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The Death-Associated Protein Kinase 1 (DAPK1) – prognostic relevance in pediatric acute lymphoblastic leukemia (ALL) and evaluation as a therapeutic target

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Abbreviations

AIEOP-BFM	Associazione Italiana Ematologia ed Oncologia Pediatrica and Berlin-Frankfurt-Münster study group
AIM-V	serum-free T-cell media
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myelogenous Leukemia
API	common mouse housekeeping gene
BCA	Bicinchoninic acid
Bcl-2	B-Cell Lymphoma 2 (apoptosis related protein)
BCP	B-Cell Precursor
BCR/ABL	Breakpoint Cluster Region/ Abelson – a tyrosine kinase encoded by a fusion gene expressed from translocation t(9;22)(Philadelphia Chromosome)
BSA	Bovine Serum Albumin
Caspase	cysteinyl-aspartate specific protease
CD	Cluster of Differentiation
cDNA	complementary DNA
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myelogenous Leukemia
Cp	Crossing point (qRT-PCR)
CpG	Cysteine-phosphate-Guanine (CpG-island)
CRAC	Cytochrome c Related Activation of Caspases
C_t	Cycle threshold (qRT-PCR)
DAPK1	Death-Associated Protein Kinase 1
DEC	Decitabine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Di-Methyl-Sulf-Oxide
DNA	Desoxyribo-Nucleic-Acid
DNAse	Enzyme that cleaves DNA
DNMT	DNA-Methyltransferase
Doxo	Doxorubicin
DTT	Dithiothreitol

ECL	Enhancing Chemiluminescence
EDTA	Ethylenediaminotetraacetic Acid
ERK	Extracellular signaling Related Kinase
ETV6-RUNX1	previous known as TEL/AML1, a fusion gene expressed from a t(12;21) chromosomal translocation
FAB	French-American-British, Classification system for hematologic diseases
FACS	Fluorescence-Activated Cell Sorting
Fas	Ligand or Receptor associated to TNF-mediated apoptosis
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FSC/SSC	forward scatter/ side scatter
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hank's Balanced Salt Solution
HCC	Hepatocellular Carcinoma
HDAC	Histone Deacetylase
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonacid (buffer substance)
HR	High Risk (ALL risk stratification)
HRP	Horseradish Peroxidase
huALL	human Acute Lymphoblastic Leukemia
huCD	human Cluster of Differentiation
IF-gamma	Interferon gamma
MAD	Mothers Against Decapentaplegic
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time Of Flight; a method of analysis in mass-spectrometry
MDS	Myelodysplastic Syndrome
MEM-alpha	Minimum Essential Medium Eagle-alpha
MES	2-(N-Morpholino)ethansulfonacid
MLC	Myosin Light Chain
MLL	Mixed Lineage Leukemia

MOPC-21	Mouse IgG1 kappa isotype control antibody
MR	Medium Risk (ALL risk stratification)
MRD	Minimal Residual Disease
MS-5	Mouse Stromal 5 (cell line)
MSP	Methylation Specific PCR
n	Quantity (n = number)
NOD	Non Obese Diabetes
NSCLC	Non Small-Cell Lung Cancer
p-value	probability
p53	Protein 53
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PAGE	PolyAcrylamide Gel Electrophoresis
PE	Phyco-Erythrine
PI	Propidium-Iodide
PMSF	Phenylmethylsulfonylfluoride
PR	Prednisone Response
PS	Phosphatidyl-Serine
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
r.p.m.	rounds per minute
RIP-Kinase	Receptor-Interacting Protein kinase
RNA	Ribonucleic acid
RNAse	Enzyme that cleaves RNA
ROS	Reactive Oxygen Species
RPMI 1640	Roswell Park Memorial Institute medium
r_s	Spearman's Rho (correlation coefficient)
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCID	Severe Combined Immunodeficiency
SD	Standard Deviation
SDS	Sodium Dodecyl-Sulfate
Ser/Thr-Kinase	Serine/Threonine Kinase
SMA	Small Body Size

SMAD	SMA + MAD
SPI	Compound used for protein lysis buffer
SR	Standard Risk (ALL risk stratification)
SYBR® - Green I	Synergy Brands (Stock Symbol) - Green I
TGF beta	Transforming Growth Factor beta
TKI	Tyrosine Kinase Inhibitor
TNF	Tumor Necrosis Factor
TRAIL	Tumor Necrosis Factor Related Apoptosis Inducing Ligand
TTL	Time To Leukemia
WHO	World Health Organization
XIAP	X-linked inhibitor of apoptosis protein
zVAD.fmk	Carbobenzoxy-L-valyl-L-alanyl-β-Methyl-L-aspart-1-yl-Fluoromethan

1. Introduction

1.1 Cancer

In 2012, worldwide leading causes of death were non-communicable diseases, like cardiovascular diseases, cancers, diabetes and chronic lung diseases accounting 68 % of overall cases. Based on this, cancer is with 14 million newly diagnosed cases and 8.2 million cancer-related deaths one of the leading causes of mortality and morbidity in the world (WHO 2016). Furthermore the World Health Organization (WHO) expects the number of new cases will rise by about 70 % over the future 20 years (WHO 2015).

Cancer can arise in nearly every tissue and affect every organ in the human body, which results in a wide spectrum of different tumor subtypes with independent underlying processes of tumor formation. Tumor development is characterized by accumulation of consecutive gene mutations (Nordling C. O. 1953) whereas Knudson postulated in 1971 that two independent mutation events, either of germline or somatic origin, are needed to convert healthy into carcinogenic cells (Knudson, JR 1971). These events either lead to gain of function products (proto-oncogenes) resulting in increased cell proliferation or in loss of function products of tumor suppressors which further promote dysregulation of cell cycle processes or cell death evading mechanisms (Croce 2008; Hanahan, Weinberg 2000; Sherr 2004). These mutations can be either inherited when they affect the reproductive cells or occur in somatic cells as an acquired mutation (Lichtenstein et al. 2000; Peto 2001). A great number of these acquired mutations occur spontaneously or were induced upon environmental factors like chemicals, radiation or intracellular ROS-species (Martincorena, Campbell 2015). Impaired DNA-repair mechanisms or mutations that remain undetected, subsequently have an impact on the DNA structure, resulting in either chromosomal changes or an altered gene sequence and lead to different protein formation with a gain or loss in protein function (Martincorena, Campbell 2015; Weinberg 2007).

1.2 Leukemia

“White blood” – when Rudolph Virchow described a patient’s blood sample in 1845 and observed a high number of white blood cells he created the terminus “leukemia” (from Ancient Greek words *leukos* for “white” and *haima* for “blood”).

Leukemias are tumors of the hematopoietic system and as a non-solid tumor they originate from hematopoietic progenitor cells in the bone marrow (Aster 2007; Pui et al. 2004).

With 80 % of all leukemic cancers, especially the acute lymphoblastic leukemia (ALL) is the most common leukemia in childhood and also the most frequent cancer in children and adolescents with the second highest number of cancer related deaths (Pui, Evans 1998; Siegel et al. 2016). Improvements of treatment regimen, supportive care and risk stratification increased cure rates of pediatric ALL over the past decades to more than 80 % (American Cancer Society 2016 a; Hunger et al. 2012; Moricke et al. 2010; Siegel et al. 2016). However, many patients encounter relapse of their disease which is associated with a poor outcome (Henze et al. 1991; Irving 2016; Malempati et al. 2007), even when risk stratification according to minimal residual disease (MRD) was assessed before (Conter et al. 2010; Schrappe et al. 2000; van Dongen et al. 1998). These patients are hard to treat because cells of relapsed leukemia cases evolved an intrinsic chemoresistance, which is not present when compared to the corresponding original diagnosed leukemia (Klumper et al. 1995; Pogorzala et al. 2015). Therefore, it is necessary to develop new approaches which allow identification of high risk patients in order to improve their therapy leading to increased patient outcome and survival.

Underlying processes that lead to pediatric ALL are manifold, while transformation of hematopoietic progenitor cells is mainly driven by inherited and environmental factors (American Cancer Society 2016 b; Greaves 1997). For example, exposure to radiation or distinct chemical compounds like benzenes can contribute to leukemogenesis, but also infections were discussed to be associated with a higher risk to develop leukemia (Greaves 1997; Kinlen 1995; Lamm et al. 1989; Preston et al. 1994). Moreover, inherited syndromes like Down syndrome or neuro-fibromatosis seem to increase the risk to develop ALL (Fong, Brodeur 1987; Moriyama et al. 2015) as well as maternal exposure to different toxins during pregnancy seems to raise the risk of MLL-rearranged pediatric ALL (Alexander et al. 2001).

“The principal pathogenetic problem in acute leukemia is a block in differentiation” (quoted verbatim: (Kumar et al. 2007)). This is caused by dysregulation of growth and anti-growth signals and mechanisms to evade apoptosis which lead to a

limitless reproductive potential of these immature leukemic blasts and their subsequent infiltration into the bone marrow (Hanahan, Weinberg 2011; Yokota 2000). Hence, the production of normal hematopoietic stem cells is suppressed by the subsequent abnormal high accumulation of immature leukemic blasts leading to a variety of different symptoms a leukemia bearing patient may present (Kumar et al. 2007). Whereas leukopenia induces bacterial infections like oral ulcer, infected tonsils or even pneumonia, the impaired production of erythrocytes causes anemia with paleness, dyspnea and fatigue. Furthermore, thrombocytopenia leads to impaired coagulation with petechia and also meninges, testes and lymph nodes might be infiltrated by the leukemic blasts (Esparza, Sakamoto 2005; Herold 2014).

To confirm diagnosis of a possible leukemia, bone marrow aspiration is performed and the subsequent verification of more than 25 % of lymphoblasts out of all nucleated cells verifies the disease (Vardiman et al. 2009). Further microscopic examination of bone marrow smears according to the French-American-British (FAB) classification, allows to classify acute lymphoblastic leukemia into three groups (L1 – L3) (Bennett et al. 1976, 1989). Immunophenotyping by fluorescence-activated cell sorting (FACS) then allows detailed characterization of B- or T- subtypes, as well as their sub-classification (Bene et al. 1995).

As genetic abnormalities are frequent in pediatric ALL, they are used for risk stratification and prognosis estimation (Armstrong, Look 2005; Pui, Evans 2006) and intensive research within the last years identified that the occurrence of genetic abnormalities can be used for diagnosis and classification of ALL patients (Arber et al. 2016; Vardiman et al. 2009). For instance, numeric and structural cytogenetic alterations resulting in an unfavorable prognosis are hypodiploidy or rearrangements of the MLL-gene (Behm et al. 1996; Pui et al. 1987). Furthermore, the chromosomal translocation t(9;22), generating the fusion protein BCR-ABL (Philadelphia-Chromosome), leads to poor prognosis, but treatment with Tyrosine-Kinase-Inhibitor Imatinib improved patient outcome over the last years (Schlieben et al. 1996; Schultz et al. 2009). On the contrary, hyperdiploidy and the translocation t(12;21), that is the most common genetic lesion in ALL and results in fusion protein ETV6-RUNX1 (former TEL-AML1) is associated with a favorable prognosis (Pui et al. 2004; Shurtleff et al. 1995; Zelent et al. 2004). Another prognostic factor is the response to prednisone, estimated on day eight of the

treatment initiation phase by analyzing white blood cell counts (prednisone response = PR) (Dordelmann et al. 1999). ALL treatment performed according to the AEIOP-BFM ALL 2000 clinical trial (**A**ssociazione **I**taliana **E**matologia ed **O**ncologia **P**ediatrica – **B**erlin **F**rankfurt **M**ünster – **A**cute **L**ymphoblastic **L**eukemia 2000), comprises risk stratification classifying patients into three different groups – standard risk (SR), medium risk (MR) and high risk (HR), based on their prednisone response and the occurrence of translocations t(4;11) and t(9;22) (Conter et al. 2010). Additionally, minimal residual disease (MRD) is defined by the amount of remaining ALL cells detected on submicroscopic level by PCR analyzing IgG/TCR rearrangements and is estimated for every patient (van Dongen et al. 1998). But even risk stratification according to MRD is not sufficient to securely detect patients with a high risk for relapse, which would allow to intensify therapy already during the initial phase of their treatment (Conter et al. 2010; Schrappe et al. 2000). Therefore, new diagnostic and therapeutic approaches are necessary to understand the underlying altered biology and pathobiology of pediatric ALL in order to allow the identification of high risk and early-relapse patients.

1.3 The NOD/SCID/huALL mouse model

In our research group a xenograft mouse model was established that detected different engraftment properties of pediatric ALL samples after transplanting primary patient material onto NOD/SCID-mice (non-obese diabetes/ severe combined immunodeficiency). It was shown, that “time to leukemia” (TTL), which describes the time from transplantation until the occurrence of first leukemia symptoms in the mice is associated with the outcome of the respective patient (Meyer et al. 2011). Leukemia samples that manifested within ten weeks were classified into the TTL^{short}-group, samples of the TTL^{long} group were characterized by the engraftment time of more than ten weeks. Moreover, there was a remarkable association between TTL^{short} and patients who encounter an early relapse which is associated with a poor outcome, whereas TTL^{long} appeared to be associated with a more favorable outcome (Meyer et al. 2011).

1.4 DAPK1

Gene expressing profiling performed in leukemia cells showing distinct engraftment in the NOD/SCID/huALL mouse model, revealed that particular molecules involved in cell death/survival pathways were significantly differently regulated when the TTL^{short} and TTL^{long} phenotype was compared (Meyer et al. 2011). Interestingly, the 'Death-Associated Protein Kinase 1-gene' (*DAPK1*-gene) was identified to be significantly downregulated in the TTL^{short}/early relapse group (Meyer et al. 2011).

DAPK1 is the most prominent member belonging to a family of Serine/Threonine (Ser/Thr)-kinases, involved in cell death associated mechanisms (Bialik, Kimchi 2006; Deiss et al. 1995). With a size of about 160 kDa, DAPK1 is a multi-domain protein, consisting out of several regions playing different roles in regulation processes and execution of its pro-apoptotic and anti-metastatic functions (Bialik, Kimchi 2006; Cohen et al. 1997; Inbal et al. 1997; Singh et al. 2016). In more detail, DAPK1 has a conserved catalytic/kinase-domain, which is regulated by an autoregulatory Ca^{2+}/Calmodulin-dependent-domain and accounts for phosphorylation of different substrates like Myosin-Light-Chain (MLC) (membrane blebbing via actin activation), Syntaxin-1 A (vesicle/membrane fusion) or even promotes autophosphorylation (Cohen et al. 1997; Bialik, Kimchi 2004; Bialik, Kimchi 2006; Shohat et al. 2001). Furthermore, adherence to actin filaments of the cytoskeleton is provided by the "cytoskeletal binding region" and a region containing several Ankyrin-repeats is involved in proper DAPK1 localization and its degradation (Bialik et al. 2004; Bialik, Kimchi 2006; Nair et al. 2013; Singh et al. 2016). Importantly, DAPK1 exhibits a death-domain, that is described to promote protein-protein interactions, as well as influence of kinase activity and apoptotic functions (Singh et al. 2016). Furthermore, bidirectional interactions with ERK (**E**xtracellular-signal-**R**egulated-**K**inase) are shown to be associated with apoptotic effects of DAPK1 (Chen et al. 2005). Moreover, DAPK1 was also described to play a role in cell death induction via autophagy while it is involved in formation of autophagic vesicles (Inbal et al. 2002; Levin-Salomon et al. 2014; Singh et al. 2016; Zalckvar et al. 2009).

Furthermore, regulation of DAPK1 is controlled by several different signals which influence either *DAPK1* transcription or the activity of its kinase-domain or death-domain (Bialik, Kimchi 2006; Singh et al. 2016). Upregulated *DAPK1* gene

expression can be induced in response to SMAD-mediated signaling via the TGF-beta pathway as well as upon activation of p53 due to DNA-damage or oncogene expression (Jang et al. 2002; Martoriati et al. 2005; Raveh, Kimchi 2001). Furthermore, several mechanisms trigger activity of DAPK1 protein like Interferon-gamma (IF-gamma) or Ceramide-6 (Deiss et al. 1995; Pelled et al. 2002). Since DAPK1 is a multifunctional protein that participates in a wide network of cell death/survival-pathways, several interactions of DAPK1 with other molecules have not been clarified yet and require more research. DAPK1 gains also clinical importance as its downregulation is described in several cancer types, like head-neck-cancers, non-small-cell-lung-cancer as well as hematopoietic malignancies (Kim et al. 2001; Kissil et al. 1997; Li et al. 2015; Matsumoto et al. 2003; Wei et al. 2015) characterizing DAPK1 as a potential tumor suppressor gene (Raveh, Kimchi 2001) and therefore a potential target for cancer therapy.

1.5 Programmed cell death

There are several types of cell death a cell can undergo, either induced by specific signaling events or by massive cell damage (Green, Llambi 2015). Necrosis, induced after trauma or infection, promotes cell swelling, plasma leakage and enzymatic digestion of cell organelles then causing a strong immune response of the surrounding tissue (Kumar et al. 2007; Yuan, Kroemer 2010). On the contrary, apoptosis, a controlled cell death program, plays an important role during embryonic development and cell-homeostasis in multicellular organisms. Furthermore, apoptosis has crucial importance in the regulation of pathologic states like DNA damage and cell injury, whereas dysregulation of apoptosis leads to tumor formation and cancer development (Alberts 2008; Brill et al. 1999; Kerr et al. 1972). Apoptosis can be induced by a wide spectrum of stimuli and finally leads to blebbing, cell shrinkage and nuclear fragmentation with DNA degradation (Elmore 2007). Upon extracellular signaling through death receptor ligands like Fas/CD95 or TNF-alpha, the extrinsic pathway is activated and initiates a signaling cascade (Ashkenazi 1998; Gaur, Aggarwal 2003). Furthermore, the intrinsic or mitochondrial pathway can be activated upon cell stress like DNA damage or deprivation of oxygen, which increases membrane permeability of the mitochondria and causes the subsequent release of cytochrome c into the cytosol

(Cai et al. 1998; Liu et al. 1996; Green, Kroemer 2004; Green, Reed 1998). Finally, both pathways result in the activation of caspases–(**c**ysteine-**asp**artic-prote**ases**) (Thornberry, Lazebnik 1998). These proteolytic enzymes ensure the correct degradation of cell organelles and the disassembly of the cell with minimal effects for the surrounding tissue (Goodsell 2000; Shalini et al. 2015).

Autophagy is a regulated process that enables the cell to remove and recycle unnecessary components engulfing them in two-membraned autophagosomes, which then fuse with lysosomes (Kroemer et al. 2009). In general, the process of autophagy is known to be a survival mechanism, but recent investigation identified autophagy to be involved in programmed cell death, proclaiming autophagic cell death as an alternative to apoptotic cell death (Shimizu et al. 2004; Tower 2015; Tsujimoto, Shimizu 2005). Finally, there are several additional variants of cell death programs where signaling pathways of apoptosis, autophagy and necrosis interconnect. For example, necroptosis involves the TNF-alpha mediated activation of RIP-kinases (**R**eceptor-**I**nteracting-**P**rotein-Kinase) which subsequently form the necrosome, leading to a regulated form of necrosis (Green, Llambi 2015; Hirsch et al. 1997; Vanden Berghe et al. 2014). In leukemia, several proteins involved in cell death programs show expression level alterations, representing potential targets to create new diagnostic and therapeutic approaches. For instance, increased expression levels of the pro-apoptotic protein BAX are associated with favorable prognosis in AML (Ong et al. 2000), whereas expression levels of the anti-apoptotic protein BCL-2 were identified to be associated with responsiveness to induction therapy in ALL (Aref et al. 2004; Kaparou et al. 2013;) and inhibitors of BCL-2 were found to induce apoptosis in CLL cells (Anderson et al. 2016).

1.6 Epigenetic regulation: DNA methylation

DNA methylation is a reversible epigenetic modification where methyl-groups are transferred onto Cytosine-residues in CpG-Islands (CpG = Cytosine and Guanine rich areas of the DNA) of the nucleotide sequence. These CpG-Islands are mostly localized in promotor-regions of distinctive genes and results in reversible repression of gene-transcription (Jones, Baylin 2002). Therefore, methylation can influence gene expression levels independent of their nucleotide sequence or their

rearrangements like DNA-deletions or -mutations (Bird 2002). In processes like aging, X-chromosome-inactivation and genomic imprinting, DNA-methylation plays a fundamental role (Johnson et al. 2012; Li et al. 1993; Sharp et al. 2011). Furthermore, epigenetic silencing via hypermethylation is a common factor in tumorigenesis of several malignancies (Burke et al. 2014; Esteller et al. 2000; Herman, Baylin 2003; Jones, Baylin 2002; Trojan et al. 2000). It can induce transcription repression of tumor suppressor genes and is in general well described to regulate gene expression of molecules involved in tumorigenesis associated cell death/survival pathways (Esteller et al. 1999; Roman-Gomez et al. 2003).

Especially DAPK1 has been described to be downregulated by epigenetic silencing in several malignancies (Katzenellenbogen et al. 1999; Kissil et al. 1997). Interestingly, in Chronic Lymphatic Leukemia (CLL) epigenetic downregulation of DAPK1 via promoter hypermethylation has been identified as a possible predisposition for CLL, when CLL-cells were compared to healthy lymphocytes (Debatin 2007; Raval et al. 2007).

DNA-methylation is controlled by the enzyme DNA-Methyltransferase (DNMT), which transfers methyl-groups from a donor-S-Adenylmethionine to a Cytosine located in a CpG-Island (Herman, Baylin 2003; Oki et al. 2007). Inhibitors of DNMTs are pyrimidine-analogs like 5-Aza-2-Hydroxy-Cytidine (Decitabine). Activated Decitabine is incorporated into the DNA and through covalent and irreversible binding to DNMT, Decitabine induces DNA hypomethylation during subsequent DNA methylation rounds (Oki et al. 2007) (Figure 1). Hence, Decitabine has been identified to reestablish epigenetic silenced tumor suppressors, therefore providing potential anti-cancer activity (Karpf et al. 2001). However, Decitabine has also cytotoxic effects with inducing cell-cycle arrest, especially at higher doses (Kantarjian, Issa 2005; Liu et al. 2011; Oki et al. 2007). Furthermore, Decitabine has been described to significantly effect and suppress tumor activity of hematopoietic malignancies. In particular, an antileukemic activity of Decitabine but also another methylation modifying drug 5-Azacytidine, could be demonstrated in AML and ALL (Karon et al. 1973; Momparler et al. 1985). However, Decitabine was just less effective in solid tumors and requires more research on this field (Aparicio, Weber 2002; Nie et al. 2014).

Moreover, Decitabine improved survival rates of patients suffering from

myelodysplastic syndrome and chronic myelogenous leukemia (Kantarjian et al. 2003; Kantarjian et al. 2006) and is further used in treatment protocols for AML in patients elder than 60 years (Nieto et al. 2016).

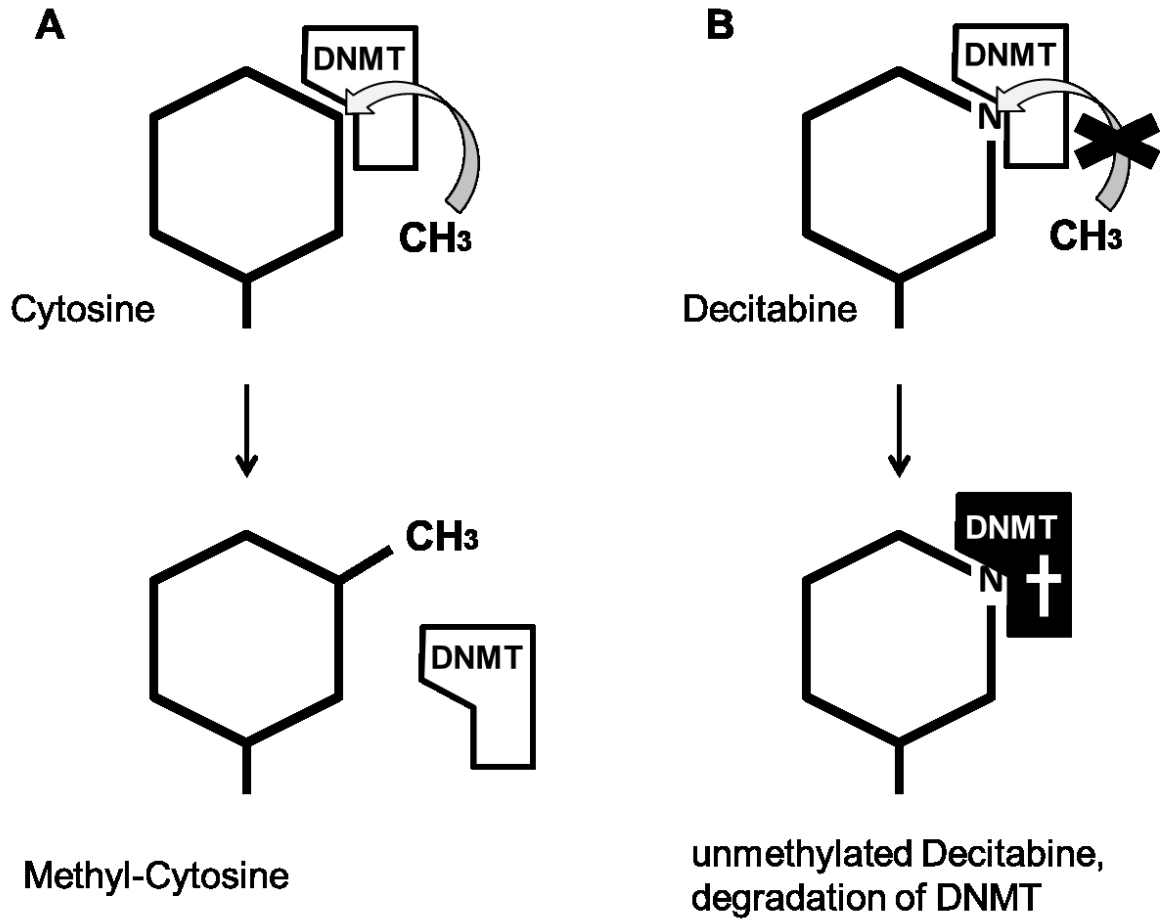

Figure 1 **Mechanism of DNMT (DNA-methyltransferase) inhibition after Decitabine treatment**

(A) Covalent binding of DNMT to a Cytosine residue of the DNA (Desoxyribonucleic Acid) nucleotide sequence results in transferring a methyl-group from a donor S-Adenosyl-Methionine to the 5'-position of the cytosine-ring and leads to formation of a methyl-cytosine. The addition of a methyl-group is required for the subsequent release of the DNMT from its covalent binding. **(B)** Decitabine that has been incorporated into the DNA covalently binds to DNMT and blocks the addition of a methyl-group, because the N-group at 5'-position of Decitabine prohibits the methyl-group transfer. This prevents DNA-methylation and DNMT is trapped and degraded.

