The role of ABR in myeloid differentiation and acute myeloid leukemia

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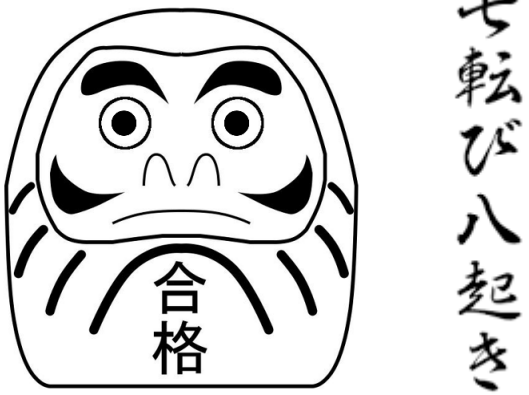

„Fall down seven times, stand up eight.“

Japanese proverb

Table of Contents

Abbreviations

7-AAD	7-aminoactinomycin D
ABR	active BCR related
ATRA	all-trans retinoic acid
ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
AML1-ETO	acute myeloid leukemia 1 protein; runt-related transcription factor 1 (RUNX1)/ Eight-twenty-one
APL	acute promyelocytic leukemia
AZA	azacitidine
BCR	breakpoint cluster region
BM	bone marrow
BSA	bovine serum albumin
C2	complement 2
C57BL/6	C57 black 6
CaLB	calcium-dependent lipid binding domain
CBFB-SMMHC	core-binding factor beta- smooth muscle myosin heavy chain
CD	cluster of differentiation
CDC42	cell division cycle 42; CDC42Hs
cDNA	complementary desoxyribonucleic acid
C/EBPα	CCAAT/enhancer binding protein alpha
CK	complex karyotype
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitors
CML	chronic myeloid leukemia

CMP	common myeloid progenitors
DH	Dbl-homology domain
DMEM	Dulbecco’s modified Eagle’s medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EYFP	Enhanced Yellow Fluorescent Protein
ETO	RUNX1 translocation partner 1
EtOH	ethanol
f	female
F4/80	Epidermal Growth Factor-like module-containing mucin-like hormone receptor-like 1
FAB	French-American-British
FACS	fluorescence-activated cell sorting
FAM	6-fluorescein amidite
FBS	fetal bovine serum
FGFR1	fibroblast growth factor receptor 1
FLT3-ITD	fms-related tyrosine kinase 3/ internal tadem duplication
FITC	fluorescein isothiocyanate
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GC	guanine- cytosine
G-CSF	granulocyte-colony stimulating factor
GDP	guanosine-diphosphate
GEF	guanine nucleotide exchange facto
Gr-1	lymphocyte antigen 6 complex, locus G; Ly-6G/Ly-6C
GTP	guanosine-5'-triphosphate
GMP	granulocyte-macrophage progenitors

Hb	hemoglobin
hnRNP E2	poly(rC)-binding protein 2; PCBP2
HRP	horseradish peroxidase
HSC	hematopoietic stem cells
HSC/P	hematopoietic stem cells/progenitors
inv	inversion
KIT	KIT proto-oncogene receptor tyrosine kinase; c-KIT; CD117; homolog of the feline sarcoma viral oncogene v-kit
LTR	long terminal repeat
m	male
+mar	addition of unknown origin, designated “marker”
M-CSF	monocyte-colony stimulating factor
MDS	myelodysplastic syndrome
miRNA, miR	micro RNA
MEP	megakaryocyte/erythroid progenitors
MN	meningioma
MPP	multipotent precursor cell
mRNA	messenger ribonucleic acid
MOI	multiplicity of infection
MYH11	Myosin-11
NK	normal karyotype
NMA	non-myeloablative
NOD/SCID	Non-Obese Diabetic/Severe Combined Immunodeficiency
NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin)
p	short arm of the chromosome
PB	peripheral blood
PCR	polymerase chain reaction
PE	Phycoerythrin

PH	pleckstrin homology domain
PLT	platelets
PMA	phorbol 12-myristate 13-acetate
PML	promyelocytic leukemia protein
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
q	long arm of the chromosome
qPCR	quantitative Real-Time PCR
Rac	ras-related C3 botulinum toxin substrate
RAR-α	retinoic acid receptor alpha
Ras	Harvey rat sarcoma virus oncogene; H-ras
Rho	rhodopsin
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
RUNX1	runt related transcription factor 1
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
ST-K	serine/threonine kinase
t	translocation
TP53	tumor protein p53
TU	transducing units
Vav	vav guanine nucleotide exchange factor
WHO	World Health Organization

Index of Figures

Index of Tables

1. Introduction

1.1 Hematopoiesis

Hematopoiesis refers to the process that generates mature blood cells of different lineages from pluripotent hematopoietic stem cells (HSCs) through a highly regulated hierarchy of successive differentiation events. Hematopoiesis is strictly controlled by a complex network of extrinsic and intrinsic stimuli, signaling pathways, growth factors, cytokines, transcription factors, and other molecular components. During fetal life hematopoiesis begins in the yolk sac followed by a phase in the liver and spleen. HSCs subsequently migrate through the fetal liver into the bone marrow (1), which remains the dominant site of hematopoiesis throughout life. HSCs represents only 0.05% of mouse bone marrow cells, and can be further subdivided into three distinct populations: long-term self-renewing HSCs, short-term self-renewing HSCs, and multipotent progenitors without detectable self-renewable potential (2).

The proliferation capacity of the cells decreases with increasing differentiation. The differentiation or maturation of hematopoietic stem cells leads to successive development-restricted stages. First, the pluripotent HSCs develop into different multipotent precursor cells (MPPs), which have lost the ability to self-renewal but still have the differentiation potential for all lines. The determined MPPs can differentiate into common lymphocyte precursor cells (CLP) and common myeloid precursor cells (CMP). Line-specific precursor cells are formed from the CLPs mature B and T lymphocytes as well as natural killer cells. The CMPs give rise to granulocyte-macrophage precursors (GMP), which differentiate into monocytes / macrophages and granulocytes, and megakaryocyte erythrocyte precursors (MEP), which differentiate into megakaryocytes or platelets and erythrocytes (3,4), (Figure 1).

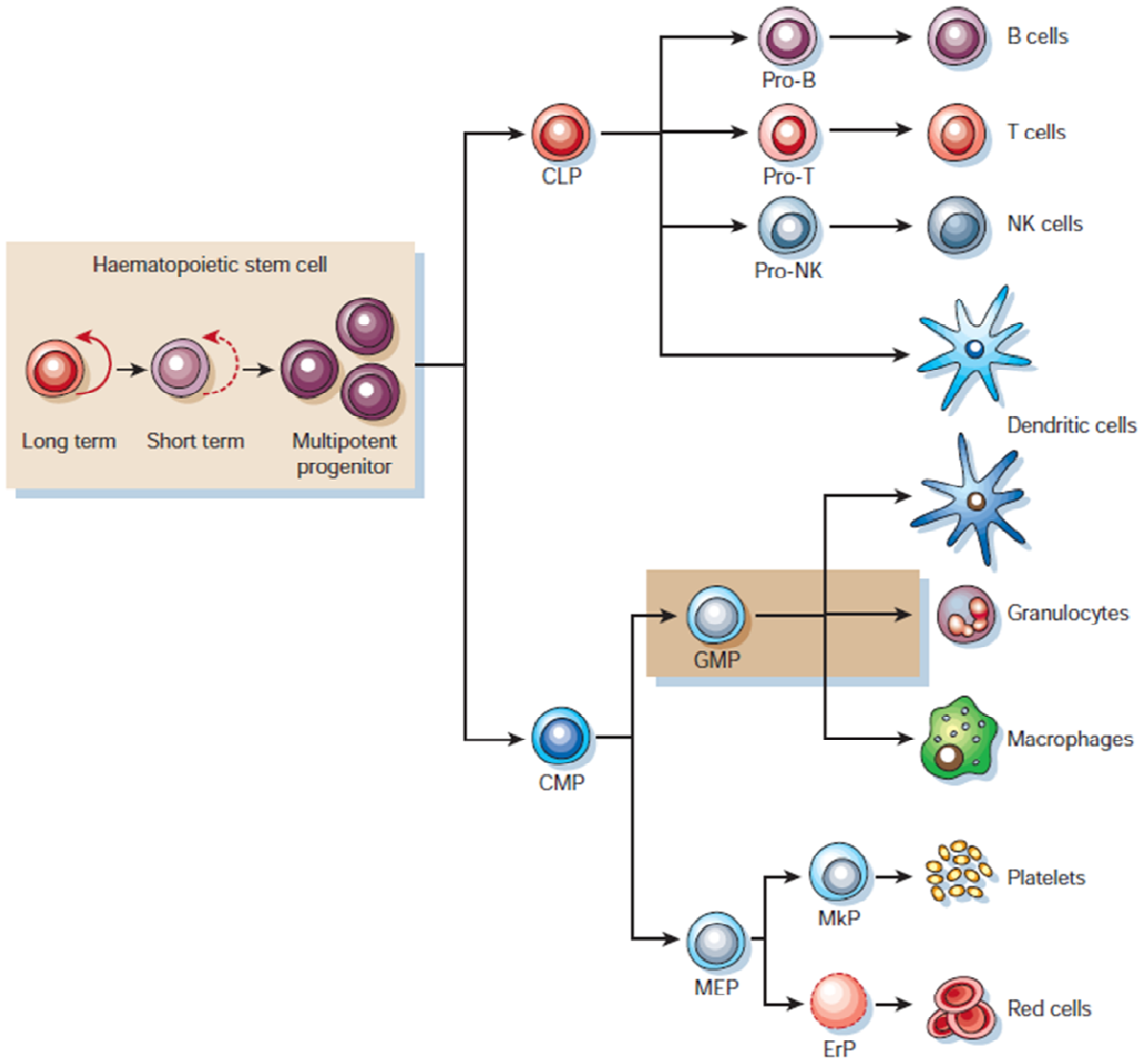

Figure 1. Hematopoietic lineage diversification. Development of hematopoietic stem cell to various lineages (2).

With increasing differentiation and the associated loss of proliferability, the different blood cells exhibit cell-line-specific gene expression. Thus, the different maturation stages and the developing cell lines express characteristic cell surface markers, known as "cluster of differentiation" (CD). The hematopoietic stem cell is characterized by the expression of the surface marker CD34 (5).

1.2 Leukemia

Cancer is the second leading cause of non-infectious diseases after cardiovascular disease, according to the World Health Organization. The causes of each type cancer are diverse so that specific diagnosis of each subtype may be essential for

the success of the treatment. The focus in cancer research lies in the identification and characterization of oncogenes and tumor suppressors, as well as their influence on cell proliferation and cell death. The disorder of normal cell differentiation is the key component in the development of many cancers.

The term leukemia stands for a heterogeneous group of hematological diseases and literally means "white blood", but is also referred as blood cancer. The disease is characterized by accumulation of hematopoietic precursor cells, which fail to undergo terminal differentiation (6). This block of differentiation is manifested in uncontrolled proliferation of hematological precursor cells in the bone marrow and lymphatic tissues, such as lymph nodes and spleen, as well as in the loss of mature functional blood and immune cells. Leukemia is classified as acute and chronic based on how quickly it progresses. Acute leukemia is fast growing and can overrun the body within a few weeks or months, while chronic leukemia is slow growing and progresses in years. In addition, acute leukemia, in which many of the blood precursor cells remain in the blast stage and lose their ability to differentiate, can be distinguished from chronic leukemia in which the precursor cells are hyperproliferative, but can be fully differentiated. According to the type of lineage which is affected, leukemia is classified as myeloid and lymphoid. Based on the course of the disease as well as the affected cell line, the acute and chronic leukemia are roughly divided into four main groups:

- Acute myeloid leukemia (AML)
- Chronic myeloid leukemia (CML)
- Acute lymphocytic leukemia (ALL)
- Chronic lymphocytic leukemia (CLL)

About 11,500 people were diagnosed with leukemia in Germany in 2010. The risk of disease for leukemia falls with children with increasing age and continues to increase continuously from about the age of 30. The absolute 5-year survival rate of leukemia patients between 2009 and 2010 was 47% (7). For a prognosis and later therapy there is a more precise diagnostic classification based on different morphological, as well as cytogenetic and molecular-genetic changes.

1.3 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a disease of myelopoiesis with malignant transformation of the hematopoietic progenitor cells, which mainly affects the differentiation of myeloid and myelomonocytic cells. It comes to a massive proliferation of immature precursor cells in the bone marrow with a displacement of the normal hematopoiesis and a flooding of the cells into the peripheral blood, known as leukocytosis. As a result of this suppression, anemia (loss of red blood cells), increased infections due to neutropenia, and an increased tendency to bleed by thrombopenia can be the consequence, and at the same time also the first signs of acute leukemia. AML occurs with an incidence of 3.7 cases per 100,000 inhabitants per year and a rising frequency in the higher age. It affects women and men similarly (8).

AML involves activation of oncogenes or deactivation of tumor suppressor genes, and a block of differentiation caused by reduced function of transcription factors. The transcription factors CCAAT/enhancer binding protein alpha (C/EBPα) and PU.1 are crucial for normal myelopoiesis and are deregulated in AML. C/EBPα$^{-/-}$ bone marrow hematopoietic cells do not effectively generate granulocyte-monocyte progenitors from the common myeloid progenitor (9). C/EBPα induces myelopoiesis via 2 major steps: 1. upregulation of myeloid-specific genes necessary for granulocytic maturation and 2. inhibition of myeloid cell proliferation (6,10).

According to French-American-British (FAB) classification, AML is divided into 9 subtypes based on the morphological appearance of the blasts and their reactivity with biochemical stains (11,12). The subtypes of the FAB classification of AML are represented in Table 1.

Table 1. French-American-British (FAB) classification of AML. Adapted from (12).

FAB-Subtype	Description	Associated translocations and rearrangements
M0	Acute myeloblastic leukemia with minimal differentiation	inv(3q26), t(3;3)
M1	Acute myeloblastic leukemia without maturation	
M2	Acute myeloblastic leukemia with maturation	t(8;21), t(6;9)
M3	Acute promyelocytic leukemia	t(15;17), t(11;17), t(5;17)
M4	Acute myelomonocytic leukemia	11q23, inv(3q26), t(3;3), t(6;9)
M4Eo	Acute myelomonocytic leukemia with abnormal eosinophils (Eo)	inv(16), t(16;16)
M5	Acute monocytic leukemia	11q23, t(8;16)
M6	Erythroleukemia	
M7	Acute megakaryoblastic leukemia	t(1;22)

According to the criteria of the World Health Organization (WHO), molecular biology features are also important for the classification of the different subtypes. The classification of AML by the WHO is therefore not entirely consistent with the FAB classification (13). The WHO divides the AML into the following four main groups by means of cytogenetic, molecular-genetic and morphological changes:

1. AML with cytogenetic and molecular-genetic aberrations
2. AML with multilinear dysplasia
3. Therapy-associated AML and myelodysplastic syndrome
4. AML without further specification or classification.

According to the WHO, the diagnosis of an AML requires the presence of pathologic myeloblasts on the nuclear cells of bone marrow and / or blood accounting for ≥ 20% (14). The FAB classification defines a blast fraction of ≥ 30% for the diagnosis of an AML in adults (11). A prognostic classification of the AML is possible due to cytogenetic examinations.

A favorable prognosis has been shown by patients with translocations such as t (8; 21) and t (15; 17) or the inversion inv (16) (15,16) as well as CEBPA mutations (17–19), whereas a high age (> 60), a secondary AML, the loss of chromosome 5 or 7, the translocations t (6; 9) (20), t (9; 22) (16) and a complex altered karyotype (21) are associated with a poor prognosis (Table 2). . Acute promyelocytic leukemia with translocation t (15; 17) has the best prognosis. The cure rate is over 80% for the treatment with vitamin A acid (ATRA) (22).

Table 2. Classification of AML according to risk group. Adapted from (23).

Risc group	Cytogenetics	Molecular characteristics
Favorable	inv(16) or t(16;16); t(8;21); t(15;17)	Normal cytogenetics with: isolated biallelic *CEBPA*-mutation *NPM1*-Mutation without *FLT3-ITD*
Intermediate	Normal karyotype Isolated +8 t(9;11) other cytogenetic abnormalities not classified as favorable or poor	*KIT* mutation in core binding factor-leukemia: inv(16) or t(16;16) t(8;21)
Poor	Complex karyotype (≥3 clonal abnormalities) Monosomal karyotype -5/-5q or -7/-7q 11q23 rearrangement s other than t(9;11) inv(3) or t(3;3) t(6;9); t(9;22)	Normal cytogenetics with: *FLT3-ITD*

1.4 Transcription Factors in Hematopoiesis: Role of C/EBPα

Transcription factors are specialized nuclear proteins that have DNA-binding domains that give them the ability to bind to specific sequences of DNA and can either stimulate or repress transcription of the related gene.

A major factor which determines the cell fate in hematopoiesis is the interplay between tissue specific transcription factors, which in turn, modulate a specific set of genes necessary for differentiation to a specific lineage (24–26). Transcription factors

play an important role in regulating major steps of hematopoiesis, such as differentiation, proliferation and survival. Several of those transcription factors have narrow expression patterns in that they are limited to a few hematopoietic lineages.
The gene encoding the transcription factor CCAAT / enhancer-binding protein alpha (C/EBPα) is encoded by an intronless gene and is located at chromosome band 19q13.1 (27). C/EBPα gene is 2783 bp long, very GC-rich (over 70%) exon, and encodes a 356 amino acid long protein (28). Measurable C/EBPα expression levels are found in adipose tissue, liver, pancreas, small and large intestine, lung, adrenal gland, skeletal muscle, prostate and placenta and in mononuclear cells of the peripheral blood (28,29). Undetectable or very low expression levels of C/EBPα were found in brain, kidney, thymus, testes and ovaries (29).
C/EBPα is the first protein discovered containing the structural motif known as leucine zipper, a periodic repetition of the amino acid leucine arranged on its alpha helix (30). C/EBPα was identified originally as a heat stable protein present in rat liver nuclei and having sequence specific DNA binding activity (31,32). The DNA sequences to which C/EBPα binds are the “CCAAT homology” common to many promoters of genes that encode mRNA, and the “enhancer core homology” common to many viral enhancers. These findings point out the role of C/EBPα as a transcriptional regulatory protein (32).

1.4.1 C/EBPα in normal hematopoiesis

Studies in the early 1990s focused on the role C/EBPα in the differentiation of adipose, liver and lung tissues. Later, the role of C/EBPα in granulopoiesis was elucidated by the finding that a large number of myeloid genes contain C/EBP binding sites in their promoters (26). The role of C/EBPα in granulopoiesis was underlined by the finding that targeted disruption of C/EBPα results in a selective early block in both granulocytic (33) and monocytic (34) maturation. The temporal expression pattern of C/EBPα during granulocytic differentiation is depicted in Figure 2 (35,36).

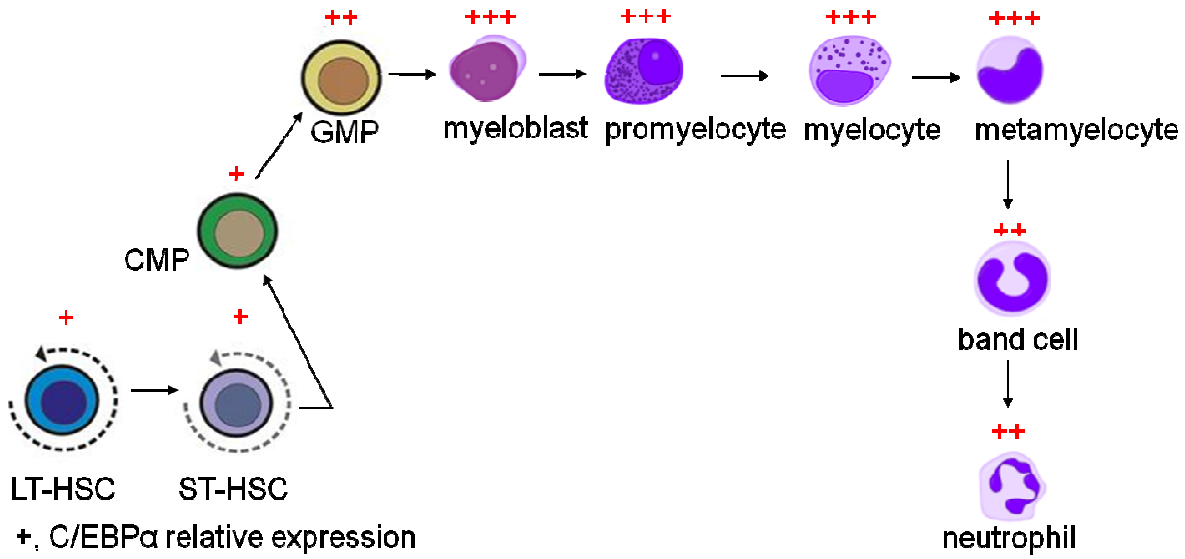

Figure 2. Expression pattern of C/EBPα expression in different stages of granulopoiesis. Relative expression levels are denoted by + according to (35,36).

Radomska et al. have shown that conditional expression of C/EBPα alone is sufficient to trigger neutrophilic differentiation (37). In addition, primary $CD34^+$ cells isolated from human bone marrow differentiate into granulocytes when transduced with a retroviral vector expressing C/EBPα (38). Loss of C/EBPα leads to an increase in HSC self-renewal in comparison to that of wild type HSC. In the hematopoietic system, a number of C/EBPα target genes have been found, including a number of primary granule protein genes (39,40). C/EBPα was also described to regulate the genes encoding the receptors for the granulocyte- (G-CSF) and monocyte-colony stimulating factor (M-CSF) (33,41–43). However, previous studies suggested that G-CSF-R was not a critical target gene, since knockout of this gene failed to show the complete granulocyte differentiation block as observed in C/EBPα knockout mice (44,45).

