The role of the non-canonical IKK complex in glioblastoma multiforme

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% (v/v)	volume/volume percentage (ml/100 ml)
% (w/v)	weight/volume percentage (g/100 ml)
°C	degree Celsius
µ	micro
2-HG	R-2 hydroxyglutarate
4EBP	eukaryotic translation initiation factor 4E-binding protein
aa	amino acids
Ack1	activated Cdc42-associated kinase
AGC	cAMP-dependent protein kinase/ protein kinase G/ protein kinase C
AP-1	activator protein 1
AraC	cytarabine
ARE	AU-rich elements
ASK1	apoptosis signal-regulating kinase 1
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
Bcl-6	B-cell lymphoma 6
BIM	bisindolyl maleimide-based, nanomolar protein kinase C inhibitors
BUB3	Budding uninhibited by benzimidazole 3
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CDK4/6	cyclin-dependent kinases 4/6
cDNA	complementary DNA
ChIP	chromatin-immunoprecipitation
Chk	Checkpoint kinase
CHX	cycloheximide
CK2	casein kinase 2
Co-IP	co-immunoprecipitation
COX2	cyclooxygenase 2
CREB	cAMP-responsive element binding protein
CTP	cytidine triphosphate
CYLD	cylindromatosis
Da	Dalton (g/mol)
DAI	DNA-dependent activator of IFN-regulatory factors
DAPK	death-associated protein kinase
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA

dsRNA	Double stranded RNA
e.g.	exempli gratia (for example)
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EGR-1	early growth response protein 1
eIF2B	eukaryotic initiation factor 2B
eIF4E	eukaryotic initiation factor 4E
Elk-1	ETS domain-containing protein
erbB2	epidermal growth factor receptor 2
ERK	extracellular signal-regulated kinase
ERα	estrogen receptor α
et al.	et alii (and others)
FACS	fluorescence activated cell sorting
Fig	figure
FITC	fluorescein isothiocyanate
FOXO	Forkhead box protein O
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBM	glioblastoma multiforme
GEF	guanine nucleotide exchange factors
GLUT	glucose transporter
GPCR	G coupled receptors
Grb2	growth-factor-receptor-bound 2
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
h(s)	hour(s)
HDAC	histone deacetylase
HER2	epidermal growth factor receptor 2
HLH	helix-loop-helix
HNSCC	head and neck cancer
Hsp27	heat shock protein 27
HuR	human antigen R
IB	immunoblotting
IDH	Isocitrate dehydrogenase
IFN	interferon
IGF-1	insulin-like growth factor 1
IKK	IκB kinase
IL	interleukin
ILK	integrin-linked kinase
IP	immunoprecipitation
IRAK	interleukin-1 receptor-associated kinase

IRF	interferon egulatory factor
ISGF3	interferon-stimulated gamma factor 3
ISRE	Interferon-stimulated response element
IκB	Inhibitor of κB
JAK	janus kinase
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
KSR	kinase suppressor of Ras
l	liter
LGI1	leucine-rich, glioma activated 1
LPS	lipopolysaccharide
LZ	leucine zipper
M	Molar (mol/l)
m	milli
MAPK	mitogen-activated protein kinase
MAPKKAP-K2	mitogen-activated protein kinase-activated protein kinase 2
MAVS	mitochondrial antiviral signaling adaptor
MCL-1	myeloid leukemia cell differentiation protein
MCL-1	myeloid leukemia cell differentiation protein
MDA5	melanoma differentiation-associated protein 5
MDM2	mouse double minute 2 homolog
MDR-1	multi-drug-resistant 1
MDR-1	multi-drug-resistant 1
MEF	mouse embryonic fibroblast
MEK	mitogen-activated protein kinase kinase
MGMT	O^6-alkylguanine DNA alkyltransferase
min	minutes
MKKK	MAP kinase kinase kinase
MLCK	myosin light chain kinase
MLK3	mixed lineage kinase 3
MLTK	MLK-like mitogen-activated protein triple kinase
MMP9	matrix metallopeptidase 9
MP1	MEK partner 1
mRNA	messenger RNA
MTIC	3-methyl-(triazen-1-yl)imidazole-4-carboxamide
mTORC	mammalian target of rapamycin complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MXI1	MAX-interacting protein
MyD88	myeloid differentiation primary response gene (88)
NAP1	NAK-associated protein 1

NEMO	NF-κB essential modulator
NFAT3	nuclear factor of activated T cells 3
NFKBIA/ IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OD	optical density
PAMP	pathogen-associated molecular pattern
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDE-4	phosphodieesterase 4
PDK1/2	phosphoinositide-dependent kinase-1 and -2
PH domain	Pleckstrin homology domain
PI	propidium iodide
PI3K	phosphatidylinositide 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphat
PIP_3	phosphatidylinositol-3,4,5-triphosphat
PKA	Protein kinase A
PKB	Protein kinase B (also referred to as Akt)
PKC	Protein kinase C
PMA	phorbol 12-myristate 13-acetate
Pol II S2	polymerase II phosphorylated at serine 2
PP2A	protein phosphatase 2A
PTEN	phosphatase and tensin homolog
Ral	Ras-like
RalGEF	Ras-like-guanine nucleotide exchange factor
Ras	Rat sarcoma
RasGEF	Ras guanine nucleotide exchange factor
RASSF1A	Ras Association Domain family 1 alpha
RB1	retinoblastoma protein 1
RIG-I	retinoic acid-inducible gene 1
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RSK	ribosomal S6 kinases
RTK	receptor tyrosine kinase
S	Serine
SAPK	Stress- activated protein kinase
sec	second
Sec5	exocyst complex component 2
SEK1	SAPK/Erk kinase 1
Shc	SHC-transforming protein

SHIP	SH2-domain-containing inositol phosphatases
shRNA	short hairpin RNA
SINTBAD	similar to NAP1 TBK1 adaptor
SOS	son of sevenless
SOX11	SRY-related HMG-box
SREBP	sterol regulatory element-binding protein
STAT	signal transducer and activator of transcription
T	Threonine
TAB	TAK1-binding protein
TAK1	TGF-β-activated kinase 1
TANK	TRAF Family Member-Associated NF-κB Activator
TBK1	TANK-binding kinase 1
TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor β
TIMP3	metalloproteinase inhibitor 3
TLR	Toll like receptor
TMZ	Temozolomide
TNFα	tumor necrosis factor-α
TPL2	tumor progression locus 2
TRADD	tumor necrosis factor receptor type 1-associated death domain protein
TRAF	tumor necrosis factor receptor-associated factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adapter-inducing IFN-β
TSC2	tuberous sclerosis 2
ULD	ubiquitin-like domain
UTR	untranslated region
V	volt
VCAM1	vascular cell adhesion molecule 1
WB	Western blot
WHO	World Health Organization
Y	Tyrosine
YB1	Y box binding protein 1
ZEB	zinc finger E-box-binding homeobox
α-KG	α-ketoglutarate

1. INTRODUCTION

1.1. Glioblastoma multiforme – one of the most lethal brain tumors

1.1.1. Classification of glioblastoma multiforme

After a long history of neurosurgery, in the beginning of the 20th century Bailey and Cushing were the first neurosurgeons to classify brain tumors histologically and to describe GBM (glioblastoma multiforme). The name derives from both, the cell type the cancer arises from, so called glia cells, and the fact that the tumors are characterized by a variable appearance in morphology and histology [1].

Glia cells are non-neuronal brain cells and constitute a group with distinct morphology and function. Whereas neurons are responsible for the transfer of information, glia cells are considered to be nutrient suppliers as well as to provide structural and metabolic support to neurons [2]. Astrocytes represent the majority of glia cells in the brain and have been found to additionally play a role in synaptic transmission and information processing [3]. Other types of glia cells are for instance microglia [4], oligodendrocytes [5], and satellite cells [6].

The WHO (World Health Organization) classification of gliomas defines three categories: astrocytic, oligodendroglial and oligoastrocytic gliomas [7]. Furthermore, a grading system that considers malignancy and histological alterations specifies four grades (WHO grade I – IV) of glioma tumors where grade I (pilocytic astrocytomas) and grade II (diffuse astrocytomas) represent low-grade gliomas with a comparatively good prognosis, whereas grade III covers anaplastic astrocytomas. WHO grade IV is referred to as GBM [8]. In general, GBM can be divided into primary and secondary glioblastoma depending on the precursor cells and genetic properties [9-11]. Primary glioblastomas develop *de novo* after multiple genetic alterations and represent the majority of all GBM cases. On the other hand, about 5% of glioblastomas are derived from lower grade astrocytomas (WHO grade II - III) and therefore display a different pattern of genetic alterations. This type is referred to as secondary glioblastoma [11].

Glioblastoma multiforme is one of the most common brain cancer types and accounts for 16% of all primary brain tumors in the United States with an incidence rate of 3.19 per

100,000 inhabitants [12]. GBM is also one of the most lethal brain tumors. Depending on grade and genetic alterations, the prognosis of glioblastoma is very poor. Ohgaki *et al.* reported a median survival of around 4.7 months after diagnosis for patients with primary GBMs and 7.8 months for patients with secondary GBMs [13]. However, the difference in survival between primary and secondary GBM is rather due to the age of patients, since secondary glioblastomas have been shown to be more prevalent in younger aged patients [11]. Younger age has been associated with longer survival in glioblastoma patients [13]. Treatment of glioblastoma with standard therapies merely prolongs the survival up to 12 – 15 months [14]. The five-year survival rate of glioblastoma patients is even less than 5% [12].

1.1.2. Genetic and molecular alterations in glioblastoma multiforme

Genetic and molecular alterations in GBM have been extensively studied within the last decades. Some widely spread changes will be subsequently described.

The tumor suppressor p53 is a transcription factor that responds to DNA damage and leads to the expression of genes that are involved in the regulation of cellular processes such as apoptosis and cell cycle inhibition [15]. Because of its key role in apoptosis, p53 mutations are involved in a wide range of cancers [16]. Here, p53 undergoes loss-of-function mutations or even deletions, resulting in increased proliferation and survival [17]. p53 mutations are also frequently detected in GBM [18]. Two-third of precursor astrocytomas exhibit *TP53* mutations and potentially develop to secondary GBM [11]. The occurrence in *de novo* developed GBM (primary GBM) is lower [11]. Also a complete loss of p53 function has been discovered in GBM [10].

Another comparatively common alteration among primary and secondary glioblastomas occurs at the *PTEN* (phosphatase and tensin homolog) gene. Mutations in this tumor suppressor have been reported in 15% (primary) - 40% (secondary) of glioblastomas [19]. Loss of PTEN functionality thereby leads to an activation of PI3K-Akt (phosphatidyl-inositide 3-kinase - Akt) signaling [20, 21]. This activation of Akt promotes cell proliferation, cell growth [22] as well as survival [23]. On the other hand, also PI3K itself can be mutated in GBM. Kita *et al.* reported a low frequency of *PIK3CA* mutations (mutations in the catalytic subunit of PI3K) in about 5% (primary) and 3% (secondary) of glioblastoma [23]. Also somatic mutations of *PI3KR1* (mutations in the regulatory subunit of PI3K) are known to be involved in gliomagenesis by activating PI3K and subsequently Akt [24]. Additionally, the amplification of the *Akt1* gene in glioblastomas has been reported by Knobbe *et al.* [25]. Taken together, genomic alterations occur at several steps

of the PI3K-Akt pathway which lead to increased survival and proliferation of degenerated glia cells.

The EGFR (epidermal growth factor receptor) regulates growth, proliferation and survival as well as angiogenesis and migration by the activation of several signaling pathways such as Akt, STAT3 and MAPK signaling [26] upon binding of ligands such as growth factors [11]. The amplification of *EGFR* can be detected in about 50% of primary glioblastomas [10], while these mutations are found less often in secondary glioblastomas [27]. Besides, the mutation variant *EGFRvIII*, where 801 base pairs are deleted, leading to continuous autophosphorylation and activation of the receptor [28], occurs in 20 – 50% of glioblastomas with *EGFR* amplification [29]. It has been shown that the amplification of the *EGFR* gene results in resistance to common chemotherapeutic treatment [30, 31].

Continued and uncontrolled proliferation is one hallmark of cancer [32]. Glioblastomas frequently show loss of the *RB1* (retinoblastoma protein 1) gene and amplification of the *CDK4* and *CDK6* (cyclin-dependent kinases) genes [9, 11, 27]. Rb1 controls the progression of the cell cycle in G_1-phase in either a HDAC (histone deacetylases) -dependent or -independent manner [33]. The direct E2F inhibition by Rb1 is abrogated upon the phosphorylation of Rb1 by the CDK4/cyclin D1 complex leading to the release of E2F1 transcription factor which activates the expression of genes controlling G_1-to-S transition [11, 34-37]. So, increased levels of phosphorylated Rb1 consequently result in ongoing cell cycle progression and thus in increased proliferation. On the other hand, active gene repression by binding of an HDAC-Rb-E2F complex to promoters of cell cycle genes plays an important role in the regulation of the cell cycle [36, 38-40]. About 25% of glioblastoma cases are marked by a loss of Rb1 expression due to promotor hypermethylation resulting in ongoing cell cycle progression and proliferation [41].

Other known defects in GBM are the loss-of-heterozygosity especially of chromosome 10 [9, 11, 42, 43], resulting in loss of tumor suppressors such as LGI1 (Leucine-rich, glioma inactivated 1) [44], BUB3 (budding uninhibited by benzimidazole 3) [45] or MXI1 (MAX-interacting protein 1) [46]. Furthermore, *IDH* (isocitrate dehydrogenase) mutations can lead to a hypermethylation of promotors resulting for instance in the repression of *CDKN2C* (p18) transcription [10, 47]. The hypermethylation is caused by the IDH product 2-HG (R-2 hydroxyglutarate) which inhibits the function of α-KG (α-ketoglutarate)-dependent enzymes such as dioxygenases that are involved in the demethylation of DNA and histones [48, 49]. Besides, heterozygous deletions of *NFKBIA* (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) have been described in glioblastomas by Bredel *et al.* This deletion is associated with increased disease

progression and seems to be associated with reduced sensitivity to the chemotherapeutic drug TMZ (Temozolomide) [50, 51]. The most frequent mutations and their prevalence in either primary or secondary glioblastoma are summarized in figure 1.1.

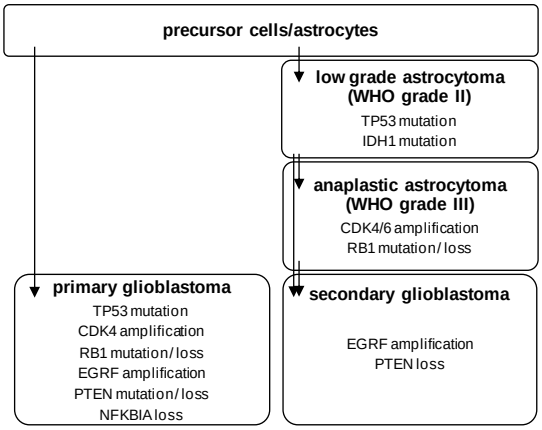


Fig. 1.1: Genetic alteration leading to initiation and progression of primary and secondary glioblastoma. Mutations as well as amplifications or loss of certain genes and their occurrence in gliomagenesis are depicted. More detailed information are given in the text. (modified from [10, 11])

Besides genetic alterations also epigenetic changes play a role in the development and progression of GBM. Especially the hypermethylation of CpG island promoters leads to inactivation of tumor suppressors such as *RB1* and *RASSF1A* (Ras Association Domain family 1 alpha), cell cycle regulators such as $p16^{INK4a}$ and $p15^{INK4b}$, apoptosis regulators including *DAPK* (death-associated protein kinase) and *TIMP3* (metalloproteinase inhibitor 3), as well as DNA repair enzymes as for example *MGMT* (O^6-alkylguanine DNA alkyltransferase) [52, 53].

The genetic and molecular heterogeneity in GBM described above does not just complicate the classification of the tumors, but does also affect treatment outcome in patients. General treatment strategies of GBM will be described in the following section.

1.1.3. Cancer therapy for glioblastoma multiforme

In general, cancer therapy consists of three main distinct approaches which are also implemented in the treatment of glioblastoma: surgery, radiotherapy and chemotherapy [54]. Surgery is one of the initial steps in the treatment of GBM [9], but is depending on the indication based on localization of the tumor, age of the patient, and diagnosis [55]. Tumor resection leads to reduction of tumor mass, relief of intracranial pressure and re-establishment of neuronal functions [9, 55]. The removal of >98% of tumor cells is associated with increased survival and improved response to radiation and chemotherapy [56]. A second important step in the treatment of cancer is radiation and has also been

shown to further prolong survival of glioblastoma patients for approximately 5 months after surgery [57]. The most studied field in the treatment of glioblastoma is chemotherapy. The standard-of-care treatment is comprised of the administration of either Gliadel or TMZ after resection [9, 58]. The chemotherapeutic drug Gliadel, also called carmustine, is an alkylating agent that can be supplied as polymer wafer for a local treatment of brain tumors after surgery and is usually combined with radiotherapy [59]. TMZ is a prodrug that gets converted into the active compound within the systemic circulation and is able to cross the blood-brain barrier [9]. The active compound (MTIC, 3-methyl-(triazen-1-yl)imidazole-4-carboxamide) causes base-pair mismatch in the DNA by methylating the O^6 position of guanine [60, 61]. The resulting DNA damage triggers apoptosis [62]. However, not just tumor cells but all rapidly dividing cells are affected by TMZ [9]. Combined therapy with surgery, radiation and chemotherapeutic treatment increases mean survival of glioblastoma patients merely about 14 months [14, 63]. Nevertheless, patients with *MGMT* methylation respond better to TMZ [64]. O^6-methyl guanine methyltransferase is an enzyme that removes alkyl groups from the O^6 of guanine thereby preventing DNA damage and compromising the cell response to TMZ [53].

A major problem in the treatment of GBM is the occurrence of innumerable resistances against radiation and/ or chemotherapeutic drugs. As already mentioned, the expression of *MGMT* interferes with TMZ treatment [64]. Most studies, mainly performed in tumor stem cells, revealed the role of efflux pumps as well as multiple dysregulated signaling pathways to lead to the development of resistances [65, 66].

At present, promising clinical studies deal with implementation of small molecular inhibitors, novel immunotherapeutic approaches, growth factor targeting and gene therapy as well as with combinations of drugs against distinct targets [9]. New treatments need to be developed based on genetic and molecular changes in every individual case. Since there are multiple, redundant signaling pathways involved in initiation and progression of GBM, a reasonable combination of therapies needs to be employed for each patient [9].

1.2. The non-canonical IKK complex in oncogenesis

The innate immune system senses PAMPs (pathogen-associated molecular patterns) and subsequently induces a variety of transcription factors that mediate the first defense against pathogens [67, 68]. NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is one of the most important transcription factors that can be triggered by bacterial and viral pathogens leading to expression of pro-inflammatory cytokines [69]. Also IRF

(interferon regulatory factor) transcription factors respond to viral pathogens and contribute to the expression of the type I *IFN* (interferon) gene as well as genes encoding pro-inflammatory cytokines [67, 70]. Both NF-κB and IRF signaling pathways depend on signal transduction via IKKs (inhibitor of κB kinases) and IKK-related kinases.