1.7 Aim of the study

This study aims to further investigate the role of DAPK1 expression in TTL^{short} compared to TTL^{long} as well as to clarify its general importance in pediatric acute lymphoblastic leukemia with special regard to its role as a potential tumor suppressor in ALL.

First, DAPK1 expression on protein levels of TTL^{short} (early relapse/poor prognosis) and TTL^{long} (good prognosis) patient samples will be analyzed and compared.

Given the reports on DAPK1-methylation in different hematological malignancies, epigenetic silencing by methylation will be addressed inclung the analysis whether the DNMT-Inhibitor Decitabine is able to restore DAPK1 expression. Therefor, ALL cell lines and patient-derived xenograft samples will be exposed to Decitabine and DAPK1 expression on transcript and protein level will be analyzed. Moreover, the effect of Decitabine treatment on cell viability will be assessed and potential cell death mechanisms will be analyzed in more detail.

Our findings will help to better understand epigenetic gene silencing processes in pediatric acute lymphoblastic leukemia and will have important implications for the development of new treatment strategies using hypomethylating agents like Decitabine.

2. Material & Methods

2.1 Material

2.1.1 Cell lines

REH	DSMZ, Germany
NALM-6	DSMZ, Germany
TANOUE	DSMZ, Germany
JURKAT	DSMZ, Germany
HeLa	DSMZ, Germany
KOPN-8	DSMZ, Germany
UoCB6	kindly provided by Dr. Rowley, Chicago, USA
RS4;11	DSMZ, Germany
018Z	established in our group
CCRF-CEM	ATCC, USA
MOLT-4	DSMZ, Germany

2.1.2 Xenograft samples (patient material)

All xenografts were derived from primary patient material and were obtained from pediatric ALL and established as patient-derived xenograft samples in a human NOD/SCID mouse model.

2.1.3 Cell culture reagents

RPMI 1640	Gibco, Thermo Fisher Sc., USA
DMEM	Gibco, Thermo Fisher Sc., USA
AIM-V	Gibco, Thermo Fisher Sc., USA
MEM alpha	Lonza, Switzerland
Fetal Calf Serum	Gibco, Thermo Fisher Sc., USA
L-Glutamine	Gibco, Thermo Fisher Sc., USA
Trypsin/ EDTA solution	Biochrom, Germany
Trypan-blue	Sigma-Aldrich, Merck, Germany
CellTiterGlo®	Progmega, USA
Bicoll separating solution	Biochrom, Germany
HBSS	Gibco Thermo Fischer Sc., USA

2.1.4 Demethylating agents, inhibitors and related materials

5-Aza-2-deoxycytidine (Decitabine)	Sigma-Aldrich, Merck, Germany
DMSO	Serva, Germany
zVAD.fmk	Bachem, Switzerland
Doxorubicin	kindly provided by the pharmacy of Ulm university, Germany

2.1.5 FACS analysis

PE-Conjugated, Affinity Purified Polyclonal Rabbit Anti-Caspase 3	1:1	BD-Pharmingen, USA
Purified Mouse Anti-Cytochrome C	1:40	BD-Pharmingen, USA
Goat F(ab')2 Anti-Mouse IgG2b-FITC, Human ads	1:20	Southern Biotech, USA
Isotype FITC (Mouse IgG2b)	1:20	Dako, Denmark
Isotype PE (Mouse gamma1) X40	1:20	BD Pharmingen, USA
IgG1, Kappa MOPC-21		Sigma-Aldrich, Merck, Germany
Saponin		Sigma-Aldrich, Merck, Germany
Annexin-V (FITC)		Roche, Switzerland
Propidium-Iodide		Sigma-Aldrich, Merck, Germany
HEPES		Biochrom, Germany
Sterofundin		B Braun, Germany
Phosphate Buffered Saline (PBS)		Biochrom, Germany
Bovine Serum Albumin (BSA)		BioFroxx, Germany
FACS Flow		BD Bioscience, USA
FACS Clean		BD Bioscience, USA
FACS Rinse		BD Bioscience, USA

2.1.6 Protein electrophoresis and Western Blot analysis

Polyacrylamid Rotiphorese 30	Carl Roth, Germany
Glycine	AppliChem, ITW, USA
Tris	Sigma-Aldrich, Merck, Germany
Triton	Sigma-Aldrich, Merck, Germany
2-Mercaptoethanol	Merck, Germany
Glycerol	J.T. Baker, Thermo Fisher Sc., USA
Ammoniumpersulfate	Serva, Germany

Temed	Serva, Germany
DTT	Sigma-Aldrich, Merck, Germany
Sodiumorthovanadate	Sigma-Aldrich, Merck, Germany
ß-Glycerophosphate	Sigma-Aldrich, Merck, Germany
Sodium fluoride	Sigma-Aldrich, Merck, Germany
Hybond ECL Nitrocellulosemembrane	GE healthcare, United Kingdom
Whatman paper	VWR Chemicals, USA
Bolt™ Bis-Tris Plus Gels	Invitrogen, Thermo Fisher Sc., USA
iBlot 2 Transfer Stacks, nitrocellulose	Invitrogen, Thermo Fisher Sc., USA
MES SDS Running buffer	novex, Thermo Fisher Sc., USA
Skim milk powder	fluka, Merck, Germany
TWEEN®	Sigma-Aldrich, Merck, Germany
Pierce™ ECL	Thermo Fisher, Sc., USA
Amersham ECL prime	GE healthcare, United Kingdom
Amersham Hyperfilm ECL	GE healthcare, United Kingdom
BCA protein assay kit	Thermo Fisher Sc., USA
Protein marker page ruler	Thermo Fisher Sc., USA
Bolt™ Sample Agent	novex, Thermo Fisher Sc., USA
Bolt™ Reducing Agent	novex, Thermo Fisher Sc., USA
Sodium azide	Sigma-Aldrich, Merck, Germany

2.1.7 Antibodies

Western Blot primary antibodies:

Mouse anti-DAPK1	1:1000	Sigma-Aldrich, Merck, Germany
Rabbit anti-SMAD1	1:1000	Abcam, United Kingdom
Mouse anti-Beta-Actin (clone AC-74)	1:10000	Sigma-Aldrich, Merck, Germany

Western Blot secondary antibodies:

Goat anti-mouse IgG conjugated to HRP		Santa Cruz, Biotechnology, USA
DAPK1	1:4000	
Beta-Actin	1:10000	

Goat anti-rabbit IgG conjugated to HRP		Santa Cruz, Biotechnology,
SMAD1	1:4000	USA

2.1.8 Real time polymerase chain reaction and related material

RNA extraction

Trizol-Reagent	ambion, Thermo Fisher Sc., USA
Chloroform	VWR Chemicals, USA
Isopropanol	VWR Chemicals, USA
Ethanol	Sigma-Aldrich, Merck, Germany
RNAse-free water	gibco, Thermo Fischer Sc., USA

cDNA preparation and Reverse Transcriptase PCR

Random Primers	Invitrogen, Thermo Fisher Sc., USA
First strand buffer	Invitrogen, Thermo Fisher Sc., USA
DTT	Invitrogen, Thermo Fisher Sc., USA
Nucleotide mix	Invitrogen, Thermo Fisher Sc., USA
RNAse out	Invitrogen, Thermo Fisher Sc., USA
SuperScript	Invitrogen, Thermo Fisher Sc., USA
RNAse/DNAse-free water	Invitrogen, Thermo Fisher Sc., USA

quantitative Real Time-PCR

LightCycler® capillaries	Roche, Switzerland
LightCycler® FastStart DNA Master^{PLUS} SYBR Green I	Roche, Switzerland

quantitative RT-PCR primers

GAPDH primer (10 pM)

forward: 9a 5’ – GGT-CT-ACT-CCT-TGG-AGG-CCA – 3’

reverse: 8s 5’ – CAG-AAC-ATC-ATC-CCT-GCC-TCT-ACT-GGC - 3’

DAPK1 primer (10 pM)

forward: 5’ – CGA-GGT-GAT-GGT-GTA-TGG-TG - 3’

reverse: 5’ – CTG-TGC-TTT-GCT-GGT-GGA – 3’

SMAD1 primer (10 pM)

forward: 5’ – AGG-AAT-GGT-AAT-TTC-TAC-TCT-TCT-GG – 3’

reverse: 5’ – TTG-TCA-CAT-TCA-TAA-TGA-CAG-GAC – 3’

2.1.9 plastic ware

Falcon 50 ml	BD Bioscience, USA
Falcon 15 ml	BD Bioscience, USA
T-25 culture flasks	Sarstedt, Germany
T-75 culture flasks	Sarstedt, Germany
T-150 culture flasks	Sarstedt, Germany
24-well tissue culture plates	Sarstedt, Germany
6-well tissue culture plates	Sarstedt, Germany
96-well tissue culture plates	Sarstedt, Germany
Reaction tubes (all sizes)	Sarstedt, Germany
Combitipps	Sarstedt, Germany
Flow cytometry tubes	Sarstedt, Germany
Pipettes Costar Strippette 5-25 ml	Corning, USA
Cryopreservation tubes	Sarstedt, Germany

2.1.10 hardware

Laminar Air Flow Hera safe	Heraeus, Germany
Incubator Hera Cell 240	Heraeus, Germany
Centrifuge 5417 R	eppendorf, Germany
Multifuge 3 s	Heraeus, Germany
eppendorf concentrator 5301	eppendorf, Germany
Mastercycler gradient	eppendorf, Germany
Thermomixer compact	eppendorf, Germany
Trans Blot SD, semi-dry transfer cell	BioRad, USA
Power Supply EV202	Consort bvba, Belgium
Magnetic Stirrer RH basic 2	IKA, Germany
Nano Drop 2000	Thermo Fisher Sc., USA

Pipetboy acu	integra biosciences, Switzerland
Pipettes 1 – 1000 µl	VWR, USA
Water bath	Memmert, Germany
Mini Gel Tank	life Technologies, USA
iBlot 2	life Technologies, USA
Fume cupboard FAZ 1	Waldner, Germany
Scale BP 310 S	Sartorius, Germany
FACSCalibur flow cytometer	BD Biosciences, Europe
qRT-PCR LightCycler 2.0	Roche, Switzerland
Elisa Reader EL-800	BioTek, USA
Developer Optimax	Protec medical systems, Germany
Scanner X736de	Lexmark, USA
Senseo Original HD7817/69	Philips, Netherlands
Microscope S 40/0.45	Leica, Germany

2.1.11 software

FlowJo 8.7	TreeStar Inc., USA
BD CellQuest Pro	BD Biosciences, Germany
Gen5 ELISA 1.07.5	Biotek, USA
LightCycler Software 2.0 Version 4.1.1.21	Roche, Switzerland
NanoDrop 2000/2000c 1.4.2	Thermo Scientific, USA
Microsoft Excel 2010	Microsoft, USA
ImageJ 1.50i	NIH, USA
Prism 6.0	GraphPad, USA

2.2 Methods

2.2.1 Cell culture methods

Cell culture of leukemia cell lines (BCP-cell lines, mature B-cell line and T-cell lines)

Leukemia cell lines were cultured in RPMI 1640 medium, supplemented with 10 % fetal calf serum and 1 % L-Glutamine. Cells were transferred in T-75 cell culture flasks and cultured in humidified air with 5 % carbon dioxide. For experiments, cells were seeded at a density of 5 x 10^5 cells/ml and cultured in 24-well tissue culture plates (measurements of cell viability, Annexin-PI analysis, apoptosis analysis and CRAC analysis). For protein and RNA isolation, cells were seeded in 6-well tissue culture plates. In all treatment experiments, cells were treated daily with the indicated concentrations of Decitabine and DMSO, respectively. Pre-incubation with zVAD.fmk was performed one hour before treatment was started. Doxorubicin was added once and incubation time lasted 48h.

Cell culture of HeLa cervical cancer cell line and MS-5 mouse stromal cell line

HeLa cell line was added to DMEM medium, also supplemented with 10% fetal calf serum and 5 % L-Glutamine, and cultured in humidified air with 5 % carbon dioxide. Cells were seeded at a density of 5 x 10^5 cells/cm^2 and after culturing for four days, cells were harvested for RNA extraction.

MS-5 mouse stromal cells were cultured in alpha-MEM medium, supplemented with 10 % fetal calf serum and 1 % L-Glutamine. Cells were seeded in T-75 cell culture flasks and grown to confluence in humidified air with 5 % carbon dioxide. For treatment experiments, cells were seeded in 96-well tissue culture plates (measurements of cell viability) or 12-well tissue culture plates (RNA extraction). Cells settled for 24 hours before being coincubated with patient-derived xenograft samples.

Determination of cell viability

Cell viability was assessed using CellTiterGlo® luminescent cell viability assay, detecting cell viability via quantification of ATP, which was used as an indicator for metabolic active cells. Briefly, the medium was discarded from cell layer and 100 µl of the prepared reagent (diluted 1 to 4 with DMEM media) was added to each well. Cells were protected from light and incubated for ten minutes at room temperature. Afterwards, cells were transferred to white light-proof multiwell plates. Finally cell viability was assessed recording luminescence by a photometer plate reader.

Establishing patient-derived xenograft samples

All leukemia xenograft samples were established utilizing the NOD/SCID/huALL xenotransplant mouse model, which is well established in our group (Meyer et al. 2011). Precisely, the xenograft samples were obtained from patients bearing newly diagnosed pediatric BCP ALL, after each patient's informed consent and the ethical review committee's agreement was given. The leukemia cells were transplanted intravenously to NOD/SCID mice (NOD.CB17-Prkdc^{scid}/J mice), obtained directly at diagnosis or cryopreserved from previously diagnosed BCP ALL. The transplanted mice were monitored for clinical signs of disease plus occurrence of human leukocytes in mouse blood by flow cytometry defined by staining for huCD45 and huCD19. As soon as first signs of leukemia manifested, the mice were sacrificed and leukemia infiltration was surveyed in peripheral blood, bone marrow and the spleen using flow cytometry measurements again to define CD19 and CD45 positive cell populations. Furthermore, we estimated the time between transplantation of patient material and overt leukemia in the mouse for each xenograft, identifying it as 'time to leukemia' (TTL). A xenograft sample was allocated to the TTL^{short} group if TTL was less than ten weeks. Otherwise xenografts showing prolonged engraftment with more than ten weeks were defined as TTL^{long}.

***Ex vivo* cell culture of patient-derived xenograft samples**

To investigate cell survival as well as response to Decitabine treatment, patient-derived xenograft samples were cultured *ex vivo* in combination with MS-5 feeder cells, which was already shown to prolong survival of primary patient material (Pal et al. 2016).

For the *ex vivo* experiments performed in this study, we exclusively used patient-derived xenograft samples obtained from spleens of the corresponding mice. In brief, cryopreserved samples were thawed quickly and washed with fresh AIM-V media. Subsequently cells were centrifuged with 1,500 r.p.m. for 5 minutes at room temperature and supernatant was discarded carefully. These washing steps were performed twice, thereafter patient derived xenograft samples were dissolved in fresh AIM-V media and preincubated at 37 °C in humidified air with 5 % carbon dioxide for 24 hours.

Simultaneously, MS-5 feeder cells were prepared for coculture with patient-derived xenograft samples. Culture media was removed carefully and cells were washed with PBS, trypsinized and centrifuged at 1,500 r.p.m. for 5 minutes at room temperature. Then supernatant was discarded and cells were seeded at a concentration of 1 x 10^4 cells/well in tissue culture plates with 96, respectively 5 x 10^4 cells/well in 24 wells and allowed to settle for 24 hours.

The next day, patient-derived xenograft cells were carefully transferred to 15 ml fresh Bicoll separating solution and centrifuged at 1,300 r.p.m. for 20 minutes at room temperature with released brakes. Furthermore, to separate healthy, intact cells from cell debris and dead cells, the mononuclear cell layer was aspirated and transferred to 50 ml Falcons containing freshly prepared PBS. Then xenograft cells were counted and coincubated with MS-5 feeder cells at a concentration of 5 x 10^5 cells/ml. Finally Decitabine treatment was performed daily and cell death of xenograft cells was assessed via flow cytometry using discrimination of FSC/SSC; cell viability of MS-5 feeder cells was defined using a luminescent viability assay.

2.2.2 Flow cytometry analysis of cells

Cell death analysis

To assess cell death rates after Decitabine and Doxorubicin treatment, cells were seeded in 24-well tissue culture plates and treatment was performed daily with the indicated concentrations. When cell death rates were assessed, the volume of each well was mixed gently and 50 µl cell suspension (tenth part) were removed carefully and transferred into a flow cytometry tube. Afterwards 150 µl Phosphate Buffered Saline (PBS) solution was added and cell death was assessed by

FSC/SSC discrimination using flow cytometry. The removed volume of 50 µl was replaced by 75 µl fresh media combined with the appropriate amount of drug.

Annexin/PI analysis

Apoptosis induction was measured using an assay, exerting the characteristics of Phosphatidylserine (PS) in lipid bilayer of plasma membranes due to changes during apoptotic signalling induction (Vermes et al. 1995). Cells were seeded at a density of 5 x 10^5 cells/ml in 6-well tissue culture plates and treated with 0.5 µM Decitabine, DMSO or 20 ng/ml Doxorubicin, respectively. For analysis, cell suspension was removed carefully, washed with Annexin-buffer (Sterofundin buffered with HEPES) and centrifuged at 1,500 r.p.m. for 5 minutes at room temperature. Afterwards supernatant was discarded and 100 µl Annexin-V (diluted 1:50 in Annexin-buffer) was added followed by light-protected incubation at room temperature for 25 minutes. Then cells were washed again with Annexin-buffer and centrifuged at 1,500 r.p.m. for 5 minutes at room temperature. Propidium-Iodide (PI) was then added immediately before acquisition by FACS analysis was performed.

CRAC analysis

The Importance of both cytochrome c release and the subsequent following caspase activation was analyzed using an assay previously established in our group (Meyer et al. 2006; Meyer et al. 2008; Queudeville et al. 2012). CRAC, which is an acronym for ***C**ytochrome c **R**elated **A**ctivation of **C**aspase-3* detected cytochrome c release and subsequent caspase-3 activation using flow cytometry. Cells were seeded in 24-well tissue culture plates and treated daily with 0.5 µM Decitabine for four days prior to pre-treatment on days zero and two with 40 µM of pan-caspase inhibitor zVAD.fmk. After two and four days of treatment, cells were transferred to flow cytometry tubes, washed with PBS solution and centrifuged at 1,500 r.p.m. for 5 minutes at room temperature. Afterwards supernatant was discarded and cells were preconditioned with a mixture of MOPC-21 and Saponin, which produces pores within the cell membrane bilayer. Light-protected incubation was then performed for 20 minutes at 4°C. After this, cells were washed with BSA-solution (containing 5 % BSA in PBS) and centrifuged with the conditions described above. Subsequently, cells were stained intracellularly with mouse anti-cytochrome c (primary antibody) and PE-conjugated anti-caspase-3 antibodies,

followed by 20 minutes light-protected incubation at 4°C. In addition to that, cells were again washed with BSA, centrifuged as described above and supernatant was discarded. The remaining cell pellet was then resuspended in BSA, stained with anti-mouse secondary antibody and incubated again for 20 minutes at 4°C, while protected from light. Finally the cells were washed with BSA solution and then centrifuged at 1,500 r.p.m. for 5 minutes at room temperature. The supernatant was discarded, the cell pellet was dissolved in 200 µl BSA and cells were analyzed performing CRAC assay via flow cytometry.

2.2.3 Analysis of gene expression

RNA extraction

Cells were seeded in 6-well tissue culture plates and treated daily with 0.5 µM Decitabine. For the indicated timepoints cells were pooled and centrifuged at 1,500 r.p.m. for 5 minutes at room temperature. Afterwards the supernatant was discarded and cells were resuspended in 500 µl Tri-Zol solution, adding 200 µl Chloroform afterwards. The suspension was vortexed for 15 seconds and allowed to settle for 2 - 5 minutes. The suspension was then centrifuged at 12,500 r.p.m. for 15 minutes at 4°C and the purified supernatant was mixed with 500 µl of ice cold Isopropanol. After this, the mixture was stored at - 80 °C over night. After removing Isopropanol, two washing steps with 100 µl 70 % Ethanol followed. Finally the obtained pellet was concentrated using vacuum centrifuge and dissolved in RNAse/DNAse-free water according to pellet size. RNA concentration was assessed using NanoDrop 2000.

cDNA synthesis/ Reverse-Transcriptase-PCR

For cDNA synthesis 500 ng of pure RNA was mixed with RNAse/DNAse-free water and 1 µl Random Primers were added to reach a total volume of 13 µl. After incubation at 85°C for three minutes, the following steps were performed on ice and Master Mix for reverse transcription was added:

5 x First strand buffer	5.0	µl
DTT	2.5	µl
Nucleotide mix	1.25	µl

RNAse/DNAse free water	1.25	µl
RNAse out	1.0	µl
SuperScript	1.0	µl
→	**12.0**	**µl**

With the added 12 µl the solution reached a total volume of 25 µl.
The mix was then put into a thermocycler and the following incubation steps were performed:

temperature	duration
37 °C	15:00 min
45 °C	30:00 min
50 °C	15:00 min
94 °C	02:00 min
4 °C	hold

After completing the procedure, 175 µl RNase/DNase-free water was added to reach a concentration of 2.5 ng/µl. The created cDNA was either stored at - 20 °C or used immediately for further experiments.

Quantitative real-time polymerase chain reaction using SYBR-Green I

To determine transcript expression levels, quantitative RT-PCR was performed using the LightCycler 2.0 system according to the manufacturer's instructions. LightCycler® Capillaries were prepared and the reaction mix brought to a total volume of 10 µl:

RNase/DNase free water	4.5	µl
LightCycler® Mix	2.0	µl
Forward Primer (10pM)	0.5	µl
Reverse Primer (10pM)	0.5	µl
cDNA	2.5	µl
→	**10.0**	**µl**

The prepared samples were covered with LightCycler®-caps and centrifuged at 1,200 r.p.m. for 1 minute at 4°C. Afterwards quantitative RT-PCR was performed on LightCycler® 2.0 and the corresponding software using the following protocol:

	temperature	duration	number of cycles	
Preincubation:	95 °C	10:00 min	Cycles:	1
Amplification :	95 °C	00:10 min	Cycles:	40
	67 °C (dependent)	00:05 min		
	72 °C	00:15 min		
Melting Curve:	95 °C	00:00 min	Cycles:	1
	65 °C	00:15 min		
	95 °C	00:00 min		
Cooling	40 °C	hold	Cycles:	1

The following annealing temperatures were used for the different primers:

GAPDH:	67°C
DAPK1:	65°C
SMAD1:	58°C

The fluorescent signal of SYBR Green I was detected for each cycle after the extension step was completed. Then LightCycler® 2.0 software was used analysing the efficiency of the performed PCR by checking the melting curves and Crossing Points and further estimation of cDNA expression levels. Gene expression levels of *DAPK1* or *SMAD1* were normalised to the expression levels of non-regulated house-keeping gene *GAPDH* and presented as relative values.

2.2.4 Protein biology methods

Protein extraction and determination

Cells were seeded in 6-well tissue culture plates and cultured as previously described. Again treatment with 0.5 µM Decitabine or DMSO was performed daily and cells were pooled at the indicated timepoints. Afterwards cells were centrifuged at 1,500 r.p.m. for 5 minutes at room temperature, supernatant was discarded and the cell pellet was dissolved in protein lysis buffer containing 30 mM TrisHCl, 150 mM NaCl, 1 % Triton X, 10 % Glycerol and a proteinase inhibitor.

Further was added 500 µM PMSF, 2 mM DTT, 1 mM Na_2VO_4, 1 mM beta-Glycerolphosphate, 5 mM sodium fluoride and SPI. The mixture was then incubated on ice for 15 minutes. After this, the cell lysate was centrifuged at 14,000 r.p.m. for 30 minutes at 4°C to remove cell debris. Finally the supernatant was extracted carefully, put into a fresh Eppendorf tub and was stored at - 20 °C or used directly for further experiments. Protein concentration was estimated using the BCA™ Protein Assay Reagent kit, according to the manufacturer's instructions.

