, L. Umans, T. Riedt, P. Bogaert, L. Haenebalcke, G. Berx, E. Dzierzak, D. Huylebroeck and J. J. Haigh (2011). "The EMT regulator Zeb2/Sip1 is essential for murine embryonic hematopoietic stem/progenitor cell differentiation and mobilization." Blood **117**(21): 5620-5630.

Granovsky, A. E. and M. R. Rosner (2008). "Raf kinase inhibitory protein: a signal transduction modulator and metastasis suppressor." Cell Res **18**(4): 452-457.

Grimwade, D., H. Walker, F. Oliver, K. Wheatley, C. Harrison, G. Harrison, J. Rees, I. Hann, R. Stevens, A. Burnett and A. Goldstone (1998). "The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The

Medical Research Council Adult and Children's Leukaemia Working Parties." Blood **92**(7): 2322-2333.

Hagan, S., R. Garcia, A. Dhillon and W. Kolch (2006). "Raf kinase inhibitor protein regulation of raf and MAPK signaling." Methods Enzymol **407**: 248-259.

Havelange, V., N. Stauffer, C. C. Heaphy, S. Volinia, M. Andreeff, G. Marcucci, C. M. Croce and R. Garzon (2011). "Functional implications of microRNAs in acute myeloid leukemia by integrating microRNA and messenger RNA expression profiling." Cancer **117**(20): 4696-4706.

He, J., Y. Li, X. Yang, X. He, H. Zhang, J. He and L. Zhang (2012). "The feedback regulation of PI3K-miR-19a, and MAPK-miR-23b/27b in endothelial cells under shear stress." Molecules **18**(1): 1-13.

Huang, L., T. Dai, X. Lin, X. Zhao, X. Chen, C. Wang, X. Li, H. Shen and X. Wang (2012). "MicroRNA-224 targets RKIP to control cell invasion and expression of metastasis genes in human breast cancer cells." Biochem Biophys Res Commun **425**(2): 127-133.

Hwang, H. W. and J. T. Mendell (2006). "MicroRNAs in cell proliferation, cell death, and tumorigenesis." Br J Cancer **94**(6): 776-780.

Jongen-Lavrencic, M., S. M. Sun, M. K. Dijkstra, P. J. Valk and B. Lowenberg (2008). "MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia." Blood **111**(10): 5078-5085.

Kim, D., O. Rath, W. Kolch and K. H. Cho (2007). "A hidden oncogenic positive feedback loop caused by crosstalk between Wnt and ERK pathways." Oncogene **26**(31): 4571-4579.

Kim, S. O., K. L. Ives, X. Wang, R. A. Davey, C. Chao and M. R. Hellmich (2012). "Raf-1 kinase inhibitory protein (RKIP) mediates ethanol-induced sensitization of secretagogue signaling in pancreatic acinar cells." J Biol Chem **287**(40): 33377-33388.

Kishore, R., S. K. Verma, A. R. Mackie, E. E. Vaughan, T. V. Abramova, I. Aiko and P. Krishnamurthy (2013). "Bone marrow progenitor cell therapy-mediated paracrine regulation of cardiac miRNA-155 modulates fibrotic response in diabetic hearts." PLoS One **8**(4): e60161.

Klein, U., M. Lia, M. Crespo, R. Siegel, Q. Shen, T. Mo, A. Ambesi-Impiombato, A. Califano, A. Migliazza, G. Bhagat and R. Dalla-Favera (2010). "The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia." Cancer Cell **17**(1): 28-40.

Kluiver, J., B. J. Kroesen, S. Poppema and A. van den Berg (2006). "The role of microRNAs in normal hematopoiesis and hematopoietic malignancies." Leukemia **20**(11): 1931-1936.

Konishi, T. (2006). "Detection and restoration of hybridization problems in affymetrix GeneChip data by parametric scanning." Genome Inform **17**(2): 100-109.

Kota, J., R. R. Chivukula, K. A. O'Donnell, E. A. Wentzel, C. L. Montgomery, H. W. Hwang, T. C. Chang, P. Vivekanandan, M. Torbenson, K. R. Clark, J. R. Mendell and J. T. Mendell (2009). "Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model." Cell **137**(6): 1005-1017.

Krek, A., D. Grun, M. N. Poy, R. Wolf, L. Rosenberg, E. J. Epstein, P. MacMenamin, I. da Piedade, K. C. Gunsalus, M. Stoffel and N. Rajewsky (2005). "Combinatorial microRNA target predictions." Nat Genet **37**(5): 495-500.

Lanotte, M., V. Martin-Thouvenin, S. Najman, P. Balerini, F. Valensi and R. Berger (1991). "NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3)." Blood **77**(5): 1080-1086.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Lorenz, K., M. J. Lohse and U. Quitterer (2003). "Protein kinase C switches the Raf kinase inhibitor from Raf-1 to GRK-2." Nature **426**(6966): 574-579.