1.2.1. The IKK-related kinases and their adaptor TANK

Both IKK-related kinases have been discovered in 1999. A yeast two-hybrid screen using TANK (TRAF family member-associated NF-κB activator) as a bait revealed TBK1 (TANK binding kinase 1) as a kinase interacting with this adaptor which modulates NF-κB function [71]. IKKε (IκB-kinase ε) has been identified in a subtractive cDNA hybridization screen of LPS (lipopolysaccharide)-treated macrophages [72]. Furthermore, Peters and colleagues identified IKKε as a homolog to IKKα and IKKβ that phosphorylates IκBα in response to PMA (phorbol-12-myristate-13-acetate) [73]. While TBK1 is expressed ubiquitously [74], IKKε has been found to be expressed in pancreas, spleen and thymus as well as peripheral blood leukocytes [72]. The transcription of IKKε is enhanced upon the exposure to LPS or viral infection, therefore IKKε is also called IKK-i (IKK-inducible) [72, 75]. Both kinases exhibit a close structural similarity to the canonical IKKs, IKKα and IKKβ. While the kinase domain of IKKε displays a similarity of 30% with IKKα and IKKβ [72, 73, 76], the TBK1 kinase domain shares 27% identity with both kinases [71, 74]. TBK1 and IKKε share 64% sequence identity among each other [77]. The domain structure of IKKε as well as TBK1 and their adaptor protein TANK is depicted in figure 1.2. The kinases are composed of an N-terminal kinase domain, a subsequent ULD (ubiquitin-like domain), a LZ (leucine zipper) and HLH (helix-loop-helix) region [78, 79]. The C-terminal coiled-coil domains facilitate binding to adaptor proteins [80] such as NAP1, SINTBAD or TANK.

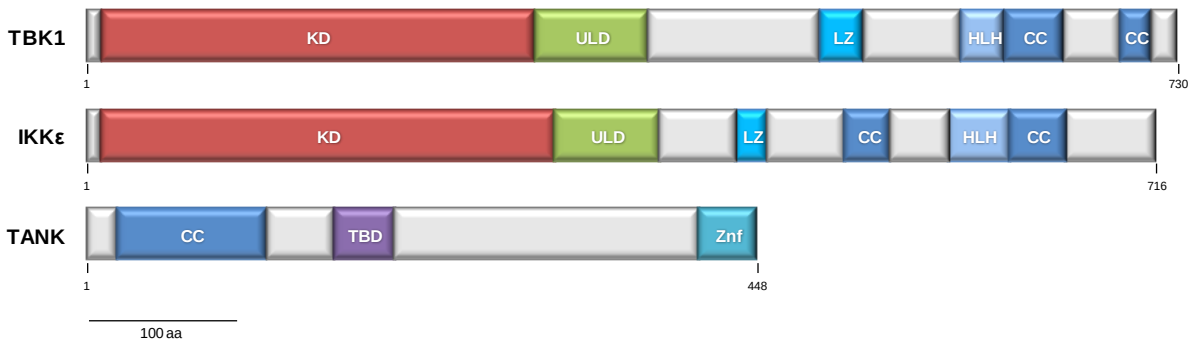


Fig. 1.2: Schematical structure of the IKK-related kinases TBK1 and IKKε as well as their adaptor TANK. Abbreviations: KD: kinase domain; ULD: ubiquitin-like domain; LZ: leucine zipper; HLH: helix-loop-helix; CC: coiled-coil; TBD: TBK1/IKKε binding domain; Znf: Zinc finger. (modified from [78, 79, 81])

Both kinases are bound by adaptor proteins such as NAP1 (NAK associated protein 1), SINTBAD (similar to NAP1 TBK1 adaptor) and TANK [81]. All three adaptors compete for the binding to the kinases [80]. TANK has first been described by Rothe *et al.* in 1996 as a protein that interacts with TRAFs (tumor necrosis factor receptor associated factors) and prevents activation of NF-κB. Therefore, TANK was first named I-TRAF (inhibitor of TRAF) [82]. The assembly of the non-canonical IKK complex by TANK is necessary to activate TBK1 and IKKε leading to phosphorylation of several downstream targets such as IRF3, IRF7 and IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha) [67, 80, 83, 84]. The impact of the complex on IRF as well as NF-κB signaling pathways will be described in detail subsequently.

1.2.2. Involvement of the non-canonical IKK complex in NF-κB and IRF transcription factor signaling pathways

The NF-κB signaling pathway is involved in the innate immune system to defend the organism against viral and bacterial pathogens [69, 85]. Ligand binding to TLRs (toll-like receptors) such as TLR1,2 and 5-9 triggers a signaling cascade via the adaptor molecule MyD88 which results in the activation of the IKK complex formed by IKKα, IKKβ and their adaptor protein NEMO (NF-κB essential modulator) [86]. The active kinases subsequently phosphorylate IκBα which retains the NF-κB dimer in the cytosol in unstimulated cells. This phosphorylation leads to the polyubiquitination and proteasomal degradation of IκBα [79, 85, 87]. The released NF-κB dimer then translocates into the nucleus and induces the expression of pro-inflammatory cytokines such as *IL-6* (interleukin 6) and *COX2* (cyclooxygenase 2) [88, 89]. TBK1 as well as IKKε are also able to phosphorylate IκBα at S36 thereby activating NF-κB target gene expression *in vitro* [71, 72]. However, in MEFs (mouse embryonic fibroblasts) deficient for IKKε and TBK1 the expression of certain NF-κB target genes is decreased, even though IκBα is degraded normally [90, 91]. Furthermore, IKKε as well as TBK1 target the NF-κB subunit p65 at residue S536 [92, 93]. Upon T cell costimulation, IKKε is able to additionally phosphorylate p65 at S468 [94] which allows IKKε to be translocated into the nucleus where it contributes to gene transcription of NF-κB target genes [95]. Furthermore, IKKε, that can translocates into the nucleus upon genotoxic stress exposure, has been found to repress apoptosis in response to DNA-damage by the phosphorylation of p65 at S468 [96]. The NF-κB signaling pathway is summarized in figure 1.3.A.

Both IKK-related kinases are more prominently involved in the activation of IRF transcription factors [97, 98]. Here, TLRs [99] as well as intracellular receptors, that recognize double stranded RNA, such as RIG-I (retinoic acid-inducible gene 1) [100] or

MDA5 (melanoma differentiation-associated protein 5) [101], or receptors detecting double stranded DNA such as DAI (DNA-dependent activator of IFN-regulatory factors) [102] are triggered by infections. Figure 1.3.B illustrates the pathway that is activated upon the stimulation of TLRs. The stimulation of TLR4 by LPS (or TLR3 by viral dsRNA) induces the recruitment of the adaptor molecules TRAM (TRIF-related adaptor molecule) and TRIF (TIR-domain-containing adapter-inducing interferon-β) [103, 104]. TRIF subsequently recruits TRAF3 which mediates the activation of the non-canonical IKK complex composed of TANK, IKKε and TBK1 [83, 104]. Besides, LPS-induced TLR4 signaling through MyD88 can lead to the recruitment of TRAF6 which also assembles with the non-canonical IKK complex. Subsequently, the IKK-related kinases TBK1 and IKKε phosphorylate cytoplasmatic IRF3, 5 or 7 [97, 98, 105, 106] leading to their homo- or heterodimerization and nuclear translocation [97, 107]. In the nucleus, IRF dimers associate with the IFN enhanceosome at ISREs (interferon-stimulated response elements) and induce the expression of type I interferon [108-110]. A second pathway activating the non-canonical IKK complex is triggered by viral infections. Here, intracellular sensors such as MDA-5 and DAI (figure 1.3.C) facilitate the activation of the non-canonical IKK complex by MAVS (mitochondrial antiviral signaling adaptor) resulting in subsequent phosphorylation of IRF transcription factors by the activated kinases TBK1 and IKKε [101, 102, 111]. This phosphorylation leads to the dimerization of IRF transcription factors which translocate into the nucleus and induce the expression of type I *IFN* gene [109, 112, 113].

Besides IRF transcription factors, both IKK-related kinases have further distinct downstream targets. TBK1 phosphorylates Sec5 at the Ral binding domain resulting in interferon induction [114]. IKKβ is phosphorylated by TBK1 in the activation loop promoting its kinase activity [74]. Furthermore, the insulin receptor is phosphorylated by TBK1 at S994 resulting in insulin resistance [115]. IKKε mediates STAT1 (signal transducer and activator of transcription 1) phosphorylation at S708 resulting in ISGF3 (interferon-stimulated gamma factor 3) stabilization [116] and also phosphorylates CYLD (cylindromatosis) at S418, thereby decreasing its deubiquitinase activity [117].

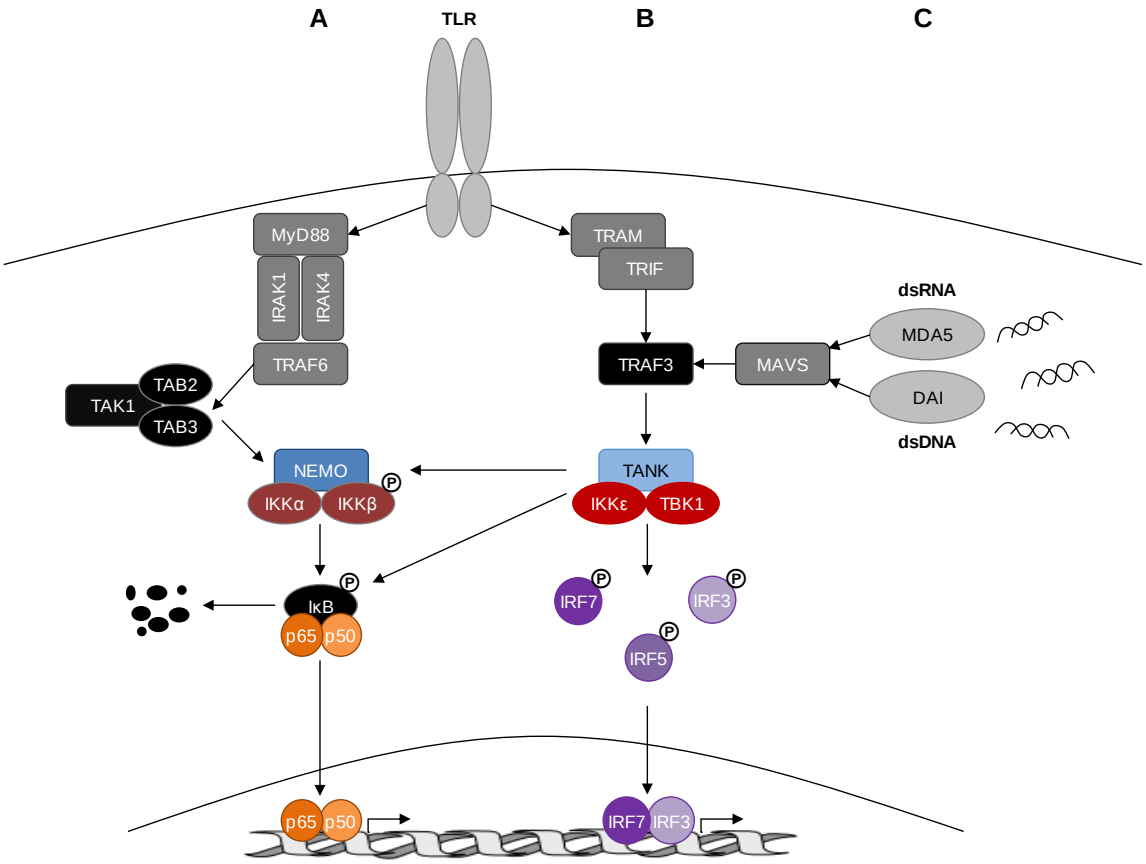


Fig. 1.3: The role of the non-canonical IKK complex in the network of NF-κB signaling. (A) The canonical NF-κB signaling is stimulated by ligand-binding to specific TLRs. This results in subsequent activation of the adaptor protein MyD88 which leads to further activation of IRAKs. Subsequently, TRAF6 gets activated and recruits the TAK1/TAB2/TAB3 complex which in turn phosphorylates and activates the canonical IKK complex consisting of NEMO, IKKα and IKKβ. The two kinases then phosphorylate IκB leading to its ubiquitination and proteasomal degradation which results in the release of NF-κB heterodimer p65/p50. The NF-κB dimer translocates into the nucleus and induces the transcription of pro-inflammatory genes. (B) In an alternative pathway, activation of TLR (e.g. TLR3 or 4) activates TRAM/TRIF adaptors that recruit TRAF3 (or TRAF6 in response to TLR4 activation) which then activates the non-canonical IKK complex consisting of an adaptor protein such as TANK and the kinases IKKε and TBK1. The two kinases phosphorylate IRF transcription factors resulting in dimerization and translocation of the IRF dimers into the nucleus where *type I interferon* expression is induced. Besides, IKKε and TBK1 have an impact on the canonical NF-κB pathway. (C) The pathway described in (B) can also be triggered by intracellular dsRNA and dsDNA originated from viruses. Here sensors such as MDA5 (dsRNA) and DAI (dsDNA) activate signaling adaptor MAVS which leads to subsequent activation of TRAF3 and the non-canonical IKK complex. Both IKK-related kinases phosphorylate cytosolic IRF transcription factors that dimerize and translocate into the nucleus where interfon type I expression is induced. Abbreviations: TLR: toll-like receptor; MyD88: myeloid differentiation primary response gene (88); IRAK: interleukin-1 receptor-associated kinase; TRAF: TNF receptor-associated factor; TAB: TAK1-binding protein; TAK1: TGF-β-activating kinase; NEMO: NF-κB essential modulator; IKK: IκB kinase; IκB: inhibitor of NF-κB; TRAM: TRIF-related adaptor molecule; TRIF: TIR domain-containing adaptor-inducing IFNβ; TANK: TRAF family member-associated NF-κB activator; TBK1: TANK binding kinase 1; IRF: interferon regulatory factor; MDA5: melanoma differentiation-associated protein 5; DAI: DNA-dependent activator of interferon-regulatory factors; MAVS: mitochondrial antiviral-signaling protein. (modified from [67, 70, 79, 118])

1.2.3. IKKε and TBK1 in cancer

The relation between NF-κB-mediated regulation of inflammation and cancer is elaborately described [119-123]. Besides, the IKK-related kinases TBK1 and IKKε recently have been shown to be involved in a wide range of signaling cascades which lead to oncogenic transformation.

TBK1 has been reported to be over-expressed in lung, colon and breast cancer and to be mutated in lung cancer [69, 79]. Moreover, TBK1 is involved in Ras-induced oncogenetic transformation. RasGEF (Ras guanine nucleotide exchange factor) can be activated by a variety of extracellular stimuli leading to the transformation of Ras into its active form. Activated Ras then activates RalGEF (Ras-like-guanine nucleotide exchange factor) leading to RalB-induced (Ras-related protein Ral-B) TBK1 activation and complex assembly with Sec5 which is part of the exocyst complex. This in turn activates Akt and leads to tumorigenic transformation [69, 79, 114, 124, 125].

IKKε attracted much importance in breast cancer. The kinase is over-expressed in about two-third of analyzed human breast cancer tissues as well as in most breast cancer cell lines [126, 127]. A copy-number amplification of *IKBKE* has been reported for 16.3% of breast cancer cell lines [127]. On the other hand, expression of CK2 (casein kinase 2) seems to be involved in increased expression of IKKε in breast cancer tissues [126]. IKKε has been found to replace activated Akt kinase and to cooperate with constitutive active MAPK pathway resulting in tumorigenesis and enhanced proliferation and survival in breast cancer cell lines [127, 128]. Moreover, IKKε-dependent NF-κB signaling is also important in breast cancer. In line with the finding that IKKε is able to phosphorylate p65 at S536, NF-κB target genes *MMP9* (matrix metallopeptidase 9) and *BCL2* (B-cell lymphoma 2) expression is enhanced in breast cancers where IKKε is over-expressed [127]. While *MMP9* is involved in the degradation of collagen IV leading to tumor progression [129], Bcl-2 mediates the inhibition of apoptosis [130]. Furthermore, IKKε phosphorylates ERα (estrogen receptor α) resulting in increased expression of cyclin D1 in breast cancer [131] which is necessary for G_1-to-S transition in the cell cycle and thereby influences proliferation ability [132].

A novel target of IKKε is STAT1 which is involved in tumorigenesis. Tenoever and colleagues showed the phosphorylation of STAT1 at S702 by IKKε and subsequent formation of the ISGF3 (interferon-stimulated gene factor 3) complex which consists of a STAT1-STAT2 heterodimer and IRF9 transcription factor [116]. The role of STAT1 in oncogenesis is contradictory. On the one hand, STAT1 transcription factors are associated with inhibited tumorigenesis. For instance, STAT1 has been shown to

negatively regulate the cell cycle by inducing p21 in ovarian cancer [133], thus blocking cell cycle progression and proliferation. On the other hand, STAT1 also enhances tumor progression. Constitutive over-expression of STAT1 has been demonstrated to reduce apoptosis in several cancer types by the induction of anti-apoptotic genes [134, 135].
Finally, both IKK-related kinases have been found to directly phosphorylate and activate Akt at T308 and S473, thereby promoting tumorigenic transformation [125, 136, 137]. The role of oncogenic Akt signaling will be described in the following chapter.

1.3. The Akt signaling pathway in cancer

1.3.1. Akt is a node in signaling and influences many cellular processes

The serine/threonine protein kinase Akt, also called protein kinase B (PKB), represents a node in signaling and is involved in the regulation of survival, proliferation, migration and metabolism [22, 138-142]. Dysregulated Akt kinase signaling is associated with the development of many diseases such as diabetes [143] and particularly cancer [144, 145].

Akt kinase, that belongs to the AGC (cAMP-dependent protein kinase/ protein kinase G/ protein kinase C) family of kinases, has been discovered independently by three groups in 1991 [22]. While Bellacosa *et al.* found Akt to be an oncogene in mouse leukemia virus AKT8 [146], Jones *et al.* as well as Coffer and colleagues used an homology-based approach to find a new cellular homolog of protein kinase C resulting in the identification of protein kinase B [147, 148]. So far, three isoforms have been identified, all share the same structural organization that is highly conserved: the N-terminal PH (pleckstrin homology) domain facilitates binding to 3-phosphoinositides which is necessary for the activation of Akt [149, 150]; the PH domain is followed by a kinase catalytic domain that is conserved among the members of AGC kinases [151]. The phosphorylation of Akt within the activation loop at T308 is crucial for the activation of the kinase [150, 152]. Furthermore, phosphorylation at S473 within the C-terminal hydrophobic tail, containing a regulatory domain, supports the full activation of Akt [142, 152, 153].

1.3.1.1. Activation of Akt

The activation of Akt is a multistep process and typically mediated by PI3K (phosphoinositide 3-kinase) which can be activated by RTK (receptor tyrosine kinase) and GPCR (G-protein-coupled receptors) [154-156]. Growth factors, cytokines as well as other extracellular stimuli like insulin or stressors such as oxidative stress trigger those receptors [22, 152, 156]. Their activation leads to the recruitment of PI3K to the plasma membrane and subsequent phosphorylation of PIP_2 (phosphatidylinositol-4,5-bisphosphat)

at the 3-OH group which results in the generation of PIP_3 (phosphatidylinositol-3,4,5-triphosphat) [157, 158]. Phosphatases such as PTEN (phosphatase and tensin homolog) or SHIP (SH2-domain-containing inositol phosphatases) mediate dephosphorylation of PIP_3 and thereby negatively regulate the activation of Akt [159-162]. PIP_3 induces the recruitment of Akt to the plasma membrane dependent on its PH domain [163, 164]. PI3K-dependent activation of Akt is typically mediated by PDK1 and 2 (phosphoinositide-dependent kinase 1 and 2) that are also recruited to the membrane by PIP_3 and facilitate the direct phosphorylation of Akt at T308 (by PDK1) and S473 (by PDK2), respectively [165-167]. Besides PDK2, Akt can be phosphorylated at S473 by ILK (integrin-linked kinase) [168, 169] as well as by autophosphorylation [170]. Phosphorylated and thereby activated Akt kinase translocates from the membrane to the cytoplasm or to the nucleus to phosphorylate its substrates [163, 171, 172]. Dephosphorylation of Akt by PP2A (protein phosphatase 2A) inactivates the kinase [171, 173]. The process of Akt activation is depicted in figure 1.4.