Western Blot analysis

For the detection of protein expression levels, proteins were separated according to their molecular weight using SDS poly acrylamide gel electrophoresis. Sodium-Dodecyl-Sulfate (SDS) is an anionic detergent which provokes neutralisation of a protein's own charge. Hence, voltage application results in separation of proteins according to their molecular weight (Weber, Osborn 1969).

50 µg of protein lysate was mixed with Bolt™ SDS Sample Buffer (4x) and Bolt™ Reducing Agent (10x), brought to a total amount of 40 µl with water and denatured for 10 minutes at 70 °C. Afterwards, the samples were loaded into precast polyacrylamide gels placed into Bolt™ MES SDS-Running buffer and separated by voltage application of 200 V and 300 mA for 45 minutes. To identify the size of proteins in particular, a protein marker containing prestained standard proteins of defined sizes was loaded on polyacrylamide gel additionally.

After adequate protein separation, proteins were blotted onto a nitrocellulose membrane using a semidry method. Either the blotting procedure was performed with the system from life technologies – using program P0 of iBlot 2 according to manufacturer's instructions, or was performed as follows. First of all, nitrocellulose membrane and four sheets of whatman paper were cut to the size of the used polyacrylamide gel and then wetted using Blotting buffer containing 48 mM TrisBase, 39 mM Glycine, 0.037% SDS and 20% Methanol. Afterwards two sheets of wetted whatman paper were positioned at the semidry blotter's bottom, followed by the nitrocellulose membrane, the polyacrylamide gel and the remaining two whatman papers. Blotting was then performed by voltage application of 25 V, 140 mA and 100 W for 90 minutes.

To prevent unspecific binding of antibodies, the nitrocellulose membrane was then incubated in Blocking buffer (5 % skim milk powder dissolved in PBS with 1%

Tween) for one hour, followed by three washing steps using PBS supplemented with 1 % Tween solution for ten minutes. The nitrocellulose membrane was then incubated with a first antibody dilution. The antibodies were diluted at indicated concentrations (see above) in PBS containing 1 % Tween, 2 % BSA and 0,02 % Sodium azide. After incubating the nitrocellulose membrane with the antibody dilution over night at 4 °C, three washing steps with PBS plus 1 % Tween were done. Finally the detection of the targeted proteins was performed using the chemiluminescent properties of luminol. To be more specific, the nitrocellulose membrane was incubated with a secondary antibody, which binds to the primary antibody but carries also the enzyme horseradish-peroxidase (HRP). This enzyme catalyzes the conversion of definitive chemiluminescent substrates to light emitting products. The incubation with a secondary antibody was performed overnight, with the antibody being diluted in 5 % skim milk powder, dissolved in PBS plus 1 % Tween. Afterwards the nitrocellulose membrane was again washed as described above and covered with ECL enhancing solution, containing the chemiluminescent substrate luminol. Then chemiluminescence was produced by targeted proteins and detected using ECL Hyper films, making the targeted proteins visible on these films.

2.2.5 Statistical analysis

Statistical analysis was performed using Mann-Whitney-U-Test, unpaired student's t-test as well as Spearman's Rho correlation analysis employing Prism 6.0 from GraphPad, USA.

3. Results

3.1 Different expression of DAPK1 in TTL^{short} and TTL^{long}

The *DAPK1* gene encodes for a Serine/Threonine-Kinase with proapoptotic features and reduced expression is described in several neoplasms, like hepatocellular carcinoma (HCC) (Matsumoto et al. 2003) and hypopharyngeal squamous cell carcinoma (Wei et al. 2015) as well as hematopoietic cancers (Kissil et al. 1997). Furthermore, results of previously published data based on a gene expression analysis revealed dysregulated apoptosis pathways in TTL^{short}/ early-relapse leukemia. Especially the expression levels of *DAPK1* appeared to be regulated significantly differently (Meyer et al. 2011). This result was further confirmed in a group of 23 patient-derived xenograft samples by quantitative RT-PCR (Queudeville et al. 2012).

Furthermore, as a first part of my study, DAPK1 protein expression was investigated in these samples by Western Blot analysis. According to differentially expressed transcripts, different protein levels were detected showing low expression in TTL^{short} and higher in TTL^{long} (Figure 2A and 2B, modified according to the original publication Queudeville, Seyfried et al. 2012, Cell death and disease). Interestingly, we also found that the DAPK1 protein expression correlates significantly with the corresponding TTL weeks with higher expression along with increasing NOD/SCID engraftment times (Figure 2C).

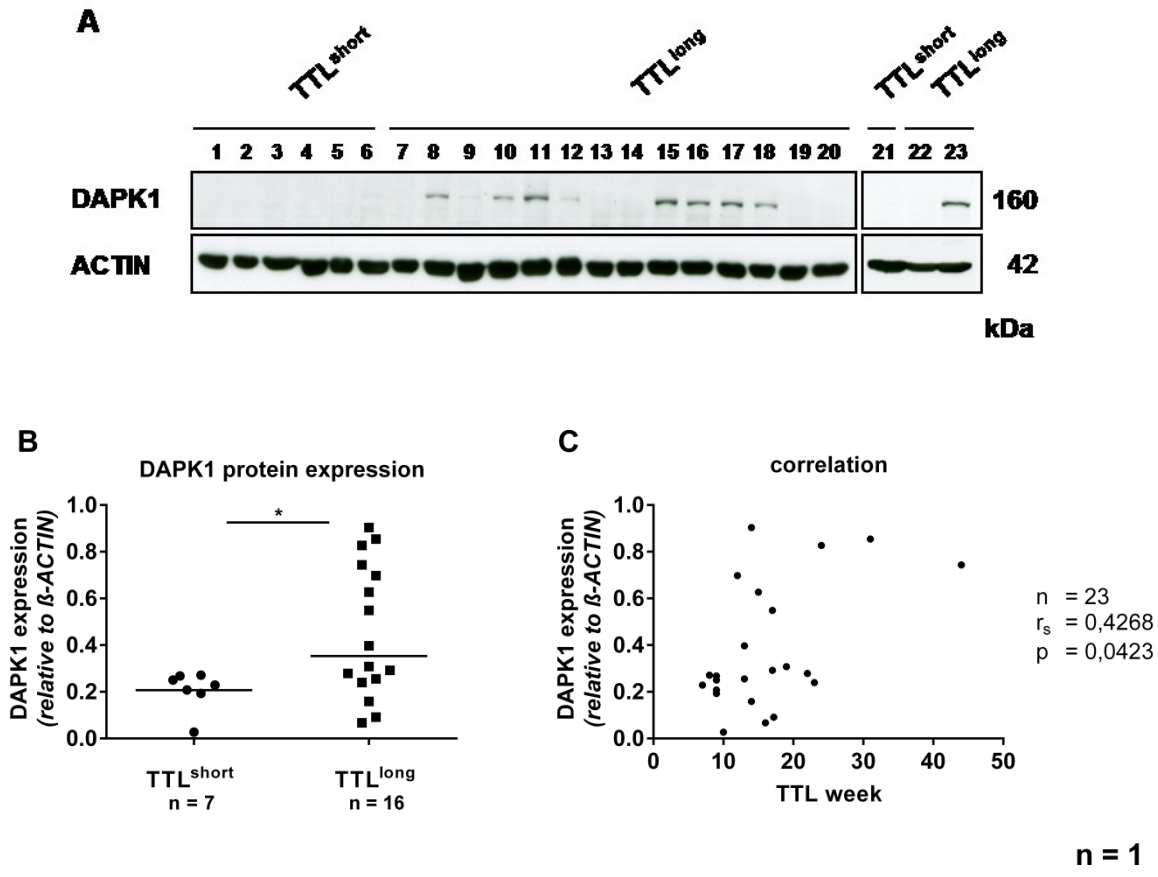

Figure 2 **DAPK1 (Death-Associated Protein Kinase 1) is expressed differently in patient-derived xenograft samples – analysis of protein levels**

(A) Western Blot analysis of 23 different patient-derived xenograft samples was performed and DAPK1 protein expression was detected. Beta-ACTIN served as a loading control and Western Blot was performed once. **(B)** Densitometric quantification of DAPK1 protein expression levels of xenograft samples shown in (A). DAPK1 expression levels of TTL^{short} and TTL^{long} (TTL = time to leukemia) were compared using the Mann-Whitney-U-Test, * $p < 0.05$. **(C)** Analysis of quantified DAPK1 protein expression levels shown in (B) and the respective TTL weeks. Statistical analysis was done using Spearman correlation, r_s is Spearman's rho, p is significance, * $p < 0.05$.

Figure derived from Queudeville et al. 2012. doi:10.1038/cddis.2012.107.

3.2 Analysis of DAPK1 expression in different leukemia cell lines

To identify the basal DAPK1 expression, we analyzed transcript and protein levels of ten different leukemic cell lines. In particular, six BCP-ALL cell lines (UoCB6, NALM-6, KOPN-8, RS4;11, REH and 018-Z), three T-ALL cell lines (JURKAT, CEM and MOLT-4) and the mature B cell line TANOUE were tested (Figure 3A). DAPK1 transcript and protein levels were assessed by quantitative RT-PCR and Western Blot analysis, respectively (Figures 3B and 3C). The HeLa cell line, derived from cervical carcinoma cells, and the patient derived leukemia xenograft X068, both known to highly express DAPK1, were used as positive controls.

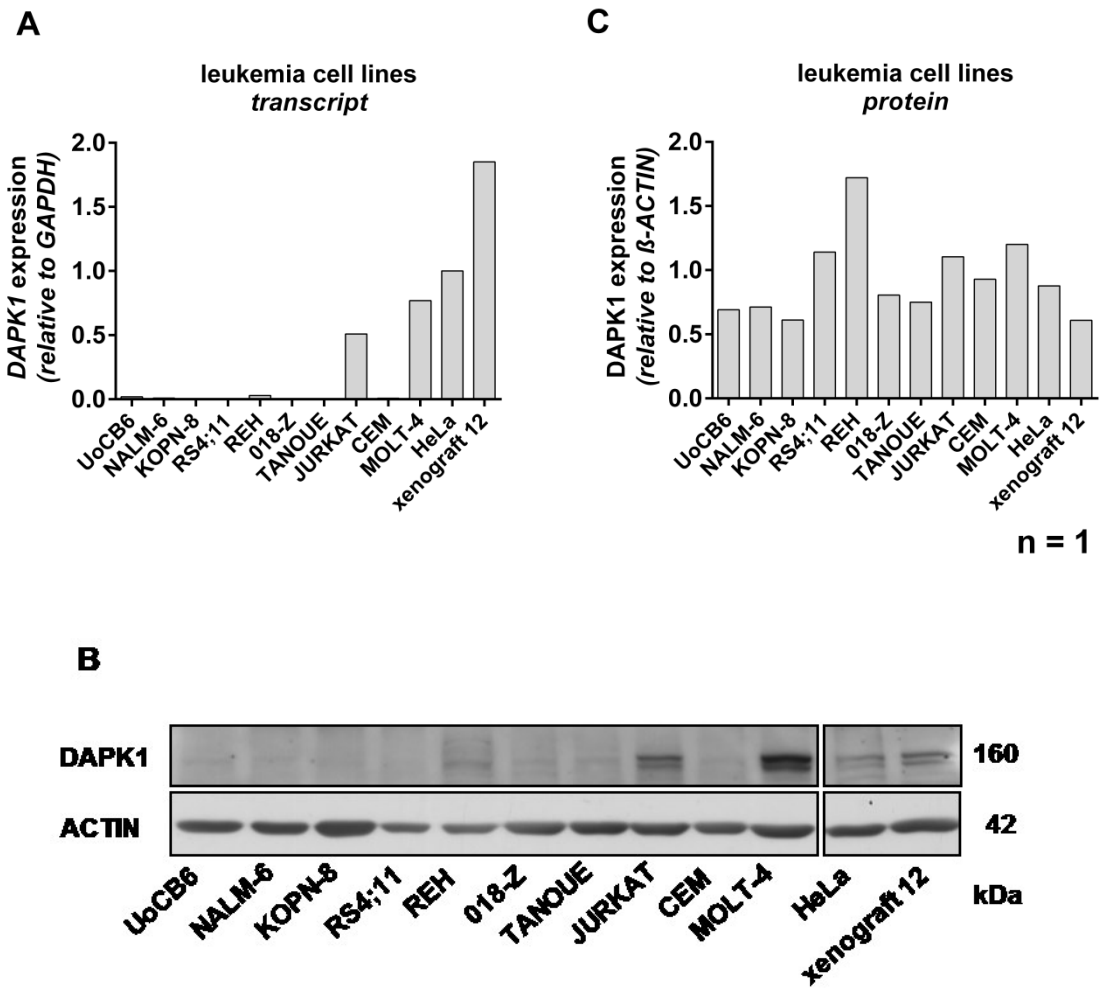

Figure 3 **DAPK1 (Death-Associated Protein Kinase 1) expression in ten different leukemia cell lines – analysis of transcript and protein levels**

(A) Quantitative RT-PCR (real-time polymerase chain reaction) analysis of *DAPK1* gene expression levels in ten different leukemia cell lines, HeLa cell line and xenograft 12 were used as positive controls. Expression levels relative to *GAPDH* (glycerinaldehyde 3-phosphat dehydrogenase) expression are shown and quantitative RT-PCR was performed once. **(B)** Western Blot analysis of DAPK1 protein expression levels tested in ten cell lines, beta-ACTIN served as a positive control. **(C)** Densitometric quantification of protein expression levels of leukemia cell lines shown in (B). Western Blot was performed once.

3.3 Treatment with the hypomethylating substance Decitabine induces cell death in ALL cell lines

3.3.1 Decitabine treatment leads to cell death induction in a dose dependent manner – analysis of four leukemic cell lines

Hypomethylating agents like Decitabine are incorporated into the DNA thereby inhibiting DNA-methyltransferase (DNMT) activity, which leads to reduced methylation during DNA replication (Stresemann, Lyko 2008). Decitabine has already been described to potently increase survival rates of patients suffering from myelodysplastic syndrome (Kantarjian et al. 2006). Since the molecular function of Decitabine is known, the connection between its demethylating properties on DNA and its positive effect on patient survival rates when used in a therapy regimen is not completely understood. However, gene silencing of tumor suppressor genes by DNA hypermethylation might play a crucial role (Daskalakis et al. 2002; Mund et al. 2006).

To determine whether Decitabine is effective in ALL cell lines, REH and NALM-6 (BCP-ALL), TANOUE (mature B-ALL) and JURKAT (T-ALL) cells were treated with increasing concentrations of Decitabine over six days. Cell viability was assessed by flow cytometry according to forward scatter/side scatter (FSC/SSC) properties. We saw an increase of cell death in all four cell lines in a dose dependent manner (Figure 4). While REH and NALM-6 cells showed the best response to Decitabine treatment, only a slight induction of cell death was detected in TANOUE cells. JURKAT cells were moderately affected by Decitabine treatment, but also showed increasing spontaneous apoptosis of control cells over time, what is probably caused by increasing cell numbers in the culture plates (Figure 4).

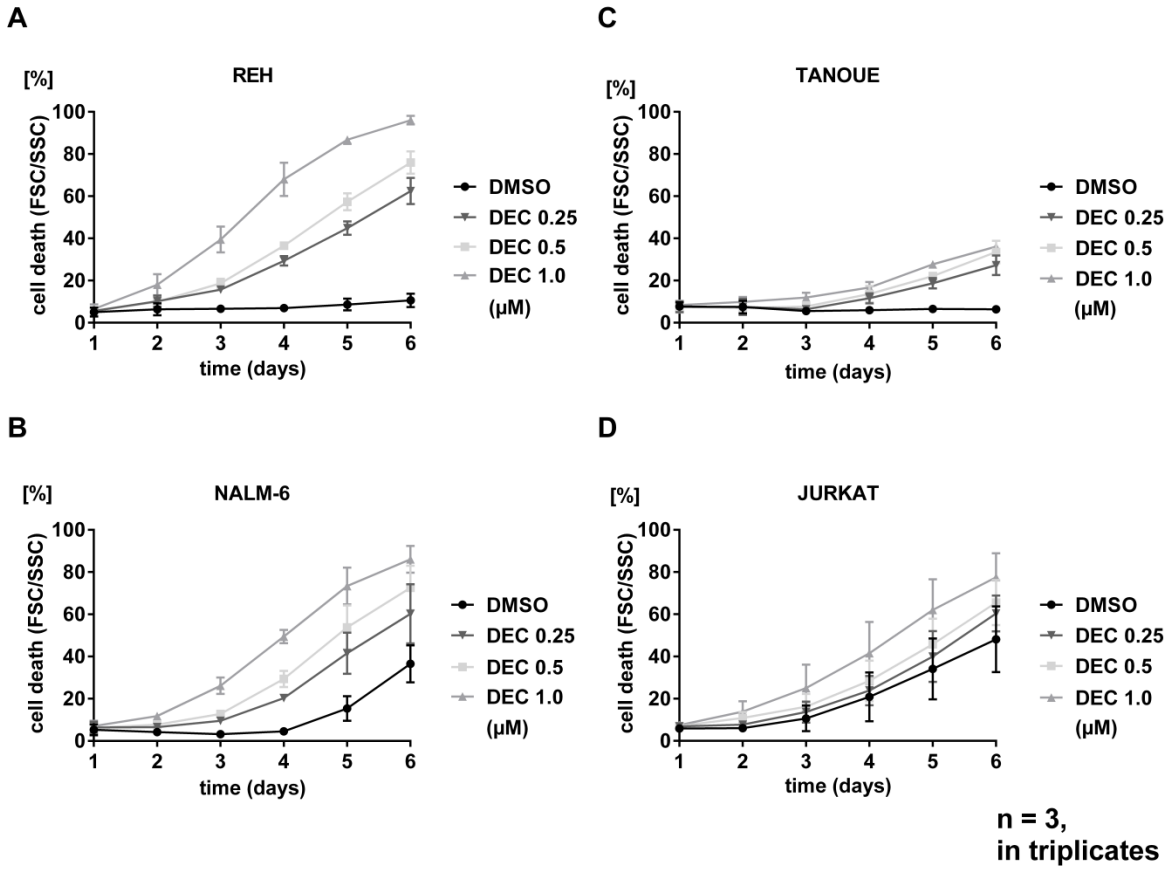

Figure 4 **Decitabine treatment leads to cell death induction in a dose-dependent manner – analysis of four leukemic cell lines**

Treatment of four leukemic cell lines, REH **(A)**, NALM-6 **(B)**, TANOUE **(C)** and JURKAT **(D)** with three different concentrations of Decitabine over six days; DMSO (dimethylsulfoxid) treated cells served as control group. Cell viability was assessed via measurement of FSC/SSC (forward scatter/ side scatter) using FACS (fluorescence-activated cell sorting) analysis. Means and standard deviation of three different experiments in triplicates are shown. Comparing control-group with each of three treatment-groups was statistical significant from day 2 until day 6 for REH, NALM-6 and TANOUE cell line; Comparing groups in JURKAT cell line shows no reliable significant result. Mann-Whitney-U-Test, result was stated significant when $p < 0.05$.

3.3.2 Effect of Decitabine treatment on DAPK1 transcript and protein expression levels in REH, NALM-6, TANOUE and JURKAT cells

To further investigate whether Decitabine treatment, beside its cell death inducing effect, also increases DAPK1 expression levels, the four leukemia cell lines REH, NALM-6, TANOUE and JURKAT were treated with 0.5 µM Decitabine and the expression of *DAPK1* transcript levels were analyzed by quantitative RT-PCR on day two, four and six. In REH and NALM-6 cells Decitabine treatment leads to increased DAPK1 expression levels compared to DMSO treated cells (Figures 5A and 5B). In TANOUE cells, although basal DAPK1 transcript expression was very low, we were able to demonstrate a strong increase of DAPK1 transcript upon Decitabine treatment (Figure 5C). Additionally, we confirmed high basal transcript expression levels of DAPK1 in DMSO control group of JURKAT cells, but a twofold increase of DAPK1 expression could be detected already 48 hours prior to Decitabine treatment (Figure 5D).

At the same points in time also DAPK1 protein levels were estimated by Western Blot analysis and we found that increased expression of DAPK1 transcript resulted in upregulation of DAPK1 protein levels in REH cells (Figure 6A). Furthermore, we found an increase of DAPK1 protein levels in NALM-6 cells as well, but not until day six, which is in contrary to REH cells (Figures 6A and 6B). However, no increase of DAPK1 protein expression could be detected in Decitabine treated TANOUE cells (Figure 6C) and just a slight increase was detected in JURKAT cells upon Decitabine treatment (Figure 6D).

Additionally, densitometric quantification of DAPK1 protein levels was performed, comparing protein expression of DAPK1 with Beta-ACTIN. We could confirm enhanced protein levels of DAPK1 in REH and NALM-6 cells over six days of treatment (Figures 7A and 7B). Also the analysis of densitometric quantified DAPK1 expression levels in JURKAT cells confirmed the slight increase seen in Western Blot analysis (Figure 7C).

Simultaneously, cell viability was assessed at days two, four and six of Decitabine treatment by flow cytometry measurements for all cell lines. We could confirm cell death inducing effect of Decitabine in REH and NALM-6 cells (Figures 8A and 8B). Furthermore, Decitabine induced cell death rates of only 40 % after six days of

treatment in TANOUE cells (Figure 8C). Aside from the relatively high rate of spontaneous apoptosis, we demonstrated Decitabine induced cell death in JURKAT cells as well (Figure 8D).

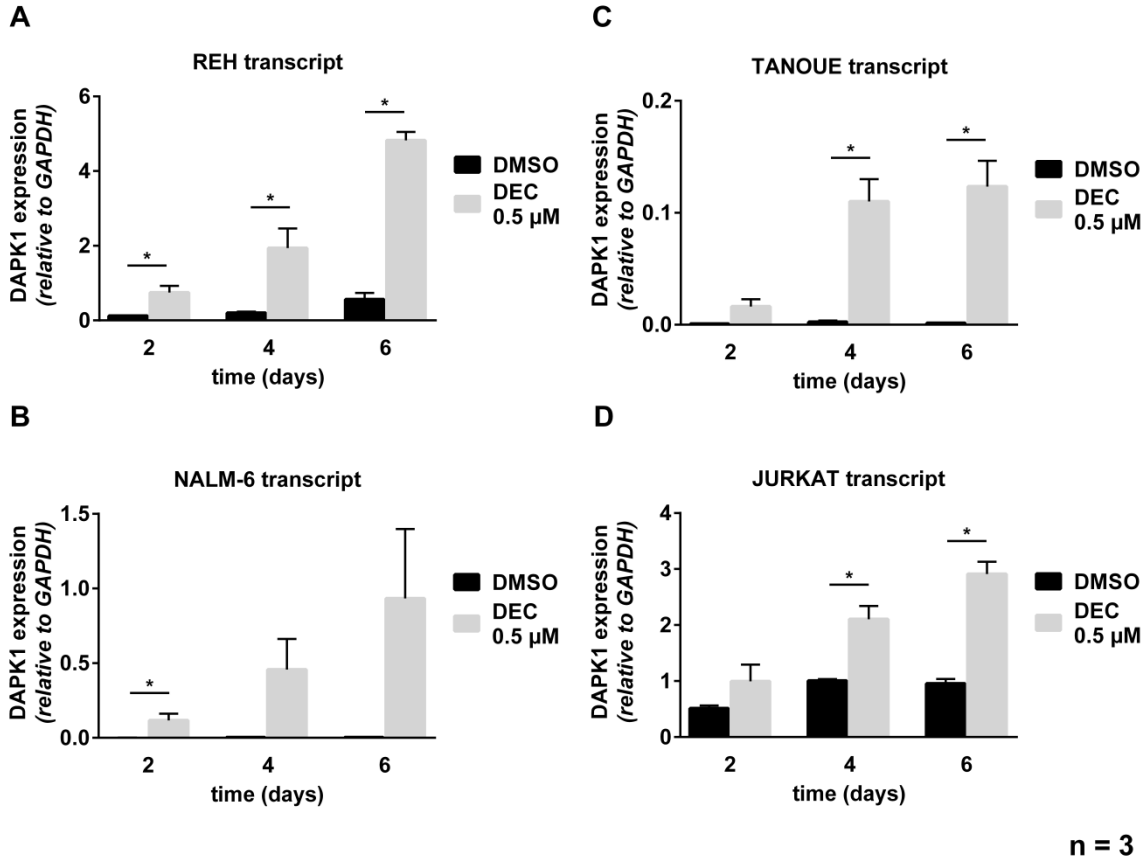

Figure 5 **Decitabine treatment enhances the expression of DAPK1 (Death-Associated Protein Kinase 1) on transcript level – analysis of four leukemic cell lines**

Treatment of four leukemic cell lines REH **(A)**, NALM-6 **(B)**, TANOUE **(C)**, JURKAT **(D)** with 0.5 µM Decitabine over six days. Quantitative RT-PCR (real-time polymerase chain reaction) of Decitabine-treated cells showing increase of DAPK1 gene expression (relative to corresponding GAPDH (glycerinaldehyde 3-phosphat dehydrogenase) expression) on day two, four and six compared to DMSO (dimethylsulfoxid) treated cells. Mean and standard deviation of three different experiments carried out in triplicates are shown. Student's t test; * $p < 0.05$.