The role of C/EBPα in granulopoiesis was underlined by the finding that non-conditional targeted block of C/EBPα results in a selective impairment in granulocytic maturation, without affecting other hematopoietic lineages (33). C/EBPα conditional knockout mice show a selective block in the transition from the CMP to GMP stage of granulopoiesis and an increase in HSC self-renewal (46). This study points out that C/EBPα is necessary for CMP to GMP transition as well as in regulating the self-renewal of HSC compartment of bone marrow during granulopoiesis. The concept of

C/EBPα as granulocyte specific transcription factor is questioned by the finding that fetal liver from C/EBPα $^{-/-}$ mice also lack mature macrophages and macrophage progenitors, suggesting that C/EBPα can have crucial role in the development of macrophages (34).
In addition to regulate the genes encoding G-CSF-R and M-CSF-R (33,41–43), C/EBPα has been shown to regulate the microRNAs 223, 34a, 30c and 182 during granulopoiesis (47–51).

1.4.2 The C/EBP family

The CCAAT/enhancer-binding proteins (C/EBPs) are a family of transcription factors that include six members C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ (52–55). Except for C/EBPζ, which lacks the basic region, each protein contains similar basic region and leucine zipper sequences at its C-terminus, which mediate DNA binding and dimerization, respectively. The C/EBP proteins form leucine zipper mediated homodimers as well as heterodimers with other C/EBP members (52–56). The N-terminal portion of each protein contains effector domains that mediate transcriptional activation, repression and autoregulatory functions. The expression pattern of each C/EBP varies in different tissues pointing out to the fact that each member could have specific roles in each cell type. In hematopoiesis, the C/EBP members shown to have specific function are C/EBPα, C/EBβ and C/EBPε. C/EBPα is necessary for early granulocytic differentiation from hematopoietic stem cell to promyelocyte and C/EBPε is necessary for terminal granulocyte differentiation, from promyelocyte to mature neutrophil (57). C/EBPβ is necessary for granulopoiesis in emergency conditions such as fungal infection or cytokine stimulation (58).

1.4.3 C/EBPα and leukemia

The early differentiation block in myeloid precursor cells and the resulting accumulation of blasts in the bone marrow as well as peripheral blood are characteristics of the AML. In the development of an AML, transcription factors are most frequently deregulated (6). A lack of C/EBPα expression or the loss of its ability to function in leukemic blasts leads to an important function of C/EBPα as a tumor

suppressor (59,60). In an AML with suppressed C/EBPα function, the transition from CMPs to GMPs is blocked, which leads to an accumulation of myeloid blasts in this stage (61). In almost 50% of all AMLs, C/EBPα is downregulated by various mechanisms (60,62,63), including its own mutations (60,64–66) (Figure 3).

A suppressed C/EBPα expression was detected in AML subtypes with different translocations. In the subtype AML-M2 with the translocation t (8; 21), the protein AML1-ETO inhibits the expression of the CEBPA mRNA (62). In addition, AML-M2 patients with t(8;21) show up to six fold less C/EBPα mRNA than AML-M2 patients with normal karyotype (62). AML-ETO does not directly bind C/EBPα promoter but inhibits C/EBPα function and autoregulation by a not known mechanism (62).

BCR-ABL, the product of the t(9;22) translocation in chronic myeloid leukemia, commonly known as Philadelphia chromosome, has been found to inhibit C/EBPα translation by interaction of the poly(rC)-binding protein hnRNP E2 with C/EBPα mRNA (67).

Another fusion protein that was found to downregulate C/EBPα in AML was AML1-MDS1-EVI1 (AME), the product of the t(3;21) translocation (68). AME was shown to induce expression of the RNA binding protein, calreticulin. This protein interacts with GCN repeats within the C/EBPα mRNA and inhibits the translation of C/EBPα protein (68). Similarly, CBFB-SMMHC fusion protein found in AML patients with inv(16) has been shown to upregulate calreticulin expression and inhibit C/EBPα (69).

PMR-RARα, the leukemic fusion protein observed in acute promyelocytic leukemia, inhibits C/EBPα by repressing C/EBPα promoter activity (70). Deregulation of the C/EBPα protein expression was found in AML subtypes with FLT3-ITD mutations (71–73). This repression can be overcome by treatment with CEP-701, a FLT3 inhibitor (72). Additionally, activation of FLT3 in AML inhibits C/EBPα function by ERK1/2-mediated phosphorylation of C/EBPα (71). Besides, hypermethylation was detected in the CEBPA promoter in AML patients (74). All these studies indicate that the myeloid master regulator C/EBPα is disrupted in acute myeloid leukemia in different ways (Figure 3).

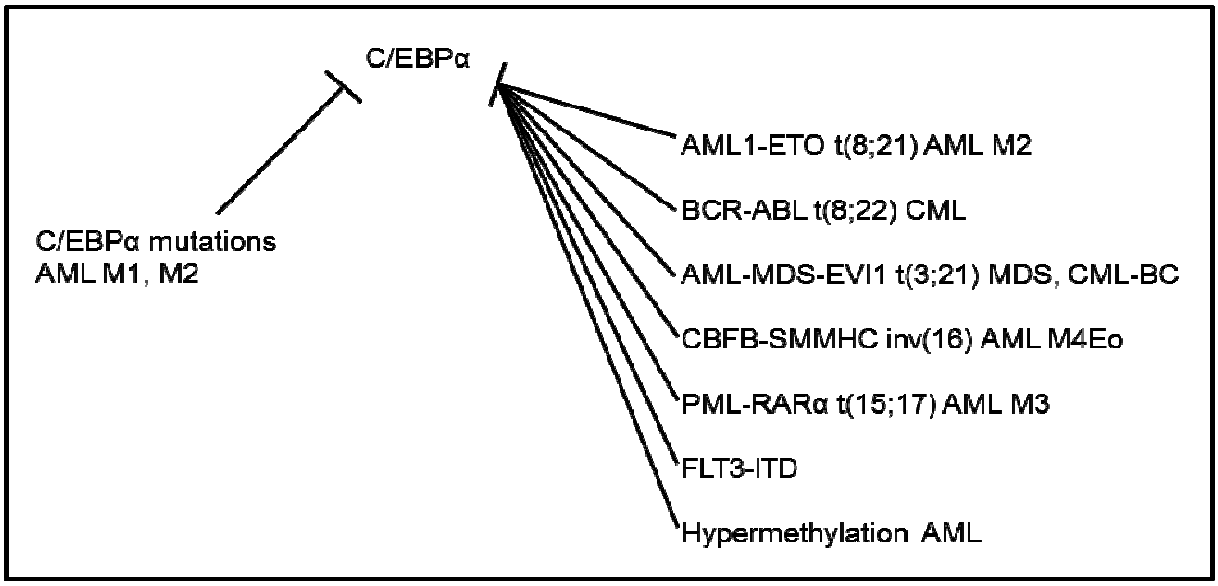

Figure 3. Repression of C/EBPα in leukemia. Several mechanisms are shown to disrupt C/EBPα expression, underlining the role of C/EBPα as tumor suppressor.

1.5 Active BCR related (ABR) gene

Active BCR related (ABR) gene is located on chromosome 17p, band p13 (75) (Figure 4). Diverse studies indicate that structural abnormalities of the short arm of chromosome 17 are associated with various clinical disorders. For example, the deletion of 17p13 occurs in Miller-Dieker syndrome, a mental retardation syndrome, in which duplications of the telomeric portion had one breakpoint located within ABR (76). Further, the loss of heterozygosity of chromosome 17p markers was reported in the progression of colorectal carcinoma (77). In CML, the Ph chromosome t(9;22) is associated with aberrant translocations involving the band 17p13 as one of the breakpoint sites: t(17;22) and t(9;17) (78,79). Moreover, a gene rearrangement have already been reported in respect to chronic myeloid leukemia (CML), including the nuclear protein P53, located at 17p13 (Figure 4), and involved in up to 30% of CML cases in blast crisis (80).

The finding of allelic deletions of DNA sequence on chromosome 17p including ABR locus in several tumors, such as medulloblastoma, a common childhood brain tumor (81,82), astrocytomas (83), and in breast cancer (84), suggest a potential tumor suppressor role for ABR. Consistently with those findings, the mutation of the P53 tumor suppressor gene has been reported in medulloblastoma and breast cancer (85,86). In addition, the loss of these markers from 17p13.3-ter region (Figure 4) was

associated with worst disease-free and overall survival outcomes in breast cancer (84).

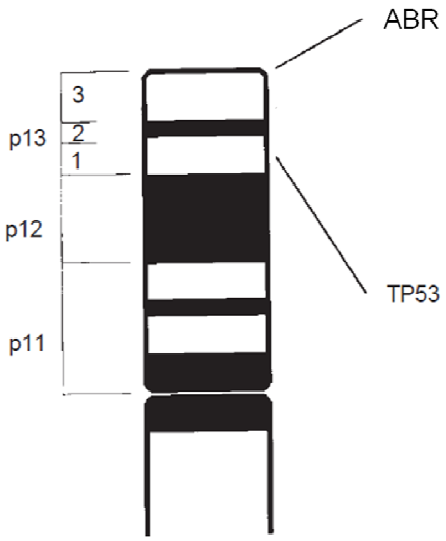

Figure 4. ABR localization on the short arm of chromosome 17. ABR is located toward the telomeric end of chromosome 17p, band p13, which also contains the tumor suppressor gene P53, also known as TP53. Adapted from (84).

The human genome contains many BCR-related sequences (87–89). However, ABR was the only identified locus which is functional (90).

Active BCR related (ABR) protein shares 68% amino acid identity with BCR (90,91). BCR gene, located on chromosome 22q, has been shown to act as a tumor suppressor in chronic myeloid leukemia, blocking leukemia formation in a NOD/SCID mouse model (92). Further, BCR expression has been reported to be downregulated in meningiomas showing loos of heterozygosity within the minimal deletion region (93) .

ABR has a differential expression pattern in various mouse tissues and is expressed at a relatively high level in the central nervous system (94,95) and in hematopoietic tissues (96). In addition, a gene expression profile from peripheral blood and bone marrow samples of AML patients identified that a significant reduced expression of ABR was strongly correlated with the major AML cytogenetic classe t(8;21) (97).

1.5.1 ABR inactivates Rac through ABR GAP domain

ABR encodes a protein consisting of three distinct functional domains: a Dbl (DH)/pleckstrin (PH) homology domain, a C2 domain and a GAP domain (96) (Figure 5).

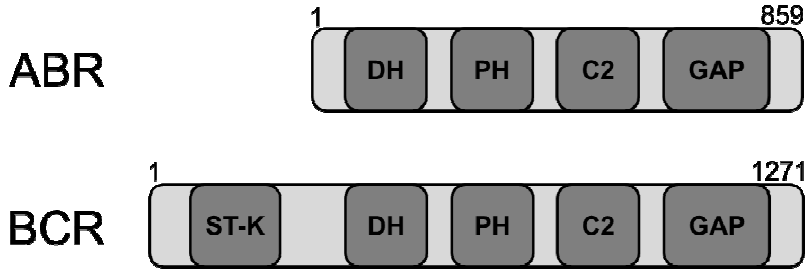

Figure 5. Domain structure of ABR and BCR. Schematic representation of ABR (upper panel) and BCR (bottom panel) domains. ABR and BCR share a Dbl (DH)/pleckstrin (PH) homology domain, a C2 domain and a GAP domain. BCR includes a serine/threonine kinase (ST-K) domain, which is absent in ABR (96)**.**

ABR belong to the class of GTPase activating proteins (GAPs), of which around 70 have been identified for Rho family members (98). Rho-family GTPases comprise a main branch of the Ras superfamily of small (~21 kDa) GTPases (99). The small GTPases of the Rho family known as key regulators of innate immune cells (75).
ABR has a GTPase-activating protein (GAP) domain that interacts with the small GTPase Rac in its active GTP-bound conformation (100,101). The interaction of ABR with Rac-GTP results in the conversion of bound GTP to GDP and the inactivation of Rac (Figure 6) (91,94,100).

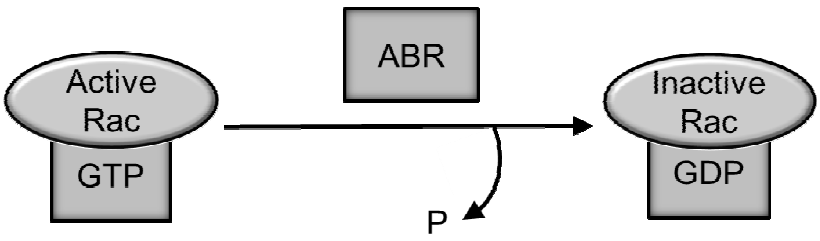

Figure 6. ABR inactivates Rac by stimulating its GTPase activity. ABR GAP domain is able to bind Rac that is in a GTP-bound conformation, resulting on hydrolysis of GTP to GDP on Rac. (96,100).

Upon hydrolysis of GTP to GDP on Rac1, Rac1-GDP (inactive state) is released from the ABR GAP domain (96). The specificity of Rac function is regulated through a

tightly controlled cycle of activation and deactivation that is mediated by upstream activators, the guanine nucleotide exchange factors (GEFs), and through deactivation by the GAPs (102,103). ABR and its related BCR act as GAPs not only for Rac1, but also for Rac2, and CDC42 (100).

Although both ABR and BCR have GAP activity, ABR lacks homology to the serine/threonine kinase domain of BCR (Figure 6). Therefore, ABR is likely to have cellular functions overlapping with but also distinct from those of BCR (96).

In vivo, ABR and the relative BCR have been shown to regulate Rac functions in cerebellum and inner ear (94,104). ABR and BCR double null mouse mutants showed glial hypertrophy in the anterior cerebellum and midbrain (94). In addition, monocytes derived from ABR and BCR double-null mice show enhanced basal Rac1 activity in assays pulling down GTP-bound Rac1 and are hyper-responsive to stimulation with epidermal growth factor and lipopolysaccharide and exhibit constitutively increased phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), which is regulated by Rac (94). ABR and BCR double-null mice abnormal motor behavior, characterized by hyperactivity and persistent circling, poor motor coordination, frequently losing their balance (104). These phenotypic features of double null mutants are all consistent with vestibular dysfunction of the inner ear (104). The deficiency of ABR or BCR in knockout mice has been shown to enhance Rac1 activity in brain and reveal a synaptic function of ABR and BCR, showing that these proteins are expressed in the brain, where they are distributed to excitatory synapses (95).

1.5.2 ABR activates Rho family proteins through ABR Dbl-homology domain

In addition to the GAP domains, both ABR and BCR contain guanine nucleotide exchange factors (GEF) (activator) (also known as Dbl-homology (DH) domains) (Figure 5), pleckstrin-homology (PH) domains and C2 domains. Guanine nucleotide-exchange factors (GEFs) are directly responsible for the activation of Rho-family GTPases in response to diverse extracellular stimuli, and ultimately regulate numerous cellular responses such as proliferation, differentiation and movement (99). The two separate GAP and Dbl-related regulatory domains exhibit distinct functional

specificities toward small GTP binding proteins. The cycling between GDP- and GTP-bound states is controlled primarily by two classes of regulatory molecule: GTPase-activating proteins (GAPs), which enhance the relatively slow intrinsic GTPase activity of Rho proteins; and guanine nucleotide-exchange factors (GEFs), which catalyze the exchange of GDP for GTP *in vivo* (99). Interestingly, ABR has a dual regulatory role inactivating Rac1 by the GAP domain (Figure 6), and activating RhoA by the Dbl-homology domain (Figure 7), acting as a dual GEF-GAP protein. In contrast to the inactive Rho-GDP, the active Rho-GTP transduces signals by interacting with downstream effectors (103,105).

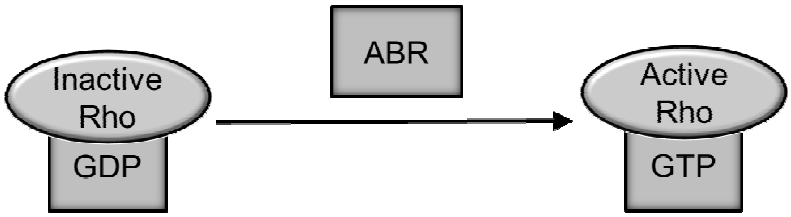

Figure 7. ABR activates RhoA through the DH domain. ABR stimulates GTP binding to Rho family (RhoA, CDC42Hs and Rac2) (91,100).

In the study from Cho et al. (2007) (106), positive evidence supporting an activity of ABR DH domains was not found in mouse macrophages either *in vitro* or *in vivo*. However, the isolated DH-domain of ABR was active in stimulating GTP binding to Rac1 and other members of the Rho family (RhoA, CDC42Hs and Rac2) *in vitro* (91,100). In addition, an ABR mutant lacking DH domain was unable to replace wild-type ABR for induction of apoptosis in human embryonic stem cells (107). Further, ABR has been reported to act as a dual GEF-GAP protein by simultaneously increasing Rho activity using its GEF domain and inactivating Cdc42 using its GAP domain during single cell wound healing *in vitro* (108). Differently from ABR DH domain, a previous study of the GEF activity of BCR DH domain revealed that the isolated DH/PH domain of BCR is associated with the activation of Cdc42 (but not Rac1 or RhoA) *in vivo* (109).

1.5.3 ABR PH domain

PH domains are almost always found to locate at a C-terminal position in relation to DH domains. PH domains can be essential for binding to Rho GTPases and evidence is raising that these linked domains cooperate to facilitate the activation of

Rho GTPases (99). For example, several studies have concluded that DH–PH fragments show greater nucleotide exchange activity than the respective DH domains alone (110,111).

ABR contains a PH domain (Figure 5) that can potentially mediate the membrane translocation of both proteins. M-CSF is known to activate phosphatidylinositol 3-kinase to generate phospholipid and therefore could provide a ligand for the PH domain-containing proteins (112). A robust increase in M-CSF-directed motility was observed in macrophages deficient for both proteins. In control cells, ABR is predominantly cytoplasmic (108). However, in response to M-CSF stimulation, ABR transiently translocated to the plasma membrane. ABR and BCR are GAPs that specifically negatively regulate Rac function *in vivo* in primary macrophages (106). A previous work by Vedham et al. (2005) (113) showed that the PH domain of Vav, a GEF for Rac, accounts for specific recruitment of Vav to the plasma membrane upon M-CSF stimulation. This finding further implicates the PH domains of ABR and BCR as potential candidates to be involved in the translocation of ABR and BCR in M-CSF stimulation. However, the PH domains of Dbl-family members have been shown to consistently bind phospholipids with low affinity and little specificity, which implies that these interactions are insufficient for membrane localization (114).

1.5.4 ABR C2 domain

The C2 (complement 2), also known as calcium-dependent lipid binding (CaLB) domains are common protein modules that were first identified in protein kinase C (115). C2 domains typically bind lipids (99) and might display calcium-dependent phospholipid binding, with calcium bridging the protein and the membrane lipids (116). The C2 domain is another modular signaling domain that can induce membrane–protein, or protein–protein, interactions, after binding several Ca^{2+} ions, although not all C2 domains are reported to be able to bind calcium (99,117). C2 domain of P120 GAP protein has been shown to interact with annexin VI (118), which is a major Ca^{2+} - and phospholipid binding protein known to have tumor suppressor activity (119). C2 domains have also been shown to be required during synaptic transmission (120). Although previous studies have reported the function of ABR GAP and Dbl homology domains (91,96,100), the function of the ABR C2 domain remains unknown.

1.5.5 ABR is able to bind to RhoGDI

Rho family-specific guanine nucleotide dissociation inhibitors (RhoGDIs) function as chaperones and are also able to insert into and extract Rac from membranes (121–127). The guanine nucleotide-dissociation inhibitors (GDIs) sequester GTPases in the cytosol in a GDP-bound state (99). A previous work has reported that RhoGDIα binds to the GAP domain of ABR and BCR (128). It is known that GAP domain binds to Rac-GTP and catalyzes conversion of the bound GTP to GDP on Rac (128). Those authors have also shown that in presence of RhoGDIα, BCR is unable to convert Rac-GTP to Rac-GDP because RhoGDI forms a direct protein complex with BCR. A similar mechanism could be likely to apply to the regulation of ABR (Figure 8) because its GAP domain shares 80% amino acid sequence identity with the BCR GAP domain and a previous study (128) showed that ABR also binds to RhoGDI in cell lysates or as purified protein. However, the RhoGDI-mediated inhibition of GAP activity for ABR is not shown in this study.

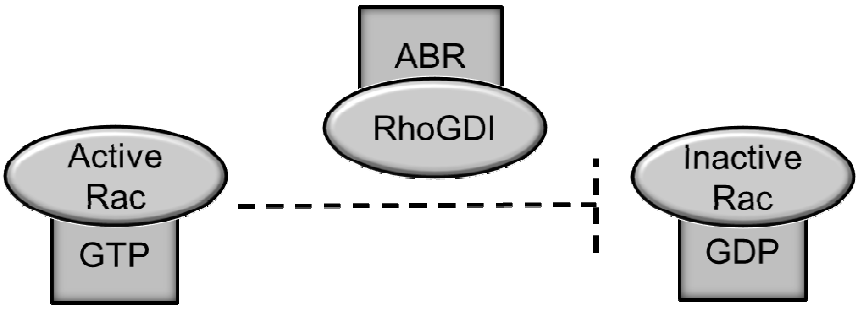

Figure 8. ABR binds to RhoGDI. Similarly to the regulation of BCR, the binding of ABR to RhoGDI is thought to prevent ABR-mediated conversion of Rac-GTP to Rac-GDP (128).