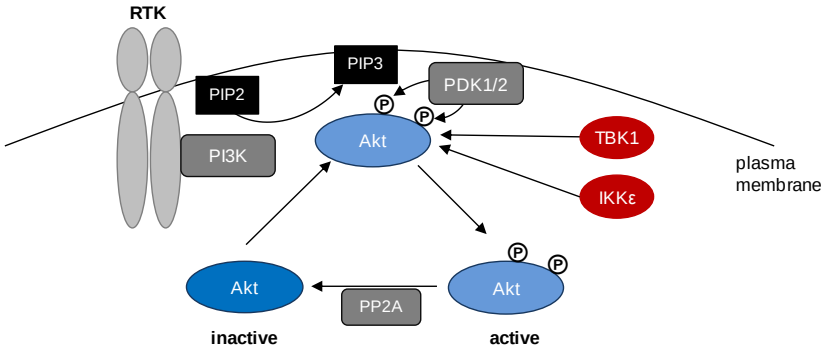


Fig. 1.4: The mechanism leading to Akt activation. Following ligand binding, RTK recruits and activates PI3K leading to phosphorylation of PIP_2 to PIP_3. PIP_3 recruits inactive Akt kinase as well as PDK1/2 to the membrane where Akt is phosphorylated by PDK1 at T308 and PDK2 at S473 for full activity. Active Akt can be inactivated by dephosphorylation by PP2A. Abbreviations: RTK: receptor tyrosine kinase; PI3K: phosphatidylinositol 3-kinase; PIP_2: phosphatidylinositol-4,5-bisphosphat; PIP_3: phosphatidylinositol-3,4,5-trisphosphat; PDK1/2: phosphoinostide-dependent kinase 1 or 2; PP2A: protein phosphatase 2A. (modified from [138, 140, 153, 174]).

Since phosphorylation of Akt at T308 and S473 is a critical step in its activation, this point was focused by many research groups, leading to the identification of many kinases that are involved in the activation of Akt. Downstream substrates of Akt as for instance mTORC (mammalian target of rapamycin complex) have been associated with the phosphorylation of Akt indicating a feedback-loop [175, 176]. More kinases involved in PI3K-independent activation of Akt are PKA (protein kinase A) in response to insulin [177, 178] or Hsp27 (heat shock protein 27) that activates Akt in response to cellular stress

[179, 180]. As already mentioned, also both IKK-related kinases, TBK1 and IKKε, have been reported to phosphorylate Akt depending on its PH domain and PI3K signaling [136].

1.3.1.2. Cellular functions of activated Akt

Once activated, Akt influences many different signaling pathways by phosphorylation of intermediates and thereby has a great impact on cellular functions. Most important downstream substrates and the consequences of their phosphorylation will be described subsequently.

Akt kinase is involved in the regulation of metabolism by phosphorylation of its downstream substrates. GSK3 (glycogen synthase kinase-3), for instance, plays a role in lipid and glucose metabolism [141]. Phosphorylation of GSK3 by Akt results in its inactivation [181]. Active GSK3 induces proteasomal degradation of its substrates such as the transcription factor SREBP (sterol regulatory element-binding protein) which initiates the expression of genes involved in cholesterol and fatty acid biosynthesis [182, 183]. Thus, activation of Akt stabilizes SREBP and enhances lipid production. The Akt substrate GSK3 also modulates glucose metabolism by inhibiting glycogen synthase [184]. Furthermore, glycolysis is increased by phosphorylation of phosphofructokinase upon Akt activation [185, 186] and activation of hexokinase [187]. Moreover, glucose uptake is modulated by Akt upon insulin stimulation in insulin responsive tissue. Whereas the transcription and translation of glucose transporters GLUT1 and GLUT3 is increased by Akt downstream target mTORC1 [188-190], GLUT4 is recruited to the plasma membrane to support glucose uptake [191-193]. The phosphorylation of transcription factor FOXO1 (Forkhead box protein O1) by Akt leads to its degradation resulting in decreased transcription of glucose-6-phosphatase [194, 195].

Other members of the transcription factor family Forkhead box such as FOXO3 and FOXO4 can be directly phosphorylated by Akt [196-198] which results in decreased transcription of genes involved in apoptosis such as *Fas ligand*, *TRAIL (*tumor necrosis factor-related apoptosis-inducing ligand*)*, *TRADD (*tumor necrosis factor receptor type 1-associated death domain protein*)*, *BIM* and *BCL-6 (*B-cell lymphoma 6*)* [198-205]. Besides Forkhead box, other transcription factors are indirectly influenced by Akt. The NF-κB transcription factor has been shown to be activated by phosphorylation of IKKα by Akt which results in transcription of anti-apoptotic and pro-survival genes [206-209]. Furthermore, CREB (cAMP-responsive element binding protein) is phosphorylated by Akt resulting in enhanced transcription of MCL-1 (myeloid leukemia cell differentiation protein) which promotes cell survival by inhibiting apoptosis [210-212]. Phosphorylation of the Akt substrate GSK3 is known to inhibit DNA binding of c-Jun and in turn of the AP-1 (activator

protein 1) transcription complex [213, 214]. Besides transcription, Akt also plays a role in translational control. Induction of mRNA translation is amongst others facilitated by Akt-dependent phosphorylation of eukaryotic translation initiation factor 4EBP (4E-binding protein) and eIF4E (eukaryotic initiation factors 4E) [215] as well as by indirect phosphorylation of eIF2B (eukaryotic initiation factor 2B) [216].

The regulation of apoptosis and cell survival are the most considerable functions of Akt in cancer. Akt has emerged as a general inhibitor of apoptosis. Besides its impact on the regulation of anti-apoptotic gene transcription (Forkhead box, NF-κB) and translational control, Akt targets several proteins involved in the apoptotic machinery. The Bcl-2 family member BAD (Bcl-2-associated death promoter) is a direct target of Akt and inhibited by phosphorylation [217-219]. Akt substrate GSK3 phosphorylates and inhibits MCL-1, a pro-survival Bcl-2 family protein [212, 220]. Furthermore, phosphorylation of pro-caspase-9 by Akt prevents its cleavage and thereby its pro-apoptotic activity [221]. Moreover, MAP (mitogen-activated protein) kinases JNK and p38 have been reported to be negatively affected by Akt. Here, the phosphorylation of upstream kinases such as ASK1 (apoptosis signal-regulating kinase 1), MLK3 (mixed lineage kinase 3) or SEK1 (SAPK/Erk kinase 1) leads to the repression of the MAPKs and eventually to the inhibition of apoptosis [222-226].

Besides apoptosis, Akt is also involved in the regulation of proliferation. Cell cycle progression and thereby cell proliferation is generally regulated by cyclins [227]. Akt promotes the expression of cyclin D1 and D3 by enhancing their translation [228]. The Akt substrate GSK3 additionally promotes the degradation of cyclin D1 by its phosphorylation at T286 which leads to the translocation of cyclin D1 into the cytosol where it gets degraded [229]. Thus, Akt, which inhibits GSK3, has a stabilizing effect on cyclin D1. Furthermore, cyclin-dependent kinase inhibitors p21 and p27 are negatively influenced by Akt leading to a relieve of cell cycle inhibition [230, 231]. Phosphorylation of p27 by Akt induces its sequestration in the cytosol thus preventing its cell cycle inhibitory effect [230, 232-234]. Also p21 translocation to the nucleus is prevented by Akt-mediated phosphorylation [235]. Moreover, expression of p21 is influenced by Akt via MDM2 (mouse double minute 2 homolog). Phosphorylation of MDM2 by Akt leads to p53 ubiquitination and subsequent degradation which in turn attenuates p21 transcription [236-238]. Important features of the complex downstream network of Akt are summarized in figure 1.5.

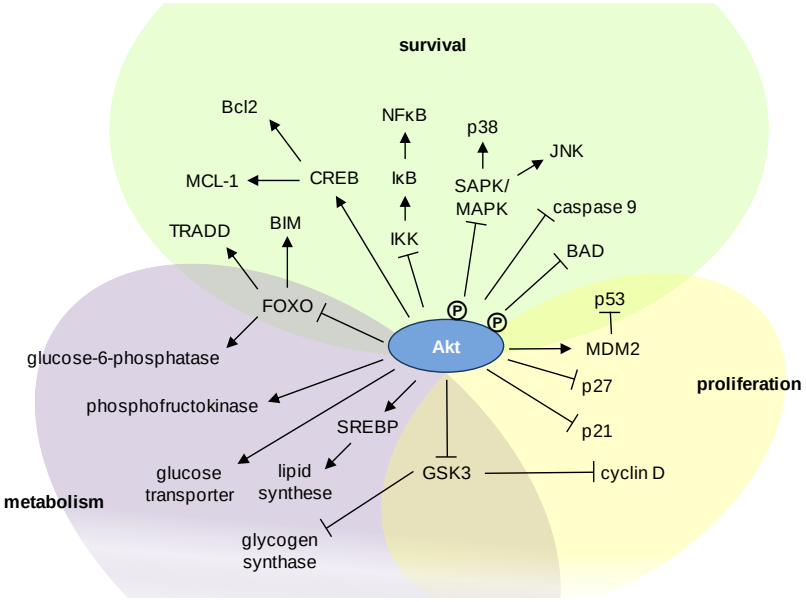


Fig. 1.5: Akt kinase is a node in signaling and mediates several cellular functions. Besides regulation of transcription, translation, cell growth, migration and angiogenesis, the major functions of Akt signaling are the promotion of cell survival by inhibition of apoptosis (green), the facilitation of proliferation (yellow) and the control of glucose and lipid metabolism (purple). A detailed description of Akt substrates and their role for certain signaling pathways is given in the text. Arrows indicate activation and blunt end arrows represent inhibition of the substrates by their phosphorylation. Abbreviations: GSK3: glycogen synthase kinase 3; MDM2: mouse double minute 2 homolog; BAD: Bcl-2-associated death promoter; JNK: c-Jun N-terminal kinases; SAPK/ MAPK: stress/ mitogen-activated protein kinases; IKK: IκB kinase; IκB: inhibitor of NF-κB, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; CREB: cAMP response element-binding protein; Bcl2: B-cell lymphoma 2; MCL-1: induced myeloid leukemia cell differentiation protein; FOXO: Forkhead box O; BIM: Bcl-2-like protein 11; TRADD: tumor necrosis factor receptor type 1-associated death domain; SREBP: sterol regulatory element-binding proteins. (modified from [22, 139, 153])

Taken together, Akt is a signaling node that influences different pathways and many cellular functions. Thereby, Akt plays a great role in tumorigenesis by promoting hallmarks of cancer such as cell survival and proliferation.

1.3.2. Akt signaling in cancer

The role of Akt in tumorigenesis has been extensively studied. Already when it was discovered as the retroviral oncogene v-Akt, a potential role in human cancer was supposed [146].

Oncogenic deregulation of Akt in human cancers is due to either gene amplifications, the activation of upstream signaling or a loss of function of negative regulators [239]. *Akt* gene amplification has been described especially for ovarian [240], gastric [241] and pancreatic cancer [242-244], whereas over-expression of Akt occurs frequently in prostate cancer [245] as well as colorectal cancer [246]. However, in most cases Akt hyperactivity is caused by dysregulation of upstream oncogenes or tumor suppressors [247]. Gene

amplification of PI3K subunit p110 (*PIK3CA*) has been observed in ovarian [248] and cervix [249] tumors. Furthermore, PI3K can be constitutively activated by active Ras [250, 251] or by over-expression of receptors such as human HER2 (epidermal growth factor receptor 2, also known as erbB2) [252, 253]. Besides, loss-of-function of tumor suppressor PTEN is a common alteration in tumors that affects Akt signaling by promoting its activation and has been shown to occur in prostate [254], lung [255], breast [256] and pancreatic cancer [257], as well as GBM [258-261].

Likewise, PI3K-independent mechanisms promote Akt activation and contribute to oncogenic transformation. The interaction of Akt with Ack1 (activated Cdc42-associated kinase) has been discovered by Mahajan *et al.* and has been shown to induce PI3K-independent phosphorylation of Akt at Y176 and subsequent phosphorylation at T308 and S473 [262]. Autoactivating Ack1 mutations resulting in activated Akt have been found in ovarian cancer [262, 263]. Furthermore, DNA double strand break response via ATM (ataxia telangiectasia mutated) has been found to activate Akt via phosphorylation at S473 in a PI3K-independent manner to support survival [264]. It is believed, that those alternative mechanisms in Akt activation serve as backup pathways that, when aberrantly activated, promote Akt-driven transformation [265].

1.4. Tumorigenic ERK1/2 signaling

Signaling by the serine and threonine protein kinases of the MAPK family regulates a large variety of cellular processes such as proliferation, apoptosis, cell cycle progression, differentiation, migration, metabolism and transcription [266-270] in response to a wide range of stimuli such as cytokines, growth factors, antigens, toxins as well as chemical and physical stresses [271, 272]. In general, MAPK pathways are three tiered cascades involving a MAP3K (MAP kinase kinase kinase) that phosphorylates MAP2K (MAP kinase kinase) that in turn phosphorylates MAPK. Three main subfamilies of MAPK namely p38, JNK (c-Jun N-terminal kinase) which is also referred as to SAPK (stress-activated protein kinase) [273], and ERK (extracellular signal-regulated kinase), are known and depicted in figure 1.6. [266, 267].

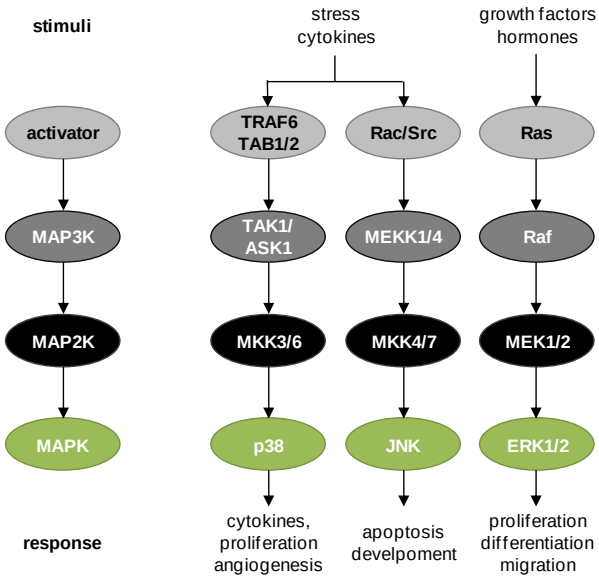


Fig. 1.6: Conventional MAPK signaling pathways proceed in three-tiered modules. Ligand binding to cell surface receptors such as GPCR or RTK lead to activation of small GTPases such as Ras, Rac, Src or other activating adaptor proteins. Their activation results in initiation of the typical three-tiered MAPK signaling and finally to the activation of either p38, JNK or ERK MAPK. Activated MAPK in turn phosphorylate and activate distinct transcription factors which induce the production of cytokines, proliferation, angiogenesis, apoptosis, development, differentiation and migration. Abbreviations: TRAF: TNF receptor associated factor; TAB: TAK1-binding protein; Ras: Rat sarcoma; MAP3K; mitogen-activated protein kinase kinase kinase; TAK1: TGF-β-activating kinase; ASK1: apoptosis signal-regulating kinase 1; MEKK = MAP3K; Raf: rapidly accelerated fibrosarcoma; MAP2K: mitogen-activated protein kinases kinase; MKK = MAP2K; MEK = MAP2K; MAPK: mitogen-activated protein kinases; JNK: c-Jun N-terminal kinases; ERK: extracellular signal-regulated kinase. (modified from [223, 274])

Both, p38 and JNK, are activated in response to pro-inflammatory cytokines or cellular stresses [275-277] by distinct three tiered MAPK signaling. While p38 plays a role in the production of cytokines, proliferation and angiogenesis [278], JNK is important for apoptosis and development [279]. Activation and consequences of ERK1/2 signaling as well as its role in cancer will be described in more detail in the following section.

1.4.1. The Ras-Raf-MEK-ERK signaling pathway

Among the three main MAP kinases, ERKs are the best studied [280]. The isoforms ERK1 (p44) and ERK2 (p42) share 84% sequence identity and fulfill more or less the same functions [281-283], so they will be referred to as ERK1/2 subsequently. The serine/threonine kinases have a size of 44 and 42 kDa, respectively, and are expressed in all tissues [266, 274, 282, 283].

The ERK1/2 signaling cascade is initiated by a variety of stimuli such as growth factors, cytokines, mitogens as well as hormones [284]. Binding of those stimuli to either RTK receptors or GPCR triggers the autophosphorylation of the receptors which creates

binding sites for the adaptor protein Shc (SHC-transforming protein) [285-287]. Interaction of Shc with Grb2 (growth-factor-receptor-bound 2) leads to the binding and activation of GEF (guanine nucleotide exchange factors) proteins such as SOS (son of sevenless) [288]. SOS gets activated and further activates small GTP binding protein Ras [289], a subfamily of small GTPases that is composed of the members H-Ras, K-Ras and N-Ras [290, 291]. Activated Ras in turn leads to the activation of Raf kinase [289, 292]. This kinase phosphorylates and thereby activates MEK1 and MEK2 which in turn phosphorylate ERK1/2 [293-295]. The phosphorylation of ERK1/2 at T202/T204 and T185/Y187 leads to dimerization and nuclear translocation where ERK1/2 phosphorylates its substrates [296]. Additionally, scaffold proteins such as KSR (Kinase suppressor of Ras) [297] enable the cytoplasmic retention of the dimers resulting in the phosphorylation of cytosolic substrates [298, 299]. Usually, scaffold proteins are required to form a multi-enzyme complex with the involved MAPKs to provide signal fidelity [299]. The classical Ras-Raf-MEK-ERK pathway and selected substrates are summarized in figure 1.7.

Besides the described classical MAPK signaling pathway, further kinases have been identified to influence the phosphorylation of ERK1/2 or its upstream kinases. PKC (Protein kinase C) [300] and MLK3 [301] phosphorylate Raf proteins which results in ERK1/2 activation. MAP3K of the p38 or the JNK pathway facilitate the activation of ERK1/2 indirectly by the phosphorylation of MEK1/2 [226, 302]. However, also kinases such as TPL2 (tumor progression locus 2) [303], MLTK (MLK-like mitogen-activated protein triple kinase) [304] or IRAK (interleukin-1 receptor-associated kinase) [305] are able to activate ERK1/2 by phosphorylation of upstream MAPKs.

So far, more than 150 substrates of ERK1/2 have been discovered [306]. In the nucleus, ERK1/2 are mainly involved in the activation of transcription factors. The direct binding and phosphorylation of Elk1 by ERK1/2 induces its binding to DNA as well as to the co-activators CBP (CREB-binding protein) and p300, thus resulting in dramatic increase of transcriptional activity [306, 307]. Elk1 induces the expression of *c-Fos*, *EGR-1* (Early growth response protein 1) [308, 309], as well as *p21* and other genes involved in cell cycle regulation and proliferation [310]. Furthermore, the c-Fos transcription factor is a substrate of nuclear ERK1/2 and the ERK1/2 substrate RSK (ribosomal S6 kinases) [311, 312]. Phosphorylation of c-Fos by ERK1/2 and RSK results in its stabilization and dimerization with c-Jun to form AP-1 transcription factor which is responsible for the expression of genes involved in proliferation and survival [313]. Another transcription factor family that is phosphorylated by ERK1/2 is FOXO. The phosphorylation of FOXO3a increases its binding to MDM2 and thereby promotes its degradation which results in the prevention of apoptosis [314].