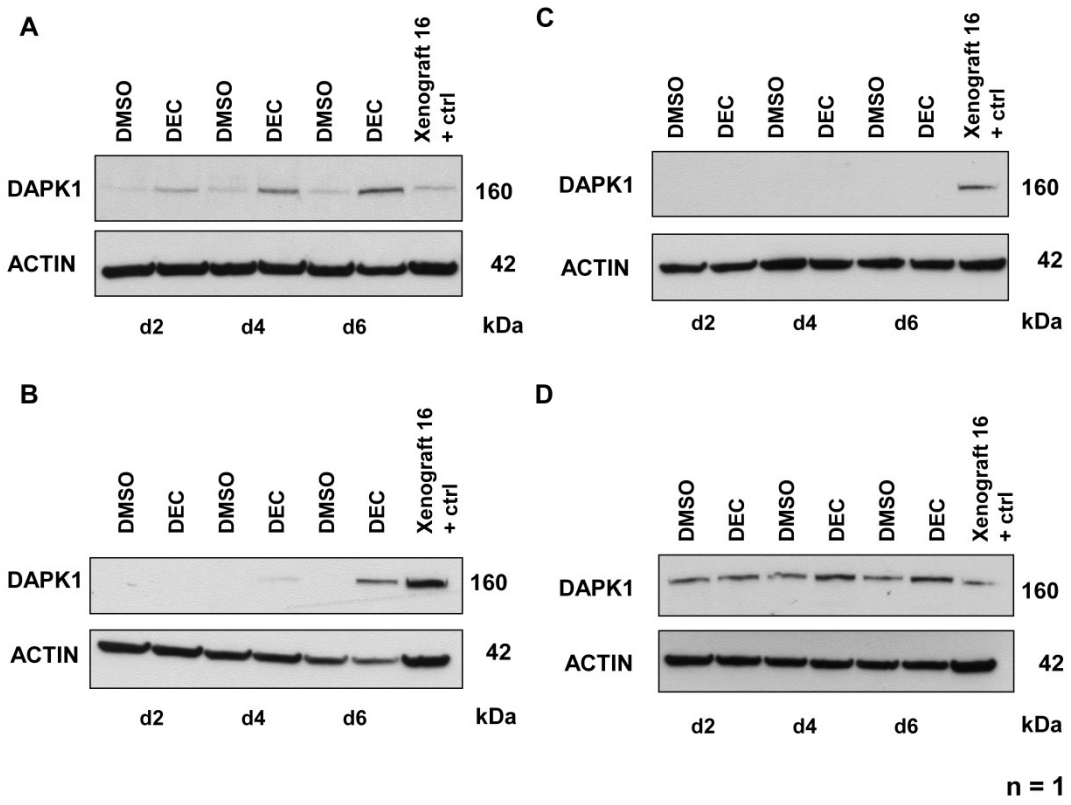

Figure 6 **Decitabine treatment enhances the expression of DAPK1 (Death-Associated Protein Kinase 1 on protein level – analysis of four leukemic cell lines**

Treatment of four leukemic cell lines REH **(A)**, NALM-6 **(B)**, TANOUE **(C)**, JURKAT **(D)** with 0.5 µM Decitabine over six days. Western Blot analysis of Decitabine treated cells on day two, four and six shows increased DAPK1 protein expression when compared to DMSO (Dimethylsulfoxid) treated control cells for REH **(A)**, NALM-6 **(B)** and JURKAT **(D)**, but no increase of DAPK1 protein expression for TANOUE **(C)**. Leukemia xenograft 16 was used as positive control. Western Blot was performed once.

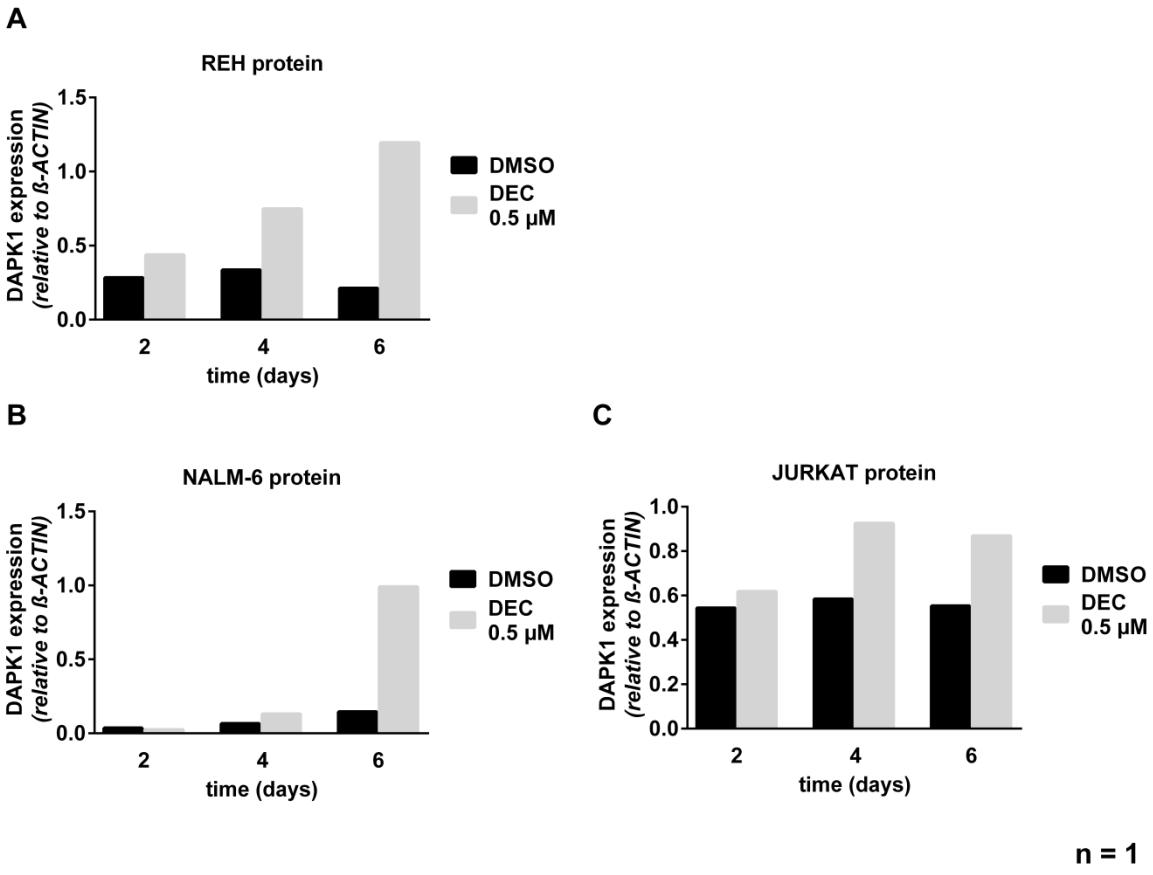

Figure 7 **Decitabine treatment enhances the expression of DAPK1 (Death-Associated Protein Kinase 1) protein levels – densitometric quantification of Western Blot analysis (Figure 6)**

Densitometric quantification of DAPK1 protein expression levels presented in Figure 6 emphasizes enhancement of DAPK1 protein level in REH **(A)**, NALM-6 **(B)** and JURKAT **(C)**. The underlying Western Blot was performed once.

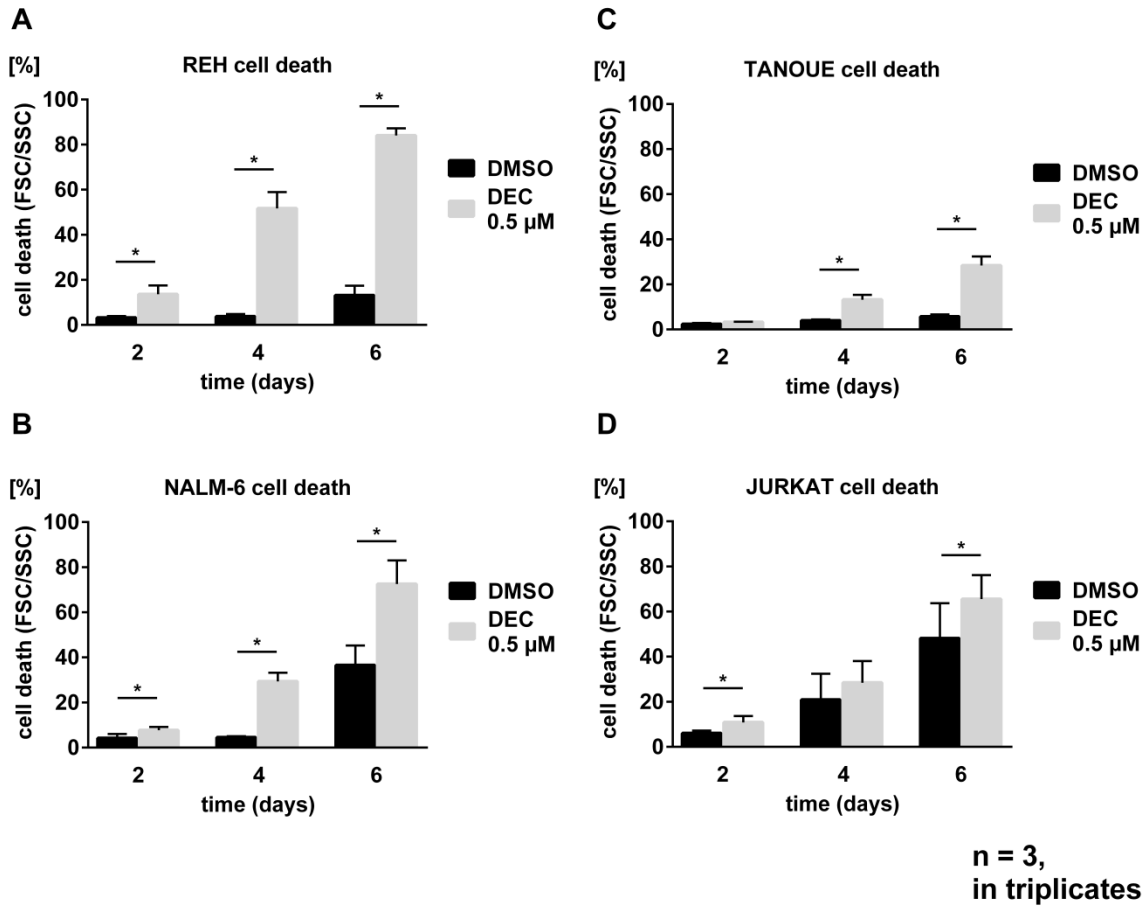

Figure 8 **Decitabine treatment leads to cell death induction in four leukemic cell lines – simultaneous analysis with estimation of transcript and protein levels**

Treatment of four leukemic cell lines, REH (A), NALM-6 (B), TANOUE (C) and JURKAT (D) with 0.5 µM Decitabine over six days leads to increased cell death compared to DMSO (Dimethylsulfoxid) treated control cells. However, control cells of JURKAT (D) are also showing increase of cell death. Cell viability was assessed by FSC/SSC (forward scatter/ side scatter) using FACS (fluorescence-activated cell sorting) analysis. Mean and standard deviation of three different experiment carried out in triplicates are shown. Mann-Whitney-U-Test; * $p < 0.05$.

3.4 Decitabine treatment triggers the apoptosis pathway

3.4.1 Analysis of Annexin/PI positivity upon Decitabine treatment

Having shown that Decitabine treatment induces cell death in a dose dependent manner as well as DAPK1 transcript and protein expression in ALL cell lines, we investigated whether Decitabine-induced cell death shows apoptotic features. As our main concern was to analyze B cell precursor leukemias, we further investigated the type of cell death triggered by Decitabine treatment in REH and NALM-6 BCP cell lines. To assess which kind of cell death is induced upon Decitabine treatment and to further discriminate between necrotic, apoptotic and living cells, an Annexin-PI analysis was performed (Fried et al. 1976; Vermes et al. 1995). Therefore fluorochrome-labelled Annexin-V for detection of Phosphatide-Serine (PS) and Propidium-Iodide (PI) was used. In living cells, Phosphatide-Serine is located in the inner side of the lipid bilayer of the cytoplasmic membrane and can not be detected from neither Annexin-V nor PI. Due to apoptotic signalling the PS is translocated (“flipped”) to the surface of the cell membrane, where it takes part in further steps of the apoptotic pathway, and can be adhered by Annexin-V, thus enabling the detection of this cell in the early phase of apoptosis. In later phases of apoptosis or in damaged cells that are necrotic or dead, loss of integrity occurs in some parts of the cytoplasmic membrane. PI can then pass through the leaky cell membrane and detect PS in the inner side of the lipid bilayer, displaying cells that are necrotic or in a late phase of apoptosis. Therefore, depending on the type of cell death that is triggered, the detection of Phosphatide-Serine differs whether necrosis or apoptosis is induced. For both REH and NALM-6 cell lines we again confirmed increased cell death upon Decitabine treatment (Figures 9A and 9C). Interestingly, Annexin-PI positive cell populations identified in treated cells indicate that Decitabine treatment induces apoptosis, rather than necrosis (Figures 9B and 9D). For positive control, we treated NALM-6 and REH cells simultaneously with Doxorubicin, since Doxorubicin has been previously described to induce apoptosis (Kalivendi et al. 2005).

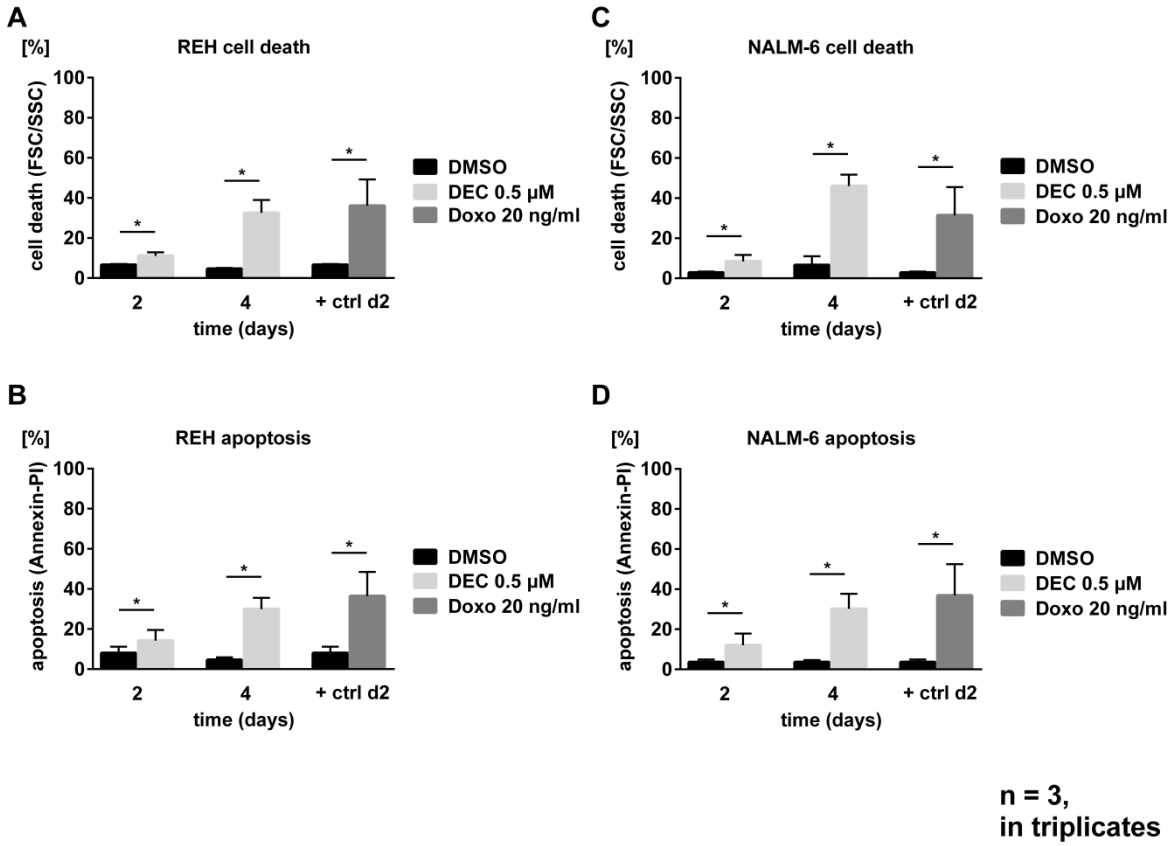

Figure 9 **Annexin-PI (Propidium-Iodide) analysis of Decitabine-induced cell death in BCP (B-Cell Precursor) cell lines**

(A) Treatment of REH cells with 0.5 µM Decitabine showed increased cell death on day four compared to DMSO-treated (Dimethylsulfoxid) cells. Doxorubicin-induced cell death was assessed on day two. Cell death was analyzed by flow cytometry using FSC/SSC (forward scatter/ side scatter) discrimination. **(B)** Decitabine treatment reveals Annexin-PI positivity compared to DMSO-treated cells, demonstrated for days two and four. Doxorubicin treated cells served as positive control, shown for day two. **(C)** and **(D)** display similar results concerning the cell death inducing effect of Decitabine as well as Annexin-PI analysis performed in NALM-6 cells. Mean and standard deviation of three independent experiments performed in triplicates are shown for Decitabine treatment. Treatment with Doxorubicin was performed two times in triplicates. Mann-Whitney-U-Test was performed for cell death analysis; * $p < 0.05$.

3.4.2 Decitabine-induced cell death shows caspase dependency

So far we have demonstrated that Decitabine-induced cell death shows apoptotic features. Furthermore we were interested, whether caspase dependency is an essential part of cell death induced by Decitabine treatment. For that we used a pharmacological approach to inhibit caspase activation. zVAD.fmk is a pan-caspase inhibitor, binding to the catalytic site of the caspase enzyme and thereby inhibiting its function. Hence REH and NALM-6 cell lines were treated with either 0.5 µM Decitabine, 40 µM zVAD.fmk or their combination for the indicated points in time and DMSO-treated cells served as control and the results are shown in Figure 10. Cell death was assessed by FSC/SSC flow cytometry measurements. For both REH and NALM-6 we detected a significant reduced Decitabine-induced cell death when combined with zVAD.fmk, indicating caspase dependency. To verify that the chosen concentrations of zVAD.fmk were effective, REH and NALM-6 cells were treated additionally with 20 ng/ml Doxorubicin, which is well described to induce caspase-dependent cell death, either with or without zVAD.fmk. We were able to achieve a significant reduction of Doxorubicin-induced cell death, when Doxorubicin was incubated with zVAD.fmk (Figure 10).

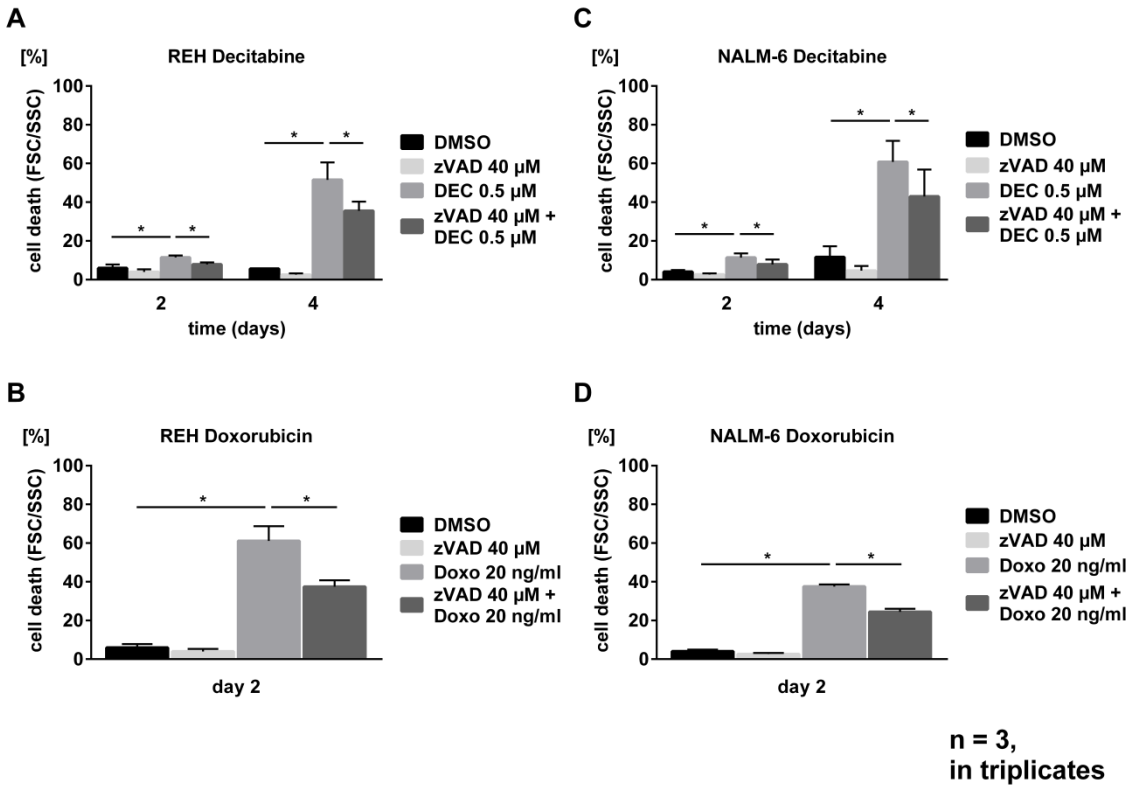

Figure 10 **Decitabine-induced cell death could be inhibited by co-incubation with zVAD.fmk (Carbobenzoxy-L-valyl-L-alanyl-β-Methyl-L-aspart-1-yl-Fluoromethan) – analysis of REH and NALM-6 cells**

(A) + (C) Treatment of REH and NALM-6 cells with Decitabine, zVAD.fmk and their combination for four days, DMSO (Dimethylsulfoxid) served as control. Cell death was assessed on days two and four using FSC/SSC (forward scatter/ side scatter) discrimination by flow cytometry. **(B) + (D)** Treatment of REH and NALM-6 cells with Doxorubicin, zVAD.fmk and their combination for two days, DMSO served as control. Cell death was assessed via FSC/SSC discrimination using flow cytometry. Mean and standard deviation of three different experiments done in triplicates are shown for Decitabine treatment. One experiment in triplicates for Doxorubicin treatment is shown. Mann-Whitney-U-Test was performed for cell death analysis; * $p < 0.05$.

3.4.3 Decitabine induces cytochrome c release and caspase activation

Till now we could show that Decitabine-induced cell death triggers Annexin-PI positivity and is at least partially caspase dependent. To investigate if cytochrome c release is involved, we performed further analysis using a flow cytometry approach again. In intact, healthy cells cytochrome c is located within the intermembrane space of mitochondria and is not present in the cytosol, therefore untraceable as long as lipid membranes of mitochondria are intact. Due to the occurrence of pro-apoptotic signalling, cytochrome c is released from the mitochondria to the cytosol, subsequently activating the apoptotic pathway which includes the activation of caspases. Therefore we performed an assay using fluorochrome labelled antibodies binding to specific epitopes on intracellular caspases and cytochrome c (Meyer et al. 2006; Meyer et al. 2008; Queudeville et al. 2012) and treated REH and NALM-6 cells with 0.5 µM Decitabine, 40 µM zVAD.fmk or their combination for four days, whereas DMSO served as control. We performed flow cytometry analysis on days two and four by measuring cell death induction via FSC/SSC discrimination and cytochrome c release into the cytosol as well as caspase-3 activation. First, we could detect increasing cell death, which can be partially inhibited by zVAD.fmk. Secondly, both cell lines showed increased caspase activation upon Decitabine treatment and increased cytochrome c release could be detected in both cell lines when treated with Decitabine (Figure 11).