1.5.6 ABR and inflammation

Previous studies of ABR have mainly focused on ABR involvement in immune regulation. The main ABR function is described as an inactivation of the small GTPase Rac1, a master molecular switch that regulates several cellular processes, including maintenance and expansion of leukemic cells (129) and regulation of immune cell functions (102). ABR has been reported to play a critical role in downregulating hypoxia-induced pulmonary hypertension by deactivating Rac1 (130). Hypoxia-treated pulmonary arterial smooth muscle cells from ABR-deficient mice also proliferated faster than those of wild type mice (130).

Mice lacking ABR are phenotypically normal and fertile. However, when they are examined in more detail under conditions that generate pathology, significant differences with control wild type animals can be measured in specific functions. For instance, the ablation of ABR results in abnormal reactivity of the innate immune system (75,94,106,130). Further, loss of ABR and BCR function leads to increased levels of activated Rac in various mouse cell types, including macrophages, astroglia and neutrophils (75,94,106,131).

1.6 Ras-related C3 botulinum toxin substrate 1 (Rac1)

1.6.1 The Rac1 molecular switch

Rac proteins are Ras related low molecular weight (20- to 30- kDa) GTP binding proteins of the Rho family (103). Rac1 is a small GTPase protein that functions as a molecular switch. It is active when it is GTP-bound and inactive in the GDP-bound conformation. Under physiological conditions Rac proteins are never permanently stable into a GTP- or GDP-bound state, but instead participate in a cycle of activation and deactivation. Rac1 is activated by guanine nucleotide exchange factors (GEFs) and inhibited by GTPase activating proteins (GAPs) (132) (Figure 9). However, little is known about how GEFs and GAPs act in concert to precisely regulate Rho GTPase signaling.

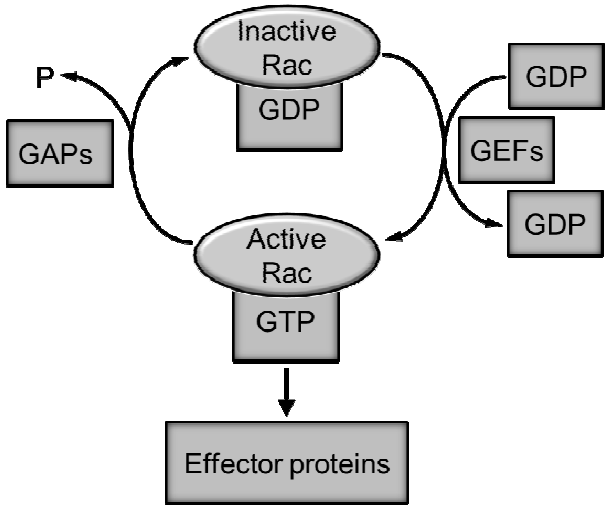

Figure 9. A biochemical model of Rac signaling. The cycle between the active, GTP-bound, and the inactive, GDP-bound, Rac GTPase is activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase-activating proteins (GAPs). The active Rho GTPases are capable of interacting with multiple effectors (133).

1.6.2 Rac and regulation of cytoskeletal organization

Rac proteins were believed to be involved primarily in the regulation of cytoskeletal organization in response to extracellular growth factors (134). As cell signal molecules, Rac proteins are involved in the cell-cell interactions by integrating extracellular signals and switching signal pathways, then further regulating actin cytoskeleton and cell migration (135). In addition to regulating actin organization, Rho GTPases coordinately activate several molecular processes which associated with cell polarity, survival, quiescence and gene transcription (136–139). In its active state, Rac1 interact with effectors and stimulate signaling pathways that control cytoskeletal dynamics, membrane trafficking and gene expression (135).

1.6.3 Rac in hematopoietic stem and progenitor cell regulation

Rac members of Rho GTPases family are important molecules regulating hematopoietic stem cells (HSCs) interactions with hematopoietic microenvironment (140). Some studies have strongly suggested that Rac1 is required for engraftment of hematopoietic progenitors into the bone marrow (BM). Rac1-deficient HSCs exhibit decreased homing in BM and impaired engraftment and reconstitution upon transplantation, which suggesting Rac1 is a key molecule regulating HSCs trafficking and residence in the BM niche (141–143). Previous studies reported that Rac proteins play crucial roles in hematopoietic cell migration and engraftment (141,143,144). In addition, the regulated Rac1 activation and deactivation cycle is implicated in anchorage-independent growth and invasiveness of the tumor cells (145). Somervaille et al. (146) reported that the progression of immortalized colony-forming cells, transformed by MLL-AF9 *in vitro* to leukemic stem cells is accompanied by enhanced engraftment potential, including elevated expression of active Rac.

1.6.4 Rac in hematologic abnormalities

The Rac signal transduction is required for the maintenance and expansion of leukemic cells by mediating their interaction with stromal cells (147). In according to those findings, a previous study reported overexpression of Rac1 in acute leukemia patients of various AML FAB subtypes, the most significant increases in Rac1 protein

expression were observed in M2 and M3 subtype (148). In this study, the authors could show that inactivation of the known ABR target Rac1 by treatment with the Rac1 inhibitor NSC23766 suppressed cell migration and growth. In addition, siRNA-mediated silencing of Rac1 in leukemia cell lines induced inhibition of cell migration, proliferation, and colony formation (148). Another study from the same group reported that deactivation of Rac1 with dominant negative-Rac1 lentivirus in primary AML samples resulted in decreased migration ability and higher levels of apoptosis induced by ectoposide (V-16). V16 is shown to be a chemotherapy drug active in AML (129,149). The etoposide forms a complex with DNA and the topoisomerase II enzyme, which aids in DNA unwinding, blocking religation of the DNA strands, and by doing so causes DNA strands to break. Therefore, this causes errors in DNA synthesis and promotes apoptosis of the cancer cell (150). A recent study from Chen et al. (2016) (151) revealed that active Rac1 can increase hematopoietic stem/progenitor cells interaction with their niche, leading to a better homing of leukemia cells. Rac1 activation further enhanced AML1-ETO9a leukemia stem cell features, such as colonial formation, quiescence and prevented leukemia cells from apoptosis. Thus, activation of Rac1 GTPase in leukemia cells is closely associated with the chemotherapy resistance, quiescence maintenance and the interaction with BM microenvironment (129,148). In fact, the activation of Rac1 GTPase has been found in myeloid-associated diseases, especially in BCR-ABL CML and MLL-AF9 AML (152–156). Interestingly, another study has reported increased levels of active Rac in AML patient samples (147).

1.6.5 Rac in myelopoiesis

The involvement of Rac in myelopoiesis has been examined *in vitro* with the use of isolated HSC/Ps from conditional knockout mice, and the data suggest that Rac1 and Rac2 regulate unique aspects of hematopoietic development. $Rac1^{-/-}$;$Rac2^{-/-}$ as well as $Rac1^{-/-}$ HSC/Ps displayed reduced proliferation in response to stem cell factor, and this is associated with reduced cyclin D1 and decreased p42/p44 extracellular signal-regulated kinase (ERK) phosphorylation (143). Increased apoptosis was noted in $Rac1^{-/-}$; $Rac2^{-/-}$ and $Rac2^{-/-}$ progenitors, which was associated with reduced serine/threonine kinase (AKT) activation after stem cell factor stimulation (143). $Rac2^{-/-}$ as well as $Rac1^{-/-}$ HSC/Ps displayed reduced proliferation in response to

stem cell factor, and this is associated with reduced cyclin D1 and decreased p42/p44 extracellular signal-regulated kinase phosphorylation (143). Increased apoptosis was noted in $Rac1^{-/-}$; $Rac2^{-/-}$ and $Rac2^{-/-}$ progenitors, which was associated with reduced AKT activation after stem cell factor stimulation.

1.7Aims of the study

AML is a heterogeneous disease characterized by a large number of genetic defects, which play an important role in the therapeutic approach and prognosis. Overexpression of oncogenes and deficiency of tumor suppressors are critical driving forces for tumorigenesis. Currently, most molecular targeted therapies have relied on the approach of designing inhibitors for selected oncogenic targets, because inactivated tumor suppressor genes have proven harder to be drug-treated. However, in recent years, reactivation of tumor suppressors by DNA methyltransferase inhibitors has been quite effective as a treatment strategy for cancer.

In the present study, we identified a novel promising gene candidate, active BCR related (ABR) gene, which we found to be downregulated in several AML subtypes. Evidences for a putative tumor suppressor gene of ABR are the allelic deletions of DNA sequence on chromosome 17p including ABR locus in several tumors, such as medulloblastoma, astrocytomas and breast cancer. In addition, a significant reduced expression of ABR was strongly correlated with the t(8;21) abnormality, found in approximately 5%–10% of all AML cases. The inactivation of Rac1 suppressed leukemic cell proliferation. Despite the known role of BCR gene, closely related to ABR in leukemia, there have been no reports showing any specific function of ABR in myeloid differentiation and leukemogenesis. The aim of this work was to gain a deeper insight into the pathogenesis of the AML and to contribute to the elucidation of disease development. Therefore in the present study, we examined the ABR expression pattern in myelopoiesis and in AML. We aimed to examine whether ABR has an influence on myeloid differentiation. Furthermore, we investigated a putative connection of ABR and the myeloid transcription factor C/EBPα in myelopoiesis. We also sought to demonstrate the influence of ABR expression on outcome of AML patients and on treatment of AML patients with azacitidine. The effect of ABR overexpression on azacitidine-induced apoptosis was analysed. We investigated whether the downregulation of ABR in AML could be caused by DNA methylation of

the ABR promoter. The findings gained in this thesis indicate the tumor suppressor potential of ABR and underline its potential role in leukemia therapeutic strategies.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment and Software

Equipment	Manufacturer / Distribution
Autoklav Model 5050 EVL	Tuttnauer
Bakterieninkubator	Memmert GmbH
Brutschrank, Heracell 150i	Thermo Scientific
CCD-Kamera (G-Box)	Syngene Bioimaging
Durchflusszytometer BD FACSCalibur	BD Pharmingen™
Elektrophoresesysteme Horizontal: Sub-Cell Model 192 Cell Vertikal: Mini Transblot®Cell	Bio-Rad Laboratories Inc.
Elektroporationsgerät Nucleofector® 2b Device	LONZA
Feinwaage AX4202	Sartorius
Geldokumentation Gene Flash	Syngene Bioimaging
Kühlzentrifuge Heraeus Fresco 17	Thermo Scientific
Lichtmikroskop Axio Vert.A1	Zeiss Deutschland
Luminometer Lumat LB9705	Berthold Technologies
MACS® cell separation columns, MACS® separator Biotec	MACS® reagents, Milteny
Mikrowelle	Severin
Milli-Q-Anlage	Millipore
Nano-Photometer	Implen
pH-Meter	Hanna Instruments
Pipetten 2µl, 10µl, 20µl, 100µl, 200µl, 1000µl	Rainin; Eppendorf
Pipettierhilfe	Integra Bioscience
Power Pac 1000	Bio-Rad Laboratories Inc.
Real Time PCR System 7500	Applied Biosystems
Real Time Thermal Cycler (Rotor-Gene™ 3000)	Corbett Research Australia
Schüttelinkubator Model 3031	GFL

Schüttler Rotamax 120	Heidolph
Sterilbank Modell HeraSafe Heraeus	Thermo Scientific
Stromversorgung Power Pac 1000	Bio-Rad Laboratories Inc.
Thermal Cycler T100™	Bio-Rad Laboratories Inc.
ThermoCycler (T Gradient Thermoblock)	Biometra®
Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell	Bio-Rad Laboratories Inc.
Ultrazentrifuge Sorvall WX (TH-641 Rotor)	Beckman
Vortexer	Fisher Scientific
Wasserbad	GFL

Software	**Manufacturer/ Distribution**
CellQuest™	BD Pharmingen™
Cyflogic 1.2.1	CyFlo Ltd
GenePix® Pro 6.0	Molecular Devices
Image J	National Institutes of Health

2.1.2 Reagents

Chemicals	**Manufacturer/ Distribution**
Acrylamid (Rotiphorese® Gel 30)	Carl Roth GmbH
Agar	Carl Roth GmbH
Ampicillin	Sigma-Aldrich
APS (Ammoniumperoxodisulfat)	Carl Roth GmbH
β-ME (β-Mercaptoethanol)	Sigma-Aldrich
Protein Assay Dye Reagent	Bio-Rad Laboratories Inc.
BSA (Bovine serum albumin) purified	SERVA Electrophoresis GmbH
CaCl2 (Calciumchlorid)	Carl Roth GmbH
DEPC (Diethylpyrocarbonat)	Carl Roth GmbH
dNTPs	QIAGEN
DTT (Dithiothreitol)	Sigma Aldrich
ECL Westernblot Detektionsreagenzien	GE Healthcare Life science, Amersham Bioscience

EDTA (Ethyldiamintetraessigsäure)	Carl Roth GmbH
Ethanol	Carl Roth GmbH
Ethidiumbromid	Sigma-Aldrich
Formaldehyd	Carl Roth GmbH
GelRed™ *Nucleic Acid Gel Stain*	Biotium Inc.
Giemsa	Carl Roth GmbH
Glycerin	Sigma-Aldrich
Glycerol	Sigma-Aldrich
HCl (Salzsäure)	Carl Roth GmbH
HEPES (Hydroxyethylpiperazinethansulfonsäure)	Carl Roth GmbH
Isopropanol	Carl Roth GmbH
May-Grünewald-Lösung	Sigma-Aldrich
Milkpowder	Carl Roth GmbH
Methanol	Carl Roth GmbH
MgCl2 (Magnesiumchlorid)	Carl Roth GmbH
NaCl (Natriumchlorid)	Carl Roth GmbH
Na2HPO4 (Natriumhydrogenphosphat)	Carl Roth GmbH
Phosphatase-, Proteinase Inhibitor Cocktails	Sigma-Aldrich
PMSF (Phenylmethansulfonylfluorid)	Fluka
Ponceau S	Sigma-Aldrich
SDS (Natriumdodecylsulfat)	Carl Roth GmbH
Temed (Tetramethylethylendiamin)	Bio-Rad Laboratories GmbH
Tris-Base (Trisaminomethan)	Carl Roth GmbH
Tris-HCl	Carl Roth GmbH
Triton-X-100	Carl Roth GmbH
TRIzol ®	Invitrogen
Tween®20	Carl Roth GmbH
Yeast	Carl Roth GmbH

Cytokines	**Manufacturer/ Distribution**
huG-CSF (*Granulocyte Colony Stimulating Factor)*	ImmunoTools GmbH
huGM-CSF (*Granulocyte-Macrophage Colony Stimulating Factor*)	ImmunoTools GmbH

IL-3 (Interleukin-3)	ImmunoTools GmbH
IL-6 (Interleukin-6)	ImmunoTools GmbH
rHuSCF (*Recombinant Human Stem Cell Factor*)	PAN™ Biotech GmbH
rHuFlt3 (*Recombinant Human Flt3-Ligand*)	PAN™ Biotech GmbH

Media and other consumables	**Manufacturer/ Distribution**
β-Estradiol	Sigma-Aldrich
CT-FBS (*Charcoal-treated fetal calf serum*)	PAA Laboratories GmbH
Chloroquin	Fluka
DMSO (Dimethylsulfioxid)	Sigma-Aldrich
DMEM (*Dulbecco's Modified Eagle Medium*)	PAA Laboratories GmbH
FBS (Fötales Kälberserum)	PAA Laboratories GmbH
IMDM (*Iscove´s Modified Dulbecco´s Medium*)	Gibco®
Penicillin/Streptomycin (P/S)	Gibco®
PBS (*Phosphate buffered saline*)	PAA Laboratories GmbH
Polybren	Takara
Puromycin	MP Biomedicals
RA (Retinoic Acid)	Sigma-Aldrich
RPMI 1640 (± Phenolrot)	PAA Laboratories GmbH

Commercial Kits	**Manufacturer/ Distribution**
AMAXA™ Cell line	Nucleofector® Kit V Lonza
Bio-Rad Protein Assay	Bio-Rad Laboratories GmbH
BLOCK-iTTM Pol II miR RNAi Expression Vector Kit	Invitrogen™
EndoFree® Plasmid Maxi Kit	QIAGEN
HiSpeed® Plasmid Maxi Kit	QIAGEN
Revert Aid™ H Minus First Strand cDNA Synthesis Kit	Thermo Scientific
LipofectaminTM LTX Reagent	InvitrogenTM
QuantiTect® SYBR Green PCR Kit	QIAGEN
QIAEX® II Gel Extraction Kit	QIAGEN
QIAprep® Spin Miniprep Kit	QIAGEN
TaqMan® MicroRNA Reverse Transcription Kit	Applied Biosystems

Enzymes	**Manufacturer/ Distribution**
DNA Polymerase I	New England Bio Labs Inc.
RNAse A	Sigma Aldrich
T4 DNA Ligase, 10X T4 DNA Ligase Buffer	Thermo Scientific
Taq-Poymerase	Promega GmbH

Markers und loading buffer	**Manufacturer/ Distribution**
6x Loading Dye Solution	Thermo Scientific
GeneRuler™ DNA Ladder Mix	Thermo Scientific
Page Ruler™ Plus Prestained Protein Ladder	Thermo Scientific

Vectors	**Source**
pEYFP-ABR	Addgene
EYFP-ABR in pCCL-cppt178-MNDU3	
VSVG	Addgene

Oligonucleotides

For detection of the expression of miRNA miR-223, and RNUB6 the specific TaqMan® MicroRNA Assays were used. All other listed primers were manually designed manually designed and purchased from Biomers.net GmbH.

Table 3. Primer sequences used for quantitative Real-Time PCR.

	Primer sequence
hu ABR forward	5´-agc cga gat atg agc ctg aa-3´
hu ABR reverse	5´-cct cga tac ccc tct tct cc-3´
mu ABR forward	5´-tga agg acg gct tcc tgg tgg a-3´
mu ABR reverse	5´-tcg gct ctg tcc ttt gtt ggc t-3´
hu CEBPA forward	5´-tgg aca aga aca gca acg ag-3´
hu CEBPA reverse	5´-ttg tca ctg gtc agc tcc ag-3´
hu GAPDH forward	5´-acc aca gtc cat gcc atc ac-3´
hu GAPDH reverse	5´-tcc acc acc ctg ttg ctg ta-3´
hu G-CSF-R forward	5´-acc tgg gca cag ctg gag tgg-3´
hu G-CSF-R reverse	5´-cag gtc gct gtg agc tgg gtc tgg-3´
hu M-CSF-R forward	5´-gtg gct gtg aag atg ctg aa-3´
hu M-CSF-R reverse	5´-cct tcc ttc gca gaa agt tg-3´
mu M-CSF-R forward	5´-gct cgg cca cta acg ccg aa-3´
mu M-CSF-R reverse	5´-ttc atg gtg gcc gtg cgt gt-3´
18s forward	5´-aaa cgg cta cca cat cca ag-3´
18s reverse	5´-cct cca atg gat cct cgt ta-3´

Table 4. siRNA duplex sequences.

	siRNA sequence
hu ABR siRNA	uac aaa gcg uuu guc gau aac uau a uau agu uau cga caa acg cuu ugu a
hu CEBPA siRNA	agc gca aca aca ucg cgg ugc gca a uug cgc acc gcg aug uug uug cgc u

Table 5. Primer sequences used for sequencing.

	Primer sequence
EGFP-C forward	5´-cat ggt cct gct gga gtt cgt g-3´
SV40pA reverse	5´-gaa att tgt gat gct att gc-3´

Antibodies

Western Blot primary antibody	**Manufacturer/ Distribution**
Anti-ABR (ab188071) rabbit, polyclonal	Abcam
Anti-C/EBPα (EP709Y) rabbit, monoclonal	Abcam
Anti-GAPDH (SC-25778) rabbit, polyclonal	Santa Cruz Biotechnology

Western Blot Secundary antibody	**Manufacturer/ Distribution**
ECL™ HRP-conjugated, monkey, polyclonal Anti-rabbit IgG	GE Healthcare Life Science
HRP- conjugated, goose, monoclonal Anti-mouse IgG (SC-2005)	Santa Cruz Biotechnology

Flow cytometry Antibodies	**Manufacturer/Distribution**
PE- conjugated anti-CD11b mouse IgG1,κ	BD Pharmingen™
PE- conjugated anti-CD34 mouse IgG1,κ	BD Pharmingen™
PE- conjugated IgG-Isotyp mouse IgG1,κ	BD Pharmingen™

2.1.3 Biological Materials

Bacteria	**Manufacturer/Distribution**
One Shot® TOP 10	Invitrogen™
Subcloning Efficiency™ *DH5α*™	Invitrogen™

C57BL/6 Mouse

The expression levels of ABR, M-CSF-R and G-CSF-R were analysed in bone marrow cells obtained from tibiae and femora from C57BL/6 mice. Mice were bred at the Animal Facility at the University of Leipzig, housed, treated and handled in accordance with the guidelines of the University Leipzig Animal Care Committee and the Regional Board of Animal Care for Leipzig. The RNA preparation as well as the

analyses of the ABR, M-CSF-R, G-CSF-R and GAPDH expression levels by means of quantitative real-time PCR were performed independently at the University Hospital Leipzig.

Human cell samples from AML patients and healthy donors

AML patient samples belonging to the cohort that received non-myeloablative hematopoietic stem cell (NMA-HSC) transplantation (157) and bone marrow samples from patients without any hematopoietic disease were obtained from University Hospital of Leipzig. Complete remission (CR) was defined according to standard morphologic criteria (158). Samples from azacitidine-treated AML patients were obtained from University Hospital of Halle (159). Azacitidine was administered as previously described (159). The study protocols used for AML patient sample collection were approved by the ethics committees of the participating centers. All patients provided written informed consent in accordance with the Declaration of Helsinki. All samples were analysed by cytogenetic and molecular genetic analysis.