The binding of ERK1/2 to scaffold proteins allows its retention in the cytosol [299] which leads to the phosphorylation of cytoplasmatic substrates such as PDE4 (phosphodieesterase 4) [315], cytosolic phospholipase A_2 [316], as well as cytoskeletal proteins such as paxillin [317] and MLCK (myosin light chain kinase) [318]. Moreover, RSK is also a cytosolic target for ERK1/2 [319, 320]. As for ERK1/2, a part of cytosolic RSK is able to translocate into the nucleus to facilitate the phosphorylation of transcription factors. Transcription factors, that are regulated by RSK, are for example CREB, NF-κB and NFAT3 (nuclear factor of activated T cells 3) [321-323]. In the cytosol, proteins involved in apoptosis such as BAD and DAPK are phosphorylated by RSK [274, 324, 325]. BAD is suppressed by this phosphorylation [326], and phosphorylated DAPK retains ERK1/2 in the cytosol, thus preventing ERK1/2 activity in the nucleus, and promotes apoptotic function [327]. How pro- and anti-apoptotic functions of ERK1/2 are linked has not been clarified so far, but seems to depend on the specificity of the stimuli as well as on the cell type. In general, cell death promoting functions of ERK1/2 are not yet well understood [274].

Some selected substrates of ERK1/2 are displayed in figure 1.7. Taken together, the Ras-Raf-MEK-ERK pathway plays a pivotal role in the regulation of apoptosis and proliferation and therefore has a major role in tumorigenesis and cancer progression.

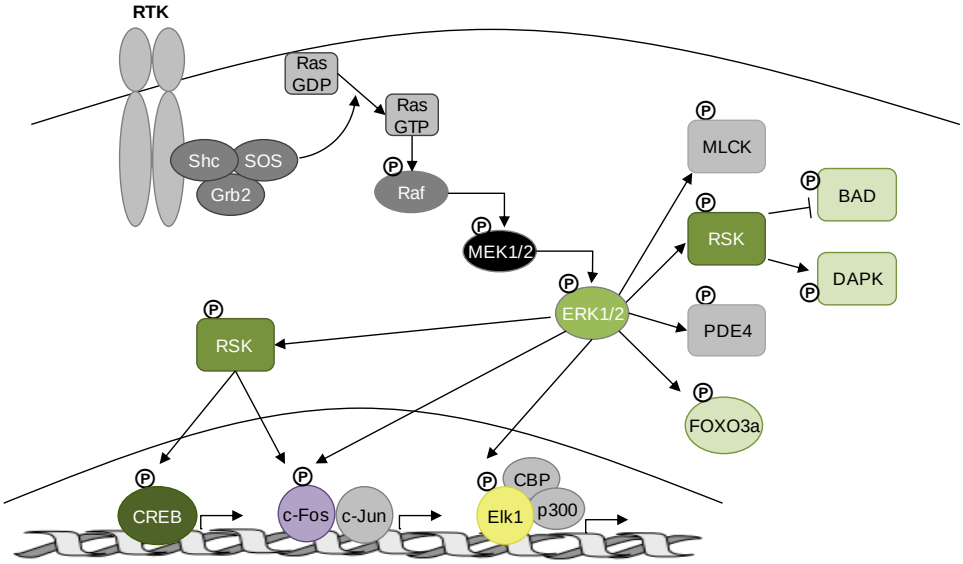


Fig. 1.7: The Ras-Raf-MEK-ERK pathway. Ligand binding to RTK leads to activation of adaptors such as Shc and Grb2 resulting in SOS activation and subsequent activation of Ras. Ras in turn phosphorylates Raf, a MAP3K inducing the three-tiered pathway of MAPK resulting in the phosphorylation and activation of ERK1/2. Examples for cytosolic substrates of ERK1/2 are PDE4, MLCK and RSK. RSK phosphorylates BAD and DAPK which are involved in the regulation of apoptosis. Nuclear substrates of ERK1/2 are the transcription factors c-Fos and Elk1 as well as CREB which is phosphorylated by ERK1/2 substrate RSK. Arrows indicate activation and blunt end arrows represent inhibition of the substrates. Abbreviations: RTK: receptor tyrosine kinase; Shc: SHC-transforming protein; Grb2: growth factor receptor-bound protein 2; SOS; Son of Sevenless; Ras: Rat sarcoma; GDP/GTP: guanosine diphosphate/ triphosphate; MEK: MAP2K; ERK:

extracellular signal-regulated kinase; MLCK: myosin light-chain kinase; RSK: ribosomal s6 kinase; BAD: Bcl-2-associated death promoter; DAPK: death-associated protein kinase; PDE4: phosphodiesterase 4; FOXO3a: Forkhead box O 3a; CREB: cAMP response element-binding protein; Elk1: ETS domain-containing protein. CBP: CREB-binding protein. (modified from [328, 329])

1.4.2. The role of Ras-Raf-MEK-ERK pathway in cancer

The Ras-Raf-MEK-ERK pathway has been shown to be important for many types of cancers. Mutations and dysregulations at several steps of the signaling cascade are known to result in increased activity of ERK1/2.

Receptors that trigger the Ras-Raf-MEK-ERK pathway can be dysregulated which leads to tumorigenesis. For instance, over-expression of EGFR has been shown in many cancers such as breast [330] lung [331] or colorectal cancer [332]. Furthermore, mutations of the receptor, which lead to constitutive activation of the receptor independent of any stimuli, have been identified [333]. The so called EGFRvIII mutation, that was first found in GBM [334-336], is lacking parts of the extracellular domain responsible for ligand binding [337] and has also been reported in breast, ovarian, prostate and lung cancer [338-340]. EGFRvIII has been demonstrated to be constitutively associated with the downstream adaptor protein Grb2 leading to Ras activation [341-343]. Other RTKs or GPCR that influence the initiation and progression of tumors are also affected in cancer by over-expression or mutation [344, 345]. In about 30% of all cancers members of the Ras family are mutated [346, 347]. K-Ras mutations occur in about 85% of tumors, N-Ras in 15% and H-Ras in less than 1% [348]. Pancreatic, lung and colon cancer are most affected by K-Ras mutations [348]. In general, mutations of Ras lead to constitutive binding of GTP which results in stimulus-independent and continuing activation of downstream cascades as Raf-MEK-ERK [346, 349]. Raf proteins play an important role in cancer as well. In particular, the family member B-Raf is known to be mutated in approximately 8% of human cancers, most frequently in thyroid cancer (45%) and melanoma (41%) [348, 350]. Most of the 40 known mutations of B-Raf result in constitutive activation and downstream signaling [348]. The prevention of Raf inactivation by conformational changes, as seen for B-Raf V600E mutation, could be a cause of constitutive activation [351, 352]. Furthermore, over-expression of wild-type B-Raf has been reported in melanoma cell lines [353]. Another family member of the Raf kinases, Raf-1, has been shown to be mutated in cancer cell lines resulting in transformation, but those mutations were not confirmed in primary tumor tissue [354-356]. Hyperactivity of Raf-1 is likely caused by over-expression and dysregulation of upstream receptors as well as oncogenic Ras [357-360]. In contrast to Ras and Raf, mutations of MEKs are rather uncommon [348, 361]. Gain-of-function

mutations leading to the activation of MEK1/2 and consequently of ERK1/2 have been described in 3% of melanomas and 2% of colon cancers [361].

Usually mutations that activate a certain signaling pathway do not occur in parallel within the same tumor because they are redundant [348]. However, all of the described mutations can lead to increased activation of ERK1/2 and thereby influence cellular functions. On the one hand, hyperactive ERK1/2 results in increased proliferation by promoting transcription factors such as c-Fos and Elk1 [307, 313]. On the other hand, reduced apoptosis and thereby increased survival is mediated by ERK1/2 substrates such as FOXO3a and RSK [314, 319].

1.5. The crosstalk between Akt and ERK1/2 signaling

The complex crosstalk between the Akt and ERK signaling cascades comprises cross-inhibition and cross-activation as well as converging of the pathways in the same substrates [362]. The initiation of both signaling pathways is facilitated by similar stimuli. Besides growth factors that trigger EGFR receptors, also IGF-1 (insulin-like growth factor 1) [363, 364] and oxidative stress [365, 366] induce Ras as well as PI3K activation [367]. Besides the activation of the Ras-Raf-MEK-ERK pathway, Ras has been demonstrated to also activate the PI3K-Akt pathway [368, 369]. Moreover, PTEN, a negative regulator of Akt, has been identified to also negatively influence Ras activation. Gu *et al.* as well as Thomas and colleagues showed PTEN to dephosphorylate Shc adaptor protein causing decreased ERK1/2 activation in glioblastoma [370, 371]. Akt has been shown to phosphorylate Raf-1 at S259 resulting in a reduced activity of Raf-1 and inhibition of ERK1/2 signaling [372, 373]. Nevertheless, this direct interaction seems not to be mandatory as Rommel and colleagues showed the interaction of Raf and Akt for differentiated myotubes but could not verify this in undifferentiated myoblasts indicating a stage-specific crosstalk [374]. Besides, Raf can be phosphorylated by PKC which is also a PI3K substrate [375, 376]. Downstream of Akt and ERK1/2 kinases, both pathways converge in some mutual substrates. Especially proteins mediating apoptosis are regulated by both signaling pathways. For instance, Akt as well as ERK1/2 phosphorylate transcription factors such as FOXO3a and CREB (see 1.3.1.2 and 1.4.1) which leads to the expression of Bcl-2 family members [218, 377, 378]. Key components of the apoptosis cascade, as for example BAD and BIM, are phosphorylated by Akt and ERK1/2, thereby repressing apoptosis [218, 379-382]. Furthermore, proliferation is regulated by both cascades. Akt and ERK1/2 downstream kinase RSK phosphorylates YB1 (Y box binding

protein 1) [383-385] as well as ERα [386-388] and thereby enhances transcription of genes involved in proliferation. Other common substrates of Akt and ERK1/2 are: TSC2 [389, 390], $p70^{S6K}$ [391], GSK3 [181, 392] and p27 [393-395]. The crosstalk between ERK1/2 and Akt signaling cascades as well as their mutual substrates are illustrated in figure 1.8.

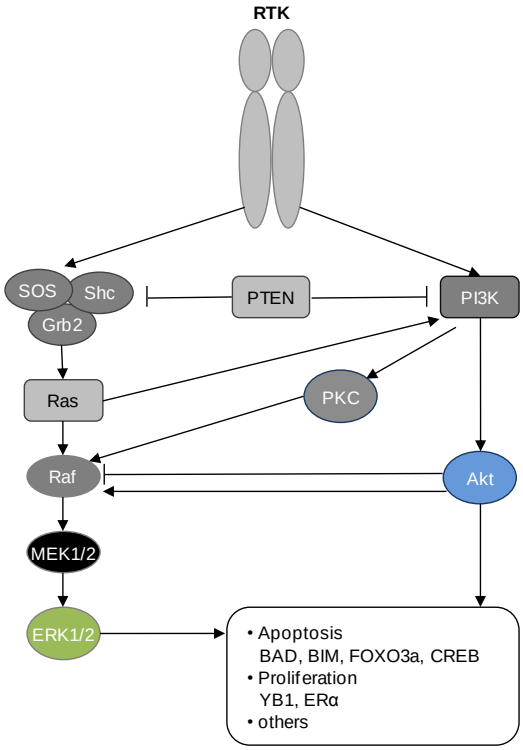


Fig. 1.8: The crosstalk between Ras-Raf-MEK-ERK and PI3K-Akt pathways. The schematic representation illustrates both pathways as shown before (Fig 1.4 and Fig. 1.7). Possible cross regulations are indicated with arrows whereas arrows stands for activation and blunt end arrows represent inhibition of the substrates. Examples of common substrates of both signaling cascades responsible for the regulation of either apoptosis or proliferation are listed in the box. Abbreviations: RTK: receptor tyrosine kinase; SOS; Son of Sevenless; Shc: SHC-transforming protein; Grb2: growth factor receptor-bound protein 2; Ras: Rat sarcoma; MEK: MAP2K; ERK: extracellular signal-regulated kinase; PTEN: phosphatase and tensin homolog; PI3K: phosphatidylinositide 3-kinases; PKC: protein kinase C; BAD: Bcl-2-associated death promoter; FOXO3a: Forkhead box O 3a; CREB: cAMP response element-binding protein; YB1: Y box binding protein 1; ERα: estrogen receptor alpha. (modified from [367, 368, 377, 396])

Another consequence of ERK1/2 and Akt signaling is the development of chemotherapeutic drug resistance. Whereas ERK1/2 mediates a resistance by increased expression of efflux pumps such as MDR-1 (multi-drug-resistant 1) [397], Akt facilitates the resistance via suppression of p53 induced apoptosis [398-400]. Additionally, the cooperation of both signaling cascades complicates cancer therapy. Since Akt and ERK1/2 signaling pathways have common substrates, they can easily fill in for the other [377]. Furthermore, other pathways such as Jak-STAT and NF-κB are also involved in the

complex interplay of signaling pathways [378, 401, 402] and therefore represent possible ways to bypass targets of chemotherapeutic drugs.

1.6. Aim of this study

Glioblastoma multiforme is one of the most common and lethal brain tumors. Many signaling pathways such as the Akt and ERK1/2 pathways have already been described to play a role in tumorigenesis. The aim of this study was to examine the expression levels of members of the non-canonical IKK complex in cancer. It was also planned to identify the importance of the non-canonical IKK complex for proliferation of glioma cell lines and to study the involved signaling cascades. In addition to knock-down experiments, the function of IKKε and TBK1 should also be revealed after their pharmacological inhibition with a small molecule inhibitor. Here, also Akt and ERK1/2 signaling cascades should be included using specific inhibitors. Furthermore, it was planned to further examine the reasons for a potential over-expression of the components of the non-canonical IKK complex by measurement of *de novo* transcription, mRNA stability and protein stability in glioma cell lines. It was then interesting to address the consequences of TANK or TBK1 knock-down on the cell cycle and cell migration. Understanding of the molecular changes in GBM can help to improve treatment and survival of patients.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Eukaryotic cell lines

Name	Description
A172	Human glioblastoma cells
A271	Human glioblastoma cells
A549	Human lung carcinoma cells
A764	Human glioblastoma cells
HeLa	Human cervix carcinoma cells
HCT116	Human colorectal carcinoma cells
HEK293T	Human embryonic kidney cells stably expressing the large T antigen of the SV40 virus
Ln229	Human glioblastoma cells
MCF7	Human breast adenocarcinoma cells
Phoenix Ampho™	Second-generation retrovirus producing, amphotropic packaging cells based on HEK293 cells
SNB19	Human glioblastoma cells
T98G	Human glioblastoma cells
U118	Human glioblastoma cells
U251	Human glioblastoma cells
U343	Human glioblastoma cells
U373	Human glioblastoma/astrocytoma cells
U87MG	Human glioblastoma/astrocytoma cells

2.1.2. *E.coli* strains

Name	Description	Source
TOP10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
XL10 Gold	Tet^r Δ(*mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI^{q}ZΔM15* Tn*10* (Tet^r) Amy Cam^r]	Stratagene

2.1.3. Antibodies

2.1.3.1. Primary antibodies for immunoblotting

Name	Species	Source
anti-Akt	rabbit polyclonal	Cell Signaling
anti-IKKε (12142)	mouse monoclonal	Abcam
anti-p44/42 MAPK (Erk1/2)	rabbit polyclonal	Cell Signaling
anti-Phospho-Akt (Ser473)	rabbit polyclonal	Cell Signaling
anti-Phospho-Akt (Thr308)	rabbit polyclonal	Cell Signaling
anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	rabbit polyclonal	Cell Signaling
anti-TANK (47632)	rabbit polyclonal	Abcam
anti-TANK (D2)	mouse monoclonal	Santa Cruz
anti-Phospho-IRF-3	rabbit monoclonal	Cell Signaling
anti-Phospho NF-κB p65 (Ser536)	rabbit monoclonal	Cell Signaling
anti-Ubiquitin (P4D1)	mouse monoclonal	Cell Signaling
anti-TBK1/NAK (D1B4)	rabbit monoclonal	Cell Signaling
anti-β-Actin (1801)	rabbit polyclonal	Abcam

2.1.3.2. Secondary antibodies for immunoblotting

Name	Species	Conjugated to	Source
anti-mouse IgG	goat	Horseradish peroxidase	Dianova
anti-rabbit IgG	goat	Horseradish peroxidase	Dianova

2.1.3.3. Antibodies used for co- and chromatin-immunoprecipitation

Name	Species	Source
anti-Akt	rabbit polyclonal	Cell Signaling
anti-IKKε (12142)	mouse monoclonal	Abcam
anti-TANK (D2)	mouse monoclonal	Santa Cruz
anti-TBK1/NAK (D1B4)	rabbit monoclonal	Cell Signaling
anti-normal IgG (2025)	mouse	Santa Cruz
anti-normal IgG (2027)	rabbit	Santa Cruz
anti-RNA polymerase II CTD phospho S2	Rabbit polyclonal	Abcam
anti-IgG	rabbit	Cell Signaling

2.1.4. Expression plasmids

cDNA/ construct/ name	Vector	Source
pHCMVG		Addgene
pMDLg/pRRE		Addgene
pRSV-Rev		Addgene
pMD2.G		Addgene
psPAX2		Addgene
shScramble	pSIREN	M.L. Schmitz
shTANK #1	pSIREN	Julia Stellzig
shTANK #2	pLL3.7	Alain Chariot
shTBK1 #1	pSIREN	Julia Stellzig
shTBK1 #2	pLKO1	Alain Chariot
TANK siBlock	290-pHAGE-hEF1aCAR-PGK Puro-Linker	Julia Stellzig

2.1.5. Synthetic oligonucleotides

2.1.5.1. Oligonucleotides for shRNA cloning

Primer	Sequence (5'→3')
sh-hTBK1-f	GATCCCCGGAGCTACTGCAAATGTCTTTCAAGAGAAGACATTTGCAGTAGCTCCTTTTTGGAAA
sh-hTBK1-r	AGCTTTTCCAAAAAGGAGCTACTGCAAATGTCTTCTCTTGAAAGACATTTGCAGTAGCTCCGGG

2.1.5.2. Oligonucleotides for real-time PCR

Primer	Sequence (5'→3')
IKKe-2-qRT-fw	GCTCAGCTCCTGGACGTGCC
IKKe-2-qRT-rev	TGCCCTGAGCTGGCTGGTCA
TBK1-qRT2-for	GGCGGAGACCCGGCTGGTAT
TBK1-qRT2-rev	ACATTTGCATAGCTCCTTGGCC
5'UTR-TANK-r	TTCCTCTTCGTCCTGTAGCA
5'UTR-TANK-f	AGGATTGTTAGAGCCTGTGGA
huActin-qPCR-f	TCCCTGGAGAAGAGCTACGA
huActin-qPCR-r	AGGAAGGAAGGCTGGAAGAG
TNF_FW	GTGATCGGCCCCCAGAGGGA
TNF_RV	ACTGGAGCTGCCCCTCAGCT
IL-6_FW	CCTGCACGGCATCTCAGCCC