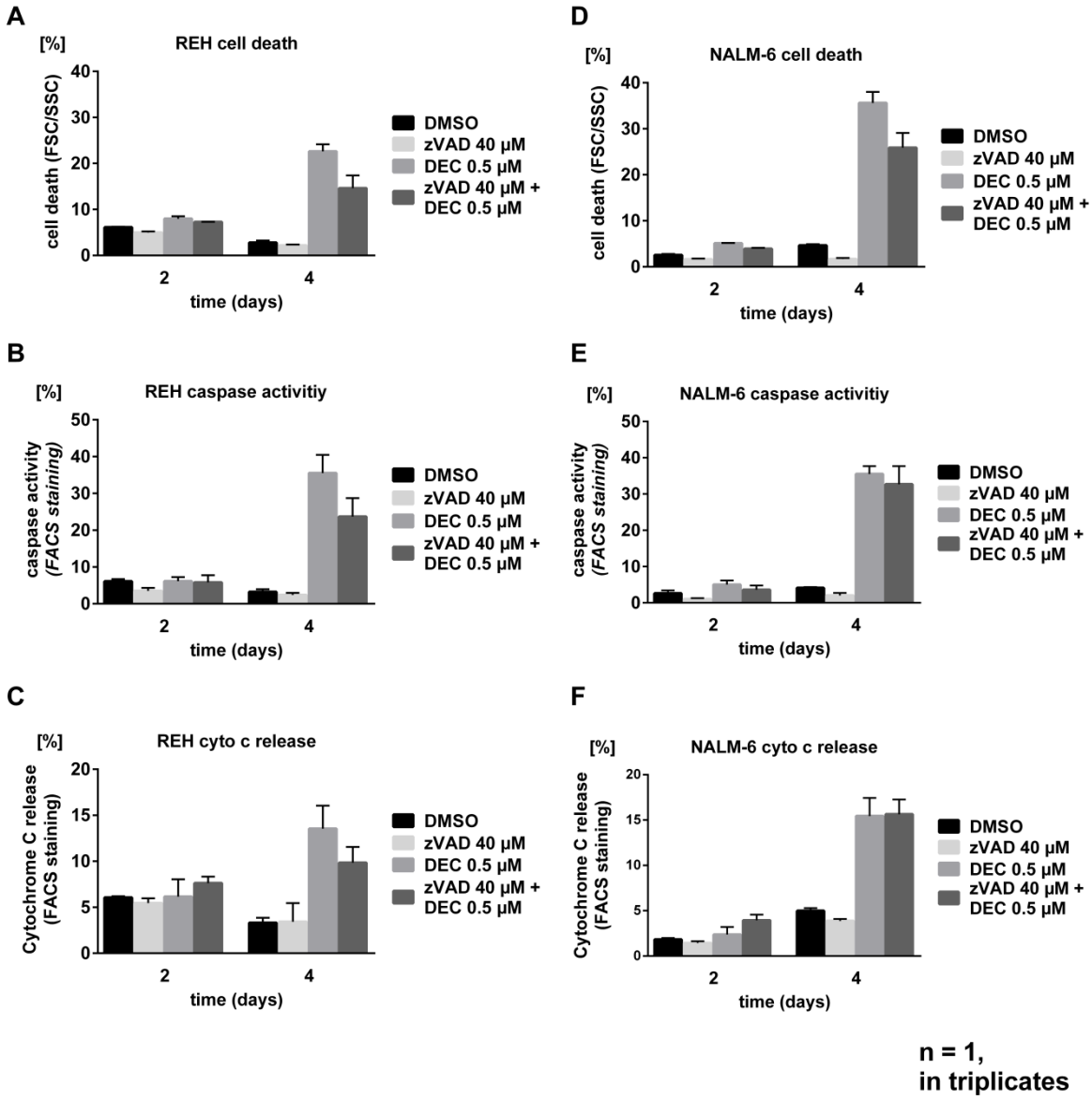

Figure 11 **Decitabine-induced cell death triggers the intrinsic apoptosis pathway in BCP (B-Cell Precursor) cell lines**

Treatment of REH and NALM-6 cells with 0.5 µM Decitabine, 40 µM zVAD.fmk (Carbobenzoxy-L-valyl-L-alanyl-β-Methyl-L-aspart-1-yl-Fluoromethan) and their combination for four days, DMSO (Dimethylsulfoxid) served as control. Flow cytometry analysis was performed on days two and four. **(A) + (D)** Decitabine treatment leads to increase of cell death in REH and NALM-6 cells, which could be partially inhibited by zVAD.fmk. Cell death was assessed using FSC/SSC (forward scatter/ side scatter) discrimination. **(B) + (E)** Decitabine treatment leads to increase of caspase activity. Analysis was done by flow cytometry staining of active caspase-3. **(C) + (F)** Decitabine treatment leads to cytochrome c release in both REH and NALM-6 cell lines. Analysis was done by flow cytometry staining for cytosolic cytochrome c. Experiment was performed once in triplicates.

3.5 Decitabine treatment induces cell death in patient-derived xenograft samples *ex vivo*

We have shown that Decitabine induced cell death in four different leukemia cell lines and increased DAPK1 expression on transcript and protein levels. To assess the effect of Decitabine on primary leukemia cells we used patient-derived xenograft samples collected frome leukemia bearing NOD/SCID mice.

3.5.1 Decitabine induces cell death in patient derived xenograft X 006

We chose one TTL^{short} xenograft to culture *ex vivo* and performed experiments where cells were treated with 0.5 µM Decitabine for six days and DMSO-treated cells served as control. Increased DAPK1 transcript levels were detected when cells were treated with Decitabine (Figure 12). Furthermore, Decitabine treatment triggered increased cell death rates, although this was also accompanied by increased cell death in DMSO-treated cells (Figure 12). Cell death was assessed using flow cytometry performing FSC/SSC discrimination.

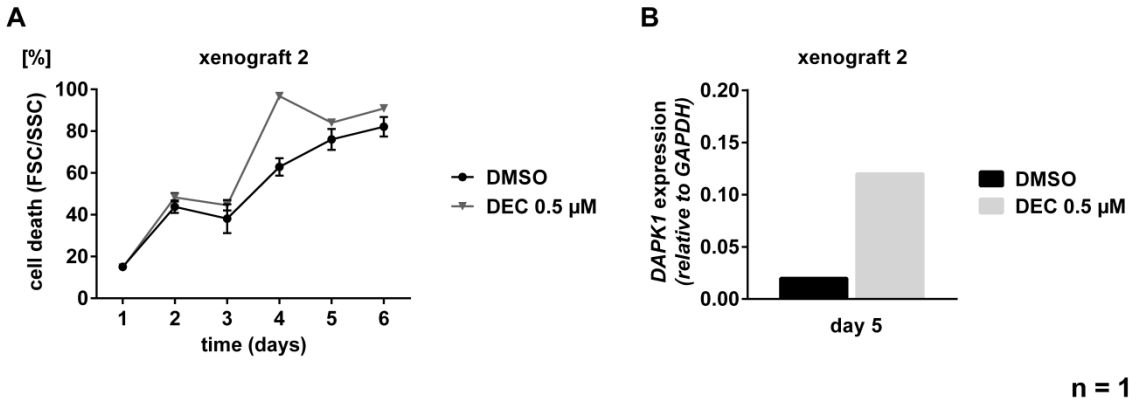

Figure 12 **Decitabine induces cell death and increase of DAPK1 (Death-Associated Protein Kinase 1) gene expression levels – analysis of patient derived xenograft 2 *ex vivo***

(A) Treatment of patient derived xenograft sample 2 with 0.5 µM Decitabine induced cell death, but was also detected in DMSO-treated (Dimethylsulfoxid) control group. Cell death was assessed using FSC/SSC (forward scatter/ side scatter) discrimination by flow cytometry **(B)** Performing quantitative RT-PCR (Real-Time Polymerase Chain Reaction) at day five reveals increase of *DAPK1* gene expression in Decitabine-treated cells compared to DMSO control group. Mean and standard deviation of one experiment performed in triplicates is shown.

3.5.2 Patient-derived xenograft samples co-cultured with mouse-derived MS-5 feeder cells – Decitabine treatment led MS-5 feeder cells unaffected from its cell death inducing effect

As described in 3.5.1, *ex vivo* culture of patient-derived xenograft samples shows limitations concerning the high rate of spontaneous apoptosis in non-treated cells, therefore we used a model of culturing patient-derived xenograft samples together with a mouse-derived feeder cell line (MS-5) to prolong survival of *ex vivo* cultured xenografts established in our laboratory (Pal et al. 2016). For that, two patient derived xenograft samples of each TTL group were cultured *ex vivo* with MS-5 feeder cells and treated with three different concentrations of Decitabine, DMSO served again as control. After four days of treatment *DAPK1* gene expression levels were assessed using quantitative RT-PCR.

In addition we had to consider that treatment with Decitabine not only has an impact on xenograft samples but might also affect viability of MS-5 feeder cells. Therefore we cultured MS-5 feeder cells alone and performed daily treatment experiments with different concentrations of Decitabine. Cell viability was assessed by a luminescent cell viability assay using microplate photometer. We could demonstrate that Decitabine caused no distinctive cell death in MS-5 feeder cells (Figure 13A). As we cannot exclude that human xenograft cells harvested for RNA-extraction might be contaminated with mouse MS-5 feeder cells, we performed further experiments to diminish these possible limitations. To evaluate if a potential contamination might also affect the detected expression levels of human *GAPDH* and *DAPK1* transcripts, we performed quantitative RT-PCR of co-cultured xenografts and MS-5 feeder cells using species-specific primers (human and mouse). For the analysis of gene expression levels via quantitative Real-Time-PCR, fluorescence intensity is measured for every cycle of the PCR-run. To put it briefly, as soon as template (cDNA), Primer and Polymerase coincided and produced newly assembled gene products, the DNA-dye SYBRGreen I intercalated into the newly generated double-stranded DNA and fluorescence was emitted prior to illumination by a beam of light of a determined wavelength (Ponchel et al. 2003). Once the measured fluorescence intensity significantly exceeds the background fluorescence, the crossing point (Cp) or cycle threshold (C_t) is achieved. Thereafter the creation of new gene products reaches the

exponential phase and gene expression levels can be quantified. Crossing points of q-RT-PCR-runs analysing target genes, like *DAPK1*, might differ between different samples. Contrariwise the crossing points of analyzed house-keeping genes, like *GAPDH*, should yield similar results among different samples.

We demonstrated that quantitative RT-PCR performed with human GAPDH primers showed crossing points of around 20 cycles when patient-derived xenograft samples or untreated xenograft X006 were analyzed. In contrast to that we obtained crossing points of around 32 cycles when MS-5 cell cDNA was used (Figure 13B). That leads to the presumption that human GAPDH primers do not detect *GAPDH* gene products in MS-5 feeder cell cDNA. Furthermore, when human DAPK1 primers were used to amplify murine MS-5 *DAPK1* transcripts Light Cycler Software stated the result as 'Invalid', indicating that also human DAPK1 primers do not create a gene product when coincided with murine MS-5 cDNA. Finally we performed quantitative RT-PCR using mouse primers to amplify a house-keeping gene API (murine housekeeping gene) using MS-5 cDNA as well as a murine cDNA obtained from black 6 mice, which served as a positive control. As we received comparable results concerning API transcript expression levels, when either cDNA of MS-5 feeder cells or a positive control black 6 mice cDNA was used, we concluded that co-culturing of patient-derived xenografts with MS-5 feeder cells is a feasible approach to test efficacy of different substances while overcoming the limitation of high rates of spontaneous apoptosis when cultured alone (Figure 13C).

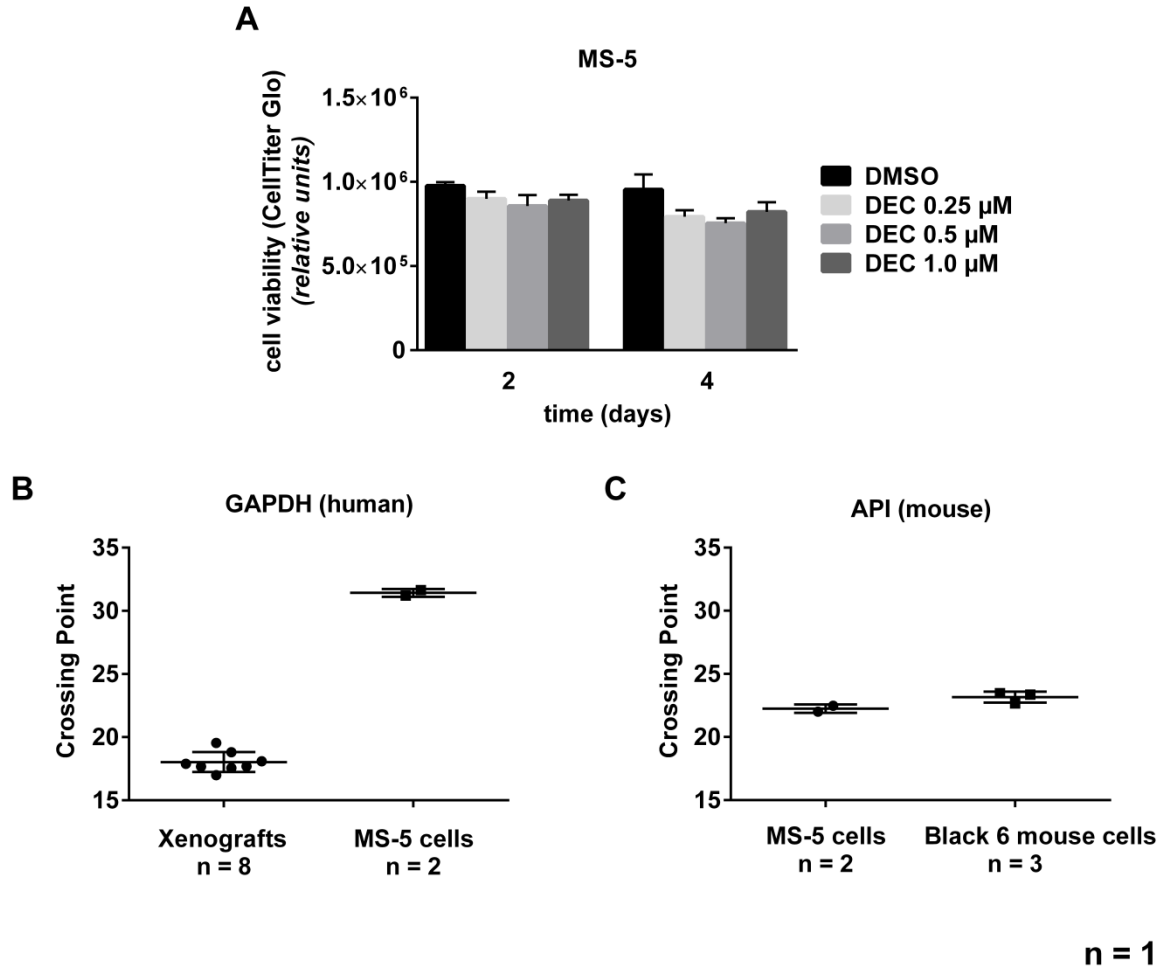

Figure 13 **Patient-derived xenograft samples co-cultured with mouse-derived MS-5 feeder cells – Decitabine treatment led MS-5 feeder cells unaffected from its cell death inducing effect**

(A) Treatment of MS-5 feeder cells with three different concentration of Decitabine for four days showed no distinctive effect on cell viability, DMSO-treated (Dimethylsulfoxid) cells served as control. Cell viability was assessed using fluorescent cell viability assay CellTiterGlo® via microplate photometer. **(B)** Quantitative RT-PCR (Real-Time Polymerase Chain Reaction) performed with human GAPDH primers showed no reaction in murine MS-5 cells, but in xenograft samples comparing the crossing points. **(C)** Quantitative RT-PCR using MS-5 cells and murine Black 6 cells with murine API primers (mouse housekeeping gene) demonstrating functional cDNA (complementary Desoxyribonucleic-acid), murine Black 6 cells served as positive control. Mean and standard deviation of (A) from one experiment performed in triplicates. Quantitative RT-PCR of (B) and (C) was performed once.

3.5.3 Decitabine treatment leads to cell death induction in a dose-dependent manner and enhances *DAPK1* gene expression – analysis of TTL^{short} and TTL^{long} patient derived xenograft samples cocultered with MS-5 feeder cells

We could demonstrate that Decitabine induced cell death in a dose-dependent manner in all four analyzed TTL^{short} xenografts (Figure 14). In addition, we detected similar results in the three analyzed TTL^{long} xenografts (Figure 15). However, TTL^{long} xenografts seem to be more sensitive to Decitabine treatment when compared to TTL^{short} xenograft samples. Furthermore, analysis of *DAPK1* gene expression revealed increased levels in both investigated TTL^{long} xenograft samples (Figure 15). In contrast, analysis of *DAPK1* gene expression levels of TTL^{short} xenograft samples showed just a slight increase upon Decitabine treatment (Figure 14).

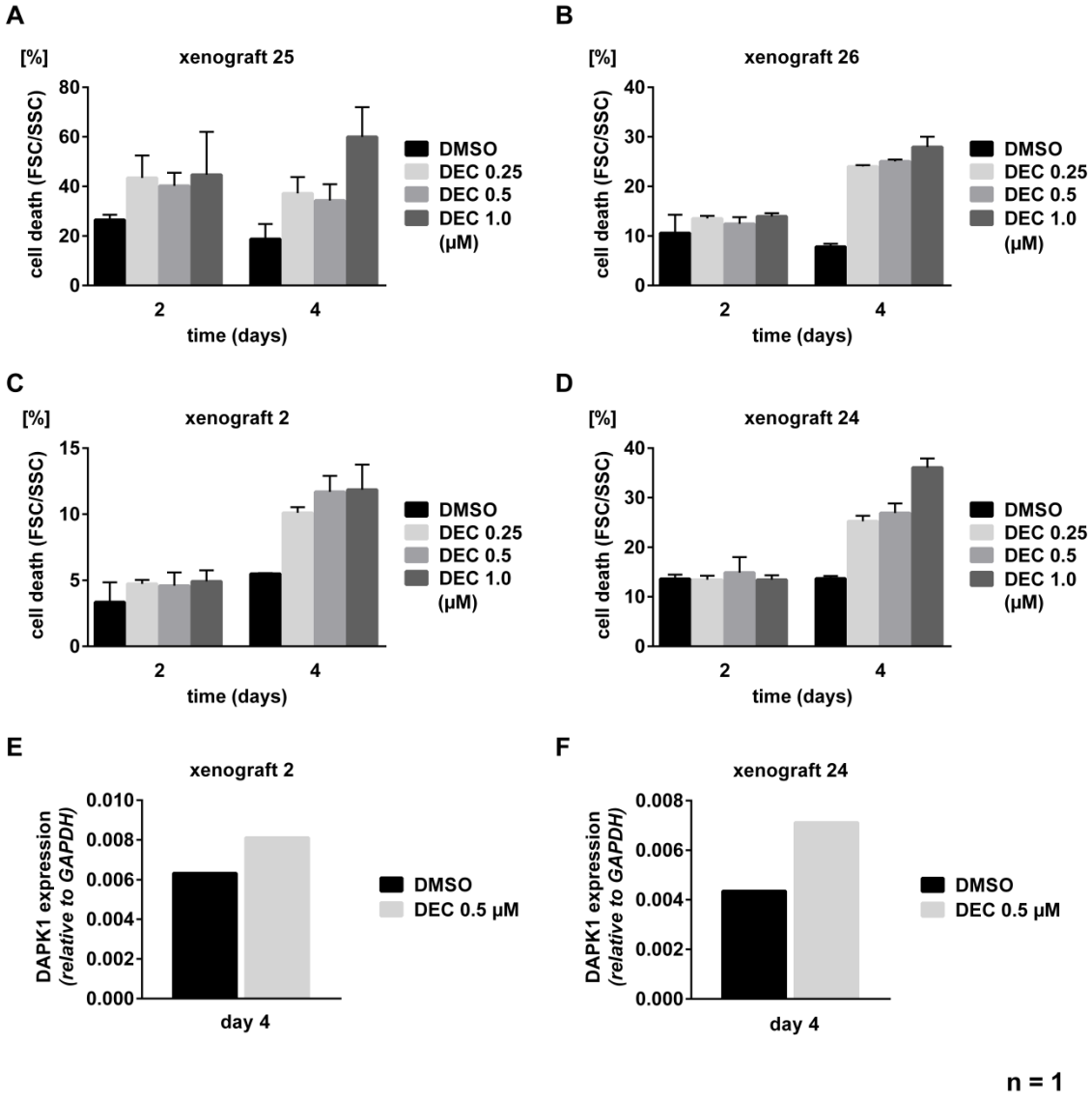

Figure 14 **Decitabine treatment leads to cell death induction in a dose-dependent manner and enhances *DAPK1 (Death-Associated Protein Kinase 1)* gene expression – TTL^{short} (TTL = time to leukemia) patient-derived xenograft samples co-cultured with MS-5 feeder cells**

(A) - (D) Treatment of four different TTL^{short} xenograft samples with Decitabine for four days leads to cell death induction in a dose-dependent manner. DMSO (Dimethylsulfoxid) treated cells served as control and cell death was assessed via FSC/SSC (forward scatter/ side scatter) discrimination by flow cytometry. **(E) + (F)** Decitabine treatment slightly enhances *DAPK1* gene expression levels in two different patient-derived TTL^{short} xenograft samples. Gene expression assessed by quantitative RT-PCR (Real-Time Polymerase Chain Reaction) performed at day four. Mean and standard deviation from one experiment performed in duplicates for xenograft 26 and performed in triplicates for xenografts 2, 24 and 25. Experiment for RNA-extraction for quantitative RT-PCR was performed once.

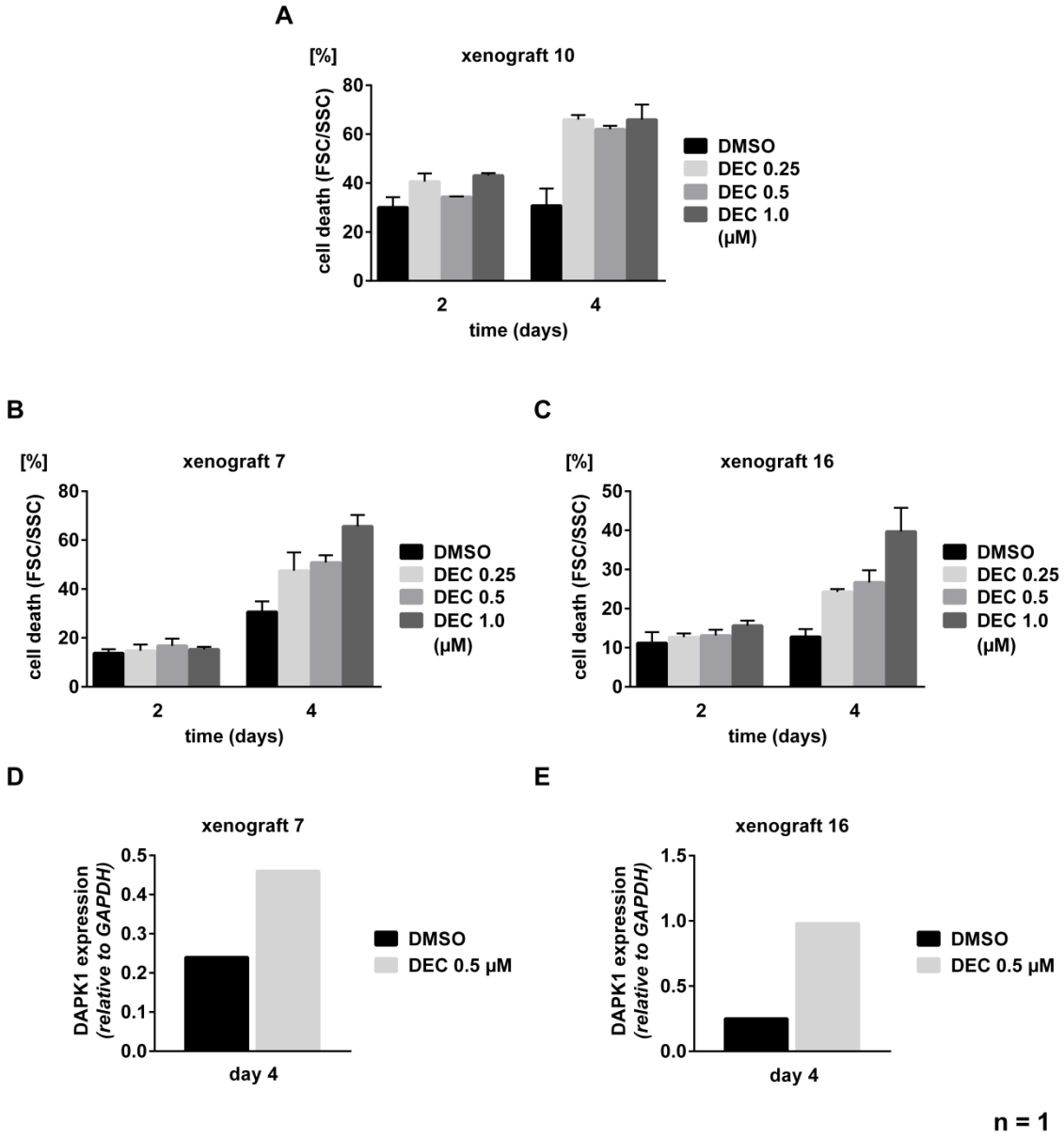

Figure 15 **Decitabine treatment leads to cell death induction in a dose-dependent manner and enhances *DAPK1 (Death-Associated Protein Kinase 1)* gene expression – TTL^{long} (TTL = time to leukemia) patient derived xenograft samples co-cultured with MS-5 feeder cells**

(A) - (D) Treatment of three different TTL^{long} xenograft samples with Decitabine for four days leads to cell death induction in a dose-dependent manner. DMSO-treated (Dimethylsulfoxid) cells served as control and cell death was assessed via FSC/SSC (sorward scatter/ side scatter) discrimination by flow cytometry. **(E) + (F)** Decitabine treatment slightly enhances *DAPK1* gene expression levels in two different patient-derived TTL^{long} xenograft samples. Gene expression assessed by quantitative RT-PCR (Real-Time Polymerase Chain Reaction) performed at day four. Mean and standard deviation from one experiment performed in duplicates for xenograft 10 and performed in triplicates for xenografts 7 and 16. Experiment for RNA-extraction (Ribonucleic-Acid) for quantitative RT-PCR was performed once.

3.6 SMAD1 is differently expressed in TTL^{short} and TTL^{long} and might regulate DAPK1 activation

3.6.1 *SMAD1* transcript expression levels in patient derived xenograft samples

SMAD1 is a molecule involved in the TGF-beta pathway and was also found to be differently regulated between TTL^{short} and TTL^{long} (Meyer et al. 2011). As SMAD molecules are transcription factors, that are described to potentially regulate DAPK1 gene expression levels (Jang et al. 2002), we investigated *SMAD1* transcript expression levels in 23 different patient-derived xenograft samples. Performing quantitative RT-PCR we could observe a significant downregulation of *SMAD1* transcript levels in TTL^{short} compared to TTL^{long} xenografts (Figure 16A). Furthermore, *SMAD1* gene expression levels correlated significantly with the initial engraftment times of the xenograft samples (time to leukemia, TTL) (Figure 16B).