Table 6. Characteristics of healthy donors and AML patients who received NMA-HSC transplantation used to analyze ABR expression.

Patient	Karyotype	Age	Gender	% PB blasts	% BM blasts	FAB subtype	FLT3-ITD
	NK	36	f	-	-	healthy	-
	NK	41	m	-	-	healthy	-
	NK	41	m	-	-	healthy	-
1	+mar,+8,-7	51	f	63	80	M1	negative
2	NK	52	f	68	95	M1	negative
3	del(5)	62	f	unknown	unknown	M2	negative
4	NK	66	m	unknown	31	M5	positive
5	8	27	m	60	64	M4	positive
6	CK	61	f	8	75	M4	negative
7	-7	73	f	82	86	M2	negative
8	NK	52	m	32	61	M2	negative
9	NK	61	f	15	61	M4	negative
10	5q-	48	f	48	94	M1	negative
11	NK	58	f	33	unknown	not classified	negative
12	NK	63	f	92	85	M2	positive
13	NK	70	m	50	84	M2	positive
14	NK	67	m	2	27	M2	negative
15	CK	72	m	86	75	M2	negative
16	t(1;7),-7	60	m	18	40	M4	unknown
17	NK	66	m	66	66	M2	negative
18	inv(16)	19	m	61	84	M4	positive
19	CK	30	f	85	95	M5	positive
20	CK	61	f	93	80	M2	positive
21	NK	57	f	28	42	M2	negative
22	NK	59	m	12	87	M5	negative
23	NK	71	m	78	88	M2	negative
24	CK	67	m	31	unknown	not classified	negative
25	inv(16)	46	f	46	40	M4	negative
26	CK	67	f	92	95	M2	negative
27	NK	62	m	77	80	M1	negative
28	inv(16)	20	f	33	45	M4	negative
29	inv(16)	63	m	97	90	M2	negative
30	-7	63	m	36	90	M2	negative
31	CK	53	f	6	50	M5	positive
32	t(11;20)	50	f	2	63	M2	negative
33	del(7),+8	62	f	26	61	M2	unknown
34	NK	65	f	44	60	M2	negative
35	NK	58	f	41	85	M4	negative
36	CK	64	f	11	45	M6	negative
37	t(X;19)	66	f	8	25	M7	negative
38	NK	68	m	unknown	unknown	M2	negative
39	NK	72	m	0	51	M2	negative
40	CK	66	m	62	87	M1	negative
41	unknown	60	f	6	57	M1	positive
42	NK	59	f	79	82	M2	positive
43	NK	51	f	12	83	M1	negative
44	NK	60	m	38	75	not classified	negative
45	NK	63	m	0	50	M1	unknown
46	CK	60	f	2	60	M2	negative
47	NK	66	f	7	34	M4	positive
48	-7	67	m	39	92	not classified	negative
49	CK	52	m	37	10	M1	negative
50	der(1;7)	59	m	20	unknown	not classified	negative
51	NK	59	f	3	26	M5	positive
52	NK	63	m	unknown	unknown	not classified	negative
53	CK	48	f	21	50	M7	negative
54	CK	62	m	12	30	M2	positive
55	t(11;19)	64	m	4	44	M4	positive
56	NK	50	m	86	85	M0	unknown
57	NK	58	m	97	94	M4	negative
58	unknown	37	f	90	90	M1	negative
59	NK	61	f	34	30	M1	negative
60	unknown	59	m	unknown	90	M2	positive
61	NK	63	m	38	50	M2	negative
62	NK	62	m	20	75	M0	negative
63	t(8;21)	53	f	22	40	M2	negative

Table 7. Genetic and morphologic features of AML patients used to analyze ABR expression according to treatment response to azacitidine.

Patient	Patient type	FAB	Gender	Age (years)	Blast count (t0), %	Blast count (t15), %
1	non-responder	M2	m	79	34	10
2	non-responder	unknown	m	62	75	75
3	non-responder	M2	m	63	24	90
4	non-responder	M4	f	68	69	21
5	non-responder	M7	f	70	70	67
6	non-responder	M1	f	70	70	80
7	non-responder	M2	m	66	80	60
8	non-responder	M1	m	73	75	90
9	non-responder	M4	f	62	90	35
10	non-responder	M4	m	68	60	70
11	non-responder	M5	f	66	83	unknown
12	non-responder	M4	m	72	57	70
13	non-responder	M1	f	72	80	50
14	non-responder	M4	m	73	81	12
15	non-responder	M2	m	65	64	11
16	responder	M2	m	77	20	≤5
17	responder	M4	f	76	30	≤5
18	responder	M2	m	74	50	≤5
19	responder	M1	m	78	25	≤5
20	responder	M4	m	70	80	≤5
21	responder	M5	f	75	70	≤5
22	responder	M5	m	61	50	≤5
23	responder	M0+M2	m	68	52	≤5
24	responder	M5	m	70	50	≤5
25	responder	M1	f	64	95	≤5
26	responder	unknown	m	76	10	≤5

FAB, French-American-British; f, female; m, male; t, time in days

2.2 Methods

2.2.1 Cell culture

The cultivation of the eukaryotic cell lines and of the mouse bone marrow cells was carried out according to standard methods in the corresponding culture media. The cells were incubated in gassing incubators at 37°C, 96% relative humidity and 5% CO_2. To determine the cell count, a dilution of the cell suspension was applied to a Neubauer counting chamber, the cells were counted in all four quadrants (each containing 16 individual squares) and then converted to the existing volume of the cell suspension.

Composition of the culture media

U937	RPMI 1640 + 10 % FBS + 1 % P/S
Mouse bone marrow cells	DMEM + 10 % FBS + 1 % P/S + dexamethasone (10^{-8} M)

2.2.2 Differentiation of the cells

The leukemic cell line U937 can differentiate to monocytic lineage upon PMA (phorbol 12-myristate 13-acetate) treatment (37). For myeloid differentiation of U937 cells, $1x10^6$ cells were induced with 10nM PMA were plated in 2 ml medium in 6-well plates and treated once with PMA. For differentiation of ABR siRNA transfected U937 cells, 1nM PMA was used. Mouse bone marrow cells from C57Bl/6 wild-type mice were seeded at $1x10^6$/mL. Differentiation of granulocytic and monocytic lineages were induced with G-CSF (1 ng/ml) or M-CSF (20 ng/ml), respectively. Treatment of the cells was done daily during the experimental process.

2.2.3 Total RNA extraction

Isolation of the whole cell RNA from mammalian cells was carried out by adding 1 ml of TRIzol®. This solution contains phenol and guanidinium thiocyanate in a single phase solution. 200 µl of chloroform were then added, the samples were carefully inverted several times and incubated for 5 min at room temperature. Phase separation into a lower red phenol-chloroform phase, a milky interphase and an

upper colorless aqueous phase took place in a cooling centrifuge at 4°C and a rate of 13,000 rpm for 15 min. The RNA dissolved in the upper phase was transferred into a new reaction vessel and thus separated from DNA and proteins, which are located in the other phases. Precipitation of the RNA was carried out by the addition of 500 µl of isopropanol. After mixing and incubating the reaction at room temperature for 10 minutes, the RNA was pelletized by centrifugation at 13,000 rpm for 10 minutes in a cooling centrifuge at 4°C. The protrusion was rejected. For washing the RNA, 1 ml of 70% ethanol was added and then centrifuged at 13,000 rpm and 4°C for 10 min. The RNA pellet was then air-dried and dissolved in RNase / DNase-free water. Storage of the extracted RNA took place until further processing at -80°C.

2.2.4 Measurement of RNA concentration

The quality and quantity determination of nucleic acids was carried out using a spectrophotometer (Nano-Photometer™). The concentration was determined by means of photometric absorption measurements at 260 nm for nucleic acids and 280 nm for proteins and calculation of the optical density (OD). An A260/A280 quotient of 1.8 (RNA) was used as a guideline for the purity of the samples.

2.2.5 Polymerase chain reaction (PCR)

The specific amplification of nucleic acids *in vitro* was achieved by the polymerase chain reaction (PCR) method. The PCR allows amplification of a DNA fragment (template) over a region flanked by two synthetic DNA oligonucleotides (primers) complementary to the template. PCR is a multi-step process. In the initial denaturation phase (5 min at 95°C, hot start), the double-stranded DNA is melted. This involves a series of 30-40 cycles with the reaction steps: denaturation (thermal melting of the template to single strands, 30-60 s at 95°C), annealing (primer hybridization to single-stranded template DNA, 10-60 s at 50-66°C) and primary extender (30-60 s at 72°C). The cyclical repetition of these steps leads to an exponential amplification of the target sequence. A final extension step (5 min at 72°C) completes unfinished extension steps. The reaction times and temperatures used are dependent on the length of the amplicons and the specific sequences. The

PCR was used for qualitative and quantitative purposes, such as RNA analysis, cloning and mutagenesis. For standard applications as well as for cloning, the heat-stable Taq DNA polymerase was used.

2.2.5.1 Reverse transcriptase

The generation of complementary DNA (cDNA) from an mRNA (messenger RNA) was performed using the retroviral reverse transcriptase. This reaction allows the use of the PCR technique for RNA analysis. For the reverse transcriptase (RT) reaction, the Revert Aid™ H Minus first Strand cDNA Synthesis kit was used according to the manufacturer's instructions. In the RT reaction, 100 ng of total RNA was used per sample. 1 µl of oligo-(dT) primers, 4 µl of 5-fold reaction buffer, 2 µl of dNTP mix (10 mM), 0.5 µl of RiboLock-RNase inhibitor and 1 µl of reverse transcriptase (200 U, Revert Aid ™ H Minus M-Mul V reverse transcriptase) and RNase / DNase-free water to a total volume of 20 µl. The mRNA was transcribed in cDNA at 42°C for 60 min. The reaction was stopped by heating at 70°C for 15 min. The resulting cDNA mixture was used directly for the subsequent PCR or stored at -20°C.

2.2.5.2 Quantitative real-time PCR (qPCR)

The quantitative real-time PCR is based on the principle of conventional PCR and enables the quantitative analysis of the DNA by fluorescence measurement in real-time. The fluorescence intensity increases proportionally to the amount of DNA formed. The QuantiTect® SYBR Green PCR kit was used for the reactions. SYBR Green I is an asymmetric cyanine dye that is inserted into the small groove of double-stranded DNA. The resulting DNA fluorescence dye complex absorbs blue light at a wavelength λ_{max} = 494 nm and emits green light at λ_{max} = 521 nm. At the end of the PCR, the exponential phase can be determined from the data obtained and a threshold is set at the beginning in order to be able to unambiguously assign each PCR measurement curve to a PCR cycle value in which the fluorescence signal exceeds this threshold value (C_T value, threshold cycle). To check the specificity of the reaction, a melting curve follows the last cycle. The PCR product is melted at a specific temperature depending on base composition and length. The reactions were

carried out in a Rotor-Gene™ 300 Real Time Thermal Cycler and Real Time PCR System 7500. The primers used are listed in section 2.1.2 (Table 3). The mRNA of the housekeeping gene GAPDH or 18S ribosomal RNA was measured to normalize the values. The calculation was carried out by means of relative quantification using the ΔΔCT method. The fold change of the expressed genes was calculated relative to the control gene according to the following formula:

$2^{-\Delta(\Delta C_T)}$ ($\Delta CT = C_{T,\ target\ gene}$, - $C_{T,\ control\ gene}$, $\Delta\ (\Delta C_T) = \Delta C_{T,\ stimulated}$ - $\Delta C_{T,\ control}$)

2.2.5.3 MicroRNA quantification through qPCR

The microRNA expression was analysed using the corresponding TaqMan® microRNA assays according to the manufacturer's data based on the method of quantitative real-time PCR. TaqMan probes consist of a fluorophore covalently attached to the 5’-end of the oligonucleotide probe and a nonfluorescent quencher (NFQ) at the 3'-end in addition to the flanking primers. The 5'-3'-exonuclease activity of the polymerase leads to the degradation of the probe at the 5 'end and to the removal of the quencher from the fluorophore. As a result, an increase in the reporter fluorescence is measured. The primers used are listed in section 2.1.2 (Table 3). For normalization in human cells, the expression of the RNUB6 was measured.

2.2.6 Transient transfection

The introduction of DNA into a eukaryotic cell with the aim of the temporary expression of a gene is known as transfection. In the transfection using electroporation, a briefly increased permeability of the cell membrane is generated through short electrical pulses, which allow a diffusion of the DNA into the cell interior. The transfections of ABR expression construct (pEYFP-ABR) as well as the siRNA oligonucleotides were carried out using an electroporation device (Nucleofector® 2b Device). $1*10^6$ U937 cells were analysed in the AMAXA™ electroporation unit using and the AMAXA™ Nucleofector Cell line Kit C according to the manufacturer's instructions.

2.2.7 Production of lentivirus

Lentiviruses are a genus within the family of retroviruses (reverse transcriptase oncoviruses). They are encapsulated positive-sense single strand RNA viruses and, in contrast to the gammaretrovirus, can also infect non-dividing, eukaryotic cells. The simple proviral genome contains three genes and two long terminal repeats. The information to control viral gene expression included three major distinct genes: gag, pol and env. Gag (group-specific antigen) encodes the matrix, capsid, and nucleocapsid proteins. Pol encodes the viral enzymes protease, reverse transcriptase (with RNase H) and integrase. Env encodes viral coat proteins. In genetic engineering for production of artificial viral vectors, coat proteins are exchanged for the improvement of stability and infection.
In transduction (also infection) by means of lentiviruses, a foreign DNA is introduced into target cells. The resulting integration of the foreign DNA into the host gene leads to the stable expression of the inserted DNA. The production of the lentiviral particles for the stable expression of the lentiviral constructs was performed in 293TN cells. Preliminarily, 50000 cells per cm^2 were applied in a 175 cm^2 cell culture flask. In the next day, the target DNA was transfected as well as the packaging plasmids psPAX2 (gag/pol) and pMD2.G (vsv-g).

Transfection batch: 20 µg target DNA
15 µg psPAX2
10 µg pMD2.G
Dissolved in 1 ml of Opti-MEM + 135 µl of PEI (1: 1000 dilution).

The mixture was stirred for 20 min. Incubated at room temperature and subsequently diluted in 11 ml of DMEM (high glucose), admixed with 2% FBS. The medium-DNA mixture is added to the medium-free cells and incubated for 3 h in the gassing incubator. Subsequently, the medium was replaced with 12 ml of fresh DMEM (high-glucose) cultivation medium. The cells were then incubated for 3 days in the gassing incubator, and a daily medium change was carried out. The resulting supernatant containing the virus particles was centrifuged at 1500 rpm for 7 min. $1*10^5$ U937 cells were infected in 24-well plates with different amounts of virus particles (0.5, 2, 8, 16, 64 and 128 µl). The amount of YFP positive cells was measured by FACS analysis.

The lentiviral titer was calculated by determination of the amount of transducing units (TU) per ml according to the following formula:

(number of YFP^+ cells) / virus amount *100

The number of transducing lentiviral particles per cell (MOI, multiplicity of infection) was calculated according to the following formula: TU / number of infected cells.
$1*10^5$ U937 cells were then infected with at least 11 MOIs and incubated in the gassing incubator. The insertion of the target DNA was examined microscopically and by FACS analysis of YFP positive cells after 3 days.

2.2.8 Flow cytometry analysis (FACS)

The flow cytometry method, also known as FACS (fluorescence-activated cell sorting), is used for the quantitative determination of surface molecules and cellular proteins, peptides and DNA. To determine the amount of specific surface proteins (antigens) such as CD11b, the cells were incubated with the corresponding dye-labelled antibodies according to the manufacturer's instructions. Basically, the principle of the antigen-antibody reaction is used. The cells thus marked were measured at the flow cytometer at the corresponding wavelengths as a function of the coupled fluorescent dye.

The determination of the transfection efficiency or transduction efficiency after lentiviral infection of cells was carried out by means of the FACS determination of the percentage amount of EYFP- (pEYFP-ABR, EYFP-ABR in pCCL-cppt178-MNDU3) cells. In addition, cell isolation by means of FACS was used to select EYFP-expressing cells. EYFP-expressing cells were isolated from the whole cell population.

The detection of apoptotic cells was performed using the phycoerythrin (PE) Annexin V Apoptosis Detection Kit I according to the manufacturer's instructions. During apoptosis the membrane phospholipids (phosphatidylserine, PS) are translocated from the inner to the outer cell membrane, thereby exposing PS to the external cellular environment. The fluorochrome-labelled $Ca2^+$-dependent phospholipid-binding protein Annexin V binds to cells with exposed PS. Since externalization of PS occurs in the earlier stages of apoptosis, Annexin V staining can identify apoptosis at

an earlier stage than assays based on nuclear changes such as DNA fragmentation. Additional late apoptotic cells were separated by the use of the dye 7-Amino-Actinomycin (7-AAD). Viable cells with intact membranes exclude 7-AAD, whereas the destroyed membranes of dead or necrotic cells are permeable to this dye. The measurement was carried out by FACS analysis. Cell differentiation was assessed by flow cytometry analysis using PE-conjugated mouse anti-human CD11b antibody (BD Biosciences), FITC-conjugated rat anti-mouse F4/80 and FITC-conjugated rat anti-mouse Gr-1 (eBioscience).

All measurements were carried out on the flow cytometer FACS Calibur or LSR II and a minimum number of 10000 events were documented. The data collected were analyzed using Cyflogic software.

2.2.9 Immunoblot analyses

Immunoblot analyses were performed as previously described(125). For ABR protein detection, a mouse monoclonal antibody anti-ABR (Abcam) and for C/EBPα protein detection, a rabbit monoclonal antibody anti-C/EBPα (Abcam) were used. Polyclonal rabbit anti-GAPDH (sc-25778; Santa Cruz Biotechnology) and b-tubulin (sc-9104) antibodies were used for normalization. The immunoreactivity was determined using an enhanced chemiluminescence method (Amersham Biosciences) according to the manufacturer's instructions. The band intensities were quantified using ImageJ software (National Institute of Health, Bethesda, MD).

2.2.10 Protein isolation and determination of concentration according to Bradford method

The production of proteins from mammalian cells was carried out by RIPA lysis. For this purpose, 1 million cells were dissolved in 100 µl of RIPA buffer, to which PMSF, DTT and protease inhibitor cocktail were each added freshly. After incubation for one hour on ice, insoluble cell components were separated by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was transferred to a new reaction vessel. Quantitative determination of the protein concentration was carried out according to the Bradford method using the Bio-Rad protein assay. The absorption at 595 nm was

measured by photometric quantification and the protein quantity was determined using a previously applied BSA calibration curve.

RIPA buffer: 150 mM NaCl
1% IGEPAL® CA-630
0.5% sodium deoxycholate
50 mM Tris; PH 8.0

+ 1/10 volume PMSF (100 mM)
+ 1/10 volume DTT (10 nM)
+ 1/10 volume protease inhibitor cocktail

2.2.11 Electrophoresis

As amphoteric macromolecules, proteins possess a different amount of positive (lysine, arginine) and negatively charged (aspartic acid, glutamic acid) as well as ionizable (e.g. histidine, cysteine) amino acid residues. The electrophoretic mobility in a porous gel matrix is therefore dependent not only on the size and shape but also on the net charge under the respective conditions. The separation of proteins by size is possible after discontinuous SDS gel electrophoresis after treatment with the anionic detergent sodium dodecyl sulfate (SDS) together with sulfur bridge-cleaving thiol reagents, such as, for example, β-mercaptoethanol. In a vertical gel electrophoresis system, the samples first pass through a collecting gel and then a separation gel, in which the proteins are separated due to their molecular size. 30-60 µg of dissolved protein, was mixed with protein loading buffer and then heat-denatured for 10 min at 95°C. The proteins and a dye-labelled standard protein mixture (PageRuler™ Plus Prestained Protein Ladder) for molecular weight estimation were applied to the gel and separated by applying a voltage of 150 V for 1 h.

Stacking gel: distilled water
1.5 M Tris-HCl (pH 8.8)
30% acrylamide
10% SDS
10% APS
TEMED

Separating gel: distilled water
0.5 M Tris-HCl (pH 6.8)
30% acrylamide
10% SDS
10% APS
TEMED

Protein loading buffer: distilled water
0.5 M Tris-HCl (pH 6.8)
10% glycerol
20% 10% SDS (w/v)
5% β-mercaptoethanol
0.05% (w/v) bromophenol blue

2.2.12 Western blot

The visualization of specific proteins in a membrane-fixed protein mixture by means of antibody binding is known as a Western blot. The separated proteins were electrophoresed by applying a voltage of 150 mA for 1 h from the SDS polyacrylamide gel to a nitrocellulose membrane. The efficiency of the protein transfer to the membrane was checked by Ponceau S staining of the membrane. Prior to the detection reaction using specific antibodies, the membrane was incubated in a 5% dry milk solution for 1 h in order to saturate excess protein binding sites and to prevent nonspecific binding of the antibodies. For the detection of the proteins, the membrane was first incubated with a specific primary antibody directed against the protein to be detected at 4°C for at least 16 h, followed by incubation with a secondary antibody directed against constant regions of the first antibody at room temperature for one hour. The secondary antibody is conjugated with the enzyme horseradish peroxidase (HRP), which is oxidized by hydrogen peroxide and subsequently reacts with luminol in this form. This in turn causes chemiluminescence, which can be detected as black color on an X-ray film or in the LI-COR C-DiGit® blot scanner. The substrate for the HRP was the WesternSure ™

Chemiluminescent substrate from LI-COR. The quantification of the band intensity was carried out using the ImageJ software.