IL-6_RV	TGCCAGTGCCTCTTTGTCGTC
Vcam_FW	ACGCTGACCCTGAGCCCTGT
Vcam_RV	ACGAGGCCACCACTCATCTCGA
TBP_FW	GAGCTGTGATGTGAAGTTTCC
TBP_RV	TCTGGGTTTGATCATTCTGTAG
HPRT1_FW	TGAGGATTTGGAAAGGGTGT
HPRT1_RV	GAGCACACAGAGGGCTACAA

2.1.5.3. Oligonucleotides for site-directed mutagenesis

Primer	Sequence (5'→3')
TANK shRNA res-1	AAGACTGAGAA**T**TA**C**GAGCAGAGAATACGTG
TANK shRNA res-2	TTCTCTGCTCGTAATTCTCAGTCTTTTGCTG

2.1.5.4. Oligonucleotides for chromatin-immunoprecipitation

Primer	Sequence (5'→3')
GAPDH-UP	TACTAGCGGTTTTACGGGCG
GAPDH-LO	TCGAACAGGAGGAGCAGAGAGCGA
ChIP_hTANK_FW1	TTTGTATGCGTGAGCGAGAG
ChIP_hTANK_RV1	CGACGATGCTATGCTGACAT
ChIP_hTANK_FW2	TCTTACCGCGGTTGGAATAC
ChIP_hTANK_RV2	CAACTGGGGAGAGGACTGAG

2.1.6. Antibiotics

Name	Final concentration	Source
Ampicillin	100 µg/ml	Sigma
Puromycin	1 – 2 µg/ml	Invitrogen
Penicillin / Streptomycin	100 IU / 100 µg/ml	Cell Concepts, PAA

2.1.7. Inhibitors

Name	Final concentration	Target	Source
1-β-D-Arabinofuranosyl-cytosine (Cytarabine, AraC)	1 µM	DNA synthesis	Calbiochem
5Z-7-oxozeaenol	1 µM	TAK1	Sigma
Actinomycin D	1 µg/ml	DNA-dependent RNA synthesis	Sigma
Akt Inhibitor VIII (Isozyme-selective, Akti-1/2)	5 µM	Akt	Calbiochem
Aphidicolin	2 µg/ml	DNA polymerase A,D	Sigma
Aprotinin	10 µl/ml	Serine proteases (Trypsin, Chymotrypsin, Plasmin)	Sigma
BX795	1 µM	IKK-related kinases	Axon Medchem
Complete ULTRA tablets	1 x	Proteases	Roche
Cycloheximide	10 µg/ml	Ribosomes	Roth
Leupeptine	10 µl/ml	Proteases (Lysosomal)	Sigma
PD98059	20 µM	MEK1/2	Cell Signaling
Temozolomide	100 µM	DNA replication	Sigma
U0126	5 µM	MEK1/2	Cell Signaling

2.1.8. Enzymes

Name	Source
Calf intestine alkaline phosphatase (CIAP)	Fermentas
Long Range PCR enzyme mix (DNA polymerase)	Qiagen
Pfu Ultra DNA polymerase	Stratagene
Restriction enzymes	Fermentas
RiboLock R1 RNase Inhibitor	Fermentas
SuperScript™ II reverse transcriptase	Invitrogen
T4 DNA Ligase	Fermentas
T4 polynucleotide kinase (PNK)	Fermentas
RNase A	Sigma

Taq DNA polymerase	Fermentas

2.1.9. Kits

Name	Source
RNeasy Mini	Qiagen
ABsolute™ qPCR SYBR green ROX mix	Thermo (ABgene)
JETquick gel extraction spin kit	Genomed
JETquick PCR purification kit	Genomed
JETstar 2.0 Plasmid Mini/Midi/Maxi	Genomed
Vybrant® MTT Cell Proliferation Assay Kit	Invitrogen
Long Range PCR kit	Qiagen
QuickChange II site-directed mutagenesis	Stratagene
Pierce® BCA protein assay kit	Thermo

2.1.10. Chemical reagents

Name	Source
1,4-Dithiothreitol (DTT)	Invitrogen
Acetic Acid	Roth
Acrylamide/ Bisacrylamide mix (Roti-phorese)	Roth
Adenosine triphosphate (ATP)	Sigma
Agarose	AppliChem
Ammonium persulfate (APS)	Bio-Rad
Becton™ krypton	BD Bioscience
Bovine serum albumin (BSA)	Sigma
Bromphenol blue	Merck
Calcium Chloride ($CaCl_2$)	Roth
Chloroform	Merck
Cupric sulfate	Roth
Deoxycholat	Sigma
deoxyribonucleotide triphosphates (dNTP) mix	Fermentas
Dimethyl 3,3-dithiobispropionimidate (DTBP)	Therma
Dimethyl sulfoxide (DMSO)	Sigma

di-Sodium hydrogen phosphate (Na_2HPO_4)	Roth
Doxycycline	Sigma
Ethanol	Roth
Ethidium bromide	Roth
Ethylendiamintetraacetic acid (EDTA)	Roth
Ficoll 400	Sigma
Gelatin	Roth
Glycerine	Roth
Glycerol	Roth
Hexadimethrine bromide (Polybrene)	Sigma
Hydrochloride acid 37% (HCl)	Roth
Isopropanol	Roth
Lithium chloride (LiCl)	Merck
Magnesium chloride ($MgCl_2$)	Merck
Magnesium sulfate ($MgSO_4$)	Merck
Methanol	Roth
N,N,N',N'-Tetramethylethylendiamine (TEMED)	Bio-Rad
Nonident P40 (NP40)	Roche
Phenylmethanesulfonyl fluoride (PMSF)	Fluka
Polyethylenimine (PEI)	Roth
Potassium chloride (KCl)	Roth
Potassium dihydrogen orthophosphate (KH_2PO_4)	Roth
Potassium hydroxide (KOH)	Merck
Propidium iodide (PI)	Sigma
Skim milk powder	Merck
Sodium azide (NaN_3)	Roth
Sodium chloride (NaCl)	Roth
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck
Sodium dodecyl sulfate (SDS)	Bio-Rad
Sodium fluoride (NaF)	Roth
Sodium hydroxide (NaOH)	Merck
Sodium orthovanadate (Na_3VO_4)	Sigma
TRIS	Roth
Triton X-100	Sigma

TRIzol®	Ambion
Tryptone	AppliChem
Tween 20	Gerbu
Yeast extract	Roth
β-Mercaptoethanol	Roth

2.1.11. Other reagents

Name	Source
Enhanced chemiluminiscence (ECL) solution	GE Healthcare
Fetal calf serum (FCS)	Cell concepts
Generuler™ 1 kb DNA ladder	Fermentas
Generuler™ 100 bp DNA ladder	Fermentas
L-Glutamine (200 mM)	Cell Concepts
Lipofectamine 2000	Invitrogen
Oligo(dT)$_{12-18}$ Primer	Invitrogen
PageRuler™ prestained protein ladder	Fermentas
Protein A/G agarose beads	Santa Cruz Biotech
Roti®fect	Roth
TrypLE^{TM} Express	Gibco
First Strand Buffer	Fermentas
T4 ligase buffer	Fermentas
FACS Flow™	BD Bioscience
FACS Clean	BD Bioscience
FACS Rinse	BD Bioscience
Trypsin/EDTA	Cell Concepts

2.1.12. Media, buffers and solutions

2.1.12.1. Media for cultivating prokaryotic cells

Bacteria were cultured either in Luria Bertani broth (LB) medium or on LB agar plates supplemented with an appropriate antibiotic. LB medium was assembled as follows:

Bacto-trypton	1% (w/v)
Yeast extract	0.5% (w/v)
NaCl	1% (w/v)

LB agar plates additionally contained 1.6% (w/v) agar.

2.1.12.2. Media for cultivating eukaryotic cells

Most eukaryotic cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), but MCF7 cells were kept in RPMI 1640 medium.

Name	Source
DMEM high glucose	Cell concepts
RPMI 1640	Cell concepts

DMEM as well as RPMI 1640 medium were supplemented with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 2 mM L-glutamine. Decomplementation of FCS was done at 56 °C for 30 min to inactivate heat-labile complement proteins.

2.1.12.3. Buffers and other solutions

All buffers that are described subsequently were prepared using deionized water when nothing else is indicated. Water used for buffers in molecular biological methods was additionally autoclaved.

Phosphate Buffered Saline (PBS) (pH 7.4)	137 mM	NaCl
	8.1 mM	Na_2HPO_4
	2.7mM	KCl
	1.5 mM	KH_2PO_4
TRIS Buffered Saline (TBS-T) (pH 7.4)	250 mM	TRIS
	1.37 M	NaCl
	50 mM	KCl
	7 mM	$CaCl_2 \cdot 2H_2O$
	1 mM	$MgCl_2 \cdot H_2O$
	0.1% (v/v)	Tween 20
TRIS-Acetate-EDTA (TAE) (pH 8.3)	0.05 M	EDTA
	2 M	TRIS
	1 M	Acetic acid
5 x SDS sample buffer	250 mM	TRIS-HCl (pH 6.8)
	15% (v/v)	β-Mercaptoethanol
	40% (v/v)	Glycerin
	10% (w/v)	SDS
	0.1% (w/v)	Bromphenol blue
SDS stacking gel (5%)	125 mM	TRIS-HCl (pH 6.8)
	5% (v/v)	Acrylamide/Bisacrylamide
	0.1% (w/v)	SDS
	0.04% (v/v)	APS
	0.3% (v/v)	TEMED

SDS separating gel (8 or 10%)	350 mM	TRIS-HCl (pH 8.8)
	8 or 10%	Acrylamide/Bisacrylamide
	0.1%	SDS
	0.04%	APS
	0.075%	TEMED
5 x SDS Running Buffer	125 mM	TRIS
	960 mM	Glycerine
	0.5% (w/v)	SDS
Transfer Buffer (Semi-dry Blot)	48.5 mM	TRIS
	39 mM	Glycerine
	20% (v/v)	Methanol
	0.038% (w/v)	SDS
NP40 lysis buffer	20 mM	TRIS-HCl (pH 7.5)
	1% (v/v)	NP40
	150 mM	NaCl
	10% (v/v)	Glycerol
	prior to use:	
	25 mM	NaF
	1 mM	Na_3VO_4
	1 mM	PMSF
	10 µg/ml	Aprotinin
	10 µg/ml	Leupeptin
TE buffer	10 mM	TRIS-HCl (pH 7.5)
	1 mM	EDTA
6 x DNA sample buffer (pH 8)	15% (w/v)	Ficoll 400
	10 mM	EDTA
	0.1% (w/v)	Bromphenol blue
ChIP-SDS buffer	1%	SDS
	10 mM	EDTA
	50 mM	TRIS-HCl (pH 8.1)
	prior to use:	
	10 µg/ml	Aprotinin
	10 µg/ml	Leupeptin
	1 mM	PMSF
ChIP Dilution buffer	0.01%	SDS
	1%	Trition X-100
	1.2 mM	EDTA
	16.7 mM	TRIS-HCl (pH 8.1)
	16.7 mM	NaCl
ChIP low salt buffer	0.1%	SDS
	1%	Triton X-100
	2 mM	EDTA
	20 mM	TRIS-HCl (pH 8.1)
	150 mM	NaCl

ChIP high salt buffer	0.1%	SDS
	1%	Triton X-100
	2 mM	EDTA
	20 mM	TRIS-HCl (pH 8.1)
	300 mM	NaCl
ChIP LiCl buffer	0.25 M	LiCl
	1%	NP40
	1 mM	EDTA
	20 mM	TRIS-HCl (pH 8.1)
	1%	Sodium deoxycholat
Annealing buffer	100 mM	Potassium acetate
	2 mM	Magnesium acetate
	30 mM	HEPES-KOH (pH 7.4)

2.2. Methods

2.2.1. Methods in cell biology

2.2.1.1. Eukaryotic cell culture

Human cell lines cultures were grown in appropriate medium in 175 cm² flasks at 37 °C in a humidified 5% CO_2 incubator. Confluent cells were trypsinized. Therefore, old medium was removed, cells were washed with 10 ml prewarmed 1 x PBS and 5 ml Trypsin was added. After incubation for 2 – 4 min. at 37 °C, cells were resuspended in complete medium to stop trypsinization. A small aliquot of cell suspension was added into a new flask with 20 ml of complete medium.

2.2.1.2. Freezing and thawing

To store cells for a longer term, they were frozen at -150 °C in 1 ml FCS with 10% (v/v) DMSO (freezing medium). Therefore cell suspension was centrifuged for 3 min at 1300 rpm and the pellet was resuspended in freezing medium. The resulting suspension was transferred into labeled freezing vials and gradually cooled down to -150 °C.

To thaw frozen cell aliquots, vials were placed in a 37 °C water bath until suspension was thawed completely. Then cells were immediately resuspended in prewarmed culture medium and pelleted to remove DMSO. Culture medium was added to the cell pellet and cells were seeded into a new flask. After cells attached to the ground, medium was changed to remove dead cells.

2.2.1.3. Transfection of eukaryotic cells

Eukaryotic cells were transfected by liposomes using either PEI, Roti®-Fect or Lipofectamine according the manufacturer's instructions. The plasmid DNA as well as transfection reagent were mixed with 100 µl DMEM each without any additives. After 5 min both solutions were mixed and further incubated for 20 min at room temperature. Meanwhile seeded cell were prepared by washing them with 2 ml of 1 x PBS and adding of fresh antibiotic-free medium. The DNA-liposome mix was added on the cells drop wise and mixed gently. The cells were incubated at 37 °C in a humidified 5% CO_2 incubator for 4 hs. Then medium was exchanged to culture medium and cells were allowed to grow for at least 24 hs.

2.2.1.4. Retro- and lentiviral infection of glioblastoma cell lines

The introduction of DNA into glioblastoma cell lines was not possible as described above. Therefore those cell lines had to be infected with retro- or lentiviruses to express desired DNA. Retroviruses were produced in Phoenix Ampho cell line by transfecting pSIREN-vector containing desired shRNA constructs with Roti®-Fect according to manufacturer's instructions. The produced retroviruses were used to silence proteins in infected glioblastoma cell lines by RNA interference. The binding of shRNA to corresponding mRNA within the cell leads to degradation of the mRNA by ribonuclease. In order to exclude off-target effects, alternative constructs for shTANK and shTBK1 were delivered by lentiviral transduction. Thus, HEK293T cells were transfected with either pLL3.7-shTANK or pLKO1-shTBK1 together with packaging plasmid psPAX2 (encoding HIV-1 Gag, Pol, Tat and Rev proteins) as well as envelope plasmid pMD2.G (encodes for VSVG) using lipofectamine according the manufacturer's instructions. Furthermore, over-expression experiments for TANK were also performed using lentiviruses to infect glioblastoma cell lines. Lentiviruses were also produced in HEK293T cells by transfecting 290-pHAGE-hEF1aCAR-PGK Puro-Linker TANK vector together with the packaging vectors pMDLg/pRRE, pRSV-Rev and pHCMVG using lipofectamine according the manufacturer's instructions. Two days after transfection of the virus producing cells, the virus containing supernatant was collected and filtered through a 0.45 µm filter. After adding Polybrene to a final concentration of 5 µg/ml, the supernatant was added to the glioblastoma cell lines for 24 hs. Then medium was changed back to culture medium. Three days after infecting glioblastoma cell lines were treated with 2 µg/ml Puromycin for at least 5 days to select cells containing the desired DNA constructs.

2.2.1.5. MTT cell viability and proliferation assays

To determine cell viability and indirectly the proliferation ability, the Vybrant® MTT cell viability assay was performed. Glioblastoma cell lines where either TANK or TBK1 was silenced or scrambled control cells were counted and 1 x 10^3 cells per well were seeded in 96-well plates. At the next day, cells were treated with either the cytostatics TMZ and AraC or the inhibitor BX795. After 3 days the assay was performed as described in the manufacturer's instructions. Water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was solubilized in 1 ml PBS by sonification and 10 µl of it was added to each well containing 100 µl new complete DMEM medium. Within 4 hs of incubation at 37 °C the living cells took up the MTT and converted it into the insoluble formazan. To stop the reaction and to lyse the cells, 100 µl SDS-HCl solution was added to each well. The plate was then further incubated at 37 °C in a humidified chamber to dissolve the formazan. The absorbance of formazan was measured 16 hs later on an Ultra Microplate Reader EL-808i at a wavelength of 562 nm.

2.2.1.6. Proliferation assays

To measure proliferation of Glioblastoma cell lines, cells were counted in a FACSCalibur. Cells were seeded at a density of 5 x 10^3 on 6 and 10 cm^2 plates, respectively, and were allowed to grow for either 72 or 120 hs. To harvest the cells, they were first washed with 1 x PBS and subsequently trypsinized with TrypLE™ Express for 5 min. Trypsinization was stopped by adding complete medium on the plate. Cell suspension was then transferred to round-bottom tubes. The number of cells was counted by FACSCalibur over a constant time of 60 sec at medium flow rate (35 ± 5 µl/min) with following settings:

Param	Detector	Voltage	Amp Gain	Mode
P1	FSC	E00	1.00	lin
P2	SSC	310	1.00	lin
P3	FL1	394		log
P4	FL2	340		log
P5	FL3	200		log

2.2.1.7. Cell migration assays

The wound-healing assay, also called scratch assay, is a method to determine cell migration where an area of a cell layer is scraped off and the migration of cells into this gap is monitored microscopically. Knock-down and control glioblastoma cells were seeded at a density of 1.5 – 2 x 10^5 cells per well in 6-well pates and grown to 80% confluence. Cells were then kept in serum-reduced complete medium supplemented with 2 µg/ml

aphidicolin to prevent proliferation. At text day, a scratch was gently made with a yellow pipette tip. The cells were washed twice with prewarmed PBS to remove loose cells and serum-reduced medium supplemented with 2 µg/ml aphidicolin was added again. Directly after scratching as well as 10 and 24 hs after pictures of the gap were taken with a life cell imaging technique using a NIKON Inverted Research Microscope Eclipse TE2000-E to retain migration. Taken pictures were analyzed using NIKON NIS-Elements AR 3.22.

2.2.1.8. Cell cycle analysis by flow cytometry

A cell cycle profile displays the DNA content of a cell population and thereby provides information about the four characteristic phases of the cell cycle. Whereas G_1 phase is characterized by a diploid set of chromosomes (2N) and S phase by an aneuploid (2N – 4N), cells in G_2 have a fourfold amount of DNA [227]. The DNA amount can be measured by flow cytometry after staining with propidium iodide, a fluorescent dye that intercalates into DNA. The measured fluorescence then is directly proportional to the DNA content of the cells. Either knock-down or control cells were seeded on 10 cm dishes and if required treated with 0.3 µM vinblastine for 24 hs to arrest the cell cycle in late G_2 phase. After collecting the culture medium in a 15 ml tube, cells were detached using TrypLE™ Express. Detached cells were then transferred to the corresponding tube and pelleted at 3,000 rpm at 4 °C for 5 min. Cells were washed with PBS and the cell pellets were taken up in 300 µl PBS. Cell were fixed by dropwise adding of 1 ml ice-cold ethanol (70%) while the suspension was vortexed to avoid the formation of cell clumps. After not less than 1 h cells were pelleted again and washed once with PBS. Cell pellets were then resuspended in 500 µl PI-TritonX100-RNaseA solution and incubated for 15 min at room temperature in the dark. To stop this staining reaction, tubes were put on ice. The emission of propidium iodide was then analyzed using a FACSCalibur with different settings for each cell line. Percentages of cells in certain cell cycle phases were determined by ModFit LT™ software.