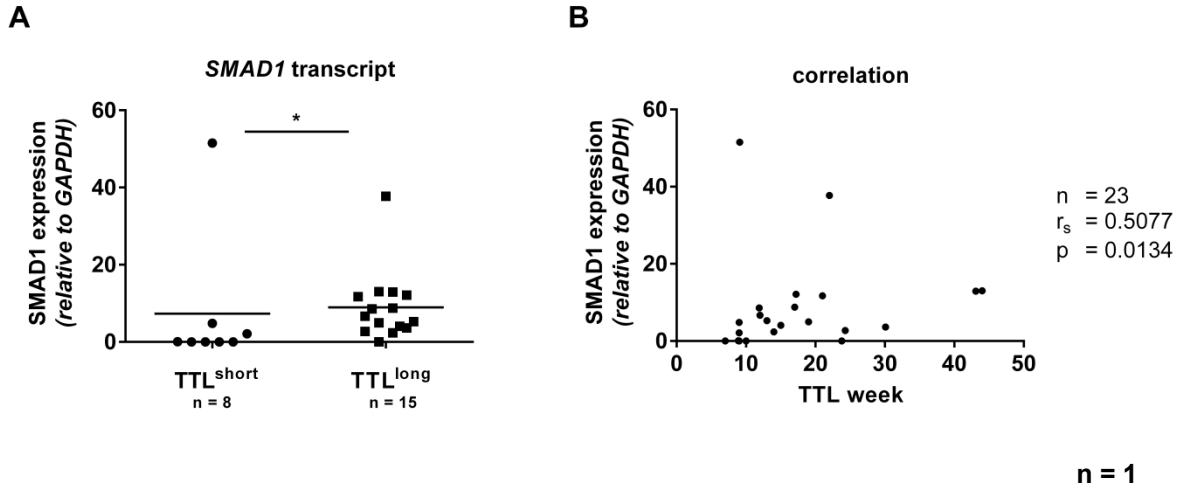

Figure 16 ***SMAD1 (SMA + MAD 1; SMA = Small Body Size, MAD = Mothers Against Decapentaplegic)*** **is differently expressed in patient-derived xenograft samples and correlates with its corresponding TTL-weeks (TTL = time to leukemia) – analysis of transcript levels**

(A) Quantitative RT-PCR (Real-Time Polymerase Chain Reaction) analysis of 23 patient-derived xenograft samples detecting *SMAD1* transcript expression (relative to *GAPDH (glycerinaldehyde 3-phosphat dehydrogenase)* expression). Mann-Whitney-U-Test. **(B)** Correlation of *SMAD1* expression levels of xenografts analyzed in (A) with corresponding TTL-number. Spearman correlation, r_s is Spearman's rho, p is significance; * $p < 0.05$. Quantitative RT-PCR was performed once.

3.6.2 SMAD1 protein expression levels in patient-derived xenograft samples

Moreover, SMAD1 protein expression was analyzed in a group of 23 different patient-derived xenograft samples by Western Blot analysis (Figure 17A). Densitometric quantification revealed significant differences of protein expression when TTL^{short} and TTL^{long} xenograft samples were compared (Figure 17B). Spearman's Rho correlation analysis showed a significant correlation of SMAD1 protein expression levels and the corresponding engraftment times (TTL), (Figure 17C).

Previously published data indicated a connection between DAPK1 and the messenger-protein-family SMAD, which take part in the TGF-beta pathway (Jang et al. 2002). Interestingly, we found an association of SMAD1 and DAPK1 protein expression showing increasing SMAD1 expression along with rising DAPK1 levels (Figure 17D), thus indicating a potential functional interplay of both molecules.

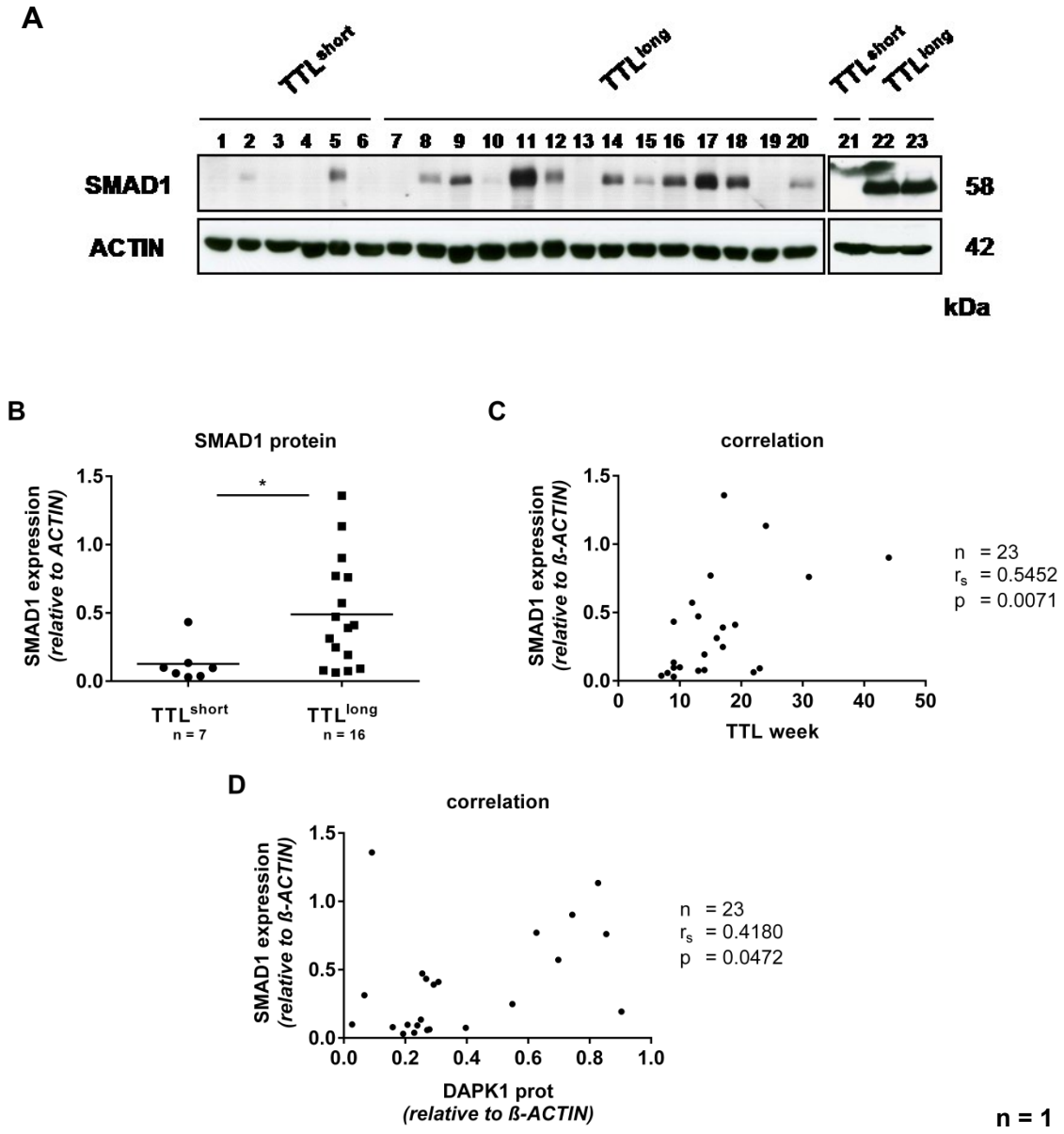

Figure 17 **SMAD1 (SMA + MAD 1; SMA = Small Body Size, MAD = Mothers Against Decapentaplegic) is differently expressed in patient-derived xenograft samples – protein levels and correlation analysis**

(A) Western Blot analysis of 23 patient-derived xenograft samples detecting SMAD1 protein expression levels, beta-ACTIN served as loading control, Western Blot was performed once. **(B)** Densitometric quantification of SMAD1 protein expression levels of xenograft samples showed in (A) and statistical analysis with Mann-Whitney-U-Test by comparing SMAD1 expression levels of TTL^{short} (TTL = time to leukemia) and TTL^{long}, p = 0.0180 **(C)** Correlation of the SMAD1 expression levels of xenograft samples analyzed in (B) with the corresponding engraftment times (TTL) and statistical analysis using Spearman correlation, p = 0.0071. **(D)** Spearman correlation of protein expression levels from DAPK1 (Death-Associated Protein Kinase 1) and SMAD1 performed in 23 patient-derived xenograft samples, r_s is Spearman's rho, p is significance; * $p < 0.05$.

4. Discussion

“The ability to modulate the life or death of a cell is recognized for its immense therapeutical potential” (quoted verbatim: Elmore 2007). All kind of cancer therapy employs this therapeutically potential and the efforts of effectively killing cancer cells with minimal side effects to healthy cells is an important aim of cancer research (Pui et al. 2001). The common and well described drugs used for treatment in ALL either block different stages of nucleotide-formation (thioguanine, mercaptopurine, methotrexate) and DNA synthesis (daunorubicin, cyclophosphamide), or produce interruption of the mitotic-spindle apparatus (vincristine) or intervene in processes of protein synthesis (asparaginase). However, these drugs all have a small therapeutic spectrum and produce a number of cytotoxic effects also in healthy cells (Pui, Evans 2006; Pui, Jeha 2007). Recently, new drugs have been identified that enable a more targeted therapy, like Tyrosine-Kinase-Inhibitors (TKI) in BCR/ABL t(22;9) positive leukemia, or the monoclonal antibody Rituximab in B-lymphocyte antigen CD20 positive ALL (Druker et al. 2001;Pui, Jeha 2007; Schultz et al. 2009; Thomas et al. 2006). The wide spectrum and the intensification of therapy approaches together with improved risk stratification and supportive therapy led to cure rates of more than 80 %, however there are still patients who encounter relapse of their disease (Pui, Evans 2006; Schrappe et al. 2000; Siegel et al. 2016). As these relapses are associated with a poor prognosis, intensive research is needed to identify new therapeutic approaches to increase survival of leukemia patients but also to improve quality of life by reducing chemotherapy induced cytotoxic effects (Pui, Evans 2006; Schrappe et al. 2012; Silverman et al. 1999).

The demand for an intensified chemotherapy depends on a patient’s individual risk for encountering an early relapse. Hence, appropriate risk stratification is important in treatment of pediatric ALL, ideally before start of induction phase of the treatment regimen was initiated. However, identification of patients with a high risk for relapse is challenging and contemporary protocols of clinical trials, including risk stratification according to detection of MRD, are not able to identify them reliably (Conter et al. 2010; Schrappe et al. 2000; van Dongen et al. 1998). Therefore, besides the necessity of new therapeutic approaches, the identification of new molecules for reliable risk stratification is strongly needed.

In this study we investigated DAPK1, a molecule that is known to be involved in apoptosis signaling and was found to be downregulated in the TTL^{short}/poor prognosis group in the NOD/SCID/huALL mouse model (Meyer et al. 2011; Queudeville et al. 2012). As DAPK1 is described to be downregulated by epigenetic silencing, the relevance of re-expressing DAPK1 with a therapeutic approach using the hypomethylating drug Decitabine in acute lymphoblastic leukemia was evaluated in this study.

4.1 DAPK1 – a pro-apoptotic molecule regulated by epigenetic silencing

Animal models in leukemia research enable the investigation of biology and genetic background of leukemia cell lines and especially primary patient material by overcoming the limitations of *in vitro* models, as well as studying human leukemia cells in a much more original setting (Jacoby et al. 2014). Our research group established a preclinical mouse model reflecting clinical features of patients bearing pediatric ALL with the engraftment properties of leukemia cells in NOD/SCID mice (TTL) (Meyer et al. 2011).

A gene expression analysis revealed dysregulation of cell death/survival pathways in the TTL^{short}-group compared to TTL^{long} (Hasan et al. 2015; Meyer et al. 2006; Meyer et al. 2008; Meyer et al. 2011; Queudeville et al. 2012; Schirmer et al. 2016) and also the pro-apoptotic tumor-suppressor-gene *DAPK1* was depicted to be downregulated in TLL^{short}.

Reduced expression of DAPK1 has been described previously in several malignancies and was found in patients suffering from head and neck cancers (Kong et al. 2005; Sanchez-Cespedes et al. 2000; Wei et al. 2015) as well as cancer of the urinary bladder (Jablonowski et al. 2011) and downregulation of DAPK1 was further identified in non-small cell lung cancer and hepatocellular carcinoma (Kim et al. 2001; Li et al. 2015; Matsumoto et al. 2003).

In the present study, we identified reduced expression of DAPK1 protein levels in the TTL^{short}-group compared to TTL^{long} in a cohort of 23 patient-derived xenograft samples (Queudeville et al. 2012) confirming previous published data identifying reduced expression of DAPK1 transcript in TTL^{short} in the same cohort as well (Meyer et al. 2011; Queudeville et al. 2012).

Furthermore, correlation analysis of DAPK1 protein expression levels and the respective engraftment times (TTL) was significant, which supports the role of

DAPK1 as a potential target for diagnostic and therapeutic approaches in pediatric acute lymphoblastic leukemia (Queudeville et al. 2012) and indicates the importance to understand the underlying processes of reduced expression levels of DAPK1 in pediatric ALL.

Epigenetic silencing of gene expression via promoter hypermethylation is a common mechanism in tumorigenesis (Herman, Baylin 2003; Jones, Baylin 2002). Furthermore, reduced DAPK1 expression caused by epigenetic silencing was described in several hematopoietic malignancies, including chronic lymphocytic leukemia (CLL) comparing CLL-cells with healthy lymphocytes (Debatin 2007; Katzenellenbogen et al. 1999; Kissil et al. 1997; Raval et al. 2007).

We could further confirm the findings of very low or even absent *DAPK1* gene expression levels, demonstrated in our analysis of six BCP-ALL cell lines and one mature B-cell line. However, two out of three T-cell lines showed much higher *DAPK1* gene expression levels, which is consistent with previously published data (Rohrs et al. 2009) as hypermethylation of DAPK1 CpG-islands is described to be more common in B-cell malignancies than in T-cell malignancies (Gutierrez et al. 2003; Katzenellenbogen et al. 1999; Nakatsuka et al. 2003).
Initiation and maintenance of DNA-methylation is regulated by DNA-Methyltransferases (DNMT). These specific enzymes regulate gene expression of distinctive genes by reversible hypermethylation of CpG-islands in gene-promoters, independent of the corresponding primary nucleotide sequence (Bird 2002; Jones, Baylin 2002). Inhibition of DNMTs by the pyrimidine-analog Decitabine results in lower DNA methylation by incorporating Decitabine into the DNA during DNA replication (Oki et al. 2007). This can induce re-expression of tumor related genes causing subsequently apoptosis induction and has been described in several tumors (Bachman et al. 1999; Karpf et al. 2001; Lavelle et al. 2003), especially in hematopoietic malignancies (Galm et al. 2006; Raval et al. 2007; Schafer et al. 2010). Furthermore, clinical trials demonstrated increased survival of patients with myelodysplastic syndrome (MDS) (Kantarjian et al. 2006) and chronic myelogenous leukemia (CML) upon Decitabine-treatment (Kantarjian et al. 2003) and approval for Decitabine-treatment of acute myelogenous leukemia (AML) in patients elder than 60 years was given recently (Nieto et al. 2016).

In the present study we selected two representative BCP-ALL cell lines (REH, NALM-6) and one mature B-ALL (TANOUE), which exhibit very low DAPK1-expression on transcript level and one T-ALL (JURKAT) representing a moderate *DAPK1* gene expression level to study Decitabine induced re-expression of DAPK1. In all four cell lines we observed a dose- and time-dependency of Decitabine-induced cell death over a period of six days, which is in line with previous published data demonstrating cell death induction upon Decitabine treatment in AML and ALL cell lines showing a time- and dose-dependency as well (Hollenbach et al. 2010; Schafer et al. 2010).

Furthermore, Decitabine is described to induce cell cycle arrest at higher doses, on the other hand low doses of Decitabine have been showed to induce hypomethylation of CpG-islands in gene-promoters (Kantarjian et al. 2006; Liu et al. 2011; Oki et al. 2007). We investigated moderate cell death induction with concentration of 0.5 µM Decitabine in all four cell lines which has been already described to be effective in the Burkitt's lymphoma cell line RAJI (Debatin 2007; Raval et al. 2007). Furthermore, we could confirm re-expression of DAPK1 transcript and protein levels upon Decitabine treatment in two BCP-cell lines, verifying the published data of Raval et al. shown in RAJI-cells (Raval et al. 2007). This has been demonstrated on B cell lines as well (Kissil et al. 1997) and matches with frequent DAPK1 hypermethylation in pediatric ALL (Gutierrez et al. 2003). Moreover, a slight increase of DAPK1 gene and protein expression levels in JURKAT T-ALL was observed, which goes in line with previous described findings that gene silencing via hypermethylation of tumor suppressor genes is more common in B-ALL than in T-ALL (Gutierrez et al. 2003; Katzenellenbogen et al. 1999). In addition, we also observed re-expression of DAPK1 in the mature B-cell line TANOUE just on transcript level, as DAPK1 protein expression remained to be unaltered upon Decitabine treatment.

Taken together, we could show that Decitabine treatment is a feasible approach for restoration of DAPK1 transcript and protein expression and is effectively inducing cell death in all tested B-ALL cell lines.

4.2 Apoptosis induction via Decitabine

Since apoptosis has been identified as a form of programmed cell death (Kerr et al. 1972) that is highly regulated by several distinctive pathways (Green, Llambi

2015) it has been recognized as an attractive pathway for cancer therapy (Ghobrial et al. 2005). Decitabine is known to induce hypomethylation of DNA, but also has cytotoxic effects and can induce cell cycle arrest via activation of the p53/p21$^{Waf1/Cip1}$ pathway (Liu et al. 2011; Oki et al. 2007, Zhu et al. 2004). The exact mode of action by which Decitabine acts remains still elusive, but several data indicate a major role for apoptosis induction upon treatment with Decitabine. Precisely, treatment of AML cell lines with Decitabine induced TRAIL-mediated extrinsic apoptosis signaling (Soncini et al. 2013) and was further associated with decreased protein levels of anti-apoptotic molecules XIAP and Bcl-2, caspase activation and collapse of mitochondrial membrane potential (Shin et al. 2012). Furthermore, induction of the intrinsic apoptosis pathway has been shown in T-cell lines after treatment with Decitabine (Ruiz-Magana et al. 2012).

In the present study we investigated apoptosis induction upon Decitabine treatment in both BCP-cell lines REH and NALM-6, using detection of Annexin/PI positivity, which is an effective approach verifying apoptosis as the observed cell death mechanism (Vermes et al. 1995) and is in line with previously published data showing Annexin/PI positivity in Decitabine-treated AML cell lines (Hollenbach et al. 2010). Furthermore, caspase activation represents the final common feature in both extrinsic and intrinsic apoptosis signaling and significantly reduced cell death induction upon combination treatment with zVAD.fmk and Decitabine was found in both REH and NALM-6 cells. Moreover, mitochondria were identified for their essential role in multi-cellular life as they control a cell's metabolism and are well known for their important role in programmed cell death, especially in apoptosis signaling (Cai et al. 1998; Green, Kroemer 2004). We identified cytochrome c release as well as caspase activation in both REH and NALM-6 cell line, suggesting that the induction of intrinsic/mitochondrial apoptosis signaling pathways plays a crucial role in Decitabine-induced cell death in BCP-cell lines, confirming results Shin et al. made analyzing Decitabine-treated AML-cell lines (Shin et al. 2012).

In summary, we identified Decitabine-induced cell death in two BCP cell lines leads to activation of apoptosis signaling, involving translocation of Phosphatidyl-Serine as well as cytochrome c release and caspase activation.

In the present study we addressed the apoptosis inducing effect of DAPK1. In addition to that, DAPK1 is also described to regulate autophagic cell death

induction (Bialik, Kimchi 2006; Inbal et al. 2002; Levin-Salomon et al. 2014; Singh et al. 2016). Hence, re-expression of DAPK1 and its role in autophagic cell death induction could be of further interest, in respect to identify new treatment approaches for pediatric ALL.

4.3 Decitabine treatment of patient-derived xenograft samples – hypermethylation of DAPK1-promoter

Investigation of leukemia cells requires functional and feasible models to increase knowledge of leukemia biology. *In vitro* studies of immortalized leukemia cell lines are a well characterized and well established approach to perform basic treatment experiments, however *in vivo* studies of primary patient material are less artificial and reflect better conditions of leukemia genesis and development (Gillet et al. 2013; Kamel-Reid et al. 1989; Lock et al. 2002). Culturing primary patient material *ex vivo* combines advantages of both systems, but suitable *ex vivo/in vitro* culture conditions were rare and not available so far (Pal et al. 2016).

In the present study, we initially investigated Decitabine-induced cell death and *DAPK1* transcripts level in a TTL^{short} patient-derived xenograft sample cultured *ex vivo* alone. However, elevated spontaneous apoptosis in the respective control group was detected as well, indicating the well-known problem of culturing primary patient samples in an *ex vivo* culture-setting (Gillet et al. 2013; Pal et al. 2016). We therefore performed co-culture experiments of *in vivo* generated patient-derived xenograft samples with the mouse stromal cell line MS-5 in an *ex vivo/in vitro* setting, established in our group.

We could demonstrate that cell death inducing effects of Decitabine were negligible in MS-5 cells, as well as detection of DAPK1 transcript levels in MS-5 cells using human DAPK1 primer sequences was not possible. Hence, we assumed that MS-5 cells were almost resistant to effects of Decitabine treatment. Furthermore, we confirmed dose-dependency of Decitabine-induced cell death in patient-derived xenograft samples which was consistent with our findings in Decitabine-treated ALL cell-lines and is in line with data published by Bhatla 2012, demonstrating dose-dependency of Decitabine-induced cell death in primary ALL samples (Bhatla et al. 2012). Moreover, we observed an increase of *DAPK1*

expression on transcript levels upon Decitabine treatment that appeared to be higher in TTL^{long} samples than in samples of the TTL^{short}-group.
We conclude that treatment with Decitabine is an effective approach to induce cell death but also increase DAPK1 transcript levels in primary ALL-samples. Furthermore, *ex vivo/in vitro* co-culture of patient-derived xenograft samples with mouse stromal cell line MS-5 is a feasible approach to investigate Decitabine drug response, while overcoming the limitations of culturing patient-derived xenograft samples *ex vivo* alone.

We identified a dose- and time-dependent cell death inducing effect of Decitabine in both ALL cell lines and ALL patient-derived xenograft samples and could show restoration of DAPK1 expression as well. Since DAPK1 expression is described to be regulated via hypermethylation (Katzenellenbogen et al. 1999; Oki et al. 2007; Raval et al. 2007), we hypothesized that differences in DAPK1 expression, which we observed comparing TTL long and short phenotypes, are due to different methylation patterns of CpG-Islands within the DAPK1 promoter. To identify hypermethylation of distinctive gene areas, “bisulfite sequencing” enables the detection of hypermethylated CpG-Islands in gene-promoters (Adusumalli et al. 2015; Herman et al. 1996; Olek et al. 1996) and microarrays like “EpiTYPER-MassARRAY” enable the analysis of methylation status at specific gene-loci. Bisulfite-enriched CpG-areas were quantified via PCR and converted into RNA *in vitro* afterwards. A base-specific cleavage of the RNA then produces fragments of different sizes and a subsequently performed MALDI-TOF (**M**atrix-**A**ssisted **L**aser **D**esorption/**I**onization-**T**ime **O**f **F**light) - mass-spectrometry-analysis detects bisulfite induced DNA-changes due to their different size compared to unmethylated RNA-fragments (Ehrich et al. 2005).
In cooperation with the research group of C. Plass in Heidelberg, analysis of DNA-methylation in CpG-Islands of seven distinctive regions of the DAPK1 promoter was performed. Since we assumed that hypermethylation of the DAPK1 promoter causes the significantly reduced expression of DAPK1 on transcript and protein level in TTL^{short} compared to TTL^{long}, 22 patient-derived xenograft samples (TTL^{short} = 8; TTL^{long} = 14) were analyzed via EpiTYPERMassARRAY and status of DAPK1 promoter methylation was estimated (Plass C., Claus R., Meyer L.H., personal communication). Either comparison of the entire methylation status or the analysis of CpG-Island methylation of each of the seven DAPK1 promoter regions

revealed no significant differences between TTL^{short} and TTL^{long}. Nevertheless, a tendency of increased methylation of DAPK1-promoter regions in TTL^{long}-xenograft samples compared to TTL^{short}-xenografts was observed, however these differences were not significant.

Taken together, these findings do not support our hypothesis that reduced DAPK1 expression levels observed in TTL^{short}-group are based on hypermethylation of *DAPK1* promoter regions. Nevertheless, patient-derived xenografts samples treated *ex vivo* with the hypermethylating agent Decitabine showed upregulation of *DAPK1* gene expression levels and cell death induction. Hence, the discrepancy between absent differences in DAPK1 promoter methylation on the one hand, and significantly different expression levels of DAPK1 on the other hand needs to be clarified. Thus, further investigations should also include mutation analysis of the *DAPK1*-gene, but also analysis of gene silencing via epigenetic alterations or genetic mutations of molecules involved in DAPK1 regulation, to further clarify how DAPK1 expression levels are regulated in B-ALL.