2.2.13 Statistical analysis

We used *Student t test* (Excel, Microsoft, USA) to determine the statistical significance of experimental results. Differences of $p \leq 0.05$ (*), $p \leq 0.01$ (**) were considered statistically significant. The results were represented as the mean ± standard deviation (SD) from of three independent experiments. The AML patients that received NMA-HSC transplantation were dichotomized into ABR high and *ABR* low expressers using a median cut. For time-to-event analyses, we calculated survival estimates using the Kaplan–Meier method, and compared groups by the log-rank test.(126) Azacitidine-treated AML patients were divided into responders and non-responders, and Kruskal test was used for statistical evaluation of *ABR* expression. The bar graphs were created with Excel and the box whisker plots with SPSS 17.

3 Results

3.1 ABR mRNA expression is repressed in AML and high ABR expression associates with improved outcome

Previous studies showed that the ABR related BCR acts as a tumor suppressor in chronic myeloid leukemia (92) and meningiomas (93). Additionally, evidences for a putative anti-oncogenic role of ABR have been shown in several tumors (81–84), including t(8;21) AML (97), in which C/EBPα is repressed by the fusion protein AML1-ETO (62). To understand its regulation in AML, the expression levels of ABR were quantified in diagnostic samples of patients with different AML subtypes. By qPCR we ascertained the ABR expression pattern in bone marrow samples of 63 primary AML patients at diagnosis and 3 healthy control bone marrow samples. Our data showed that ABR expression is strikingly repressed in bone marrow of AML patients independent of the subtype (Figure 10).

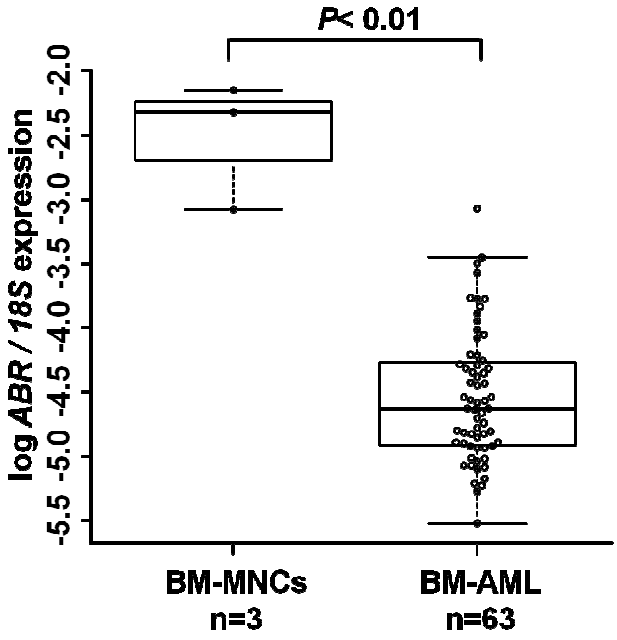

Figure 10. Reduced ABR expression in AML patients. qPCR for ABR was carried out using bone marrow cells derived from AML patients at diagnosis (n=63). Values represent the log base 2 transcript abundancies normalized to 18S and further to the expression level of bone marrow mononuclear cells (BM-MNCs) from healthy donors (n=3).

A recent treatment approach for AML is the allogeneic hematopoietic stem cell transplantation (HSCT) after non-myeloablative conditioning, which uses a low dose

of total body irradiation to enable older patients to undergo HSCT (157). By using the median cut algorithm in a subset of 36 *de novo* AML patients who received non-myeloablative hematopoietic stem cell (NMA-HSC) transplantation in complete remission, we observed that patients with high ABR expression showed a significant longer overall survival than patients with low ABR expression (Figure 11).

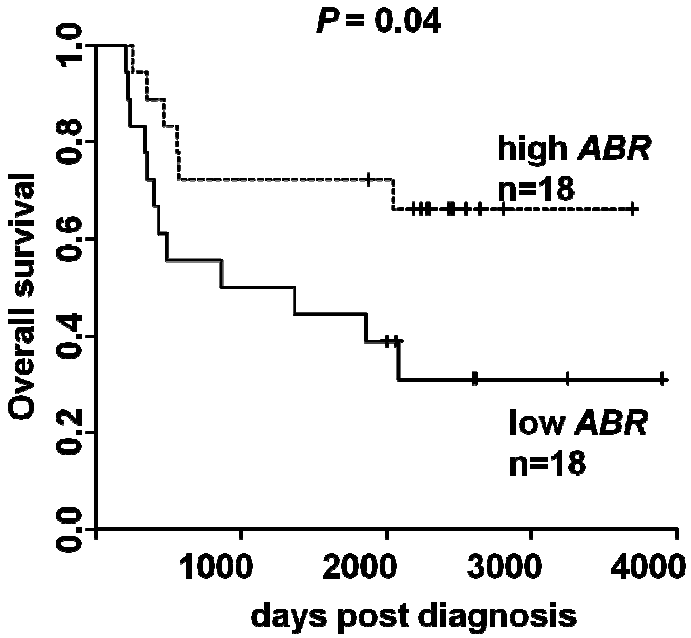

Figure 11. High ABR expression is associated with improved outcome in AML. Overall survival of the *de novo* AML patients in complete remission who received non-myeloablative hematopoietic stem cell (NMA-HSC) transplantation in complete remission (n=36) is presented in Kaplan–Meier plots. ABR expression levels are cut at median. Log rank test was used for statistical evaluation.

We analysed the clinical and molecular characteristics of 63 primary AML patients at diagnosis according to ABR expression. Our data show that high ABR expression in AML correlates with favorable clinical and molecular patient characteristics in AML, such as a significant lower percentage of blasts in peripheral blood (P=.006) and high expression of miR-181a (P<.001). In addition, we observed tendencies towards a lower number of white blood cells (WBC) (P=.06), and a lower blast number in bone marrow (P=.06) (Table 8). We did not observe a significant difference in the mutational status of CEBPA and FLT3 between high and low ABR expressers (Table 8). However, a low ABR expression was associated with mutated NPM1 (p=.04).

Table 8. Comparison of clinical and molecular characteristics of AML patients that received NMA-HSC transplantation according to ABR expression. The patients were dichotomized into ABR high and ABR low expressers using a median cut.

Characteristic	Low *ABR* (n=31)	High *ABR* (n=32)	p value
Median age, y (range)	61 (21-74)	61 (38-73)	.29
Gender (male/female), n	15/16	16/16	1
Median Hb, g/dL (range)	9.2 (5.8-14.9)	9.0 (4.3-15.7)	.79
Median PLT, 10^9/L (range)	56 (2-179)	48 (1-192)	.48
WBC at diagnosis	8.1 (1-385)	4.6 (0.7-140)	.06
% blasts in PB	50 (2-97)	21 (0-97)	.006
% blasts in BM	80 (27-95)	60 (10-94)	.06
Normal karyotype, n	13	13	1
CEBPA mut	3 (10%)	3 (10%)	1
FLT3-ITD	8 (26%)	5 (16%)	.53
NPM1 mut	11 (37%)	4 (13%)	.04
high miR-181a-1	9 (29%)	23 (71%)	<.001
high miR-181a-2	8 (26%)	24 (75%)	<.001
de novo AML, n	22	20	.60

Abbreviations: y, years; Hb, hemoglobin; PLT, platelets; FLT3-ITD, internal tandem duplication of the FLT3 gene. The median expression value was used as a cut point. It was calculated based on the expression levels assessed by qPCR. P-values compare patients who have low ABR expression versus high ABR expression by log rank test.

Genetic and morphological characteristics of all AML patients who received NMA-HSC transplantation and healthy donors are shown on Table 6.

3.2 ABR is increased during myeloid differentiation of mouse bone marrow cells

It is known that ABR protein has monocyte-colony stimulating factor (M-CSF)-induced activity in mouse bone marrow macrophages (106). A previous study could show that C/EBPα has the capacity to direct monocytic development from myeloid progenitors (9). In addition, C/EBPα is able to activate the M-CSF-receptor (M-CSF-R) promoter (43). To investigate the role of ABR in monopoiesis, we analysed the ABR expression during monocytic differentiation of mouse bone marrow cells. The cells were cultured in presence of M-CSF (20 ng/ml) for seven days. Total RNA was isolated and quantified using qPCR method. We could demonstrate that ABR expression was significantly increased upon M-CSF-stimulated differentiation of mouse bone marrow cells (Figure 12A). The differentiation status of the cells was evaluated by qPCR based measurement of the monocytic marker M-CSF-R. The mouse bone marrow cells showed an up to 19 fold induction of M-CSF-R (Figure

12B). Additionally, myeloid differentiation was assessed by flow cytometry using the

myeloid surface marker CD11b and mouse monocytic marker F4/80 (Figure 12C).

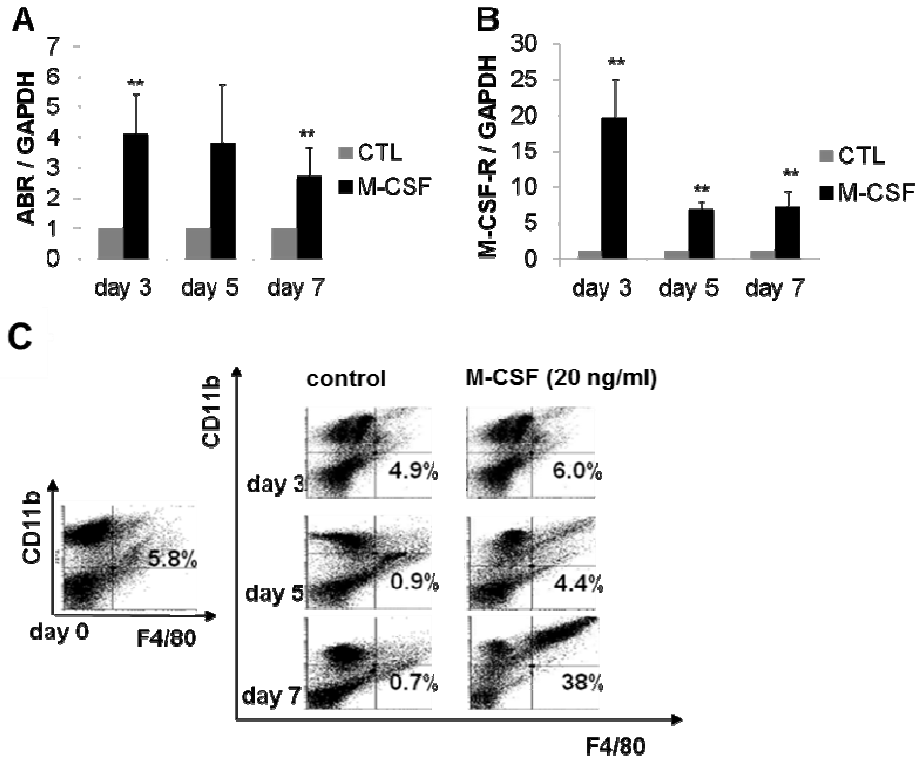

Figure 12. ABR expression is increased during M-CSF-induced monocytic differentiation of mouse bone marrow cells. Bone marrow cells derived from wild-type C57Bl/6 mice were treated with M-CSF for 7 days. (A-B) qPCR was carried out with oligonucleotides for ABR and M-CSF-R, respectively. Values were normalized to GAPDH and further to the expression level of control treated cells (CTL). (C) FACS analysis was performed using myeloid CD11b and mouse specific macrophage (F4/80) antibodies. Total data are represented as mean ± SD from 3 representative experiments (**$p \leq .01$).

The myeloid transcription factor C/EBPα plays a critical role in granulocytic differentiation, elucidated by the finding that targeted disruption of C/EBPα results in a block of granulocytic maturation (33). Further, C/EBPα is able to activate the granulocyte-colony stimulating factor receptor (G-CSF-R) promoter (41). To access the role of ABR in granulopoiesis, we analysed the ABR expression during granulocytic differentiation of mouse bone marrow cells. The cells were cultured in presence of G-CSF (1 ng/ml) for seven days. Total RNA was isolated and quantified using qPCR method. We could demonstrate that ABR expression was significantly increased upon G-CSF-stimulated differentiation of mouse bone marrow cells (Figure 13A). The differentiation status of the cells was evaluated by qPCR based measurement of the granulocytic marker G-CSF-R. The mouse bone marrow cells showed an up to 14 fold induction of G-CSF-R (Figure 13B). Additionally, myeloid

differentiation was assessed by flow cytometry using the myeloid surface marker CD11b and mouse granulocytic marker Gr-1 (Figure 13C).

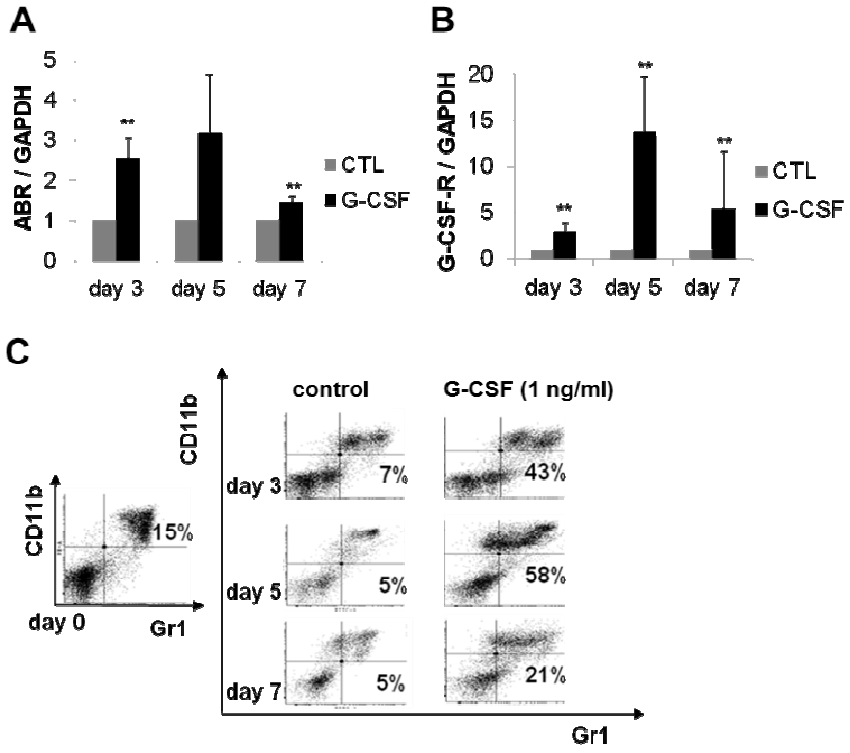

Figure 13. ABR expression is increased during G-CSF-induced granulocytic differentiation of mouse bone marrow cells. Bone marrow cells derived from wild-type C57Bl/6 mice were treated with G-CSF for 7 days. (A-B) qPCR was carried out with oligonucleotides for ABR and G-CSF-R, respectively. Values were normalized to GAPDH and further to the expression level of control treated cells (CTL). (C) FACS analysis was performed using myeloid CD11b and mouse specific granulocyte (Gr1) antibodies. Total data are represented as mean ± SD from 3 representative experiments (**$p \leq .01$).

3.3 ABR expression is increased during PMA-induced myeloid differentiation

A previous study has shown that M-CSF stimulation is associated with ABR-induced inactivation of Rac in mouse bone marrow macrophages (106). Moreover, our data show that ABR expression is induced during monocytic and granulocytic differentiation mediated by M-CSF and G-CSF, respectively. To further understand how ABR activity is biologically significant in the context of myelopoiesis, we carried out differentiation experiments in U937 cells. U937 cells can differentiate to macrophages under PMA (phorbol 12-myristate 13-acetate) induction (160). The

expression of the myeloid surface marker CD11b was used to measure cell differentiation 48 hours after the PMA treatment by immunofluorescence (Figure 14A). The expression levels of ABR and M-CSF-R were further examined by quantitative real-time PCR. We observed that ABR and M-CSF-R expression levels are increased significantly during differentiation (Figure 14B-C).

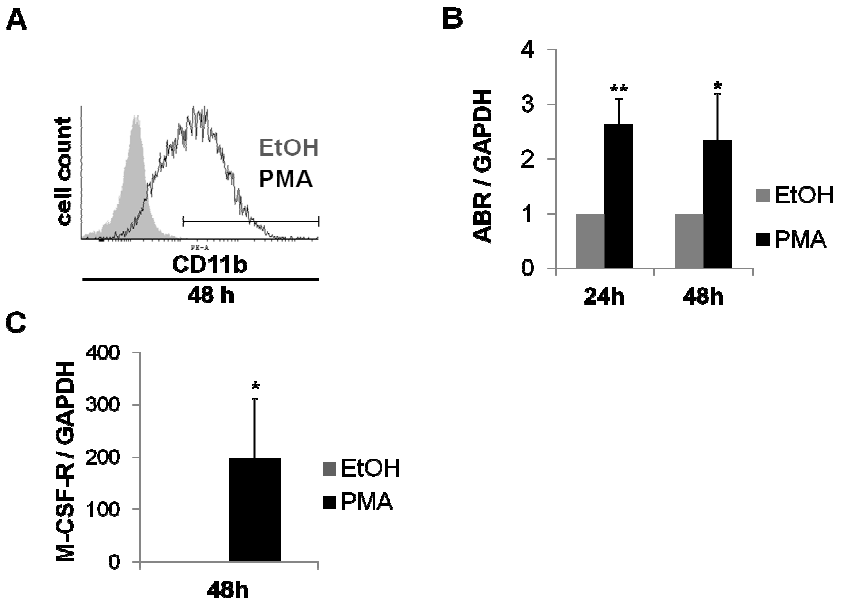

Figure 14. ABR expression is increased during PMA-induced myeloid differentiation. (A-C) U937 cells were treated with PMA (10nM) for the determined time points. (A) FACS analysis was performed using CD11b antibody. (B-C) Total RNA was analysed by qPCR with oligonucleotides for ABR (B) and M-CSF-R (C). Values were normalized to GAPDH and further to the expression level of control treated cells (ethanol, EtOH). Total data are represented as mean ± SD from 3 representative experiments (**$p \leq .01$, *$p \leq .05$).

3.4 ABR knockdown reduces C/EBPα expression levels and PMA-induced myeloid differentiation

Our data show that ABR is increased during myeloid differentiation (Figures 12-14). Diverse studies have shown the loss of ABR gene in several tumors (81–84) and that induced BCR expression was able to block leukemia formation in a CML NOD/scid mouse model (92,161). Moreover, the oncoprotein BCR-ABL regulates the expression of C/EBPα by inducing poly(rC)-binding protein hnRNP E2-which inhibits the translation of CEBPA mRNA (67). Thus, we hypothesized that the BCR related ABR might be an important player in myeloid differentiation upstream to C/EBPα. To investigate this, we performed ABR knockdown by specific siRNA. ABR knockdown

was verified by western blot and showed a 40% reduction of the ABR protein 24 hours after siRNA treatment in comparison to control. Analysis of CEBPα protein revealed a 30% reduction of the protein level 24 hours after siRNA mediated knock down of ABR (Figure 15).

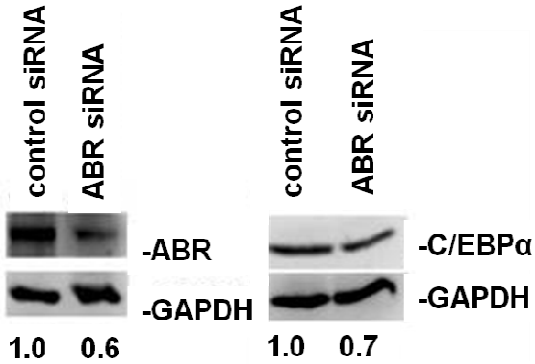

Figure 15. The inhibition of ABR reduces the expression of C/EBPα. U937 cells were transfected with ABR siRNA or control siRNA. Total protein was analysed by western blot analysis with ABR and C/EBPα antibodies. Values below the gel image indicate the protein levels normalized to GAPDH.

In contrast to the well-known BCR, which is shown to block leukemogenesis in mice (92,161), relatively little is known about the role and function of ABR in the context of myeloid differentiation and leukemia. To further analyse the role of ABR in differentiation, ABR expression was blocked by specific siRNA. For this purpose, U937 cells were transiently transfected with ABR siRNA or control siRNA. Six hours after transfection, the cells were stimulated with 1nM PMA. We found that a block of ABR expression resulted in a 53% reduction of C/EBPα expression levels after PMA treatment (Figure 16).

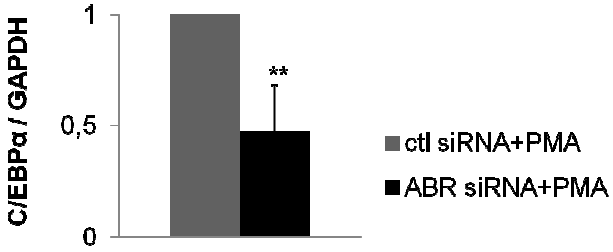

Figure 16. ABR knockdown reduces C/EBPα expression levels after PMA-induced myeloid differentiation. U937 cells were transfected with ABR siRNA or control siRNA. Six hours after transfection, cells were stimulated with PMA (1nM) for 48 hours. (D) qPCR was carried out with oligonucleotides for C/EBPα. Values were normalized to GAPDH and further to the expression level of control treated cells (ctl). Total data are represented as mean ± SD from 3 representative experiments (**$p \leq .01$).

As our data show that ABR expression is increased during PMA-induced myeloid differentiation (Figure 14B), we investigated the effect of an ABR block during differentiation. Therefore, leukemic U937 cells were transfected with ABR siRNA or control siRNA, respectively. Six hours after sIRNA transfection, the cells were treated with 1 nM PMA for 48 hours. Flow cytometry analysis revealed that ABR knockdown in leukemic U937 cells leads to a significant 45% reduction of the surface marker CD11b in comparison to the control siRNA transfected cells after PMA treatment (Figure 17).