2.2.2. Methods in biochemistry

2.2.2.1. Preparation of protein extracts

In order to analyze expression levels of proteins as well as their phosphorylation state they first need to be extracted from either eukaryotic cells or patient samples.

2.2.2.1.1. Protein isolation from patient samples

Patient tissue of glioblastoma multiforme, astrocytoma grade III and normal brain were kindly provided by Prof. Dr. Axel Pagenstecher. Frozen tissue was cutted in a cryo bench, weighted and transferred to microcentrifuge tubes. To extract the proteins, 10 x volume of

cold NP40 extraction buffer containing a mix of protease inhibitors (1 x Complete ULTRA tablet and 5 µg/ml Pepstatin A) was added and the tissue was homogenized by using a Turrax. Samples were frozen immediately on dry ice and transferred to -80 °C.

2.2.2.1.2. Determination of protein concentration

To determine the protein concentration of homogenized patient tissues, the Pierce® BCA protein assay was performed in 96-well plates following the manufacturer's instructions. BSA stocks (2 mg/ml) were used to prepare a set of nine diluted standards ranging from 0 – 2000 µg/ml. The working reagents were prepared by mixing 50 parts BCA reagent A with one part of BCA reagent B. In each well of a 96-well plate 10 µl of either the standards or the sample with unknown protein concentration was mixed with 200 µl of the working reagent. After incubating the plate at 37 °C for 30 min the plate was cooled down to room temperature. The absorbance was measured on an Ultra Microplate Reader EL-808i with a wavelength of 562 nm within 10 min after cooling. Each measurement was carried out in triplicates. The standard curve was used to determine the protein concentration of the samples extracted from patient tissue. Before Western blot analysis of the samples, they were mixed with 5 x SDS sample buffer to a final concentration of 1 x SDS.

2.2.2.1.3. Lysate preparations from eukaryotic cells

Proteins from glioblastoma cell lines were extracted by SDS lysis to get proteins from all the compartments within the cell and even membrane-bound proteins. Cells were first washed once with ice-cold PBS, harvested by scraping and then transferred to microcentrifuge tubes. After pelleting the cells at 3,000 rpm for 5 min, cells were resuspended in 1 x SDS sample buffer. After heating the suspension to 95 °C they were sonicated twice for 20 sec to shear the genomic DNA. The samples were then heated again to 95 °C for 5 min and frozen at -80 °C.

2.2.2.2. Co-immunopecipitation

The interaction of cellular proteins were determined by co-immunoprecipitation. To increase the binding stability of the interactions, the proteins were crosslinked before the actual precipitation. For the crosslinking, cells were first washed once with cold 1 x PBS on the dish. A freshly prepared 0.5 mM DTBP solution in 1 x PBS was added to the cells and incubated for 30 min at room temperature. After removing the DTBP solution cells were washed two times with PBS containing 200 mM TRIS/HCl (pH 7.5) for 10 min to stop the crosslinking reaction. Then cells were harvested by scraping in PBS with TRIS/HCl (200 mM, pH 7.5). Harvested and washed cells were lysed under native conditions by

resuspending the pellet in NP40 buffer containing protease inhibitors and incubated on ice for 20 min. Nuclear DNA was shared by two times sonification for 20 sec. To spin down cellular debris, the sonicated lysate was centrifuged 10 min at 13,000 rpm and the supernatant was transferred to new tube. After taking out 10% of the lysate as input control, the samples were incubated with A/G sephasose for 60 min at 4 °C to prevent nonspecific binding. After spinning down the beads by centrifugation, the supernatants were transferred to a new tube and 1 - 2 µg of primary antibody or control IgG were added. The antibodies were allowed to bind the proteins in the samples overnight at 4 °C on a spinning wheel. At the next day, 25 µl of A/G sepharose was added and samples were again incubated at 4 °C gently rocking for 2 hs. The supernatant was discarded and the beads were washed 5 times with NP40 buffer containing protease inhibitors. To elute bound proteins from the beads they were boiled at 95 °C in 2 x SDS sample buffer for 4 min. The eluates were then further analyzed by SDS-PAGE and Western Blot.

2.2.2.3. Polyacrylamide gel electrophoresis

In order to separate equally charged proteins by their molecular weight a discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Samples were mixed with 5 x SDS sample buffer to a final concentration of 1 x SDS. Cells were already lysed in 1 x SDS sample buffer as described in 2.2.2.1.3. This sample buffer contains SDS to denature and negatively charge the proteins as well as β-mercaptoethanol to reduce disulfide bonds. SDS polyacrylamide gel contained a separating gel with an acrylamide concentration between 8 and 15%, depending on the expected size of the proteins, and a stacking gel containing 5% acrylamide which is necessary to focus the proteins in a single sharp band. This stacking of proteins was performed at 80 V. Once the separation of proteins started, the current was increased up to 120 V. To estimate the approximate size of various proteins, a pre-stained protein marker was loaded on the polyacrylamide gel next to the samples. Proteins were visualized by Western blotting.

2.2.2.4. Western blot and immune detection

Western Blot is a method to detect proteins by transferring them on a membrane and identify the proteins by binding of specific antibodies. Separated proteins from the SDS-PAGE were transferred to and immobilized on polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer method. Therefore, the PVDF membrane was activated by methanol for a few seconds and whatman papers were incubated in transfer buffer. Two of those whatman papers were placed into the electroblotting device. The activated PVDF membrane, the SDS polyacrylamide gel and another whatman paper were added on top. After removing carefully air bubbles between the layers, the device

was closed and a constant voltage of 24 V was applied. Depending on percentage of the SDS polyacrylamide gel, the size of the protein of interest and the number of gels per device, the transfer was performed for 40 to 195 min. The membranes with the transferred proteins were blocked by incubating for at least 30 min in blocking solution containing either 5% skim milk powder or BSA dissolved in TBS-T. After washing the membrane twice in TBS-T, the incubation with primary antibodies was carried out overnight at 4 °C. After washing three times with TBS-T for 10 min., the membranes were incubated with secondary peroxidase-coupled antibody for 1 h at room temperature. The non bound antibody was removed from the membranes by washing again three times for 10 min with TBS-T. Proteins with bound antibodies were then detected by autoradiography using an enhanced chemiluminiscence (ECL) system.

2.2.3. Methods in molecular biology

2.2.3.1. Preparation of competent *E.coli*

E.coli stains were used to amplify plasmid DNA in high copy numbers. To ensure the uptake of the DNA bacteria were made chemically competent using the $CaCl_2$ method. An Erlenmeyer flask with 10 ml LB medium was inoculated with *E.coli* and incubated overnight shaking at 37 °C. Next day, additional 90 ml of prewarmed LB medium were added and cells were allowed to grow further for approximately 70 min. Bacteria were cooled down and harvested by centrifugation at 4,000 g for 15 min at 4 °C. Cell pellets were resuspended in 50 ml ice-cold 0.1 M $CaCl_2$ solution and incubated at 4 °C for 30 min. Bacteria were again pelleted by centrifugation and resuspended in 3 ml of a 10% glycerol/0.1 M $CaCl_2$ solution. Competent *E.coli* were aliquoted at 100 µl and stored at -80 °C.

2.2.3.2. Competent *E.coli* transformation

Various chemically competent *E.coli* stains were transformed to amplify plasmids. An aliquot of 50 µl of *E.coli*, which was thawed on ice, was mixed with 1 µg of the DNA and incubated for 20 min on ice. To induce the uptake of DNA into the bacteria via heat-shock, they were placed at 42 °C for 90 sec. After incubating the suspension for additional 5 min on ice, LB medium was added and the bacteria were allowed to grow shaking for 1 h at 37 °C. Finally the transformed bacteria were plated out on LB agar plates supplemented with appropriate antibiotic to select for transformed cells. Plates were incubated overnight at 37 °C.

2.2.3.3. Plasmid DNA Purification

Isolation of DNA from transformed E.coli was either performed by miniprep to identify the right clones or by maxiprep for large-scale preparations of highly pure DNA for transfection of eukaryotic cells. Both protocols follow the principles of alkaline lysis by Birnboim and Doly [403]. Minipreps were done by inoculation of bacteria in 3 ml of LB medium and further growth overnight at 37 °C. Next day, they were transferred to a tube and pelleted by centrifugation for 5 min at 5,000 rpm. LB medium was aspirated and bacteria were resuspended on ice in 200 µl buffer P1. To lyse the cells, 200 µl of buffer P2 was added and mixture was incubated for 5 min at room temperature. After neutralization with 200 µl precooled buffer P3, tubes were incubated for 20 min. on ice. Thereafter the solution was centrifuged 10 min at 13,000 rpm and the supernatant was transferred to a new tube. To precipitate the DNA 350 µl isopropanol was added and the well mixed solution was centrifuged 15 min at 13,000 rpm. The supernatant was discarded and DNA washed with 500 µl 70% ethanol. DNA was pelleted, air-dried and redissolved in 30 µl TE buffer.

For Maxiprep the Jetstar 2.0 Plasmid Mini/Midi/Maxi kit was used according the manufacturer's instructions. Pelleted bacteria cells, that were grown overnight at 37 °C in 250 ml LB medium supplemented with antibiotics, were resuspended in 10 ml buffer E1 containing 100 µg/ml RNase. Suspension was then mixed with 10 ml buffer E2 in order to lyse cells. After incubation for 5 min at room temperature, lysates were neutralized by adding 10 ml buffer E3. Cell debris was spinned down and supernatant was applied to a Jetstar 2.0 column pre-equilibrated with 30 ml buffer E4. Once lysate ran through the column by gravity flow, it was washed once with 60 ml of buffer E5. After elution of DNA from the column by adding 15 ml of buffer E6, DNA was precipitated with 0.7 volumes of isopropanol and centrifuged for at least 30 min at 12,000 x g. The formed pellet was washed once with 5 ml of 70% ethanol and recentrifuged. Air dried DNA was dissolved in a suitable volume of TE buffer and quantified spectrophotometrically.

2.2.3.4. Introduction of DNA fragments in vector molecules – cloning

Plasmids are commonly used to introduce a desired DNA into eukaryotic cells. This certain DNA first has to be cloned into a suitable vector.

2.2.3.4.1. Polymerase chain reaction

The DNA fragment that will be cloned into a vector can be produced either by amplification by PCR (polymerase chain reaction) method or by annealing of two oligonucleotides. Polymerase chain reaction is a common method to amplify defined DNA

sequences. To minimize the risk of unwanted point mutations the Long Range PCR Kit of Qiagen was used that contains a DNA polymerase with an 3'-5' exonuclease activity for proof-reading. The PCR reaction was set up on ice as followed:

Template DNA	20 ng
10x LongRange PCR buffer	5 µl
dNTP mix	0.5 mM
forward primer	0.4 µM
reverse primer	0.4 µM
Long Range PCR enzyme mix	2 units
RNase-free water	to a total volume of 50 µl

The amplification of the DNA in a Bio-Rad thermocycler was carried out under following conditions:

Initial activation:	3 min	95 °C
Denaturation:	15 sec	95 °C
Annealing	30 sec	55 - 62 °C (~5 °C below T_m of primers)
Extension:	1 min/kb	68 °C

After 35 cycles an additional elongation step at 68 °C for 5 - 7 min was added. The annealing temperature depends on the melting point of the designed primers that usually contain a further non-complementary sequence coding for specific endonuclease restriction sites that are used to ligate the DNA into a vector that was opened with the same restriction enzymes.

For smaller DNA fragments it is also possible to anneal oligonucleotides to get fragments that can be ligated into an opened vector. Therefore 5 µg of each oligonucleotide were mixed in annealing buffer to a total volume of 50 µl. The mixture was incubated 5 min at 95 °C and then slowly cooled down by shutting off the heating block. After cooling down of the annealed oligonucleotides to room temperature, they were stored at -20 °C.

2.2.3.4.2. Restriction enzyme digestion, agarose gel electrophoresis and gel extraction

Vectors are used as vehicles to bring desired DNA into eukaryotic cells. Both DNA fragments and vectors first need to be cut with certain restriction endonucleases to generate compatible ends before they can be ligated. Restriction enzymes commonly used for digestions recognize and cut short palindromic sequences of 4 to 7 base pairs. In order to digest DNA the following mixture was prepared on ice and incubated for 2 hs at the enzyme specific optimal temperature:

DNA	3 µg
10 x restriction buffer	1 µl
restriction enzyme 1 (10 U/µl)	0.5 µl
restriction enzyme 2 (10 U/µl)	0.5 µl
MilliQ water	to a total volume of 10 µl

The digested products were analyzed by agarose gel electrophoresis. To separate DNA fragments according to their size, 0.8 – 2% (w/v) agarose was melted in TAE buffer. To visualize the DNA under UV light 0.5 µg/ml ethidium bromide was added to the liquid gel. Ethidium bromide intercalates in DNA double strands and emits fluorescent light when exposed to UV light. Before loading DNA samples on the gel they were mixed with 6 x Loading Dye. Then the gels was run at a constant voltage of 80 V in TAE buffer.

For further cloning desired fragments were excised from the agarose gel under reduced UV exposure using a clean scalpel. The JETquick gel extraction kit was used according to the manufacturer's instructions to isolate the DNA from the gel slice. Therefore the excised gel was melted at 50 °C in buffer L1 for 15 minutes. The mixture was loaded on a spin column where the DNA binds to the resin. After washing the DNA with solution L2, it was eluted in 30 µl sterile water and stored at -20 °C.

2.2.3.4.3. Dephosphorylation and 5'-phosphorylation of DNA

To obviate the religation of plasmid DNA that was digested with just one restriction enzyme, the 5'-phosphates were removed using calf intestine alkaline phosphatase (CIAP). Following mixture was therefore prepared:

Linearized plasmid DNA (1 µg/µl)	1 - 3 µl
10 x CIAP reaction buffer	1 µl
CIAP (1 U/µl)	1 µl
MilliQ water	to a total volume of 10 µl

This mixture was then incubated at 37 °C for 30 min. After stopping the reaction by heating at 85 °C for 10 min the plasmid DNA could be used for ligation reaction or stored at -20 °C.

The dephosphorylation of plasmid DNA necessitate the 5'-phosphorylation of the DNA fragment that shall be ligated into the vector. The transfer of γ-phosphate of ATP to the 5'-OH group the DNA was accomplished by T4 polynucleotide kinase (PNK) according the subsequent procedure. First, the following mixture was prepared:

Purified PCR products	1 – 5 µl
10 x reaction buffer A	2 µl
ATP (10 mM)	2 µl
PNK (10 U/µl)	1 µl
MilliQ water	to a total volume of 20 µl

The reaction mixture was incubated at 37 °C for 20 min. Heating the solution at 75 °C for 10 min inactivates the kinase. Products can be directly used for ligation reaction or stored at -20 °C. Sometimes it can be useful to repeat the agarose gel electrophoresis and gel extraction at this step to remove the enzyme when it is not completely inactivated.

2.2.3.4.4. Ligation of DNA fragments

Finally the opened and dephosphorylated plasmid DNA and the 5'-phosphorylated DNA fragment can be ligated to create a new plasmid used for transferring DNA into eukaryotic cells.

The ligation reaction was set up with different molar ratios of plasmid to insert to assure the best outcome. Vector:Insert mixtures with ratios ranging between 1 : 1 and 1 : 10 were mixed with 1 µl 10 x T4 DNA ligase buffer and 1 µl T4 DNA ligase (1 U/µl) and were filled up with sterile water to a final volume of 10 µl. Samples were then either incubated for 2 hs at room temperature or slowly cooled down to 4 °C overnight. Ligation reaction mix was directly used for the transformation of appropriate *E.coli* stains and remaining mixture was stored at -20 °C.

2.2.3.4.5. Site-directed point mutagenesis

In order to alter the function of proteins, single amino acids can be changed by mutating one or several base pairs in the sequence of the protein of interest. Besides it is possible to create proteins that are resistant to silencing by certain siRNA or shRNA by introducing a silent mutation that has no influence on the amino acid sequence and thereby does not alter the function of the protein.

To introduce silent mutations the site-directed mutagenesis was performed using the QuickChange II site-directed mutagenesis kit from Stratagene according the manufacturer's suggestions. The design of two synthetic complementary primers containing the single point mutations was carried out according to the manufacturer's instructions. The following mixture was prepared for a typical PCR reaction:

Template DNA (100 ng/µl)	1 µl
10 x reaction buffer	5 µl
Quick solution	3 µl
dNTP mix (10 mM)	1 µl
Forward primer (10 µM)	1.2 µl
Reverse primer (10 µM)	1.2 µl
***Pfu ultra* DNA polymerase**	1 µl
MilliQ water	to a total volume of 50 µl

The linear amplification of PCR products containing the desired mutation was performed with the following PCR parameters for 18 cycles:

Initial activation	2 min	95 °C
Denaturation	50 sec	95 °C
Annealing	30 sec	60 °C
Extension	1 min/kb	68 °C

After the 18th cycle an additional elongation step at 68 °C for 5 min was carried out to ensure the generation of full-length products. To digest the methylated template DNA, 1 µl of *Dnp*I restriction enzyme (10 U/µl) was added and the samples were incubated for 2 hs at 37 °C. Unmethylated DNA resulting from polymerase chain reaction is not affected by this digestion. Finally the PCR product was directly transformed into highly competent XL10 Gold *E.coli* that were provided with the kit.

2.2.3.5. RNA extraction

2.2.3.5.1. TRIzol® RNA extraction from patient tissue

In order to isolate total RNA from patient samples, frozen tissue was prepared as described for protein extraction in 2.2.2.1.1 and then homogenized in 500 µl TRIzol®. After adding of 100 µl chloroform and vigorously agitation, samples were incubated at room temperature for phase separation. Samples were then centrifuged for 10 min at 13,000 rpm at 4 °C. The mixtures should be separated in three phases where the RNA resides in the upper aqueous phase which was transferred to a new tube. RNA was then precipitated by adding of 250 µl isopropanol (100%) and incubated at room temperature for 10 min. Precipitated RNA was pelleted by centrifugation for 10 min at 13,000 rpm and washed with 150 µl 70% ethanol. The pellet was air dried and resuspended in 20 µl RNase-free water. Samples were stored at -80 °C.

2.2.3.5.2. RNA isolation from eukaryotic cells

Total RNA was isolated from eukaryotic cells using the RNeasy kit from Qiagen following the manufacturer's instructions. Cells were washed with PBS and harvested by scraping. Cells were lysed by adding 600 µl of RLT buffer containing β-Mercaptoethanol (10µl/ml)

and passing them trough a blunt 20-gauge needle for at least five times. Lysates were then mixed with 600 µl ethanol (70%) and loaded on a provided spin column. The column was first washed once with 700 µl RW1 buffer and afterwards twice with 500 µl RPE buffer. To remove remaining ethanol of buffer RPE, the column was centrifuged without adding further buffers. RNA was then eluted by adding 30 µl RNase-free water on the column and centrifugation. RNA concentration and purity was measured in a spectrophotometer, afterwards 1.5 µl RiboLock R1 was added to prevent RNA degradation. RNA was stored at -80 °C.

2.2.3.6. Synthesis of complementary DNA

Before the expression of mRNA of interest can be analyzed by semi-quantitative real-time PCR, mRNA was transcribed into cDNA. The CDNA synthesis was performed using the SuperScript™ II reverse transcriptase kit by Invitrogen. The following reaction mix was set up:

Template RNA	1 µg
dNTP mix (10 mM)	1 µl
Oligo(dT)$_{12-18}$ primer	1 µl
MilliQ water	to a total volume of 12 µl

By using Oligo(dT)$_{12-18}$ primer, that bind to the poly(A)-tail of mRNAs, it is ensured that whole mRNA is transcribed into cDNA. For certain questions it can be necessary to use specific primers for any mRNA. The reaction mixture described above was incubated at 70 °C for 5 min and immediately put on ice. Then following reagents were added to each reaction:

5 x First-strand buffer	4 µl
DTT (100 mM)	2 µl
RiboLock R1	1 µl
SuperScript II reverse transcriptase	1 µl
MilliQ water	to a total volume of 20 µl

Samples were the further incubated in a thermocycler at 42 °C for 55 min. The reaction was stopped by heat inactivation at 70 °C for 15 min. The resulting cDNA was diluted to a final volume of 100 µl with sterile water and stored at -20 °C.