Marcucci, G., M. D. Radmacher, K. Maharry, K. Mrozek, A. S. Ruppert, P. Paschka, T. Vukosavljevic, S. P. Whitman, C. D. Baldus, C. Langer, C. G. Liu, A. J. Carroll, B. L. Powell, R. Garzon, C. M. Croce, J. E. Kolitz, M. A. Caligiuri, R. A. Larson and C. D. Bloomfield (2008). "MicroRNA expression in cytogenetically normal acute myeloid leukemia." N Engl J Med **358**(18): 1919-1928.

Martel, F., D. Grundemann and E. Schomig (2002). "A simple method for elimination of false positive results in RT-PCR." J Biochem Mol Biol **35**(2): 248-250.

Meltzer, P. S. (2005). "Cancer genomics: small RNAs with big impacts." Nature **435**(7043): 745-746.

Mi, S., J. Lu, M. Sun, Z. Li, H. Zhang, M. B. Neilly, Y. Wang, Z. Qian, J. Jin, Y. Zhang, S. K. Bohlander, M. M. Le Beau, R. A. Larson, T. R. Golub, J. D. Rowley and J. Chen (2007). "MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia." Proc Natl Acad Sci U S A **104**(50): 19971-19976.

Moffit, J. S., K. Boekelheide, J. M. Sedivy and J. Klysik (2007). "Mice lacking Raf kinase inhibitor protein-1 (RKIP-1) have altered sperm capacitation and reduced reproduction rates with a normal response to testicular injury." J Androl **28**(6): 883-890.

Mrozek, K., G. Marcucci, D. Nicolet, K. S. Maharry, H. Becker, S. P. Whitman, K. H. Metzeler, S. Schwind, Y. Z. Wu, J. Kohlschmidt, M. J. Pettenati, N. A. Heerema, A. W. Block, S. R. Patil, M. R. Baer, J. E. Kolitz, J. O. Moore, A. J. Carroll, R. M. Stone, R. A. Larson and C. D. Bloomfield (2012). "Prognostic significance of the European LeukemiaNet standardized system for reporting cytogenetic and molecular alterations in adults with acute myeloid leukemia." J Clin Oncol **30**(36): 4515-4523.

O'Neil, J. and A. T. Look (2007). "Mechanisms of transcription factor deregulation in lymphoid cell transformation." Oncogene **26**(47): 6838-6849.

Odabaei, G., D. Chatterjee, A. R. Jazirehi, L. Goodglick, K. Yeung and B. Bonavida (2004). "Raf-1 kinase inhibitor protein: structure, function, regulation of cell signaling, and pivotal role in apoptosis." Adv Cancer Res **91**: 169-200.

Ohyashiki, J. H., K. Ohyashiki, K. Toyama, N. Kimura, J. Minowada, A. J. Kinniburgh and A. A. Sandberg (1989). "T-cell receptor gene rearrangement and its expression in human myeloid leukemia cell lines." Cancer Genet Cytogenet **37**(2): 193-200.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.

Ren, G., S. Baritaki, H. Marathe, J. Feng, S. Park, S. Beach, P. S. Bazeley, A. B. Beshir, G. Fenteany, R. Mehra, S. Daignault, F. Al-Mulla, E. Keller, B. Bonavida, I. de la Serna and K. C. Yeung (2012). "Polycomb protein EZH2 regulates tumor invasion via the transcriptional repression of the metastasis suppressor RKIP in breast and prostate cancer." Cancer Res **72**(12): 3091-3104.

Rio, D. C., M. Ares, Jr., G. J. Hannon and T. W. Nilsen (2010). "Purification of RNA using TRIzol (TRI reagent)." Cold Spring Harb Protoc **2010**(6): pdb prot5439.

Rivas-Ruiz, R., J. Moreno-Palacios and J. O. Talavera (2013). "[Clinical research XVI. Differences between medians with the Mann-Whitney U test]." Rev Med Inst Mex Seguro Soc **51**(4): 414-419.

Rommer, A., K. Steinleitner, H. Hackl, C. Schneckenleithner, M. Engelmann, M. Scheideler, I. Vlatkovic, R. Kralovics, S. Cerny-Reiterer, P. Valent, H. Sill and R. Wieser (2013). "Overexpression of primary microRNA 221/222 in acute myeloid leukemia." BMC Cancer **13**: 364.

Rosmarin, A. G., Z. Yang and K. K. Resendes (2005). "Transcriptional regulation in myelopoiesis: Hematopoietic fate choice, myeloid differentiation, and leukemogenesis." Exp Hematol **33**(2): 131-143.