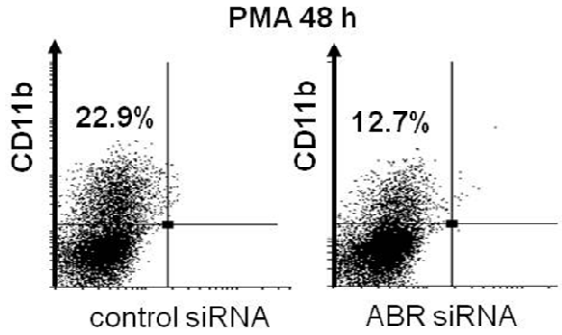

Figure 17. ABR knockdown leads to a reduction of PMA-induced myeloid differentiation. U937 cells were transfected with ABR siRNA or control siRNA. Six hours after transfection, cells were stimulated with PMA (1nM) for 48 hours. FACS analysis was performed using CD11b antibody. The dot plots show an example of the flow cytometry results.

3.5 ABR increases myeloid differentiation

Furthermore, we investigated the influence of an augmented ABR expression on myeloid differentiation. U937 cells were transduced with lentiviral vector EYFP-ABR in pCCL-cppt178-MNDU3 followed by PMA-treatment for 48h. The results indicated a 44% increase in the CD11b positive cell population by ABR overexpression (Figure 18). These findings illustrate a role of ABR in PMA-induced myeloid differentiation.

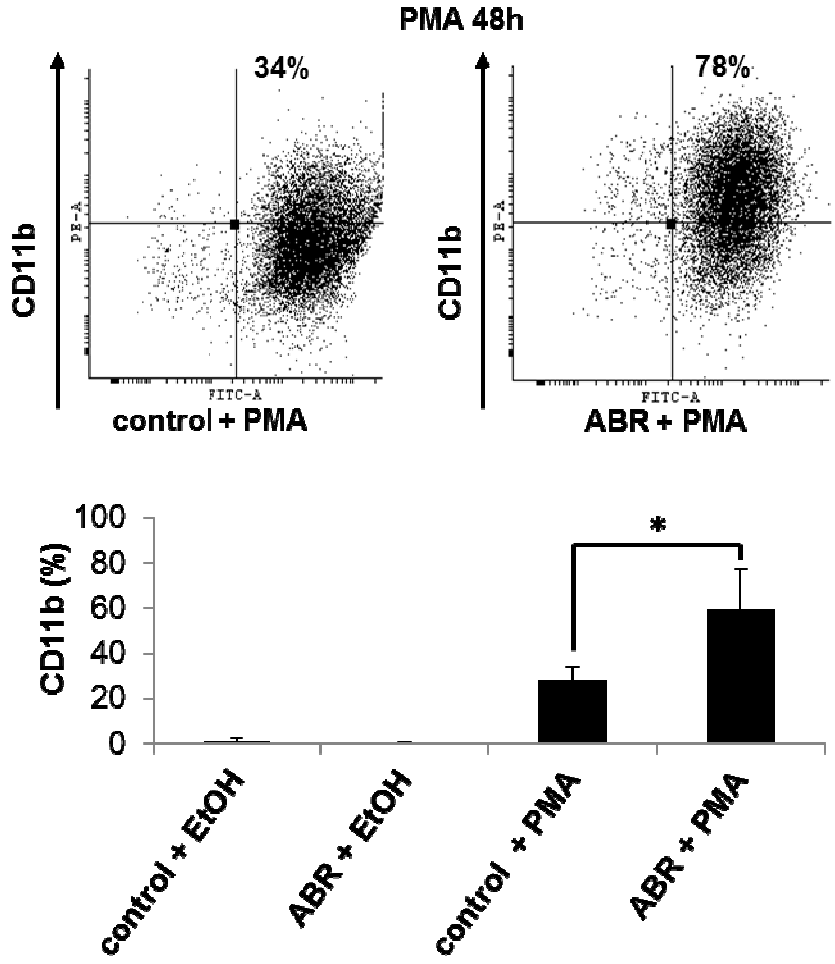

Figure 18. ABR increases PMA-induced myeloid differentiation. U937 cells were stably transfected with lentiviral vector EYFP-ABR in pCCL-cppt-MNDU3 and treated with PMA (10nM) or vehicle (ethanol, EtOH) for 48h. FACS analysis was performed using CD11b antibody. Total data are represented as mean ± SD from 3 representative experiments (*$p \leq$, .05).

3.6 ABR induces C/EBPα expression and thereby increases the expression of M-CSF-R, G-CSF-R and miR-223

Further, we examined the ability of ABR to regulate the expression of C/EBPα. Thus, we performed transient overexpression of ABR and measured resulting C/EBPα protein and mRNA expression levels. Here, we could demonstrate that enforced ABR expression for 24 hours leads to a 2.4-fold enhance of C/EBPα protein level (Figure 19A) and 2.6-fold increase of C/EBPα mRNA in U937 cells (Figure 19B).

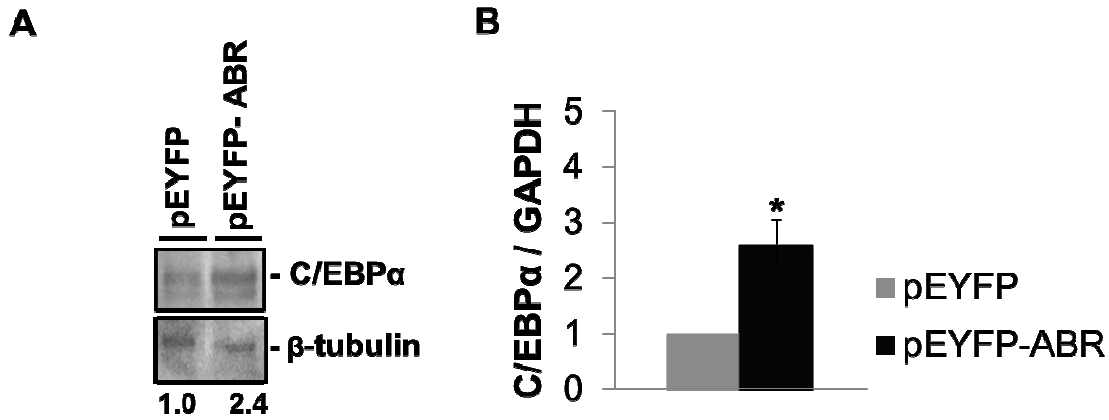

Figure 19. ABR induces transcription factor C/EBPα expression. (A-B) U937 cells were transfected with pEYFP-ABR or pEYFP-empty vector for 24 hours. (A)Total protein was analysed by western blot analysis with C/EBPα antibody. Values below the gel image indicate C/EBPα protein levels normalized to β-tubulin. (B) Total RNA was analysed by qPCR with oligonucleotides for C/EBPα. Values were normalized to GAPDH. Total data are represented as mean ± SD from 3 representative experiments (*$p \leq .05$).

Thus, we hypothesize that ABR is able to induce C/EBPα targets. To further investigate this regulatory network, U937 cells were co-transfected with ABR or control vector and siRNA (control siRNA or C/EBPα siRNA). After 24 hours, the RNA of the cells was isolated and the expression levels of C/EBPα and its targets M-CSF-R, G-CSF-R and miR-223 were measured by qPCR. The co-expression of ABR and control siRNA for 24 hours leads to a 3.1-fold C/EBPα, 2.7-fold M-CSF-R, 1.8-fold for G-CSF-R and 2.9-fold for miR-223 induction in U937 cells (Figure 20 A-D, second columns). Conversely, the co-transfection of C/EBPα siRNA and empty vector or ABR for 24 hours blocked ABR-mediated induction of C/EBPα and its targets M-CSF-R, G-CSF-R and miR-223 (Figures 20 A-D, third and fourth columns). These results indicate that ABR induces the expression of M-CSF-R, G-CSF-R and miR-223 through C/EBPα.

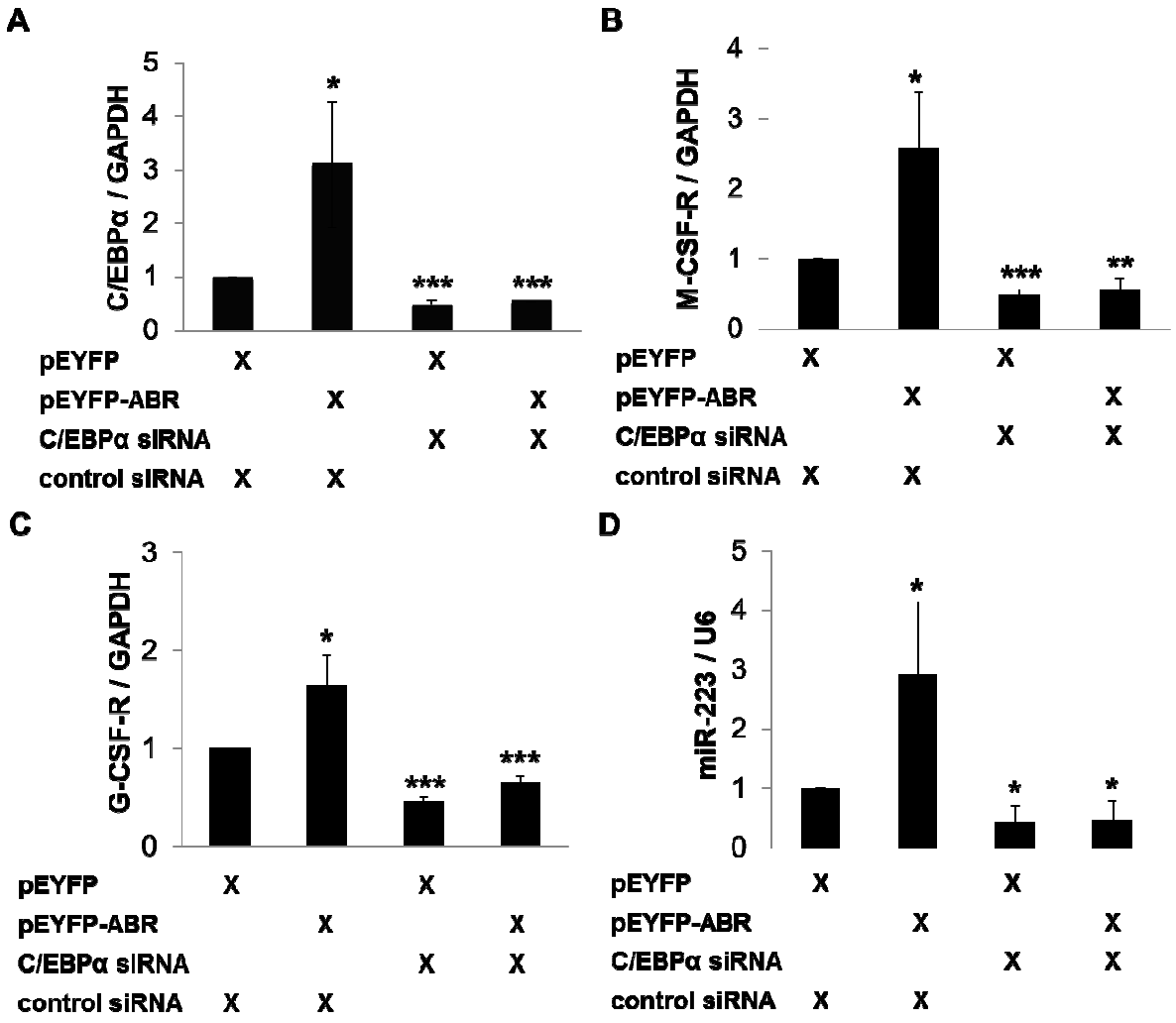

Figure 20. ABR induces C/EBPα expression and thereby increases the expression of M-CSF-R, G-CSF-R and miR-223. (A-D) U937 cells were co-transfected with pEYFP-ABR or pEYFP-empty vector along with C/EBPα siRNA or control siRNA for 24 hours. Total RNA was analyzed by qPCR with oligonucleotides for C/EBPα, M-CSF-R, G-CSF-R and miR-223. Values were normalized to GAPDH and further to the expression level of cells transfected with pEYFP and control siRNA. Total data are represented as mean ± SD from 3 representative experiments (***$p \leq .001$, **$p \leq .01$ *$p \leq .05$).

3.7 Treatment of leukemic cells with the Rac1 inhibitor NSC23766 resulted in an increased expression of C/EBPα

Our data show that ABR is able to induce C/EBPα expression (Figures 19 and 20 A). In addition, several studies reported that ABR inactivates Rac1 protein (91,94,100). Thus, we were interested to know whether Rac inhibition could affect C/EBPα expression. Therefore, we treated U937 cells with Rac inhibitor NSC23766 and analysed C/EBPα expression by qPCR. Interestingly, the inactivation of Rac1 via

treatment with the Rac1 inhibitor NSC23766 for 12 hours resulted in a significant increase of C/EBPα mRNA up to 87% (Figure 21). These data further support a role of ABR in the C/EBPα regulation.

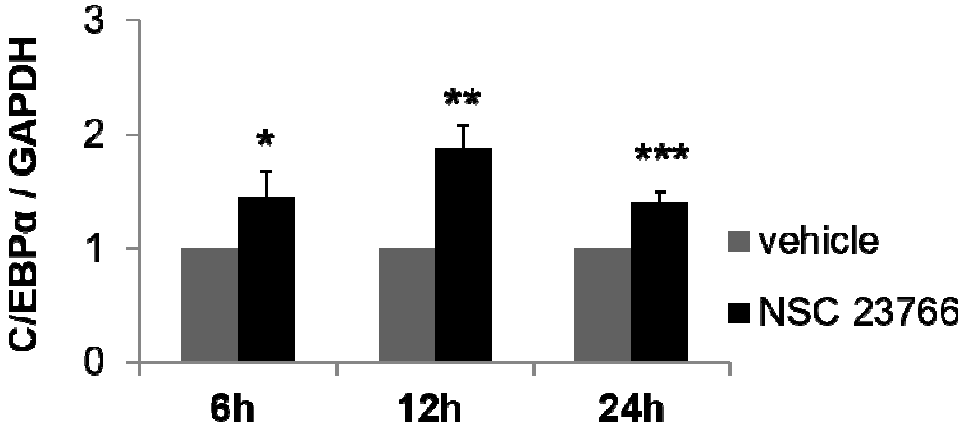

Figure 21. Treatment of leukemic cells with the Rac1 inhibitor resulted in an increased expression of C/EBPα. U937 cell were treated with 100µM Rac1 inhibitor NSC23766 for the determined time points. Total RNA was analysed by qPCR with oligonucleotides for C/EBPα. Values were normalized to GAPDH and further to the expression level of control treated cells (H_2O). Total data are represented as mean ± SD from 3 representative experiments (***$p \leq .001$, **$p \leq .01$ *$p \leq .05$).

3.8 High ABR expression associates with response to azacitidine treatment in AML patients

As our data show that ABR is a favorable prognostic factor in AML, we were interested in the impact of ABR expression on AML treatment. Thus, we aimed to examine the ABR expression pattern in azacitidine-treated AML patients. To this end, patients were divided into two groups (responders and non-responders) after azacitidine therapy. Genetic and morphologic features of each azacitidine-treated AML sample are shown in section 2.1.3, Table 7. Response was defined as a blast clearance (≤5%) in the bone marrow on day 15 after start of the first azacitidine treatment cycle (159), which was achieved in 11 of 26 patients (42%) (Table 9). Bone marrow samples from 26 AML patients (>60 years) were collected at diagnosis and ABR expression was quantified by qPCR (Figure 22). We found that a high ABR expression significantly correlated with a better response after azacitidine treatment (Figure 22). These observations suggest that ABR is a relevant target for the treatment of leukemia.

Table 9. Characteristics of AML patient samples used for ABR expression analysis according to azacitidine-treatment response.

Characteristic	Non-responders (n=15)	Responders (n=11)
Median age, y (range)	68 (62-79)	74 (61-78)
Gender, n (male / female)	9 / 6	8 / 3
Day 0 blasts, median (range)	70 (24-90)	50 (10-95)
Day 15 blasts, median (range)	63,5 (10-90)	≤5

y, years; n, number

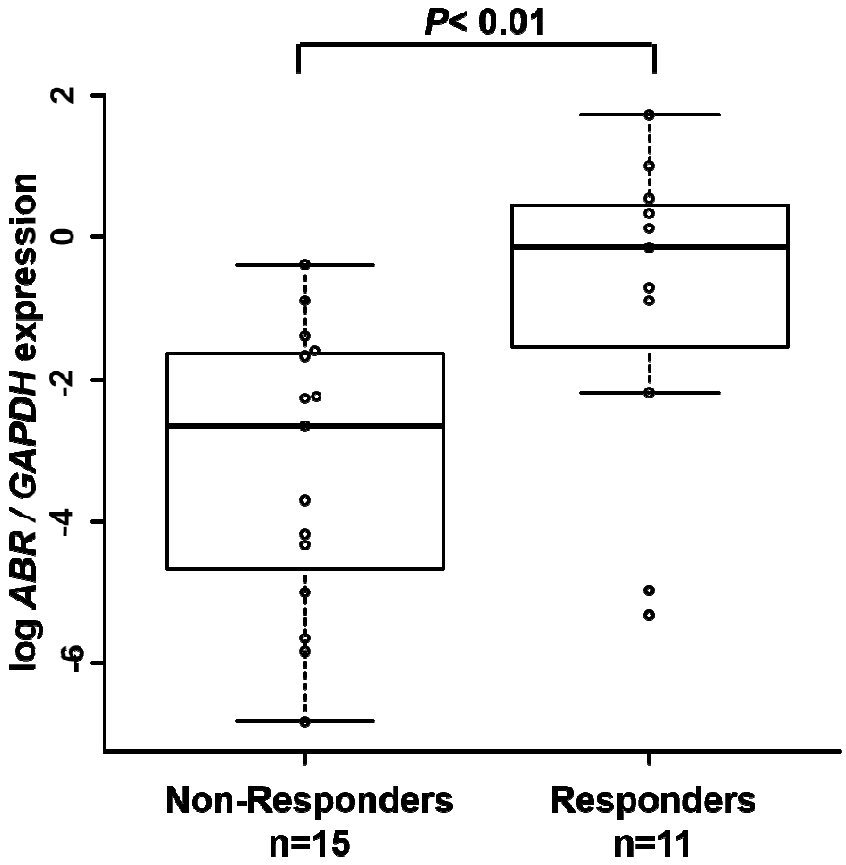

Figure 22. Response to azacitidine treatment in AML patients associates with high ABR expression. qPCR for ABR was carried out using bone marrow cells derived from AML patients at diagnosis (n=26). Values represent the log base 2 transcript abundancies normalized to GAPDH. Kruskal test was used for statistical evaluation.

3.9 ABR increases azacitidine-induced apoptosis

A previous study in mouse leukemic cells demonstrated that the majority of azacitidine (85%) is incorporated into RNA (162). In agreement with those findings, in the human AML cell line KG-1a, azacitidine has been shown to incorporate into both RNA and DNA, with predominant incorporation into RNA in comparison with DNA (163). Previous studies showed that azacitidine induces apoptosis in MLL-rearranged AML cell line THP-1 (164) and FLT3-ITD AML cell line Molm 14 (165). As we hypothesize that ABR could act as a tumor suppressor gene in AML, we were interested to evaluate the impact of ABR expression on azacitidine treatment in leukemic cells. To investigate the apoptotic effect of azacitidine, U937 cells were treated with azacitidine for 48 hours. Apoptotic cells were measured by flow cytometry after staining with Annexin V and 7-AAD. U937 cells treated with 5 µM azacitidine exhibited on average a 14% increase in apoptosis (Figure 23).

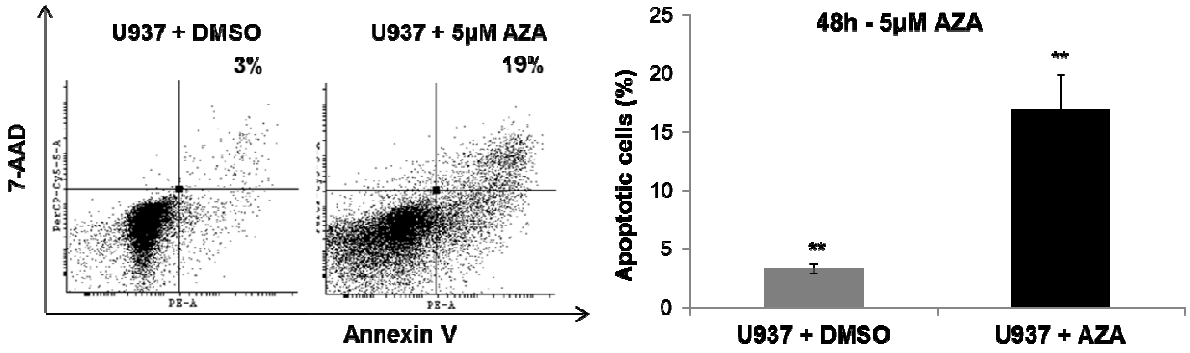

Figure 23. Induction of apoptosis in U937 cells by azacitidine treatment. Flow cytometry analysis for Annexin V and 7-AAD staining in U937 cells to analyse apoptosis. Cells were treated with 5 µM azacitidine (AZA) or DMSO for 48 hours. Total data are represented as mean ± SD from 3 representative experiments (**$p \leq .01$).

Azacitidine induces tumor suppressor gene expression (166), including transcription factors associated with myeloid differentiation (164). It is known that DNA methylation of C/EBPα in AML is a rare event associated with favorable cytogenetic subgroups inv(16) and t(15;17) (167). To address the effect of azacitidine on the expression of ABR and C/EBPα in U937 cells, we treated those cells with azacitidine for 48 hours. The RNA of the cells was isolated and the expression levels of ABR and C/EBPα were measured by qPCR. We could show that azacitidine leads to an increase of mRNA expression levels of ABR and C/EBPα in U937 cells (Figure 24 A-B). These

results suggest that a putative epigenetic modification may be responsible for a reduced expression of ABR in AML.