2.2.3.7. Semi-quantitative real-time polymerase chain reaction

The semi-quantitative real-time PCR is a common method to quantify changes of gene expression of target genes between different samples. By adding the fluorescent reagent SYBR green to the reaction that intercalates with double stranded DNA the amplification of synthesized DNA can be measured.

Real-time PCRs were performed by preparing the following reaction mixtures per well in triplicates on a 96-well plate:

Template cDNA (10 ng/µl)	2 µl
Absolute SYBR green ROX mix	12,5 µl
Forward primer (5 µM)	0.5 – 1.5 µl
Reverse primer (5 µM)	0.5 – 1.5 µl
MilliQ water	to a total volume of 25 µl

Following, amplification and analyzes were executed using an Applied Biosystems 7300 real-time PCR system with the following parameters for PCR reaction:

Initial activation:	15 min	95 °C	
Denaturation:	15 sec	95 °C	
Annealing	30 sec	60 °C	40 cycles
Extension:	30 sec	72 °C	

Data were normalized to the mRNA levels of the house-keeping genes *β-Actin* or *Tbp* and *Hprt1* and relative changes in expression levels compared to any calibrator (e.g. with shScramble silenced cells, untreated cells or healthy brain samples) were calculated by the ΔΔCt method.

2.2.3.8. Chromatin-immunoprecipitation

In order to analyze the *de novo* transcription of the *Tank* gene in different glioblastoma cell lines, a chromatin-immunoprecipitation (ChIP) was performed. Thereby, active polymerase II, which was bound to the DNA, was precipitated. Co-precipitated DNA was then determined by real-time PCR. To crosslink the proteins that are bound to the DNA, 10 ml of a 1% formaldehyde solution was added to confluent grown cells and incubated for 10 min at room temperature. Formaldehyde was then neutralized by adding 1 ml of 1.25 M glycine, followed by an incubation at room temperature for 2 minutes. After washing cells were washed twice with ice-cold PBS, they were harvested by scraping and pelleted by centrifugation at 1,500 rpm for 5 min. Pelleted cells were lysed in 1,600 µl ChIP-SDS buffer supplemented with Aprotinin (10 µg/ml), Leupeptin (10 µg/ml) and PMSF (1 mM), and incubated for 10 min on ice. To shear genomic DNA, lysates were sonicated intervallic (4 times for 1 minute with 1 min breaks). Cell debris was removed by centrifugation of the lysate for 20 min at 13,200 rpm. The supernatant was transferred to a new tube. Aliquots of the lysates were frozen at -80 °C and 100 µl was taken as input control. To examine the quality of DNA shearing as well as the amount of DNA, the input control was first reverse crosslinked. Therefore, input was mixed with 100 µl ChIP TE buffer and 2 µl RNase A and incubated at 37 °C for 30 minutes. Following, 5 µl of a 10% SDS solution and 5 µl Proteinase K was added and further incubation at 37 °C for at

least 4 hs and subsequently at 65 °C for 6 hs. DNA was then isolated using the Jet PCR Purification Kit according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry. The shearing of DNA was controlled by agarose gel electrophoresis. Fragments should appear between 300 - 800 bp. To precipitate active polymerase II, cell lysates were thawed on ice and mixed with ChIP dilution buffer to a final volume of 1 ml. To reduce non-specific binding at the A/G sepharose beads, lysates were pre-cleaned by adding the beads and subsequent incubation for 1 h at 4 °C in a rotating wheel. Beads were pelleted and supernatant was agitated with 35 µl A/G sepharose as well as 2 µg of either active polymerase II or IgG antibody. The mixture was incubated rotating overnight at 4 °C. At the next day, beads were washed consecutively with 1 ml ChIP low salt buffer, 1 ml ChIP high salt buffer, 1 ml ChIP LiCl buffer and two times with 1 ml ChIP TE buffer. Then beads were resuspended in 100 µl ChIP TE buffer. Finally, samples were reverse crosslinked as described before and DNA was extracted. Purified DNA was then used as template for real-time PCR analysis using specific primers for either GAPDH or TANK whereas two distinct regions of the *Tank* gene were chosen for the analysis. All data were fist normalized to IgG as negative control for each ChIP pull-down and eventually to GAPDH to exclude differences between the cell lines.

3. RESULTS

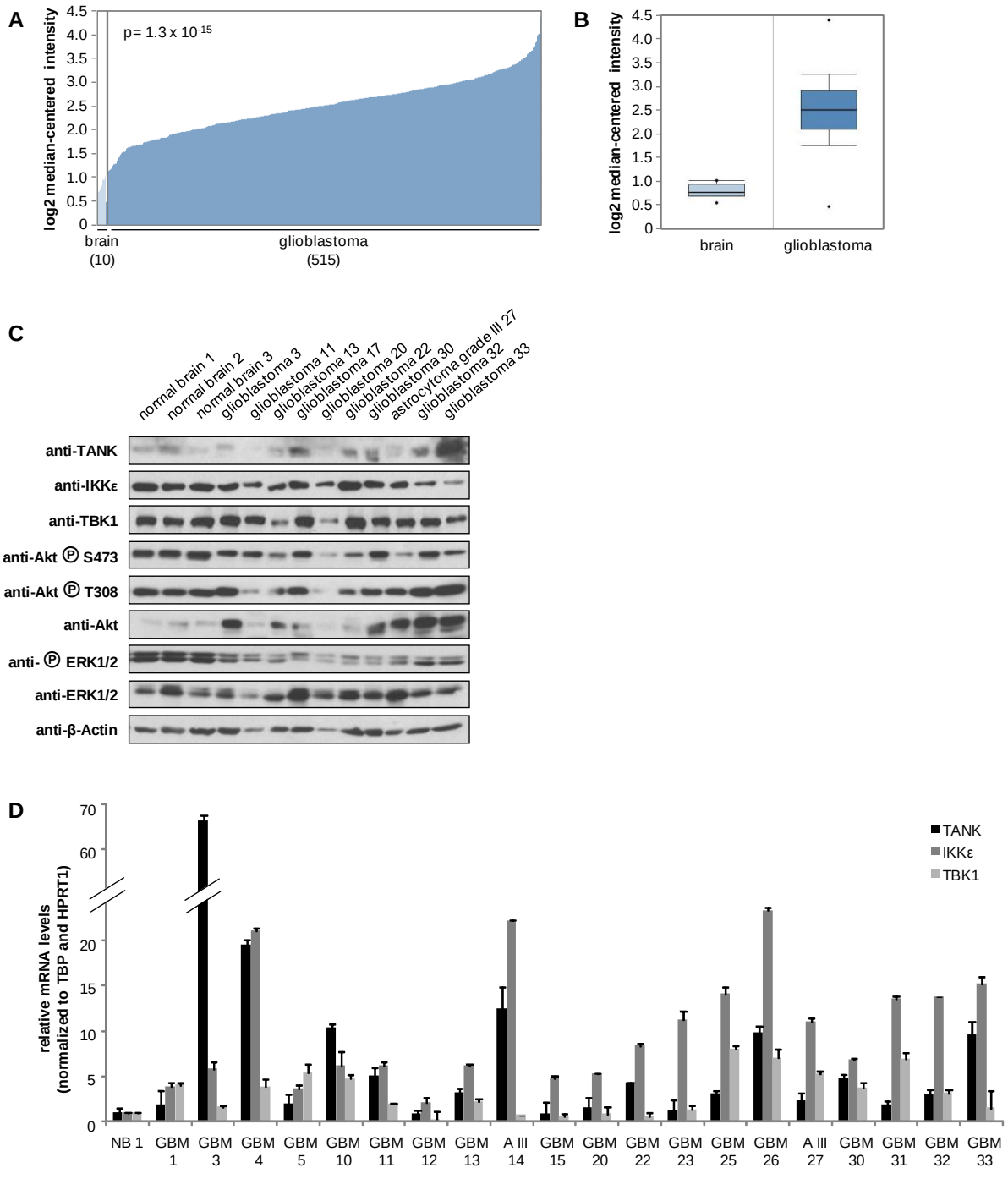
3.1. The expression of the non-canonical IKK complex in glioblastoma multiforme

3.1.1. Protein and mRNA levels of the non-canonical IKK complex in primary patient tissue samples

The kinases of the non-canonical IKK complex are already known to be involved in the oncogenesis of different types of cancer such as breast, lung and colon cancer [404]. A database research using the integrated cancer database Oncomine (http://www.oncomine.org) turned out an over-expression of the adaptor protein TANK (Fig. 3.1.A-B). Here, an *in silico* expression analysis was performed by comparing different microarray studies of various normal and glioma tissue samples. In order to confirm the expression of the adaptor protein TANK as well as the two IKK-related kinases TBK1 and IKKε in primary glioma tumors, proteins were extracted from patient tissue samples, that were kindly provided by Prof. Dr. Axel Pagenstecher, and analyzed by Western blot (Fig. 3.1.C). Elevated protein levels of TANK but also of TBK1 and IKKε indicate that some cancers show over-expression of components of the non-canonical IKK complex in GBM and astrocytoma grade III. Considerable fluctuation of the measured protein levels further indicate a great variance between different patients. In addition, the expression of Akt and ERK1/2 kinases was examined, since both play a crucial role in the development and progression of GBM (see 1.1.2). Akt kinase as well as ERK1/2 also show increased protein levels in some gliomas compared to normal brain as well as a great variance among the patients. Akt is constitutively phosphorylated at T308 and S473. Additionally, ERK1/2 was phosphorylated in the investigated primary brain tissues. The phosphorylation of both kinases is higher in normal brain tissue compared to glioblastomas/astrocytomas whereas the expression is elevated in glioblastomas/astrocytomas tissue. The complete set of analyzed patient samples are shown in supplementary figure S1, further illustrating variable expression and phosphorylation levels.

In parallel, RNA was isolated from the patient tissue samples and mRNA levels of TANK, TBK1 and IKKε were determined by real-time PCR (Fig. 3.1.D). Consistent with the findings for protein levels, also the mRNA levels were elevated in glioblastoma/astrocytoma patient samples in comparison with normal brain tissue. The

variance of mRNA levels was even more striking compared to protein levels. Besides TANK, also mRNA levels of IKKε varied a lot whereas mRNA levels of TBK1 show less variability among the analyzed patient samples. To visualize and compare this variance, mRNA levels were displayed as boxplots (Fig. 3.1.E) indicating increased mRNA levels of IKKε and TANK and to a lesser extent of TBK1 compared to normal brain. The variance between the values was most remarkable for TANK.



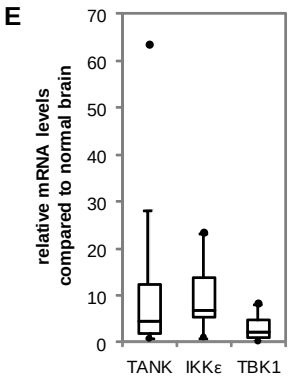


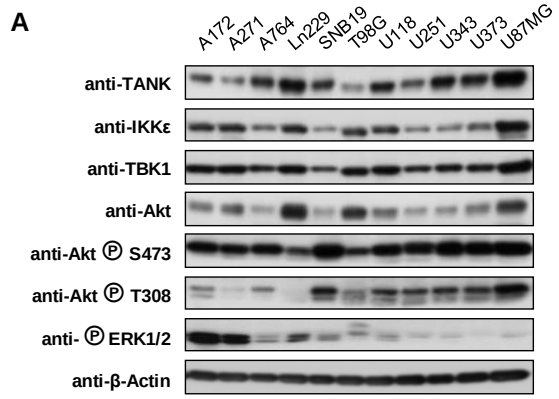
Fig. 3.1.: Expression analysis of members of the non-canonical IKK complex in primary patient tissues. (A) The expression of TANK was analyzed by an *in silico* expression analysis of normal brain (light blue, n=10) and GBM patient samples (dark blue, n=515) using the database Oncomine. Each bar represents an individual tissue sample (Reporter ID: 209451_at; Nucleotide Acc. No.: U59863). (B) The data of (A) were summarized in boxplots. The box illustrates the interquartile range (75^{th} to 25^{th} percentile). The upper part of the bar shows the 90^{th} percentile and lower bar the 10^{th} percentile. The dots stands for outliners. The graph was created using the Oncomine 3.0 software. (C) Patient tissue samples of normal brain (NB), glioblastoma multiforme (GBM) and astrocytoma grade III (A III) were (if possible) divided in two parts and either protein or RNA was extracted. Proteins were extracted from patient tissues by homogenizing of the material in NP40 lysis buffer and using a Turrax. Endogenous protein expression was then analyzed by Western blot using the indicated antibodies for endogenous proteins. More GBM and A III patient samples are shown in supplementary figure S 1. (D) Total RNA was extracted by TRIzol® and translated into cDNA using Oligo(dT) primers. The amount of mRNA was quantified by real-time PCR using specific primers for either TANK, TBK1 or IKKε. Expression levels of NB were set as 1. Housekeeping genes *Tbp* and *Hprt1* were used to normalize values. Error bars express the standard deviation of two experiments performed in triplicates. The variation of mRNA levels of TANK, TBK1 and IKKε were further visualized in boxplots (E), in which the box represents the interquartile range with median, error bars show the overall range of values and dots symbolize outliners.

Taken together, the analysis of patient tissues from GBM and astrocytoma grade III validate an over-expression of the non-canonical IKK complex in some cases, but apart from that, highly variable levels of the proteins were found. As during resection of cancer tissue usually transformed and non-tumorigenic cells are gathered together, expression levels might be biased. Therefore, also glioblastoma cell lines were analyzed for the expression of the non-canonical IKK complex.

3.1.2. Protein and mRNA levels of the non-canonical IKK complex in glioma cell lines

Next, glioblastoma cell lines were analyzed and protein as well as mRNA levels of TANK, TBK1 and IKKε were determined. Therefore, 11 glioma cell lines were lysed and analyzed by Western blot (Fig. 3.2.A). The levels of TANK protein were elevated in most of the glioma cell lines. The expression was especially high in the cell lines Ln229, U118, U343, U373 and U87MG. Both IKK-related kinases were as well expressed, but the observed high variability in TANK protein levels was not seen for the kinases. Moreover, protein levels of Akt and its phosphorylation status were analyzed. The kinase was expressed in

all examined cell lines, but Ln229 and T98G showed remarkable high protein levels of Akt. The residue S473 is highly phosphorylated among all cell lines whereas the phosphorylation at the main activation site T308 was found in 9 of 11 cell lines. In contrast, the phosphorylation of ERK1/2 was inversely correlated with Akt T308. Furthermore, the expression of the non-canonical IKK complex was examined on mRNA levels (Fig. 3.2.B). Thus, total RNA was isolated from all analyzed glioma cell lines and real-time PCR was performed to detect mRNA levels of TANK, TBK1 and IKKε. Reflecting the results observed for protein levels (Fig. 3.2.A), mRNA levels of TANK were exaggerated and highly variable. The mRNA levels of TBK1 and IKKε showed less variability (see also supplementary Fig. S2). The variance of mRNA levels is further visualized as boxplots in figure 3.2.B. Since TANK forms a complex with IKKε and TBK1 [67, 81, 405], it was interesting to investigate whether this complex also occurs in GBMs. To address this question, co-immunoprecipitation experiments were performed (Fig. 3.2.D). Two cell lines were chosen and endogenous proteins were precipitated with anti-TANK antibody. The following analysis of the eluates by Western blot revealed the co-precipitation of TBK1 and IKKε in both cell lines, confirming the formation of the non-canonical IKK complex with the adaptor protein TANK. As the IKK-related kinases can phosphorylate Akt [125, 137], it was also interesting to test whether Akt co-immunoprecipitates with IKKε and TBK1. However, co-immunoprecipitation experiments failed to detect such an interaction.



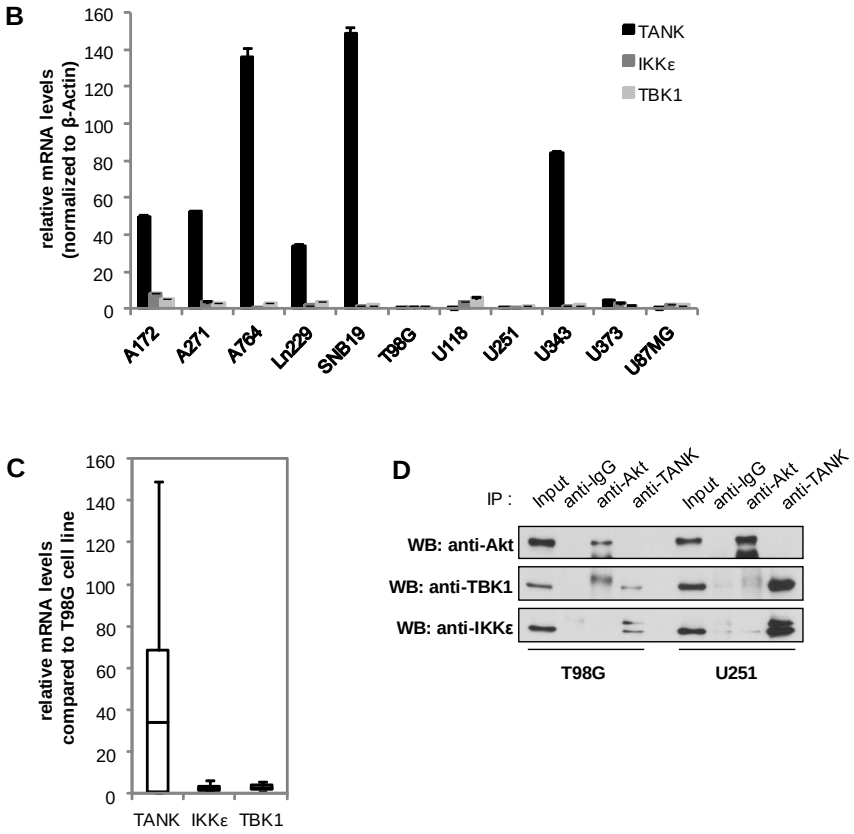


Fig. 3.2.: Expression analysis of members of the non-canonical IKK complex in glioma cell lines. (A) Proteins were extracted from 11 glioma cell lines in 1 x SDS sample buffer and analyzed by Western blot using the indicated antibodies to detect either endogenous proteins or their phosphorylation. (B) Total RNA was extracted and translated into cDNA using Oligo(dT) primers. The amount of mRNA was further semi-quantitatively analyzed by real-time PCR using specific primers for either TANK, TBK1 or IKKε. Expression levels of T98G were set as 1 after normalization to the housekeeping gene *β-Actin*. Error bars express the standard deviation of three independent experiments performed in triplicates. The variation of mRNA levels of TANK, TBK1 and IKKε were further visualized in boxplots (C), in which the box represents the interquartile range with median, error bars show the overall range of values and dots symbolize outliners. (D) T98G and U251 cells were lysed and endogenous proteins were precipitated using anti-Akt and anti-TANK antibodies as well as anti-IgG as negative control. Eluates were analyzed by Western blot using the indicated antibodies, input samples of the cell lysates are shown to confirm expression of the proteins.