Ru, P., R. Steele, P. Newhall, N. J. Phillips, K. Toth and R. B. Ray (2012). "miRNA-29b suppresses prostate cancer metastasis by regulating epithelial-mesenchymal transition signaling." Mol Cancer Ther **11**(5): 1166-1173.

Rucker, F. G., A. C. Russ, S. Cocciardi, H. Kett, R. F. Schlenk, U. Botzenhardt, C. Langer, J. Krauter, S. Frohling, B. Schlegelberger, A. Ganser, P. Lichter, T. Zenz, H. Dohner, K. Dohner and L. Bullinger (2013). "Altered miRNA and gene expression in acute myeloid leukemia with complex karyotype identify networks of prognostic relevance." Leukemia **27**(2): 353-361.

Schaar, D. G., D. J. Medina, D. F. Moore, R. K. Strair and Y. Ting (2009). "miR-320 targets transferrin receptor 1 (CD71) and inhibits cell proliferation." Exp Hematol **37**(2): 245-255.

Schneider, C. A., W. S. Rasband and K. W. Eliceiri (2012). "NIH Image to ImageJ: 25 years of image analysis." Nat Methods **9**(7): 671-675.

Schroeder, A., O. Mueller, S. Stocker, R. Salowsky, M. Leiber, M. Gassmann, S. Lightfoot, W. Menzel, M. Granzow and T. Ragg (2006). "The RIN: an RNA integrity number for assigning integrity values to RNA measurements." BMC Mol Biol **7**: 3.

Somech, R., E. N. Gal-Yam, S. Shaklai, O. Geller, N. Amariglio, G. Rechavi and A. J. Simon (2007). "Enhanced expression of the nuclear envelope LAP2 transcriptional repressors in normal and malignant activated lymphocytes." Ann Hematol **86**(6): 393-401.

Stanford, W. L., J. B. Cohn and S. P. Cordes (2001). "Gene-trap mutagenesis: past, present and beyond." Nat Rev Genet **2**(10): 756-768.

Stec, J., J. Wang, K. Coombes, M. Ayers, S. Hoersch, D. L. Gold, J. S. Ross, K. R. Hess, S. Tirrell, G. Linette, G. N. Hortobagyi, W. Fraser Symmans and L. Pusztai (2005). "Comparison of the predictive accuracy of DNA array-based multigene classifiers across cDNA arrays and Affymetrix GeneChips." J Mol Diagn **7**(3): 357-367.

Steudemann, C., S. Bauersachs, K. Weber and G. Wess (2013). "Detection and comparison of microRNA expression in the serum of Doberman Pinschers with dilated cardiomyopathy and healthy controls." BMC Vet Res **9**: 12.

Theroux, S., M. Pereira, K. S. Casten, R. D. Burwell, K. C. Yeung, J. M. Sedivy and J. Klysik (2007). "Raf kinase inhibitory protein knockout mice: expression in the brain and olfaction deficit." Brain Res Bull **71**(6): 559-567.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman (2002). "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." Genome Biol **3**(7): RESEARCH0034.

Vardiman, J. W., J. Thiele, D. A. Arber, R. D. Brunning, M. J. Borowitz, A. Porwit, N. L. Harris, M. M. Le Beau, E. Hellstrom-Lindberg, A. Tefferi and C. D. Bloomfield (2009). "The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes." Blood **114**(5): 937-951.

Vermeulen, J., F. Pattyn, K. De Preter, L. Vercruysse, S. Derveaux, P. Mestdagh, S. Lefever, J. Hellemans, F. Speleman and J. Vandesompele (2009). "External oligonucleotide standards enable cross laboratory comparison and exchange of real-time quantitative PCR data." Nucleic Acids Res **37**(21): e138.

Yeung, K., P. Janosch, B. McFerran, D. W. Rose, H. Mischak, J. M. Sedivy and W. Kolch (2000). "Mechanism of suppression of the Raf/MEK/extracellular signal-regulated kinase pathway by the raf kinase inhibitor protein." Mol Cell Biol **20**(9): 3079-3085.

Yeung, K., T. Seitz, S. Li, P. Janosch, B. McFerran, C. Kaiser, F. Fee, K. D. Katsanakis, D. W. Rose, H. Mischak, J. M. Sedivy and W. Kolch (1999). "Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP." Nature **401**(6749): 173-177.

Yeung, K. C., D. W. Rose, A. S. Dhillon, D. Yaros, M. Gustafsson, D. Chatterjee, B. McFerran, J. Wyche, W. Kolch and J. M. Sedivy (2001). "Raf kinase inhibitor protein interacts with NF-kappaB-inducing kinase and TAK1 and inhibits NF-kappaB activation." Mol Cell Biol **21**(21): 7207-7217.