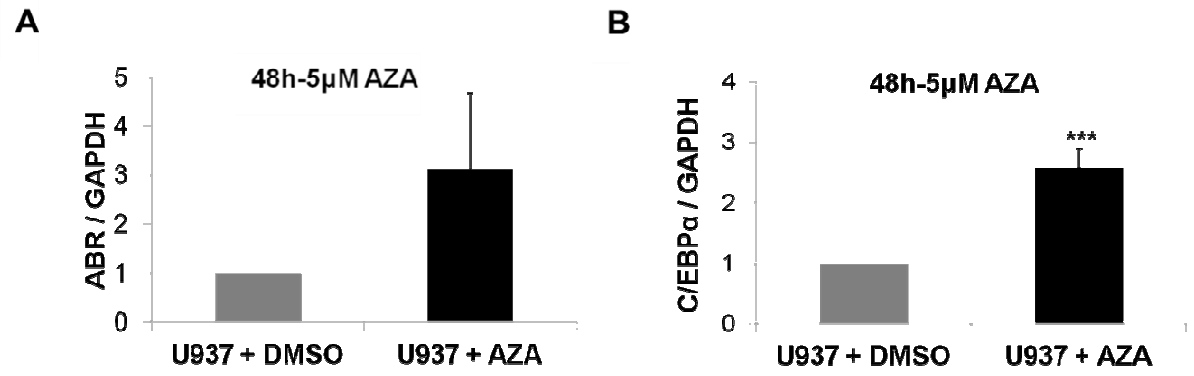

Figure 24. Azacitidine treatment increases the expression of ABR and CEBPA. (A-B) qPCR analysis of ABR (A) and CEBPA (B) expression in non-transduced U937 cells after azacitidine treatment (5µM). Total data are represented as mean ± SD from 3 representative experiments (****p* ≤ .001).

Azacitidine can induce functional expression of aberrantly silenced genes in cancer, causing growth arrest and apoptosis in tumor cells (166). To investigate the effect of ABR ectopic expression in azacitidine therapy, we stably overexpressed ABR in leukemic U937 cells and treated the cells with 15 µM azacitidine. ABR significantly enhanced the proapoptotic effect of azacitidine (2.7 fold with 15 µM azacitidine for 48 hours) in comparison to control (Figure 25).

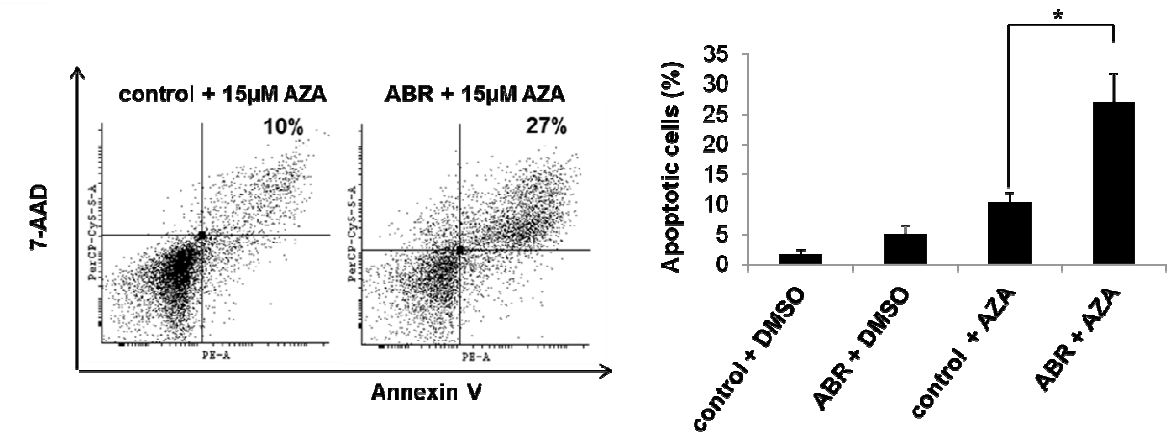

Figure 25. ABR overexpression enhances azacitidine-induced apoptosis. Flow cytometry analysis for Annexin V and 7-AAD staining in U937 cells stably expressing ABR or control sequence (A) to analyse apoptosis. Cells were treated with 15 µM azacitidine or DMSO for 48 hours. Total data are represented as mean ± SD from 3 representative experiments (**p* ≤ .05).

3.10 Analysis of the DNA methylation data for ABR in AML patient samples from TCGA data set

Promoter hypermethylation plays a major role in cancer through transcriptional silencing of critical growth regulators such as tumor suppressor genes (166). To investigate whether downregulation of ABR in AML is caused by DNA methylation of the ABR promoter, we analyzed DNA methylation data for ABR utilizing The Cancer Genome Atlas (TCGA) AML patient data set from The GDC Data Portal. The analysis was performed on 194 acute myeloid leukemia samples in 255 methylation sites using the Expander software (168,169). The patients were dichotomized into ABR high (red) and ABR low (blue) expressers using a median cut (Figure 26). The sum of methylation intensity per patient group and for each methylation site was calculated. In the upper panel of Figure 26, DNA methylation distribution of ABR gene is shown on all ABR methylation sites. In the lower panel, the heatmap plot represents the DNA methylation data between patients with high (red) and low (blue) ABR expression. The color scales for the heatmap reflect normalized log base 2 abundances. All values of the methylation sites of 1 patient are divided by the average of all methylation sites of this patient, so that a log 2-fold change (fc) of 1 means no change while fc>1 means higher methylated and fc <1 means lower methylated. Surprisingly, the ABR promoter region is not methylated in both low and high ABR expressing patients while a large portion of the open reading frame is methylated in both groups (Figure 26). These findings suggest that repression of ABR in AML is not due to methylation. We could also observe that the intron 16 of ABR contains the mature miRNA-3183 (Figure 26), also known as novel sol-miR-39 (170).

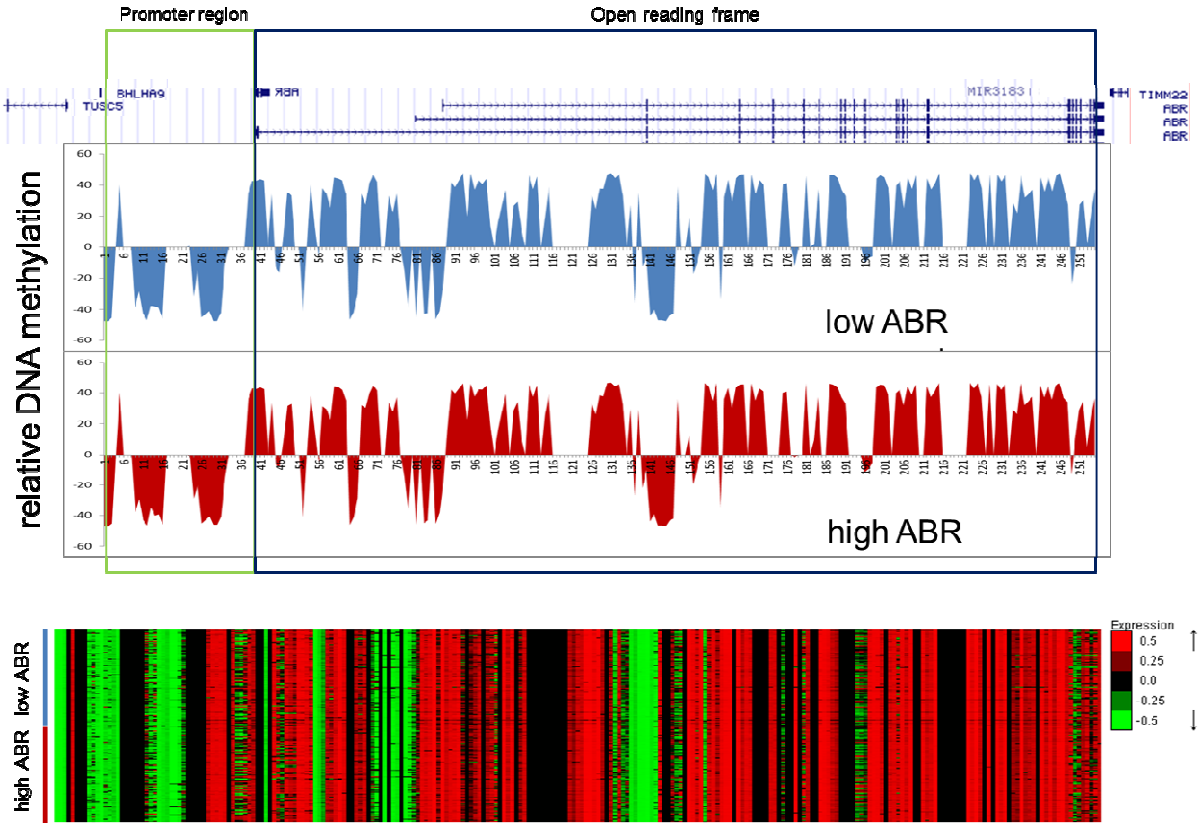

Figure 26. DNA methylation data for ABR in AML patients from The Cancer Genome Atlas (TCGA) data set. The patients (n=194) were divided into ABR high (red) and ABR low (blue) expressers. Upper panel: DNA methylation distribution of ABR gene. The peaks scores represent the sum of methylation values of the ABR high or ABR low expressing patients. Peaks with positive values represent hypermethylation and the negative peaks hypomethylation. Lower panel: Heatmap plot illustrating DNA methylation data between patients with high and low ABR expression. Each column represents a methylation site of ABR and each row represents a patient.

4 Discussion

Hematopoiesis is a dynamic and highly complex process. The development from the pluripotent stem cell to the differentiated, functional cell of a particular cell type is ensured by the cooperation of various signals in the cell. A disturbance of these regulatory mechanisms can lead to an imbalance between differentiation and proliferation, which consequently can lead to a lack of maturation with a simultaneous large increase in undifferentiated precursor cells. Tumor suppressor genes play a role in the regulation of these mechanisms. Deregulation of tumor suppressor genes is also associated with the development of neoplasias, including leukemias (171,172). For instance, the transcription factor C/EBPα is one of the main regulators of normal hematopoiesis (173) and is misregulated in nearly half of all acute myeloid leukemias by various mechanisms (60,62,63).

BCR gene, closely related to ABR, acts as a tumor suppressor in chronic myeloid leukemia (92) and has overlapping functions with ABR. Furthermore, BCR act as a negative regulator of the BCR-ABL oncoprotein in chronic myeloid leukemia (92), inhibiting C/EBPα translation (67). The active BCR related (ABR) gene is located in a chromosomal region in which structural aberrations are associated with diverse diseases, including chronic myeloid leukemia (76–80). In addition, diverse studies show an association of ABR deletion with several non-hematopoietic tumors (81,83,84). These observations suggest that ABR is a relevant target for the treatment of leukemia and possibly of several other types of cancer. Therefore, in the present work, the role of ABR was investigated in the myeloid system and in acute myeloid leukemia.

4.1 The importance of ABR in AML

Acute myeloid leukemia (AML) in adults is still associated with poor outcome (174). In the development of AML, the deregulation of transcription factors is the most frequent event (6). In addition, AML involves activation of oncogenes or deactivation of tumor suppressor genes, and a block of differentiation caused by reduced function of transcription factors (6,10).

An important tumor suppressor protein in the myeloid differentiation is the

transcription factor C/EBPα. Experimental data from mice models as well as AML patient samples suggest that the loss of function or expression of C/EBPα provides a platform on which AML develops (10). In the different subtypes of the AML, approximately 50% of the cases have a lack of expression, dysregulation or mutation of C/EBPα (60,62–65). It has already been shown in a mouse model that C/EBPα plays a major role in hematopoiesis and leukemogenesis. In AML patients with CEBPA mutations, homozygous individuals showed a disorder of the myeloid differentiation of precursor cells in the bone marrow and thus developed an AML (175). Mutations in one or both alleles of CEBPA are observed in about 10% of patients with acute myeloid leukemia (AML) and are predominant in AML patients with normal karyotype (59,60). In young AML patients, these are found in 13% to 19% of cases (60,176).

The translation of C/EBPα mRNA is inhibited by BCR-ABL, an intracellular, constitutively active tyrosine kinase (67). In addition, the oncoprotein BCR-ABL has been shown to downregulate the expression of G-CSF-R, possibly through negative regulation of C/EBPα (67). It is also known that the ABR relative BCR act as a tumor suppressor in CML (92,161), possibly by antagonizing the effects of BCR-ABL in early stages of CML (177). In addition, BCR-induced gene expression strongly inhibits the oncogenic effects of BCR-ABL in NOD/SCID mice, allowing 80% survival (161).

A previous study has shown that the activity of Rac is increased in growth factor dependent murine myeloid precursor cell line 32Dcl3 ectopically expressing BCR-ABL (178). Additionally, SCID mice, deficient in functional B and T lymphocytes, injected with those cells expressing BCR-ABL plus dominant-negative N17Rac proteins had markedly prolonged survival and reduced ability of leukemic cells to home to bone marrow and spleen of mice (178). Thus, this study has identified the known ABR target Rac as a BCR-ABL downstream effector with an important role in leukemogenesis (178). Consistently with these findings, enhanced migration and interaction of AML cells with bone marrow stroma were highly dependent of Rac activity, which was found to be increased in AML cells (147). Conversely, the inhibition of Rac1 has been shown to prevent clonal expansion of leukemic stem cells (147).

Functional investigations of tumor suppressor genes during leukemia development

and maintenance are of great public interest and might open a new era of novel treatment strategies for cancer in general. Although the relative BCR is demonstrated to act as a tumor suppressor in leukemia (92), a specific function of ABR in leukemia has not been addressed. Therefore, in the present study, we utilize AML patient samples as models to investigate ABR expression pattern in myelopoiesis and leukemia. This study provides the first evidence that repression of ABR is a common phenomenon in AML from different subtypes (Figure 10). Those results are supported by a previous study, which described a reduction of ABR expression in t(8;21) AML (97), one of the most common karyotypic abnormalities in AML. In the subtype AML-M2 with the translocation t (8; 21), the fusion protein AML-ETO inhibits C/EBPα function and autoregulation by a unknown mechanism (62). Because Rac1 is downregulated by ABR (106), a reduction of ABR expression might contribute to the accumulation of Rac1 in AML. Consistently, activation of Rac protein has been found in different myeloid associated diseases, including AML (147,152,153,156). Due to the reduced ABR expression in various AML subtypes as well as the tumor suppressive effect of ABR, Rac1 is no longer effectively inactivated by ABR and thus a massive increase in oncogenic factors might occur.

An important recent finding is that active Rac1 can promote leukemia development in AML-ETO-9a mouse model by a better homing of leukemic cells in niche which further enhancing their leukemia stem cell features, such as colonial formation, quiescence, and preventing leukemia cells from apoptosis (149). Further, Rac1 inhibitor treatment either *in vivo* or *in vitro* could effectively block the homing of leukemia cells (149). Those findings are supported by other studies, which reported that Rac1 is required for the engraftment phase of hematopoietic reconstitution in mice (141,143) Similarly, MLL-AF9–transduced murine leukemic stem cells showed upregulated levels of active Rac (146).

By analyzing *de novo* AML patient in complete remission who received HSC transplantation, we clearly see a prognostic correlation between a high ABR expression and a longer survival of the patients (Figure 11). In accordance with this, we observed that high ABR expression in AML at diagnosis is associated with favorable clinical and molecular patient characteristics in AML, such as a significant lower blast percentage in the peripheral blood, a trend of lower blast number in the bone marrow and a significant high miR-181a expression (Table 8).

Diverse publications illustrate the expression pattern of miR-181 in hematopoiesis and leukemia. Higher levels of miR-181a expression have been previously shown to directly correlate with higher odds of achieving a complete remission and lower risk of experiencing relapse and/or death in patients with cytogenetically normal AML. (179,180). A previous study reported that high levels of miR-181a were found in normal hematopoietic stem-progenitor cells, indicating that this miRNA may also play a regulatory role in earlier steps of hematopoiesis (181). Furthermore, Schwind et al. (180) found a negative correlation between the expression levels of miR-181a, ID1, an inhibitor of hematopoietic differentiation, and TCF-4, a transcription factor promoting neoplastic transformation (182). Previous studies have underlined the importance of Rac1 in the activation of TCF-4 (183,184). In agreement with those findings, active Rac1 has been shown to physically interact with TCF-4 in colon cancer cells (184). In addition, activation of Rac1 resulted in aberrant induction of the Wnt pathway as observed in different cancers (184–186). Similarly to ABR and Rac1, miR-181a expression has been shown to be associated with native immunity-mediated processes. The expression of TLR4 and IL1B negatively correlated with miR-181a expression, predicted to be direct targets of miR-181a (180). High expression of miR-181a associates with a less aggressive disease possibly by downregulating TLR4 and IL1B genes, that modulate the innate immune response to microbial pathogens in the normal host, but also when upregulated may support survival and proliferation of malignant blasts in AML patients (187–190). Several publications emphasize the importance of Rac1 in expression and activation of TLR-4 in different pathogenesis (191–193).

In addition, we found that a low ABR expression was associated with mutated NPM1. NPM1 mutation in the absence of fms-like tyrosine kinase 3 internal tandem duplications (FLT3-ITD) is associated with a relatively favorable prognosis. However, NPM1 is a late driver mutation, (194) identified as being frequently overlapped with FLT3-ITD, which is associated with an unfavorable prognosis (195). Moreover, the persistence of NPM1 mutation was associated with a significantly higher risk of relapse (196). Those data suggest that ABR is a key mediator of tumor suppressor function in AML.

Approximately 50 % of AML cases have reduced CEBPA mRNA caused by various mechanisms (Figure 3) (60,62,63) including its own mutations (62,64–66). In

addition, CEBPA promoter hypermethylation, evident in 37 % of AML cases, leads to silencing of CEBPA expression (197). Thus, a reduced number of patients in the analysed cohort had no downregulation of CEBPA mRNA caused by known mechanisms, making difficult the finding of a correlation between ABR and CEBPA mRNA expression in the AML samples studied.

Our data indicated that AML patients with higher ABR expression levels respond better to clinical azacitidine-based therapy (Figure 23). Conversely, a reduced ABR expression correlated with a disturbance of myeloid differentiation (Figure 17) and worse outcome in AML (Figures 11, 23). This study provides the first evidence that repression of ABR is a common phenomenon in AML from different subtypes. The available results of this work suggest that ABR might function as a tumor suppressor gene in AML.

A previous study revealed that ABR is able to bind to RhoGDI in cell lysates or as purified protein through its GAP domain (128). The fact that expression of constitutively active Rac has been reported to cause displacement of endogenous proteins bound to RhoGDI, resulting in their degradation (198) could be a reason for the downregulation of ABR in AML, in which accumulation of active Rac has been reported (147). Moreover, previous studies have pointed that high expression of RhoGDI1 is associated with poor prognosis in different cancers, such as colorectal cancer and oral squamous cell carcinoma (199,200).

ABR gene is ubiquitously expressed in mouse tissues, including bone marrow (96). ABR has been previously reported as a negative regulator of immune reaction. In mouse cells of the innate immune system, such as neutrophils and macrophages, the lack of a functional ABR protein results in abnormal reactivity of the innate immune system (75,96). Mice lacking ABR exhibit an increased susceptibility to several inflammatory diseases, such as endotoxemia, septic shock, asthma, and pulmonary hypertension, demonstrating that ABR activity is required for appropriate control of innate immune response *in vivo* (75,130,201). Diverse studies suggested a putative tumor suppressive role of ABR in several cancers (81,83,84). These observations suggest that ABR is a relevant target for the treatment of different types of cancer. Taken together, we discovered ABR as a new critical player in myeloid differentiation. We show that myeloid differentiation inducers lead to increased expression levels of

the ABR and CEBPA targets M-CSF-R and G-CSF-R in human and mouse myelopoiesis. A reduction of ABR expression leads to decreased C/EBPα levels and a minimized myeloid differentiation (Figure 27A). In AML we observed a downregulation of ABR expression. Conversely, high ABR expression 1) enhanced myeloid differentiation; 2) correlated with a favorable prognosis in AML and 3) increased azacitidine-induced apoptosis (Figure 27B). Further, a response to azacitidine treatment correlated with a high ABR expression. Our data indicate the functional role of ABR as a new player in myelopoiesis and AML. Because ABR has been shown to control innate immune responses and ABR deletion has been found in several human cancers, targeted treatments that increase endogenous levels of ABR might also provide novel therapeutic strategies to enhance the treatment response for patients not restricted to AML, as the immune system is able to recognize and attack cancer cells.

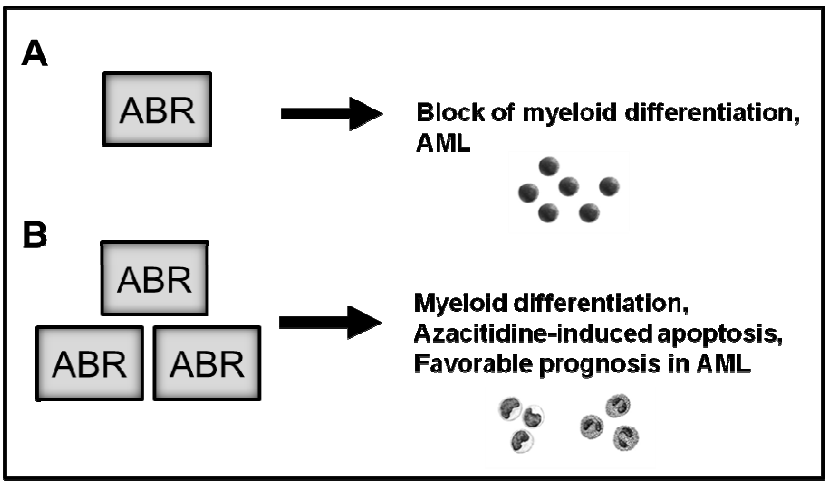

Figure 27. Schematic representation of a model for the role of ABR in normal myelopoiesis and in AML. A knockdown of ABR expression resulted in block of myeloid differentiation. In accordance to this, ABR expression is reduced in AML (A). Conversely, ABR overexpression enhanced myeloid differentiation and azacitidine-induced apoptosis. A high ABR expression correlated with better outcome in AML (B).