4.4 Suggestion of a possible interaction: SMAD1 and DAPK1

DAPK1 is a pro-apoptotic and anti-metastatic kinase and several molecules are described to influence DAPK1 gene expression levels, or to be involved in DAPK1 signaling, inducing apoptotic and autophagic cell death (Bialik, Kimchi 2006; Inbal et al. 1997; Singh et al. 2016). Previous studies described that gene expression levels of *DAPK1* can be influenced by SMAD-mediated (SMAD = SMA (Small Body Size) + MAD (Mothers Against Decapentaplegic)) signaling leading to apoptosis induction (Jang et al. 2002). The SMAD-family is a group of messenger molecules/transcription factors mediating intracellular signaling of the TGF-beta (Transforming-Growth-Factor beta) pathway, thus regulating several processes of cell homeostasis, including proliferation, differentiation and cell survival (Attisano, Wrana 2002). Furthermore, alterations of the TGF-beta pathway, including TGF-receptors or SMAD-molecules were described in human cancers, including hematologic malignancies (Dong et al. 2006; Samanta, Datta 2012). Furthermore, the relevance of SMAD1 for the anti-proliferative effect of TGF-beta signaling has been described for B-cell lymphoma previously (Bakkebø et al. 2010). Since we could not confirm our original hypothesis that DAPK1 expression levels are

regulated by hypermethylation of its promoter and thereby leading to DAPK1 gene silencing within the TTL^{short} group, we analyzed SMAD as a possible interaction partner of DAPK1. We could confirm that in addition to the pro-apoptotic molecule DAPK1, SMAD1 was also found to be downregulated in TTL^{short}/early relapse group (Meyer et al. 2011). SMAD1 transcript and protein levels were significantly downregulated in a cohort of 23 patient-derived xenograft samples, which significantly correlated with their respective number of TTL-weeks. Furthermore, significant correlation of DAPK1 protein expression levels with SMAD1 protein expression with increasing DAPK1 expression along with increasing SMAD1 was found in the group of 23 patient-derived xenograft samples.

Taken together, the reduced expression of DAPK1 and SMAD1 on transcript and protein level in TTL^{short}/ high risk/ early relapse acute lymphoblastic leukemia might indicate that DAPK1 expression is indirectly regulated by SMAD1, which expression in turn might be controlled by methylation and both might be important prognostic factors that possibly may have relevance for identification of pediatric high risk-ALL. However, the underlying processes of their signaling interactions as well as their potential role for therapeutic approaches remain still elusive and has to be further investigated.

4.5 Outlook

The development of a wide spectrum of different drugs used for treatment of pediatric acute lymphoblastic leukemia, improved risk stratification and supportive care resulted in cure rates of more than 80 % (Kaatsch 2010; Pui, Evans 2006; Siegel et al. 2016). Nevertheless, one fifth of the patients do not benefit from these progresses, as they encounter relapse of their disease, mostly associated with poor prognosis (Conter et al. 2010; Schrappe et al. 2000). The significant downregulation of expression levels in TTL^{short}/early relapse identified DAPK1 and also SMAD1 as possible candidates to allow identification and prediction of patients with pediatric acute lymphoblastic leukemia that have a high risk for an early relapse.

Here, we demonstrated that cell death induction in ALL cell lines as well as in patient-derived xenograft samples was achieved when treated with the hypomethylating drug Decitabine, which was further accompanied with re-

establishment of DAPK1 expression levels. Since established protocols used to treat pediatric acute lymphoblastic leukemia always consists of poly-chemotherapeutic approaches, the inclusion of Decitabine for high risk patients might be a possible starting point. It has already been shown that combination of established chemotherapy with drugs that affect epigenetic alterations like DNMT-Inhibitors or histone deacetylase inhibitors (HDAC-Inhibitors) could increase the treatment efficacy of refractory advanced non-small lung cancer (Juergens et al. 2011). This could be also a promising approach for pediatric ALL treatment, as the known intrinsic chemoresistance of relapsed leukemia cells (Klumper et al. 1995; Pogorzala et al. 2015) may be reversed upon combination treatment with established chemotherapy and HDAC-Inhibitors and/or DNMT-Inhibitors, as previously published data demonstrated (Bhatla et al. 2012).

Taken together, our results of reduced DAPK1 expression associated with high risk patients indicate a potential relevance of reduced expression of tumor suppressor genes in pediatric leukemias. Furthermore, we demonstrated the ability of Decitabine to restore the expression of reduced DAPK1 in leukemic cells and displayed its ability in apoptosis induction, suggesting it as a potential therapy approach in treatment of acute lymphoblastic leukemia.

5. Summary

In the present study, we referred to previous published data that identified different engraftment phenotypes of patient-derived ALL-samples (Acute Lymphoblastic Leukemia) transplanted on NOD/SCID mice (Non Obese Diabetes/ Severe Combined Immunodeficiency), which demonstrated a connection between patient outcome and engraftment properties. A gene expression analysis of patient-derived xenograft samples identified altered expression levels of apoptosis-related genes, including the pro-apoptotic molecule DAPK1 (Death-Associated Protein Kinase 1) which was significantly downregulated in the TTL^{short}/ early relapse group (TTL = time to leukemia) compared to TTL^{long} on transcript level. As a first result of the present study we could confirm these findings and found reduced expression levels of DAPK1 protein in TTL^{short} compared with TTL^{long} in the same cohort as well.

Out of a group of ten ALL-cell lines we identified two BCP-ALL (B-Cell Precursor-Acute Lymphoblastic Leukemia) (REH, NALM-6) and one mature B-ALL (TANOUE) as low expressing DAPK1 ALL cell lines and one T-ALL (JURKAT) that shows moderate expression levels of DAPK1. Treatment experiments with the DNMT-Inhibitor Decitabine were performed, showing a time- and dose-dependency of Decitabine-induced cell death in all four cell lines.

Furthermore, we analyzed expression levels of DAPK1 upon Decitabine-treatment in all four cell lines. Upregulation of *DAPK1* transcript expression was observed in all four cell lines whereas DAPK1 protein levels were only increased in REH, NALM-6 and JURKAT cells.

Assessing Annexin/PI staining, mitochondrial cytochrome c release and subsequent caspase activation we showed that Decitabine induced apoptosis in BCP-cell lines REH and NALM-6.

In line with the cell line data in patient-derived xenograft samples also a dose- and time dependent cell death induction upon Decitabine treatment was found. Moreover, DAPK1 gene expression was markedly enhanced in patient-derived xenograft samples, upon exposure to 0.5 µM Decitabine.

However, analysis of DAPK1 promoter methylation performed in cooperation with the research group of C. Plass in Heidelberg revealed no significant difference of methylation status comparing TTL^{short} and TTL^{long}. Accordingly to this contradictory result, promoter hypermethylation seems to be not the reason for reduced DAPK1

gene expression in the TTL^{short} group.

Therefore, it is also not clear whether increased DAPK1 expression and apoptosis induction observed upon Decitabine treatment is due to demethylation of the DAPK1 specific promoter.

We additionally analyzed SMAD1 (SMA + MAD; SMA = Small Body Size; MAD = Mothers Against Decapentaplegic) expression levels, since SMAD-molecules were previously described to possibly interact with DAPK1 and SMAD1 was also identified to be downregulated in TTL^{short} phenotype. Expression of both SMAD1-transcript and protein levels revealed to be significantly downregulated in the TTL^{short}/ early relapse group and were found to be significantly associated with engraftment times (TTL) and a significant correlation was found for DAPK1 and SMAD1 protein levels.

Our results demonstrate that DAPK1 may have relevance for risk stratification and prognosis estimation in pediatric high-risk ALL. Furthermore, the integration of Decitabine in treatment regimen of relapsed acute lymphoblastic leukemia might be a promising approach.

Taken together, low DAPK1 expression might contribute to lower apoptosis signaling in TTL^{short} ALL and increased expression upon Decitabine exposure might point to methylation as regulating mechanism, thus indicating a future marker for prognostication and target for therapy. However, additioinal investigations, particularly with respect to Decitabine and the specific role of DAPK1 in inducing apoptosis (inhibitor studies) need to be addressed before.

6. Publication bibliography

1. Adusumalli, S., Mohd Omar, M. F., Soong, R. and Benoukraf, T.: Methodological aspects of whole-genome bisulfite sequencing analysis. *Briefings in bioinformatics* 16: 369–379 (2015)
2. Alberts, B.: Molecular biology of the cell. 5th ed. New York: Garland Science, 1115–1129 (2008)
3. Alexander, F. E., Patheal, S. L., Biondi, A., Brandalise, S., Cabrera, M. E., Chan, L. C., Chen, Z., Cimino, G., Cordoba, J. C., Gu, L. J., Hussein, H., Ishii, E., Kamel, A. M., Labra, S., Magalhaes, I. Q., Mizutani, S., Petridou, E., Oliveira, M. P. de, Yuen, P., Wiemels, J. L. and Greaves, M. F.: Transplacental chemical exposure and risk of infant leukemia with MLL gene fusion. *Cancer research* 61: 2542–2546 (2001)
4. American Cancer Society a: http://www.cancer.org/cancer/leukemia-acutelymphocyticallinadults/detailedguide/leukemia-acute-lymphocytic-risk-factors (10.11.2016)
5. American Cancer Society b: http://www.cancer.org/cancer/leukemiainchildren/detailedguide/childhood-leukemia-survival-rates (10.11.2016)
6. Anderson, M. A., Deng, J., Seymour, J. F., Tam, C., Kim, S. Y., Fein, J., Yu, L., Brown, J. R., Westerman, D., Si, E. G., Majewski, I. J., Segal, D., Heitner Enschede, S. L., Huang, D. C. S., Davids, M. S., Letai, A. and Roberts, A. W.: The BCL2 selective inhibitor venetoclax induces rapid onset apoptosis of CLL cells in patients via a TP53-independent mechanism. Blood 127: 3215–3224 (2016)
7. Aparicio, A. and Weber, J. S.: Review of the clinical experience with 5-azacytidine and 5-aza-2'-deoxycytidine in solid tumors. *Current opinion in investigational drugs* 3: 627–633 (2002)

8. Arber, D. A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M. J., Le Beau, M. M., Bloomfield, C. D., Cazzola, M. and Vardiman, J. W.: The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* 127: 2391–2405 (2016)

9. Aref, S., Salama, O., Al-Tonbary, Y. and Mansour, A.: Assessment of bcl-2 expression as modulator of fas mediated apoptosis in acute leukemia. Hematology 9: 113–121 (2004)

10. Armstrong, S. A. and Look, A. T.: Molecular genetics of acute lymphoblastic leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 23: 6306–6315 (2005)

11. Ashkenazi, A.: Death Receptors. Signaling and Modulation. *Science* 281: 1305–1308 (1998)

12. Aster J. C.: The Hematopoietic and Lymphoid Systems. In: Kumar V., Abbas A. K., Fausto N., Mitchell R. N. (Editors) Robbins basic pathology. 8. ed. Philadelphia Pa.: Saunders Elsevier, 444-451 (2007)

13. Attisano, L. and Wrana, J. L.: Signal transduction by the TGF-beta superfamily. *Science* 296: 1646-1647 (2002)

14. Bachman, K. E., Herman, J. G., Corn, P. G., Merlo, A., Costello, J. F., Cavenee, W. K., Baylin, S. B. and Graff, J. R.: Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. *Cancer research* 59: 798–802 (1999)

15. Bakkebo, M., Huse, K., Hilden, V. I., Smeland, E. B. and Oksvold, M. P.: TGF-beta-induced growth inhibition in B-cell lymphoma correlates with Smad1/5 signalling and constitutively active p38 MAPK. BMC immunology 11: 57 (2010)

16. Behm, F. G., Raimondi, S. C., Frestedt, J. L., Liu, Q., Crist, W. M., Downing, J. R., Rivera, G. K., Kersey, J. H. and Pui, C. H.: Rearrangement of the MLL gene confers a poor prognosis in childhood acute lymphoblastic leukemia, regardless of presenting age. *Blood* 87: 2870–2877 (1996)

17. Bene, M. C., Castoldi, G., Knapp, W., Ludwig, W. D., Matutes, E., Orfao, A. and van't Veer, M. B.: Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia* 9: 1783–1786 (1995)

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

19. Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A., Gralnick, H. R. and Sultan, C.: Proposals for the classification of chronic (mature) B and T lymphoid leukaemias. French-American-British (FAB) Cooperative Group. *Journal of clinical pathology* 42: 567–584 (1989)

20. Bhatla, T., Wang, J., Morrison, D. J., Raetz, E. A., Burke, M. J., Brown, P. and Carroll, W. L.: Epigenetic reprogramming reverses the relapse-specific gene expression signature and restores chemosensitivity in childhood B-lymphoblastic leukemia. *Blood* 119: 5201–5210 (2012)

21. Bialik, S. and Kimchi, A.: DAP-kinase as a target for drug design in cancer and diseases associated with accelerated cell death. *Seminars in cancer biology* 14: 283–294 (2004)

22. Bialik, S. and Kimchi, A.: The death-associated protein kinases: structure, function, and beyond. *Annual review of biochemistry* 75: 189–210 (2006)

23. Bialik, S., Bresnick, A. R. and Kimchi, A.: DAP-kinase-mediated morphological changes are localization dependent and involve myosin-II phosphorylation. *Cell death and differentiation* 11: 631–644 (2004)

24. Bird, A.: DNA methylation patterns and epigenetic memory. *Genes & development* 16: 6–21 (2002)

25. Brill, A., Torchinsky, A., Carp, H. and Toder, V.: The Role of Apoptosis in Normal and Abnormal Embryonic development. *Journal of Assisted Reproduction and Genetics* 16: 512–519 (1999)

26. Burke, M. J. and Bhatla, T.: Epigenetic modifications in pediatric acute lymphoblastic leukemia. Frontiers in pediatrics 2: 42 (2014)

27. Cai, J., Yang, J. and Jones, D.: Mitochondrial control of apoptosis. The role of cytochrome c. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1366: 139–149 (1998)

28. Chen, C.-H., Wang, W.-J., Kuo, J.-C., Tsai, H.-C., Lin, J.-R., Chang, Z.-F. and Chen, R.-H.: Bidirectional signals transduced by DAPK-ERK interaction promote the apoptotic effect of DAPK. *The EMBO journal* 24: 294–304 (2005)

29. Cohen, O., Feinstein, E. and Kimchi, A.: DAP-kinase is a Ca2+/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. *The EMBO journal* 16: 998–1008 (1997)

30. Conter, V., Bartram, C. R., Valsecchi, M. G., Schrauder, A., Panzer-Grumayer, R., Moricke, A., Arico, M., Zimmermann, M., Mann, G., Rossi, G. de, Stanulla, M., Locatelli, F., Basso, G., Niggli, F., Barisone, E., Henze, G., Ludwig, W.-D., Haas, O. A., Cazzaniga, G., Koehler, R., Silvestri, D., Bradtke, J., Parasole, R., Beier, R., van Dongen, Jacques J M, Biondi, A. and Schrappe, M.: Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. *Blood* 115: 3206–3214 (2010)

31. Croce, C. M.: Oncogenes and cancer. *The New England journal of medicine* 358: 502–511 (2008)

32. Daskalakis, M., Nguyen, T. T., Nguyen, C., Guldberg, P., Kohler, G., Wijermans, P., Jones, P. A. and Lubbert, M.: Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2'-deoxycytidine (decitabine) treatment. *Blood* 100: 2957–2964 (2002)

33. Debatin, K.-M.: Chronic lymphocytic leukemia: keeping cell death at bay. *Cell* 129: 853–855 (2007)

34. Deiss, L. P., Feinstein, E., Berissi, H., Cohen, O. and Kimchi, A.: Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes & development* 9: 15–30 (1995)

35. Dong, M. and Blobe, G. C.: Role of transforming growth factor-beta in hematologic malignancies. *Blood* 107: 4589–4596 (2006)

36. Dordelmann, M., Reiter, A., Borkhardt, A., Ludwig, W. D., Gotz, N., Viehmann, S., Gadner, H., Riehm, H. and Schrappe, M.: Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood* 94: 1209–1217 (1999)

37. Druker, B. J., Sawyers, C. L., Kantarjian, H., Resta, D. J., Reese, S. F., Ford, J. M., Capdeville, R. and Talpaz, M.: Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *The New England journal of medicine* 344: 1038–1042 (2001)

38. Ehrich, M., Nelson, M. R., Stanssens, P., Zabeau, M., Liloglou, T., Xinarianos, G., Cantor, C. R., Field, J. K. and van den Boom, D.: Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America* 102: 15785–15790 (2005)

39. Elmore, S.: Apoptosis: a review of programmed cell death. *Toxicologic pathology* 35: 495–516 (2007)

40. Esparza, S. D., and Sakamoto, K. M.: Topics in pediatric leukemia - acute lymphoblastic leukemia. *MedGenMed* 7: 23-23 (2005)

41. Esteller, M., Avizienyte, E., Corn, P. G., Lothe, R. A., Baylin, S. B., Aaltonen, L. A. and Herman, J. G.: Epigenetic inactivation of LKB1 in primary tumors associated with the Peutz-Jeghers syndrome. *Oncogene* 19: 164–168 (2000)

42. Esteller, M., Sanchez-Cespedes, M., Rosell, R., Sidransky, D., Baylin, S. B. and Herman, J. G.: Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer research* 59: 67–70 (1999)

43. Fong, C. T. and Brodeur, G. M.: Down's syndrome and leukemia: epidemiology, genetics, cytogenetics and mechanisms of leukemogenesis. *Cancer genetics and cytogenetics* 28: 55–76 (1987)

44. Fried, J., Perez, A. G. and Clarkson, B. D.: Flow cytofluorometric analysis of cell cycle distributions using propidium iodide. Properties of the method and mathematical analysis of the data. *The Journal of cell biology* 71: 172–181 (1976)

45. Galm, O., Herman, J. G. and Baylin, S. B.: The fundamental role of epigenetics in hematopoietic malignancies. *Blood reviews* 20: 1–13 (2006)

46. Gaur, U. and Aggarwal, B. B.: Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. *Biochemical pharmacology* 66: 1403–1408 (2003)

47. Ghobrial, I. M., Witzig, T. E. and Adjei, A. A.: Targeting apoptosis pathways in cancer therapy. *CA: a cancer journal for clinicians* 55: 178–194 (2005)

48. Gillet, J.-P., Varma, S. and Gottesman, M. M.: The clinical relevance of cancer cell lines. *Journal of the National Cancer Institute* 105: 452–458 (2013)

49. Goodsell, D. S.: The molecular perspective: caspases. *The oncologist* 5: 435–436 (2000)

50. Greaves, M. F.: Aetiology of acute leukaemia. *The Lancet* 349: 344–349 (1997)

51. Green, D. R. and Kroemer, G.: The pathophysiology of mitochondrial cell death. *Science* 305: 626–629 (2004)

52. Green, D. R. and Llambi, F.: Cell Death Signaling. *Cold Spring Harbor perspectives in biology* 7: a006080 (2015)

53. Green, D. R. and Reed, J. C.: Mitochondria and apoptosis. *Science* 281: 1309–1312 (1998)

54. Gutierrez, M. I., Siraj, A. K., Bhargava, M., Ozbek, U., Banavali, S., Chaudhary, M. A., El Solh, H. and Bhatia, K.: Concurrent methylation of multiple genes in childhood ALL: Correlation with phenotype and molecular subgroup. *Leukemia* 17: 1845–1850 (2003)

55. Hanahan, D. and Weinberg, R. A.: Hallmarks of cancer: the next generation. *Cell* 144: 646–674 (2011)

56. Hanahan, D. and Weinberg, R. A.: The hallmarks of cancer. *Cell* 100: 57–70 (2000)

57. Hasan, M. N., Queudeville, M., Trentin, L., Eckhoff, S. M., Bronzini, I., Palmi, C., Barth, T., Cazzaniga, G., Te Kronnie, G., Debatin, K.-M. and Meyer, L. H.: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6: 1382–1395 (2015)

58. Henze, G., Fengler, R., Hartmann, R., Kornhuber, B., Janka-Schaub, G., Niethammer, D. and Riehm, H.: Six-year experience with a comprehensive approach to the treatment of recurrent childhood acute lymphoblastic leukemia (ALL-REZ BFM 85). A relapse study of the BFM group. *Blood* 78: 1166–1172 (1991)

59. Herman, J. G. and Baylin, S. B.: Gene silencing in cancer in association with promoter hypermethylation. *The New England journal of medicine* 349: 2042–2054 (2003)

60. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D. and Baylin, S. B.: Methylation-specific PCR. A novel PCR assay for methylation status of CpG islands. *Proceedings of the National Academy of Sciences* 93: 9821–9826 (1996)

61. Herold, G.: Innere Medizin 2014. Eine vorlesungsorientierte Darstellung; unter Berücksichtigung des Gegenstandskataloges für die Ärztliche Prüfung; mit ICD 10-Schlüssel im Text und Stichwortverzeichnis. Köln: Selbstverl. 92-98 (2014)

62. Hirsch, T., Marchetti, P., Susin, S. A., Dallaporta, B., Zamzami, N., Marzo, I., Geuskens, M. and Kroemer, G.: The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. *Oncogene* 15: 1573–1581 (1997)

63. Hollenbach, P. W., Nguyen, A. N., Brady, H., Williams, M., Ning, Y., Richard, N., Krushel, L., Aukerman, S. L., Heise, C. and MacBeth, K. J.: A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. *PloS one* 5: e9001 (2010)

64. Hunger, S. P., Lu, X., Devidas, M., Camitta, B. M., Gaynon, P. S., Winick, N. J., Reaman, G. H. and Carroll, W. L.: Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 30: 1663–1669 (2012)

65. Inbal, B., Bialik, S., Sabanay, I., Shani, G. and Kimchi, A.: DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *The Journal of cell biology* 157: 455–468 (2002)

66. Inbal, B., Cohen, O., Polak-Charcon, S., Kopolovic, J., Vadai, E., Eisenbach, L. and Kimchi, A.: DAP kinase links the control of apoptosis to metastasis. *Nature* 390: 180–184 (1997)

67. Irving, J. A. E.: Towards an understanding of the biology and targeted treatment of paediatric relapsed acute lymphoblastic leukaemia. *British journal of haematology* 172: 655–666 (2016)

68. Jablonowski, Z., Reszka, E., Gromadzinska, J., Wasowicz, W. and Sosnowski, M.: Hypermethylation of p16 and DAPK promoter gene regions in patients with non-invasive urinary bladder cancer. *Archives of medical science : AMS* 7: 512–516 (2011)

69. Jacoby, E., Chien, C. D. and Fry, T. J.: Murine models of acute leukemia: important tools in current pediatric leukemia research. *Frontiers in oncology* 4: 95 (2014)

70. Jang, C.-W., Chen, C.-H., Chen, C.-C., Chen, J.-y., Su, Y.-H. and Chen, R.-H.: TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nature cell biology* 4: 51–58 (2002)

71. Johnson, A. A., Akman, K., Calimport, S. R. G., Wuttke, D., Stolzing, A. and Magalhaes, J. P. de: The role of DNA methylation in aging, rejuvenation, and age-related disease. *Rejuvenation research* 15: 483–494 (2012)

72. Jones, P. A. and Baylin, S. B.: The fundamental role of epigenetic events in cancer. *Nature reviews. Genetics* 3: 415–428 (2002)

73. Juergens, R. A., Wrangle, J., Vendetti, F. P., Murphy, S. C., Zhao, M., Coleman, B., Sebree, R., Rodgers, K., Hooker, C. M., Franco, N., Lee, B., Tsai, S., Delgado, I. E., Rudek, M. A., Belinsky, S. A., Herman, J. G., Baylin, S. B., Brock, M. V. and Rudin, C. M.: Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. *Cancer discovery* 1: 598–607 (2011)

74. Kaatsch, P.: Epidemiology of childhood cancer. Cancer treatment reviews 36: 277–285 (2010)

75. Kalivendi, S. V., Konorev, E. A., Cunningham, S., Vanamala, S. K., Kaji, E. H., Joseph, J. and Kalyanaraman, B.: Doxorubicin activates nuclear factor of activated T-lymphocytes and Fas ligand transcription: role of mitochondrial reactive oxygen species and calcium. *The Biochemical journal* 389: 527–539 (2005)

76. Kamel-Reid, S., Letarte, M., Sirard, C., Doedens, M., Grunberger, T., Fulop, G., Freedman, M. H., Phillips, R. A. and Dick, J. E.: A model of human acute lymphoblastic leukemia in immune-deficient SCID mice. *Science* 246: 1597–1600 (1989)

77. Kantarjian, H. M. and Issa, J.-P. J.: Decitabine dosing schedules. *Seminars in hematology* 42: 17-22 (2005)

78. Kantarjian, H. M., O'Brien, S., Cortes, J., Giles, F. J., Faderl, S., Issa, J.-P., Garcia-Manero, G., Rios, M. B., Shan, J., Andreeff, M., Keating, M. and Talpaz, M.: Results of decitabine (5-aza-2'deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. *Cancer* 98: 522–528 (2003)

79. Kantarjian, H., Issa, J.-P. J., Rosenfeld, C. S., Bennett, J. M., Albitar, M., DiPersio, J., Klimek, V., Slack, J., Castro, C. de, Ravandi, F., Helmer, R. 3., Shen, L., Nimer, S. D., Leavitt, R., Raza, A. and Saba, H.: Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer* 106: 1794–1803 (2006)

80. Kaparou, M., Choumerianou, D., Perdikogianni, C., Martimianaki, G., Kalmanti, M. and Stiakaki, E.: Enhanced levels of the apoptotic BAX/BCL-2 ratio in children with acute lymphoblastic leukemia and high-risk features. Genetics and molecular biology 36: 7–11 (2013)