Yoon, S. and R. Seger (2006). "The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions." Growth Factors **24**(1): 21-44.

Yun, J., C. A. Frankenberger, W. L. Kuo, M. C. Boelens, E. M. Eves, N. Cheng, H. Liang, W. H. Li, H. Ishwaran, A. J. Minn and M. R. Rosner (2011). "Signalling pathway for RKIP and Let-7 regulates and predicts metastatic breast cancer." EMBO J **30**(21): 4500-4514.

Zaidi, S. K., C. R. Dowdy, A. J. van Wijnen, J. B. Lian, A. Raza, J. L. Stein, C. M. Croce and G. S. Stein (2009). "Altered Runx1 subnuclear targeting enhances myeloid cell proliferation and blocks differentiation by activating a miR-24/MKP-7/MAPK network." Cancer Res **69**(21): 8249-8255.

Zebisch, A., L. Cerroni, C. Beham-Schmid and H. Sill (2003). "Therapy-related leukemia cutis: case study of an aggressive disorder." Ann Hematol **82**(11): 705-707.

Zebisch, A., A. P. Czernilofsky, G. Keri, J. Smigelskaite, H. Sill and J. Troppmair (2007). "Signaling through RAS-RAF-MEK-ERK: from basics to bedside." Curr Med Chem **14**(5): 601-623.

Zebisch, A., M. Haller, K. Hiden, T. Goebel, G. Hoefler, J. Troppmair and H. Sill (2009). "Loss of RAF kinase inhibitor protein is a somatic event in the pathogenesis of therapy-related acute myeloid leukemias with C-RAF germline mutations." Leukemia **23**(6): 1049-1053.

Zebisch, A., P. B. Staber, A. Delavar, C. Bodner, K. Hiden, K. Fischereder, M. Janakiraman, W. Linkesch, H. W. Auner, W. Emberger, C. Windpassinger, M. G. Schimek, G. Hoefler, J. Troppmair and H. Sill (2006). "Two transforming C-RAF germ-line mutations identified in patients with therapy-related acute myeloid leukemia." Cancer Res **66**(7): 3401-3408.

Zebisch, A., A. Wolfler, I. Fried, O. Wolf, K. Lind, C. Bodner, M. Haller, A. Drasche, D. Pirkebner, D. Matallanas, O. Rath, K. Blyth, R. Delwel, E. Taskesen, F. Quehenberger, W. Kolch, J. Troppmair and H. Sill (2012). "Frequent loss of RAF kinase inhibitor protein expression in acute myeloid leukemia." Leukemia **26**(8): 1842-1849.

Zhu, S., M. L. Si, H. Wu and Y. Y. Mo (2007). "MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1)." J Biol Chem **282**(19): 14328-14336.

Zipper, H., H. Brunner, J. Bernhagen and F. Vitzthum (2004). "Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications." Nucleic Acids Res **32**(12): e103.

6. Appendix

Tables

Figures

Oligonucleotide primers

Table 3. Oligonucleotide primers used for PCR and qPCR analysis.

Primer	Sequence 5' to 3'
RKIP F2	GAG CCC TGG CCG GTC TCC CTT GTC CCA AAC TTT
RKIP R2	CCA AAA GGG TCT TTG AGC ACC AGA GGA CAT CCG
RKIP R4	AGA CTT CCG TGT CCG GAT GAT AGA TAG CCT CTC C
RKIP forward	CTA CAC CTT GGT CCT GAC AGA
RKIP reverse	GAG CCC ACA TAA TCG GAG AGG
B2M forward	GAG TAT GCC TGC CGT GTG
B2M reverse	AAT CCA AAT GCG GCA TCT
GUSB forward	GAA AAT ACG TGG TTG GAG AGC TCA TT
GUSB reverse	CCG AGT GAA GAT CCC CTT TTT A
EZH2 forward	AAT CAG AGT ACA TGC GAC TGA GA
EZH2 reverse	GCT GTA TCC TTC GCT GTT TCC
SNAI1 forward	TCG GAA GCC TAA CTA CAG CGA
SNAI1 reverse	AGA TGA GCA TTG GCA GCG AG
SLUG forward	CGA ACT GGA CAC ACA TAC AGT G
SLUG reverse	CTG AGG ATC TCT GGT TGT GGT
BACH1 forward	TCT GAG TGA GAA CTC GGT TTT TG
BACH1 reverse	CGC TGG TCA TTA AGG CTG AGT AA
NFE2L2 forward	TCC AGT CAG AAA CCA GTG GAT
NFE2L2 reverse	GAA TGT CTG CGC CAA AAG CTG
YY1 forward	AAG AGC GGC AAG AAG AGT TAC
YY1 reverse	CAA CCA CTG TCT CAT GGT CAA TA