4.2 ABR, a new player in myelopoiesis

ABR deactivates the small GTPase Rac1, a master molecular switch that regulates several cellular processes, including maintenance and expansion of leukemic cells (147) and regulation of immune cell functions (102). A previous study has focused on ABR involvement in multiple aspects of mouse macrophage function, including

morphology, macrophage-colony-stimulating factor (M-CSF)-induced motility, and phagocytosis (106). Additionally, ABR translocated to the plasma membrane upon M-CSF stimulation in mouse bone marrow macrophages transduced with ABR lentivirus (106). Although the ABR relative BCR is demonstrated to act as a tumor suppressor in leukemia (92), a specific function of ABR in myelopoiesis and leukemia has not been addressed. Therefore, in the present study, we utilize human leukemic cells, mouse bone marrow cells and AML patient samples as models to investigate the role of ABR in myelopoiesis and leukemia.

To further understand how ABR activity is biologically significant in the context of myelopoiesis, we carried out differentiation experiments in U937 cells. U937 cells are a good model system for studying myeloid differentiation in general, as they can differentiate to macrophages under PMA induction (160). Our study identifies a novel function of ABR, showing that ABR stable overexpression of ABR enhances myeloid differentiation of leukemic U937 cells (Figure 18). Conversely, a block of ABR prevents myeloid differentiation of U937 cells (Figure 17) and leads to repression of the myeloid transcription factor C/EBPα (Figures 15-16), which plays a central role in myeloid differentiation and is functionally disrupted in leukemia. We also observed that ABR expression levels are increased during myelopoiesis of mouse bone marrow cells treated with M-CSF (Figure 12) or G-CSF (Figure 13). In summary, these results demonstrate that ABR is a new critical player in myeloid differentiation.

4.3 ABR as a novel inducer of myeloid transcription factor C/EBPα

It has become increasingly clear that the C/EBPα network with other proteins plays an important role not only in lineage commitment and differentiation in the hematopoietic system but also in the pathogenesis of AML. It is already known that the ABR relative BCR act as a negative regulator of the BCR-ABL oncoprotein, inhibiting leukemia formation in a CML NOD/scid mouse model (92,161). In addition, a previous study revealed that the oncoprotein BCR-ABL downregulates the expression of C/EBPα (67). The same study reported that ectopic expression of BCR-ABL in myeloid precursor cells leads to transcriptional suppression of G-CSF-R, possibly through down-modulation of C/EBPα, the master regulator of granulocytic differentiation (67). Thus, we hypothesized that the highly related ABR might be an

important player in myeloid differentiation upstream to C/EBPα. Relatively little is known about new protein players upstream to C/EBPα that might contribute to myelopoiesis. Therefore, in the present study, we used the human myeloid U937 cells as a model to identify a putative connexion between ABR and C/EBPα in ABR overexpressing cells.

C/EBPα functions as a key mediator of myelopoiesis, which is a key step disrupted in distinct subtypes of AML (65). The receptors for G-CSF (G-CSF-R) and M-CSF (M-CSF-R) as well the miR-223 are direct targets of C/EBPα (41,43,47). In the present study, we observed that several myeloid differentiation inducers lead to increased expression levels of the ABR and CEBPA targets M-CSF-R and G-CSF-R, in human and mouse myelopoiesis (Figures 12-14). Conversely, a reduction of ABR expression leads to decreased C/EBPα levels (Figures 15-16) and a minimized myeloid differentiation (Figure 17).
A previous study from our group has shown that C/EBPα acts as a tumor suppressor gene by upregulating miR-223 in normal granulopoiesis (48). In the present study we show that ABR induces transcription factor C/EBPα (Figures 19 and 20A) and thereby increases the expression of M-CSF-R, G-CSF-R and miR-223 (Figures 20B-21), suggesting that ABR expression could have a significant role in myeloid differentiation and in leukemia through induction of C/EBPα and its target genes.

In line with this, the treatment of leukemic cells with the Rac1 inhibitor NSC23766 resulted in an increased expression of CEBPA (Figure 22), suggesting that ABR might induce CEBPA expression via Rac1 repression. Those findings suggest that Rac inhibition by ABR might have an indirect impact on reducing CEBPA gene transcription. Taken together, we conclude that ABR is a biologically and functionally important and relevant player upstream to C/EBPα in myeloid differentiation.

4.4 ABR expression and the use of azacitidine for AML treatment

The cytosine analogue azacitidine is a drug used for epigenetic cancer therapies. This compound functions as a DNA methyltransferase inhibitor and has been shown to reactivate epigenetically silenced tumor suppressor genes *in vitro*. However, the mode of action of these drugs in patients is not clear and apparently clinical responses are influenced both by epigenetic alterations and by apoptosis induction

(202). Previous studies reported the use of azacitidine in chemotherapy for treatment of AML (203,204). In addition, a recent study reported that the addition of azacitidine to intensive chemotherapy for unselected older patients with AML does not appear to be beneficial (159). Our data show that response to azacitidine treatment is associated with high ABR expression in elderly patients with AML (Figure 23). These data highlight the role of ABR in therapy response and suggest that ABR expression pattern might be a possible reason why some patients respond better to azacitidine therapy while others do not.

Additionally, we could show that ABR stable overexpression enhances azacitidine-induced apoptosis (Figure 26). This result is supported by a previous study which shows that the inactivation of Rac1 in leukemia increases chemotherapy-induced apoptosis (129,151), supporting a possible mechanism of ABR action in AML therapy via inhibition of Rac1. In addition, another study reveals that a block of ABR expression inhibited apoptosis and promoted colony formation from dissociated human embryonic stem cells (107). In addition, the same study reveals that ABR GAP domain plays a role for induction of apoptosis in human embryonic stem cells, underlining the importance of ABR-mediated Rac inhibition for the induction of apoptotic process (107).

The promoter methylation of genes closely associated with myeloid differentiation results in a significant blockade of cell differentiation. Besides genetic aberrations, epigenetic modifications, such as DNA methylation, have been shown to initiate or increase malignant transformation (205–207). Further, DNA methylation is frequently abnormal in AML as examined by previous studies of individual genes and genome wide (206,208–212). Thus, the identification of hypermethylated genes that become demethylated and reactivated by drug treatment and the establishment of statistically robust associations between epigenetic reactivation events and patient responses represent an important research area. Therefore, we analysed the mRNA expression of ABR and CEBPA after azacitidine treatment. We could show that azacitidine treatment increased the expression levels of ABR and CEBPA in U937 cells (Figure 25), suggesting that a potential epigenetic alteration could be responsible for downregulation of ABR in AML. Therefore, to determine if aberrant DNA promoter methylation affects ABR expression, we utilized the dataset from The Cancer Genome Atlas Research Network (TCGA), in which the genome-wide methylation

status is available (213). Surprisingly, we found ABR promoter region is not methylated in both low and high ABR expressing patients, while a large region of the open reading frame is methylated in both groups (Figure 27). Thus, the downregulation of ABR in AML is probably not caused by DNA methylation of the ABR promoter. Methylation of CpG islands spanning promoter regions is associated with control of gene expression. However, it is considered that methylation of exonic CpG islands is not related to gene expression, because such exonic CpG islands are usually distant from the promoter. In contrast, previous studies have shown that methylation of a transcribed portion of CpG-rich non-mammalian gene locating near a promoter can reduce mammalian gene promoter activity (214–216).

Previous studies in mouse and human leukemic cells demonstrated that azacitidine incorporated into RNA to a larger extent than into DNA (162,163). In a mouse leukemia cell line L1210, Li et al. (162) demonstrated that AZA incorporated into RNA and DNA at a ratio of approximately 85:15, respectively. In addition, a previous report revealed that methylation status of promoter regions of CEBPA did not correlate with the CEBPA expression level in azacitidine-treated MLL-rearranged AML cell line THP-1, suggesting that azacitidine did not induce C/EBPα expression by enhancing the demethylation of the CEBPA promoter (164). Moreover, it should be highlighted that a large number of genetic pathways are likely to be affected by epigenetic therapies, thereby making the effect of their deregulation difficult to predict.

The DNA methylation status of C/EBPα core promoter has been previously reported as a rare event in AML (74,217). However, aberrant DNA methylation has been found in the most upstream promoter region of C/EBPα, which has been found to be densely methylated in 51% of AML patients (167). This aberrant DNA methylation was strongly associated with two favorable cytogenetic subgroups: inv(16) and t(15;17) (167). Surprisingly, while epigenetic treatment with DNA-demethylating agent azacitidine increased C/EBPα mRNA levels in leukemia cell lines, C/EBPα protein levels were decreased by miRNA-124, which is frequently silenced by epigenetic mechanisms in leukemia cell lines, becomes upregulated after epigenetic treatment, and targets the C/EBPα 3´untranslated region (167). It is already shown that C/EBPα is post-transcriptionally downregulated in AML by fusion proteins (62,67–72) or miRNAs (47–50,167,218). Thus, our findings suggest ABR downregulation in AML AML is not due to methylation. We speculate that ABR repression in AML might

occur in a post-translational manner, through expression of constitutively active Rac previously reported in AML (147), which might cause displacement of proteins bound to RhoGDI, possibly ABR (Figure 8), resulting in their degradation (198).

4.5 Outlook

In the present dissertation, we could assign a role for ABR in myelopoiesis and in AML. The functional relevance of ABR in leukemia was demonstrated by the identification of ABR as a new player upstream of C/EBPα in myeloid differentiation. In addition to the findings, there are still questions to be clarified regarding the mechanism by which inhibition of Rac1 by ABR might impact CEBPA gene transcription. Rac1 is involved in regulating the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) (219), a transcription factor activated by tyrosine kinases through several mechanisms (220). In acute myeloid leukemia, constitutive activation of STAT pathway has been reported in various studies (219,221,222). In addition, phosphorylation of STAT3 results in the induction of c-myc proto-oncogene product (Myc) (219). Several studies have shown that transcription factor Myc is able to negatively regulate C/EBPα expression and block C/EBPα transactivation function (223–225). Consistently with those findings, Rac1 inhibition with siRNA or Rac1 inhibitor NSC23766 has been shown to repress Myc transcription (226). It is therefore tempting to speculate that ABR-mediated Rac inhibition might induce CEBPA gene transcription via Myc downregulation.

The findings on the functional relevance of ABR in myeloid differentiation and AML provide initial indications that ABR might function as tumor suppressor in myeloid cells. In addition to the studies carried out within the scope of this thesis, no further studies have been carried out with ABR in the hematopoietic system. However, evidence for a putative tumor suppressor role of ABR has been reported in various malignant tissues (81,83,84).

Further mechanisms might cooperate with ABR during activation of C/EBPα in myelopoiesis. ABR and the tumor suppressor P53 are located toward the telomeric end of chromosome 17p, band p13 (Figure 4) (84). The P53 protein functions as a transcription factor, regulating downstream genes involved in cell cycle arrest, DNA

repair and programmed cell death (227–229). Deletion of 17p including ABR locus (81,82) was consistent with mutation of the P53 tumor suppressor gene in medulloblastoma and breast cancer (85,86). In addition, the loss of these markers from 17p13.3-ter region (Figure 4) was associated with worst outcome in breast cancer (84). In CML, the Ph chromosome t(9;22) is associated with aberrant translocations involving the band 17p13 as one of the breakpoint sites: t(17;22) and t(9;17) (78,79). Moreover, a gene rearrangement have already been reported in respect to chronic myeloid leukemia (CML), including the nuclear protein P53, located at 17p13 (Figure 4), and involved in up to 30% of CML cases in blast crisis (80). The progression of CML from the chronic to the acute phase involves frequent aberrations in chromosome 17, to which ABR and p53 have been mapped (80).

P53 mutations are closely related to older age and 17p deletion in AML through unbalanced translocations such as t(5;17) (230,231). P53 gene mutations are found in less than 10% of *de novo* AML (230,232). However, patients with P53 mutation are generally resistant to chemotherapy and have very short survival (233). A strong correlation between P53 mutations associated with loss of heterozygosity (LOH) and a typical form of dysgranulopoiesis has been reported in AML and myelodysplastic syndrome (234,235). Detection of P53 mutations is important because, in patients with LOH, response to chemotherapy will be extremely short and, in those cases, allogenic bone marrow transplantation should be considered (231). Loss of P53 function confers genomic instability, impaired apoptosis and diminished cell cycle restraint (236–241).

A previous study indicates that CEBPA gene transcription can be directly activated by the tumor suppressor P53 and myeloid transcription factor Krüppel-like factor 4 (KLF4), suggesting a p53-KLF4-CEBPA axis. In AML patient cells, the incidence of p53 mutations is low; however, suppression of P53 function is a frequent event (242) with concomitant reduction of KLF4 and CEBPA protein levels (243). Inactivation of the p53–KLF4–CEBPA axis in leukemic cell leads to loss of cell-cycle control, inadequate response to apoptotic signals and loss of myeloid differentiation (243).

P53 deficiency has been shown to increase Rac1 activity in primary mouse embryonic fibroblasts dependent on elevated phosphatidylinositide 3-kinase activity, and this collaboration is sufficient to promote proliferation and transformation in these cells (244). In addition, a block of ABR target Rac1 by expressing the dominant-

negative Rac1 or using the Rac1 inhibitor NSC23766 in lymphoma cells was able to abrogate the hyperproliferative phenotype of P53-deficient lymphoma cells to an extent that is similar to reconstitution of functional P53 (245). Thus, elevated Rac1 activity in P53-defective lymphocytes may act cooperatively with P53 deficiency to promote cell-cycle progression and survival in B- and T-cell transformation (245).

Further, *in vivo* xenografts displayed decreased lymphoma development when Rac1 was suppressed (245). Those data suggest that ABR might interfere with the proliferative phenotype of P53 deficient AML patients. Inactivation of P53 function including other mechanisms than rare mutations is a frequent event in patients with cytogenetically normal AML and APL (242,246). In this context, further functional studies would have to be carried out in order to show a possible regulation of ABR in these patients.

The functional investigations carried out within the scope of the dissertation were carried out exclusively *in vitro*. Thus the investigation of the ABR effects in an AML mouse model would be of great interest for the detection of the biological relevance of the ABR. Here we show that ABR overexpression sensitized leukemic U937 cells to azacitidine therapy, leading to increased apoptosis of those cells. Thus, ABR could delay the outbreak of the disease in leukemic mice treated with azacitidine and contribute for the development of less invasive treatment approaches for the treatment of AML.

5 Summary

Acute myeloid leukemia (AML) is a phenotypically and genetically heterogeneous disease with great variability in response to therapy and poor outcome in adults. AML involves activation of oncogenes or deactivation of tumor suppressor genes, and a block of differentiation caused by reduced function of transcription factors. The transcription factor CCAAT / enhancer binding protein alpha (C/EBPα) is a major regulator in granulopoiesis and often disrupted in AML. In almost 50% of all AMLs, C/EBPα is functionally disrupted by various mechanisms including its own mutations.

ABR (Active BCR related) protein deactivates Rac1 (ras-related C3 botulinum toxin substrate 1), which plays an essential role in regulating normal hematopoiesis and in leukemia. BCR gene, closely related to ABR, acts as a tumor suppressor in chronic myeloid leukemia and has overlapping functions with ABR. Evidence for a putative tumor suppressor role of ABR has been shown in several solid tumors, in which deletion of ABR is present.

In the present work ABR was identified as a new player upstream to C/EBPα during myelopoiesis. Overexpression of ABR leads to an induction of transcription factor C/EBPα, and thereby increases the expression of M-CSF-R, G-CSF-R and miR-223, suggesting that ABR expression could have a significant role in myeloid differentiation and in leukemia through induction of CEBPA and its target genes. In the investigation of the importance of ABR for leukemogenesis, reduced expression levels of ABR were found in AML among different subtypes. Furthermore, a block of ABR leads to a reduction of myeloid differentiation and a repression of the myeloid transcription factor C/EBPα. Conversely, stable overexpression of ABR enhances myeloid differentiation. Inactivation of the known ABR target Rac1 by treatment with the Rac1 inhibitor NSC23766 resulted in an increased expression of C/EBPα. For clinical therapy of AML the DNA methyltransferase inhibitor azacitidine is used. . The studies show that the response of patients to azacitidine therapy correlates with a significantly higher ABR expression. A significant longer overall survival of AML patients was associated with higher ABR expression levels. This is the first report showing that ABR expression plays a critical role in both myelopoiesis and AML. These data indicate the tumor suppressor potential of ABR and underline its potential role in leukemia therapeutic strategies.

6 Zusammenfassung

Die Akute myeloische Leukämie (AML) ist eine phänotypisch und genetisch heterogene Erkrankung mit einer sehr variablen Reaktion auf Therapie und schlechter Prognose bei Erwachsenen. Bei der AML werden Onkogene aktiviert, Tumorsuppressorgene deaktiviert und die Differenzierung aufgrund reduzierter Funktion von Transkriptionsfaktoren blockiert. Der Transkriptionsfaktor CCAAT / enhancer binding protein alpha (C/EBPα) ist ein wichtiger Regulator in der Granulopoese und bei AML oft dereguliert. In fast 50% aller AMLs wird C/EBPα durch verschiedene Mechanismen einschließlich seiner eigenen Mutationen herunterreguliert.

Das ABR (Active BCR Related) - Protein deaktiviert Rac1 (ras-related C3 botulinum toxin substrate 1), welches eine wesentliche Rolle bei der Regulierung der normalen Hämatopoese und bei der Leukämie spielt. Das eng mit ABR verwandte BCR-Gen wirkt als Tumorsuppressor bei chronischer myeloischer Leukämie und hat funktionale Gemeinsamkeiten mit ABR. Eine vermutliche Tumorsuppressor-Rolle von ABR wurde in mehreren soliden Tumoren mit ABR-Deletion gezeigt.

In der vorliegenden Arbeit konnte ABR als ein neuer Mitspieler von C/EBPα während der Myelopoese identifiziert werden. Es zeigte sich, dass ABR den Transkriptionsfaktor C/EBPα induziert und dadurch die Expression von M-CSF-R, G-CSF-R und miR-223 erhöht. Dies deutet darauf hin, dass die Expression von ABR eine bedeutende Rolle bei der myeloiden Differenzierung und Leukämie durch die Induktion von CEBPA und seinen Zielgenen spielen könnte. Untersuchungen zur Bedeutung von ABR für die Leukämogenese zeigten in verschiedenen Subtypen der AML durchgängig ein vermindertes Expressionsniveau von ABR. Weiterhin konnte nachgewiesen werden, dass der Block von ABR zu einer Reduktion der myeloiden Differenzierung sowie Repression des myeloiden Transkriptionsfaktors C/EBPα bewirkt. Im umgekehrten Fall führt eine stabile Überexpression von ABR zur einer gesteigerten myeloiden Differenzierung. Die Inaktivierung des bekannten ABR-Ziels Rac1 durch die Behandlung mit dem Rac1-Inhibitor NSC23766 erhöht die Expression von C/EBPα. In der klinischen Therapie von AML wird der DNA-Methyltransferase-Inhibitor Azacitidin eingesetzt. Die Untersuchungen zeigen, dass die Reaktion von Patienten auf die Azacitidin-Therapie mit einem signifikant höheren ABR-Expression korreliert. Die Ergebnisse dieser Arbeit unterstreichen zudem den Zusammenhang

zwischen signifikant längerem Gesamtüberleben der AML-Patienten und höheren ABR-Expressionsniveaus. In der vorliegenden Arbeit konnte somit erstmals eine entscheidende Rolle der Expression von ABR in der Myelopoese und AML nachgewiesen werden. Diese Ergebnisse zeigen das Tumorsuppressorpotential von ABR und unterstreichen seine mögliche Rolle in Strategien der Leukämie-Therapie.

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Selbstständigkeitserklärung

Diese Dissertation ist von der Naturwissenschaftlichen Fakultät I - Biowissenschaften der Martin- Luther-Universität Halle-Wittenberg genehmigt worden.

Hiermit erkläre ich, Carolina Yaeko Namasu, dass ich die vorliegende Dissertation selbständig und ohne unzulässige Hilfe Dritter verfasst habe und keine anderen, als die angegebenen Quellen und Hilfsmittel verwendet habe. Die aus fremden Quellen übernommenen Gedanken oder Graphiken sind unter Angabe der Quelle gekennzeichnet.

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Halle (Saale), 19.07.2017 Carolina Yaeko Namasu

Erklärung über bisherige Promotionsversuche

Die vorliegende Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt. Ich erkläre, dass ich keine früheren Promotionsversuche unternommen habe und dass an keiner anderen Universität ein Promotionsverfahren anhängig ist.

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Publications

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2. Hartmann JU, Bräuer-Hartmann D, Wurm AA, Kardosova M, **Namasu CY**, Gerloff D, Katzerke C,, Bill M, Schwind S, Müller-Tidow C, Niederwieser D, Alberich-Jorda M, Behre G. MiR-143 targets ERK5 in granulopoiesis and predicts outcome of patients with acute myeloid leukemia. Manuscript under review.

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