81. Karon, M., Sieger, L., Leimbrock, S., Finklestein, J. Z., Nesbit, M. E. and Swaney, J. J.: 5-Azacytidine: a new active agent for the treatment of acute leukemia. *Blood* 42: 359–365 (1973)

82. Karpf, A. R., Moore, B. C., Ririe, T. O. and Jones, D. A.: Activation of the p53 DNA damage response pathway after inhibition of DNA methyltransferase by 5-aza-2'-deoxycytidine. *Molecular pharmacology* 59: 751–757 (2001)

83. Katzenellenbogen, R. A., Baylin, S. B. and Herman, J. G.: Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. *Blood* 93: 4347–4353 (1999)

84. Kerr, J. F., Wyllie, A. H. and Currie, A. R.: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer* 26: 239–257 (1972)

85. Kim, D. H., Nelson, H. H., Wiencke, J. K., Christiani, D. C., Wain, J. C., Mark, E. J. and Kelsey, K. T.: Promoter methylation of DAP-kinase: association with advanced stage in non-small cell lung cancer. *Oncogene* 20: 1765–1770 (2001)

86. Kinlen, L. J.: Epidemiological evidence for an infective basis in childhood leukaemia. *British journal of cancer* 71: 1–5 (1995)

87. Kissil, J. L., Feinstein, E., Cohen, O., Jones, P. A., Tsai, Y. C., Knowles, M. A., Eydmann, M. E. and Kimchi, A.: DAP-kinase loss of expression in various carcinoma and B-cell lymphoma cell lines: possible implications for role as tumor suppressor gene. *Oncogene* 15: 403–407 (1997)

88. Klumper, E., Pieters, R., Veerman, A. J., Huismans, D. R., Loonen, A. H., Hahlen, K., Kaspers, G. J., van Wering, E. R., Hartmann, R. and Henze, G.: In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia. *Blood* 86: 3861–3868 (1995)

89. Knudson, A. G., JR: Mutation and cancer: statistical study of retinoblastoma. *Proceedings of the National Academy of Sciences of the United States of America* 68: 820–823 (1971)

90. Kong, W.-J., Zhang, S., Guo, C., Zhang, S., Wang, Y. and Zhang, D.: Methylation-associated silencing of death-associated protein kinase gene in laryngeal squamous cell cancer. *The Laryngoscope* 115: 1395–1401 (2005)

91. Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., Blagosklonny, M. V., El-Deiry, W. S., Golstein, P., Green, D. R., Hengartner, M., Knight, R. A., Kumar, S., Lipton, S. A., Malorni, W., Nunez, G., Peter, M. E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B. and Melino, G.: Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell death and differentiation* 16: 3–11 (2009)

92. Kumar V., Abbas A. K., Fausto N. and Mitchell R. N.: Cell Injury, Cell Death, and Adaptations. In: Kumar V., Abbas A. K., Fausto N. and Mitchell R. N. (Editors) Robbins basic pathology. 8. ed. Philadelphia Pa.: Saunders Elsevier, 9-12 (2007)

93. Lamm, S. H., Walters, A. S., Wilson, R., Byrd, D. M. and Grunwald, H.: Consistencies and inconsistencies underlying the quantitative assessment of leukemia risk from benzene exposure. *Environmental health perspectives* 82: 289–297 (1989)

94. Lavelle, D., DeSimone, J., Hankewych, M., Kousnetzova, T. and Chen, Y.-H.: Decitabine induces cell cycle arrest at the G1 phase via p21WAF1 and the G2/M phase via the p38 MAP kinase pathway. *Leukemia Research* 27: 999–1007 (2003)

95. Levin-Salomon, V., Bialik, S. and Kimchi, A.: DAP-kinase and autophagy. *Apoptosis : an international journal on programmed cell death* 19: 346–356 (2014)

96. Li, E., Beard, C. and Jaenisch, R.: Role for DNA methylation in genomic imprinting. *Nature* 366: 362–365 (1993)

97. Li, Y., Zhu, M., Zhang, X., Cheng, D. and Ma, X.: Clinical significance of DAPK promoter hypermethylation in lung cancer: a meta-analysis. *Drug design, development and therapy* 9: 1785–1796 (2015)

98. Lichtenstein, P., Holm, N. V., Verkasalo, P. K., Iliadou, A., Kaprio, J., Koskenvuo, M., Pukkala, E., Skytthe, A. and Hemminki, K.: Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *The New England journal of medicine* 343: 78–85 (2000)

99. Liu, Q.-Y., Chen, D.-W., Xie, L.-P., Zhang, R.-Q. and Wang, H.-Z.: Decitabine, independent of apoptosis, exerts its cytotoxic effects on cell growth in melanoma cells. *Environmental toxicology and pharmacology* 32: 423–429 (2011)

100. Liu, X., Kim, C. N., Yang, J., Jemmerson, R. and Wang, X.: Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86: 147–157 (1996)

101. Lock, R. B., Liem, N., Farnsworth, M. L., Milross, C. G., Xue, C., Tajbakhsh, M., Haber, M., Norris, M. D., Marshall, G. M. and Rice, A. M.: The nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse model of childhood acute lymphoblastic leukemia reveals intrinsic differences in biologic characteristics at diagnosis and relapse. *Blood* 99: 4100–4108 (2002)

102. Malempati, S., Gaynon, P. S., Sather, H., La, M. K. and Stork, L. C.: Outcome after relapse among children with standard-risk acute lymphoblastic leukemia: Children's Oncology Group study CCG-1952. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 25: 5800–5807 (2007)

103. Martincorena, I. and Campbell, P. J.: Somatic mutation in cancer and normal cells. *Science* 349: 1483–1489 (2015)

104. Martoriati, A., Doumont, G., Alcalay, M., Bellefroid, E., Pelicci, P. G. and Marine, J.-C.: dapk1, encoding an activator of a p19ARF-p53-mediated apoptotic checkpoint, is a transcription target of p53. *Oncogene* 24: 1461–1466 (2005)

105. Matsumoto, H., Nagao, M., Ogawa, S., Kanehiro, H., Hisanaga, M., Ko, S., Ikeda, N., Fujii, H., Koyama, F., Mukogawa, T. and Nakajima, Y.: Prognostic significance of death-associated protein-kinase expression in hepatocellular carcinomas. *Anticancer research* 23: 1333–1341 (2003)

106. Meyer, L. H., Eckhoff, S. M., Queudeville, M., Kraus, J. M., Giordan, M., Stursberg, J., Zangrando, A., Vendramini, E., Moricke, A., Zimmermann, M., Schrauder, A., Lahr, G., Holzmann, K., Schrappe, M., Basso, G., Stahnke, K., Kestler, H. A., Te Kronnie, G. and Debatin, K.-M.: Early relapse in ALL is identified by time to leukemia in NOD/SCID mice and is characterized by a gene signature involving survival pathways. *Cancer cell* 19: 206–217 (2011)

107. Meyer, L. H., Karawajew, L., Schrappe, M., Ludwig, W.-D., Debatin, K.-M. and Stahnke, K.: Cytochrome c-related caspase-3 activation determines treatment response and relapse in childhood precursor B-cell ALL. *Blood* 107: 4524–4531 (2006)

108. Meyer, L. H., Queudeville, M., Eckhoff, S. M., Creutzig, U., Reinhardt, D., Karawajew, L., Ludwig, W.-D., Stahnke, K. and Debatin, K.-M.: Intact apoptosis signaling in myeloid leukemia cells determines treatment outcome in childhood AML. *Blood* 111: 2899–2903 (2008)

109. Momparler, R. L., Rivard, G. E. and Gyger, M.: Clinical trial on 5- AZA-2′-deoxycytidine in patients with acute leukemia. *Pharmacology & Therapeutics* 30: 277–286 (1985)

110. Moricke, A., Zimmermann, M., Reiter, A., Henze, G., Schrauder, A., Gadner, H., Ludwig, W. D., Ritter, J., Harbott, J., Mann, G., Klingebiel, T., Zintl, F., Niemeyer, C., Kremens, B., Niggli, F., Niethammer, D., Welte, K., Stanulla, M., Odenwald, E., Riehm, H. and Schrappe, M.: Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. *Leukemia* 24: 265–284 (2010)

111. Moriyama, T., Relling, M. V. and Yang, J. J.: Inherited genetic variation in childhood acute lymphoblastic leukemia. *Blood* 125: 3988–3995 (2015)

112. Mund, C., Brueckner, B. and Lyko, F.: Reactivation of epigenetically silenced genes by DNA methyltransferase inhibitors: basic concepts and clinical applications. *Epigenetics* 1: 7–13 (2006)

113. Nair, S., Hagberg, H., Krishnamurthy, R., Thornton, C. and Mallard, C.: Death associated protein kinases: molecular structure and brain injury. *International journal of molecular sciences* 14: 13858–13872 (2013)

114. Nakatsuka, S.-i., Takakuwa, T., Tomita, Y., Hoshida, Y., Nishiu, M., Yamaguchi, M., Nishii, K., Yang, W.-I. and Aozasa, K.: Hypermethylation of death-associated protein (DAP) kinase CpG island is frequent not only in B-cell but also in T- and natural killer (NK)/T-cell malignancies. *Cancer science* 94: 87–91 (2003)

115. Nie, J., Liu, L., Li, X. and Han, W.: Decitabine, a new star in epigenetic therapy: the clinical application and biological mechanism in solid tumors. *Cancer letters* 354: 12–20 (2014)

116. Nieto, M., Demolis, P., Behanzin, E., Moreau, A., Hudson, I., Flores, B., Stemplewski, H., Salmonson, T., Gisselbrecht, C., Bowen, D. and Pignatti, F.: The European Medicines Agency Review of Decitabine (Dacogen) for the Treatment of Adult Patients With Acute Myeloid Leukemia: Summary of the Scientific Assessment of the Committee for Medicinal Products for Human Use. *The oncologist* 21: 692–700 (2016)

117. Nordling C. O.: A new theory on cancer-inducing mechanism. *British journal of cancer* 7: 68–72 (1953)

118. Oki, Y., Aoki, E. and Issa, J.-P. J.: Decitabine--bedside to bench. *Critical reviews in oncology/hematology* 61: 140–152 (2007)

119. Olek, A., Oswald, J. and Walter, J.: A Modified and Improved Method for Bisulphite Based Cytosine Methylation Analysis. *Nucleic Acids Research* 24: 5064–5066 (1996)

120. Ong, Y. L., McMullin, M. F., Bailie, K. E. M., Lappin, T. R. J., Jones, F. G. C. and Irvine, A. E.: High bax expression is a good prognostic indicator in acute myeloid leukaemia. British journal of haematology 111: 182-189 (2000)

121. Pal, D., Blair, H. J., Elder, A., Dormon, K., Rennie, K. J., Coleman, D. J. L., Weiland, J., Rankin, K. S., Filby, A., Heidenreich, O. and Vormoor, J.: Long-term in vitro maintenance of clonal abundance and leukaemia-initiating potential in acute lymphoblastic leukaemia. *Leukemia* 30: 1691–1700 (2016)

122. Pelled, D., Raveh, T., Riebeling, C., Fridkin, M., Berissi, H., Futerman, A. H. and Kimchi, A.: Death-associated protein (DAP) kinase plays a central role in ceramide-induced apoptosis in cultured hippocampal neurons. *The Journal of biological chemistry* 277: 1957–1961 (2002)

123. Peto, J.: Cancer epidemiology in the last century and the next decade. *Nature* 411: 390–395 (2001)

124. Plass, C., Claus R., Meyer L.H.: (personal communication)

125. Pogorzala, M., Kubicka, M., Rafinska, B., Wysocki, M. and Styczynski, J.: Drug-resistance Profile in Multiple-relapsed Childhood Acute Lymphoblastic Leukemia. *Anticancer research* 35: 5667–5670 (2015)

126. Ponchel, F., Toomes, C., Bransfield, K., Leong, F. T., Douglas, S. H., Field, S. L., Bell, S. M., Combaret, V., Puisieux, A., Mighell, A. J., Robinson, P. A., Inglehearn, C. F., Isaacs, J. D. and Markham, A. F.: Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. *BMC biotechnology* 3: 18 (2003)

127. Preston, D. L., Kusumi, S., Tomonaga, M., Izumi, S., Ron, E., Kuramoto, A., Kamada, N., Dohy, H., Matsuo, T. and Matsui, T.: Cancer incidence in atomic bomb survivors. Part III. Leukemia, lymphoma and multiple myeloma, 1950-1987. *Radiation research* 137: 68-97 (1994)

128. Pui, C. H. and Evans, W. E.: Acute lymphoblastic leukemia. *The New England journal of medicine* 339: 605–615 (1998)

129. Pui, C. H., Williams, D. L., Raimondi, S. C., Rivera, G. K., Look, A. T., Dodge, R. K., George, S. L., Behm, F. G., Crist, W. M. and Murphy, S. B.: Hypodiploidy is associated with a poor prognosis in childhood acute lymphoblastic leukemia. *Blood* 70: 247–253 (1987)

130. Pui, C.-H. and Evans, W. E.: Treatment of acute lymphoblastic leukemia. *The New England journal of medicine* 354: 166–178 (2006)

131. Pui, C.-H. and Jeha, S.: New therapeutic strategies for the treatment of acute lymphoblastic leukaemia. *Nature reviews. Drug discovery* 6: 149–165 (2007)

132. Pui, C.-H., Campana, D. and Evans, W. E.: Childhood acute lymphoblastic leukaemia – current status and future perspectives. *The Lancet Oncology* 2: 597–607 (2001)

133. Pui, C.-H., Relling, M. V. and Downing, J. R.: Acute lymphoblastic leukemia. *The New England journal of medicine* 350: 1535–1548 (2004)

134. Queudeville, M., Seyfried, F., Eckhoff, S. M., Trentin, L., Ulrich, S., Schirmer, M., Debatin, K.-M. and Meyer, L. H.: Rapid engraftment of human ALL in NOD/SCID mice involves deficient apoptosis signaling. *Cell death & disease* 3: e364 (2012)

135. Raval, A., Tanner, S. M., Byrd, J. C., Angerman, E. B., Perko, J. D., Chen, S.-S., Hackanson, B., Grever, M. R., Lucas, D. M., Matkovic, J. J., Lin, T. S., Kipps, T. J., Murray, F., Weisenburger, D., Sanger, W., Lynch, J., Watson, P., Jansen, M., Yoshinaga, Y., Rosenquist, R., Jong, P. J. de, Coggill, P., Beck, S., Lynch, H., La Chapelle, A. de and Plass, C.: Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. *Cell* 129: 879–890 (2007)

136. Raveh, T. and Kimchi, A.: DAP kinase-a proapoptotic gene that functions as a tumor suppressor. *Experimental cell research* 264: 185–192 (2001)

137. Rohrs, S., Romani, J., Zaborski, M., Drexler, H. G. and Quentmeier, H.: Hypermethylation of Death-Associated Protein Kinase 1 differentiates natural killer cell lines from cell lines derived from T-acute lymphoblastic leukemia. *Leukemia* 23: 1174–1176 (2009)

138. Roman-Gomez, J., Castillejo, J. A., Jimenez, A., Barrios, M., Heiniger, A. and Torres, A.: The role of DNA hypermethylation in the pathogenesis and prognosis of acute lymphoblastic leukemia. *Leukemia & lymphoma* 44: 1855–1864 (2003)

139. Ruiz-Magana, M. J., Rodriguez-Vargas, J. M., Morales, J. C., Saldivia, M. A., Schulze-Osthoff, K. and Ruiz-Ruiz, C.: The DNA methyltransferase inhibitors zebularine and decitabine induce mitochondria-mediated apoptosis and DNA damage in p53 mutant leukemic T cells. *International journal of cancer* 130: 1195–1207 (2012)

140. Samanta, D. and Datta, P. K.: Alterations in the Smad pathway in human cancers. Frontiers in bioscience (Landmark edition) 17: 1281–1293 (2012)

141. Sanchez-Cespedes, M., Esteller, M., Wu, L., Nawroz-Danish, H., Yoo, G. H., Koch, W. M., Jen, J., Herman, J. G. and Sidransky, D.: Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer research* 60: 892–895 (2000)

142. Schafer, E., Irizarry, R., Negi, S., McIntyre, E., Small, D., Figueroa, M. E., Melnick, A. and Brown, P.: Promoter hypermethylation in MLL-r infant acute lymphoblastic leukemia: biology and therapeutic targeting. *Blood* 115: 4798–4809 (2010)

143. Schirmer, M., Trentin, L., Queudeville, M., Seyfried, F., Demir, S., Tausch, E., Stilgenbauer, S., Eckhoff, S. M., Meyer, L. H. and Debatin, K.-M.: Intrinsic and chemo-sensitizing activity of SMAC-mimetics on high-risk childhood acute lymphoblastic leukemia. *Cell death & disease* 7: e2052 (2016)

144. Schlieben, S., Borkhardt, A., Reinisch, I., Ritterbach, J., Janssen, J. W., Ratei, R., Schrappe, M., Repp, R., Zimmermann, M., Kabisch, H., Janka-Schaub, G., Bartram, C. R., Ludwig, W. D., Riehm, H., Lampert, F. and Harbott, J.: Incidence and clinical outcome of children with BCR/ABL-positive acute lymphoblastic leukemia (ALL). A prospective RT-PCR study based on 673 patients enrolled in the German pediatric multicenter therapy trials ALL-BFM-90 and CoALL-05-92. *Leukemia* 10: 957–963 (1996)

145. Schrappe, M., Hunger, S. P., Pui, C.-H., Saha, V., Gaynon, P. S., Baruchel, A., Conter, V., Otten, J., Ohara, A., Versluys, A. B., Escherich, G., Heyman, M., Silverman, L. B., Horibe, K., Mann, G., Camitta, B. M., Harbott, J., Riehm, H., Richards, S., Devidas, M. and Zimmermann, M.: Outcomes after induction failure in childhood acute lymphoblastic leukemia. *The New England journal of medicine* 366: 1371–1381 (2012)

146. Schrappe, M., Reiter, A., Ludwig, W. D., Harbott, J., Zimmermann, M., Hiddemann, W., Niemeyer, C., Henze, G., Feldges, A., Zintl, F., Kornhuber, B., Ritter, J., Welte, K., Gadner, H. and Riehm, H.: Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM Study Group. *Blood* 95: 3310–3322 (2000)

147. Schultz, K. R., Bowman, W. P., Aledo, A., Slayton, W. B., Sather, H., Devidas, M., Wang, C., Davies, S. M., Gaynon, P. S., Trigg, M., Rutledge, R., Burden, L., Jorstad, D., Carroll, A., Heerema, N. A., Winick, N., Borowitz, M. J., Hunger, S. P., Carroll, W. L. and Camitta, B.: Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 27: 5175–5181 (2009)

148. Shalini, S., Dorstyn, L., Dawar, S. and Kumar, S.: Old, new and emerging functions of caspases. *Cell death and differentiation* 22: 526–539 (2015)

149. Sharp, A. J., Stathaki, E., Migliavacca, E., Brahmachary, M., Montgomery, S. B., Dupre, Y. and Antonarakis, S. E.: DNA methylation profiles of human active and inactive X chromosomes. *Genome research* 21: 1592–1600 (2011)

150. Sherr, C. J.: Principles of tumor suppression. *Cell* 116: 235–246 (2004)

151. Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C. B. and Tsujimoto, Y.: Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nature cell biology* 6: 1221–1228 (2004)

152. Shin, D. Y., Park, Y.-S., Yang, K., Kim, G.-Y., Kim, W.-J., Han, M. H., Kang, H. S. and Choi, Y. H.: Decitabine, a DNA methyltransferase inhibitor, induces apoptosis in human leukemia cells through intracellular reactive oxygen species generation. *International journal of oncology* 41: 910–918 (2012)

153. Shohat, G., Spivak-Kroizman, T., Cohen, O., Bialik, S., Shani, G., Berrisi, H., Eisenstein, M. and Kimchi, A.: The pro-apoptotic function of death-associated protein kinase is controlled by a unique inhibitory autophosphorylation-based mechanism. *The Journal of biological chemistry* 276: 47460–47467 (2001)

154. Shurtleff, S. A., Buijs, A., Behm, F. G., Rubnitz, J. E., Raimondi, S. C., Hancock, M. L., Chan, G. C., Pui, C. H., Grosveld, G. and Downing, J. R.: TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. *Leukemia* 9: 1985–1989 (1995)

155. Siegel, R. L., Miller, K. D. and Jemal, A.: Cancer statistics, 2016. *CA: a cancer journal for clinicians* 66: 7–30 (2016)

156. Silverman, L. B., Gelber, R. D., Young, M. L., Dalton, V. K., Barr, R. D. and Sallan, S. E.: Induction failure in acute lymphoblastic leukemia of childhood. *Cancer* 85: 1395–1404 (1999)

157. Singh, P., Ravanan, P. and Talwar, P.: Death Associated Protein Kinase 1 (DAPK1): A Regulator of Apoptosis and Autophagy. *Frontiers in molecular neuroscience* 9: 46 (2016)

158. Soncini, M., Santoro, F., Gutierrez, A., Frige, G., Romanenghi, M., Botrugno, O. A., Pallavicini, I., Pelicci, P., Di Croce, L. and Minucci, S.: The DNA demethylating agent decitabine activates the TRAIL pathway and induces apoptosis in acute myeloid leukemia. *Biochimica et biophysica acta* 1832: 114–120 (2013)

159. Stresemann, C. and Lyko, F.: Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *International journal of cancer* 123: 8–13 (2008)

160. Thomas, D. A., Faderl, S., O'Brien, S., Bueso-Ramos, C., Cortes, J., Garcia-Manero, G., Giles, F. J., Verstovsek, S., Wierda, W. G., Pierce, S. A., Shan, J., Brandt, M., Hagemeister, F. B., Keating, M. J., Cabanillas, F. and Kantarjian, H.: Chemoimmunotherapy with hyper-CVAD plus rituximab for the treatment of adult Burkitt and Burkitt-type lymphoma or acute lymphoblastic leukemia. *Cancer* 106: 1569–1580 (2006)

161. Thornberry, N. A. and Lazebnik, Y.: Caspases: enemies within. *Science* 281: 1312–1316 (1998)

162. Tower, J.: Programmed cell death in aging. *Ageing research reviews* 23: 90–100 (2015)

163. Trojan, J., Brieger, A., Raedle, J., Esteller, M. and Zeuzem, S.: 5'-CpG island methylation of the LKB1/STK11 promoter and allelic loss at chromosome 19p13.3 in sporadic colorectal cancer. *Gut* 47: 272–276 (2000)

164. Tsujimoto, Y. and Shimizu, S.: Another way to die: autophagic programmed cell death. *Cell death and differentiation* 12 Suppl 2: 1528–1534 (2005)

165. van Dongen, J. J. M., Seriu, T., Panzer-Grümayer, E. R., Biondi, A., Pongers-Willemse, M. J., Corral, L., Stolz, F., Schrappe, M., Masera, G., Kamps, W. A., Gadner, H., van Wering, E. R., Ludwig, W.-D., Basso, G., Bruijn, M. A. C. de, Cazzaniga, G., Hettinger, K., van der Does-van den Berg, Anna, Hop, W. C. J., Riehm, H. and Bartram, C. R.: Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. *The Lancet* 352: 1731–1738 (1998)

166. Vanden Berghe, T., Linkermann, A., Jouan-Lanhouet, S., Walczak, H. and Vandenabeele, P.: Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nature reviews. Molecular cell biology* 15: 135–147 (2014)

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

168. Vermes, I., Haanen, C., Steffens-Nakken, H. and Reutelingsperger, C.: A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of immunological methods* 184: 39–51 (1995)

169. Weber, K. and Osborn, M.: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *The Journal of biological chemistry* 244: 4406–4412 (1969)

170. Wei, D.-M., Liu, D.-Y., Lei, D.-P., Jin, T., Wang, J. and Pan, X.-L.: Aberrant methylation and expression of DAPk1 in human hypopharyngeal squamous cell carcinoma. *Acta oto-laryngologica* 135: 70–78 (2015)

171. Weinberg, R. A.: The biology of cancer. New York, NY: GS Garland Science, 475–517 (2007)

172. WHO 2015: http://www.who.int/mediacentre/factsheets/fs297/en/ (10.11.2016)

173. WHO 2016: http://www.who.int/mediacentre/factsheets/fs310/en/index2.html (10.11.2016)

174. Yokota, J.: Tumor progression and metastasis. *Carcinogenesis* 21: 497–503 (2000)

175. Yuan, J. and Kroemer, G.: Alternative cell death mechanisms in development and beyond. *Genes & development* 24: 2592–2602 (2010)

176. Zalckvar, E., Berissi, H., Mizrachy, L., Idelchuk, Y., Koren, I., Eisenstein, M., Sabanay, H., Pinkas-Kramarski, R. and Kimchi, A.: DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. *EMBO reports* 10: 285–292 (2009)

177. Zelent, A., Greaves, M. and Enver, T.: Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia. *Oncogene* 23: 4275–4283 (2004)

178. Zhu, W.-G., Hileman, T., Ke, Y., Wang, P., Lu, S., Duan, W., Dai, Z., Tong, T., Villalona-Calero, M. A., Plass, C. and Otterson, G. A.: 5-aza-2'-deoxycytidine activates the p53/p21Waf1/Cip1 pathway to inhibit cell proliferation. *The Journal of biological chemistry* 279: 15161–15166 (2004)

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