In summary, the results for mRNA and protein levels are consistent with the findings for patient tissue samples, indicating TANK to be dysregulated in GBM.

3.1.3. The interplay of gene transcription and mRNA stability in the regulation of TANK expression

The measured mRNA levels of TANK do not exactly correlate with protein levels in each cell line. For instance, the cell line U87MG shows high protein levels and low mRNA levels for TANK. The A764 cell line contains high mRNA levels and moderate protein levels of TANK (Fig. 3.2. A and C). In order to explain this discrepancy of mRNA and protein levels of TANK in the glioma cell lines, mechanisms of mRNA and protein regulation were

examined. In general, the steady-state level of a certain mRNA is defined by mRNA synthesis and simultaneously occurring mRNA decay [406, 407]. Similarly, the steady-state levels of proteins is regulated by the relative rates of protein synthesis and decay [408].

To determine ongoing transcription of the *Tank* gene as possible explanation for considerable differences in mRNA levels of TANK between distinct glioma cell lines, a chromatin-immunoprecipitation was performed (Fig. 3.3.A). Since elongation of the transcript is associated with the occupancy of the gene by active RNA polymerase II, that is phosphorylated at S2, this occupation can be seen as indicator for active transcription [409]. Thus, a chromatin-immunoprecipitation using a specific antibody for RNA polymerase II phosphorylated at S2 (Pol II S2p) was performed using cell lines with high (A764) and low (U251, U87MG) mRNA levels of TANK. The amount of precipitated genomic DNA was measured by real-time PCR using specific primers that cover two distinct regions in intron 1 (TANK#1 and TANK#2). In line with the findings for mRNA levels, the *de novo* transcription of TANK was higher in A764 cells when compared to U251 and U87MG cells that showed reduced *de novo* transcription and steady-state mRNA levels. To measure the stability of TANK mRNA, the analyzed cell lines were treated with Actinomycin D to inhibit the *de novo* transcription. The amount of mRNAs was measured at various time points, as displayed in figure 3.3.B. The relative mRNA stability of TANK was different for every cell line. In U87MG cells associated with high protein but low mRNA levels of TANK (Fig. 3.2. A and C), a high mRNA stability was observed. In contrast, A764 cells with high mRNA levels (Fig. 3.2.C) revealed a high rate of mRNA decay resulting in lower protein levels (Fig. 3.2.A). Although the mRNA stability of TANK was higher in the U251 cell line than observed in A764 (Fig. 3.3.B), the protein level in U251 was lower in the end (Fig. 3.2.A). Interestingly, the mRNA stability of TBK1 was similar in all analyzed cell lines (Fig. 3.3.B, right panel). Additionally, a possible regulation of protein levels by protein turnover was analyzed. Through the inhibition of the protein synthesis with cycloheximide (CHX) the protein decay can be followed. Three glioma cell lines were treated for various periods with CHX and the protein abundance was examined by Western blot (Fig. 3.3.C). U251 and U87MG cells displayed a stable protein level of TANK and TBK1 within the observed time, indicating that TANK and TBK1 are not regulated at protein levels to an appreciable extent. Only TBK1 protein levels decrease after 12 hs treatment with CHX in U87MG cells. The T98G cell line with low TANK protein levels was chosen as a positive control for the CHX treatment. Here TANK and TBK1 were already degraded after 2 hs treatment with CHX.

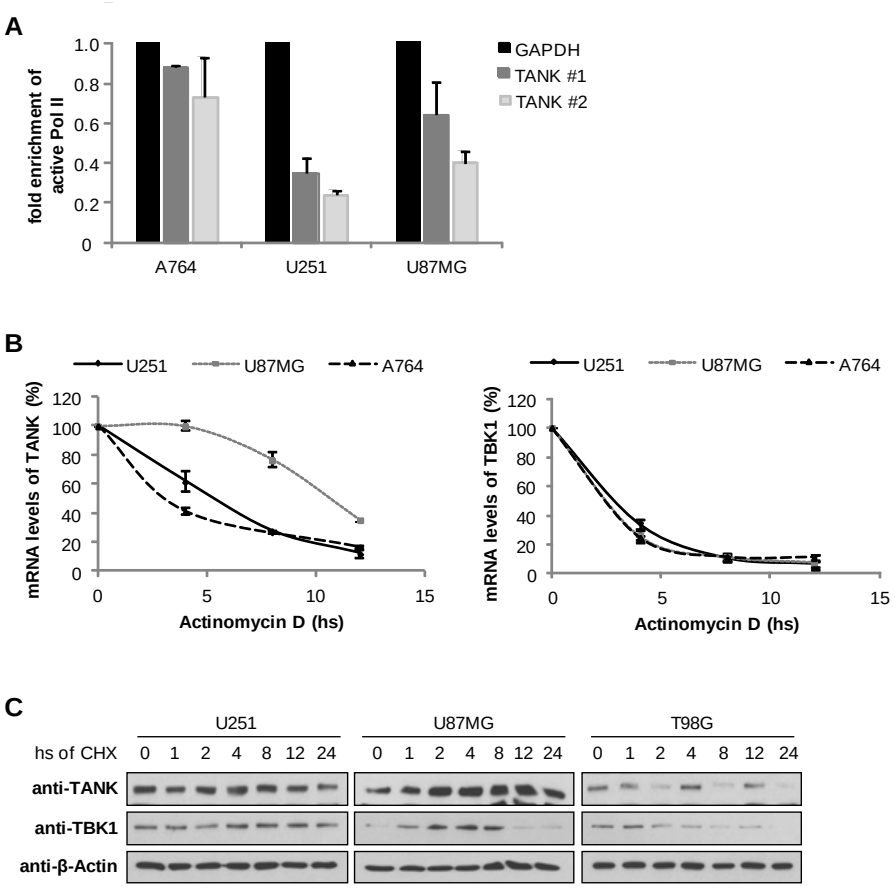


Fig. 3.3.: The interplay of gene transcription and mRNA stability in the regulation of TANK protein levels. (A) A764, U251 and U87MG cell were analyzed for *de novo* transcription of the *Tank* gene by ChIP. After crosslinking of proteins to the DNA, ChIP assays were performed using either a Pol II S2p antibody or an unspecific IgG control antibody. Binding of actively elongating polymerase to two distinct regions of the *Tank* gene as well as *Gapdh* as housekeeping gene was determined by real-time PCR using specific antibodies. The amount of Pol II S2p associated with the indicated genomic region is calculated as fold enrichment over IgG control and enrichment in *Gapdh* gene was set as 1 to compare different cell lines among each other. (B) A764, U251 and U87MG cells were treated with 1 µg/ml Actinomycin D for the indicated times. Total RNA was extracted and translated into cDNA using Oligo(dT) primers. The mRNA stability of either TANK (left panel) or TBK1 (right panel) mRNA was examined by real-time PCR using specific primers. Values were calculated by the ΔΔCt method and normalized to *ß-Actin*. Untreated cells were set as 100%. Mean values ± SD of three independent experiments performed in triplicates are shown. (C) The protein stability of TANK and TBK1 was assessed in U251, U87MG and T98G cells by treating the cells with 50 µg/ml Cycloheximide (CHX) for the indicated times. Cells were lysed in 1 x SDS sample buffer and lysates were analyzed by Western blot using the indicated antibodies. Anti-β-Actin was used to confirm equal protein loading.

In summary, these findings show that the variable levels of the TANK protein in glioma result from differences at all analyzed gene expression levels (*de novo* transcription, mRNA stability and protein stability).

3.2. The influence of TANK and TBK1 on cellular functions of glioma cell lines

3.2.1. The role of the non-canonical IKK complex in proliferation

Cancer cells are usually characterized by high proliferation rates. Several factors dysregulating the healthy balance between proliferation and apoptosis are already known [410]. Proliferation assays were performed, in order to validate the role of the non-canonical IKK complex in this context, and revealed a great diversity among the glioma cell lines (Fig. 3.4).

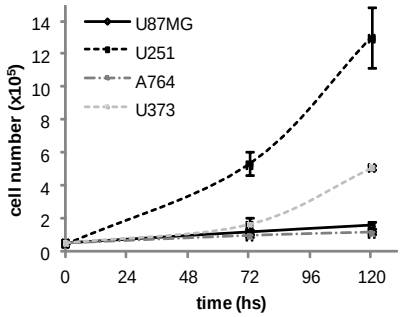
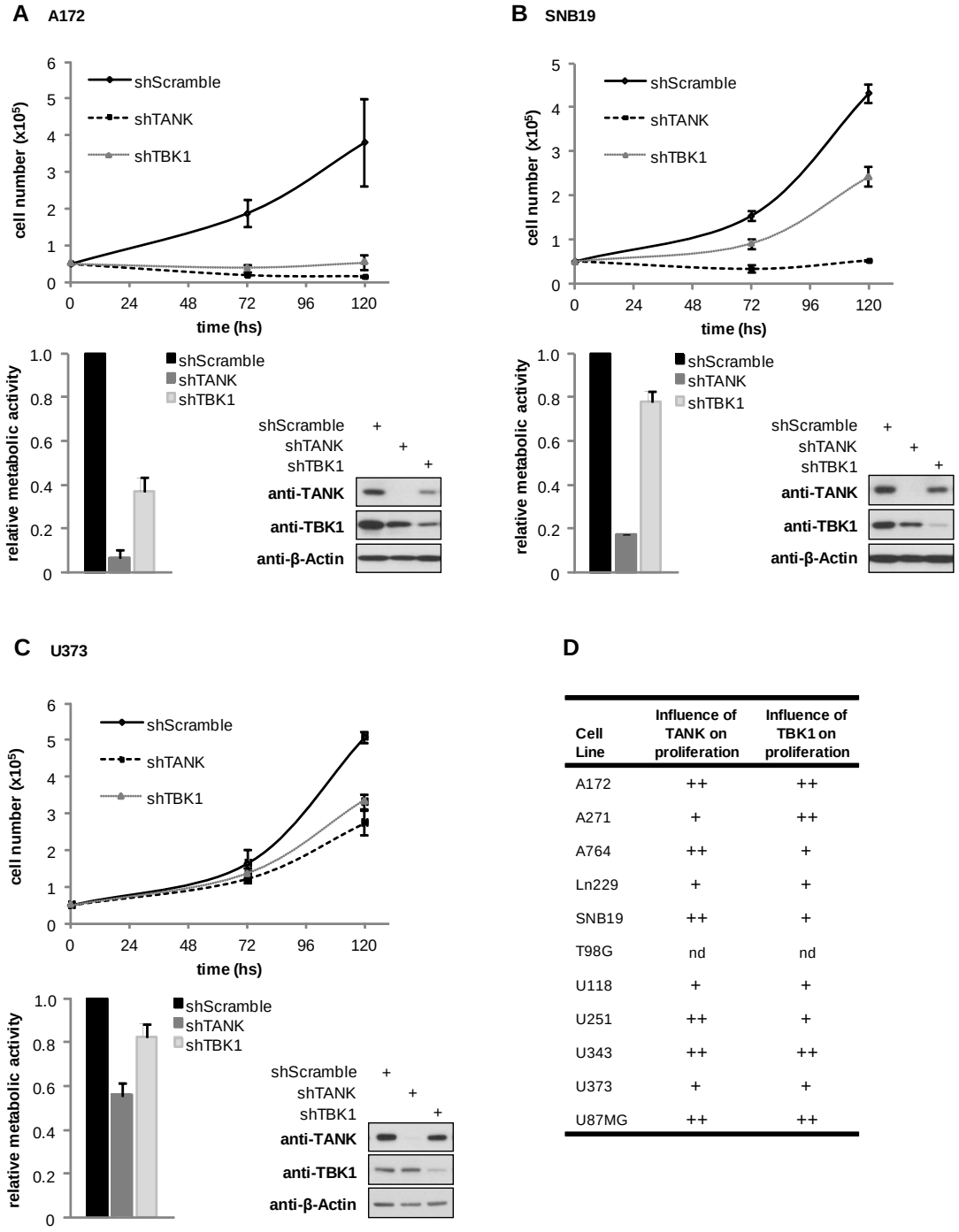


Fig. 3.4.: The proliferation of different glioma cell lines. Diverse glioma cells were seeded at a density of $5x10^4$ cells. Number of cells was determined with a FACSCalibur 72 and 120 hs after seeding. Mean values ± SD are shown of three independent experiments.

Subsequently, components of the complex were silenced and the effects on the cell proliferation were determined. Retroviral knock-down was performed for the adaptor TANK as well as the kinase TBK1. Clark and colleagues demonstrated that the absence of the adaptor destroys the complex and impairs the function of TBK1 whereas the function of IKKε is completely abrogated [411]. TANK or TBK1 were silenced in 11 glioma cell lines using retroviral gene transfer of shRNA to induce RNA interference. Proliferation assays were performed as described above. Additionally, MTT assays were carried out for all cell lines. By measuring the metabolic activity of cells, the MTT assay provides information about the cell viability and proliferation. The effects of either TANK or TBK1 silencing on glioma cell lines are summarized in figure 3.5.D. The results shown in figure 3.5.A-C exemplify the proliferation and MTT assays for three cell lines. The full data set is depicted in supplementary figure S3. The proliferation ability of A172 cells was affected remarkably by silencing either TANK or TBK1. The same was true for the metabolic activity measured by MTT assay (Fig. 3.5.A). The cell lines U343 and U87MG showed the same effects (Fig S3). TBK1 had a much lesser impact on proliferation and metabolism in the SNB19 cell line when compared to TANK (Fig. 3.5.B). This was also seen in the cell lines A764 and U251. Interestingly, just the A271 cell line showed the opposite - a major decrease in proliferation when TBK1 was silenced compared to the TANK knock-down. However, the U373 cell line represents a group of 3 cell lines where

the effect of silencing TANK or TBK1 on proliferation were low (Fig. 3.5.C). Only in the T98G cell line the influence of TANK or TBK1 on proliferation was not detectable probably due to an insufficient knock-down.



Cell Line	Influence of TANK on proliferation	Influence of TBK1 on proliferation
A172	++	++
A271	+	++
A764	++	+
Ln229	+	+
SNB19	++	+
T98G	nd	nd
U118	+	+
U251	++	+
U343	++	++
U373	+	+
U87MG	++	++

Fig. 3.5.: The role of TANK and TBK1 in the proliferation ability of glioma cell lines. (A) A172 cells were retrovirally transduced to express shRNAs leading to a knock-down of TANK, TBK1 or a scrambled shRNA as a control. Transduced cells were selected with puromycin and used to determine cell proliferation (upper graphs) or alternatively cell viability (lower left graph). Proliferation was measured by seeding cells at a density of $5x10^4$ cells, followed by determination of cell numbers after 72 and 120 hs using a FACSCalibur. Error bars show standard deviations from three independent experiments. Aliquots of the cells were lysed and tested by Western blotting for efficient knock-down (lower right). MTT assays were performed by seeding

knock-down cells at a density of 1x10^3 cells/well, followed by further growth for 4 days and performance of the MTT assay according to the manufacturer's instructions. Relative metabolic activity of control cells was arbitrarily set as 1, error bars show standard deviations of three independent experiments. (B) Experiments of A were performed for SNB19 cells. (C) Experiments of A were performed for U373 cells. (D) Results from proliferation assays from all cell lines are summarized. Symbols indicate strength of influence on proliferation: - no, + low, ++ great influence and nd not detectable.

Taken together, 10 out of 11 examined cell lines display an impact of TANK or TBK1 knock-down on proliferation. Thus, these experiments revealed an important role of the non-canonical IKK complex in proliferation of glioma cell lines.

Unspecific side effects of shRNA constructs, also called off-target effects, are a well described problem of RNA interference [412] that is usually excluded by confirming the effects with a second shRNA construct containing a distinct binding sequence. Within the scope of this work, off-target effects were excluded using alternative shRNAs for either TANK or TBK1 (Fig. 3.6). For that purpose, U251 cells were infected with the viruses carrying the distinct shRNAs to silence either TANK (Fig. 3.6.A) or TBK1 (Fig. 3.6.B) and proliferation assays were performed to determine the functional outcome. Decreased proliferation rates were observed for TANK or TBK1 knock-down to a comparable extent for each construct, indicating that the observed effect is not due to artificial side effects.

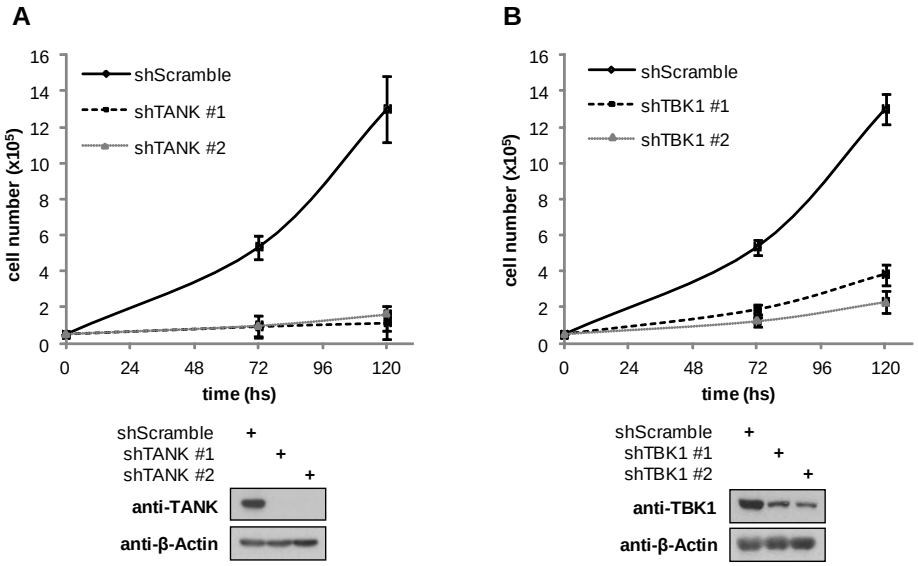


Fig. 3.6.: shRNAs for TANK and TBK1 do not induce unspecific side effects. U251 cells were infected with either retro- (#1) or lentiviruses (#2) to express shRNA targeting either TANK (A) or TBK1 (B) as well as scrambled shRNA as control. After selection with puromycin, cells were seeded at a density of 1x10^4. The amount of cells was determined with FACSCalibur 72 and 120 hs after seeding to further follow the proliferation. Mean values ± SD are shown of three independent experiments. An aliquot of cells was lysed in 1 x SDS sample buffer and analyzed by Western blot for efficient knock-down.

3.2.2. The role of the non-canonical IKK complex in cell cycle progression

A role of the non-canonical IKK complex in the regulation of cell cycle progression has been described before [69]. The decreased proliferation ability after silencing TANK or TBK1 raises the question whether those effects are caused by an impact of either TANK or TBK1 on the cell cycle.

For that purpose, cell cycle profiles were recorded for three selected glioma cell lines by propidium iodide staining and subsequent analysis by flow cytometry (Fig. 3.7). The distribution of cells within the cell cycle phases was illustrated in stacked-bar graphs (middle panels). The cell cycle profiles for A764 cell line (Fig. 3.7.A) did not reveal any differences between TANK-depleted cells and the control cells. Also U251 cells (Fig. 3.7.C) showed just slight changes. Here, the silencing of TANK led to an increasing number of cells in G_1 and S phase. In contrast, U373 cells (Fig. 3.7.B) revealed an accumulation of cells in S phase after the knock-down of TANK. For most of the investigated cell lines no changes in cell cycle profile after TBK1 knock-down were observed, apart from A764 where the knock-down of TBK1 led to slightly increased cell number in G_1 phase.

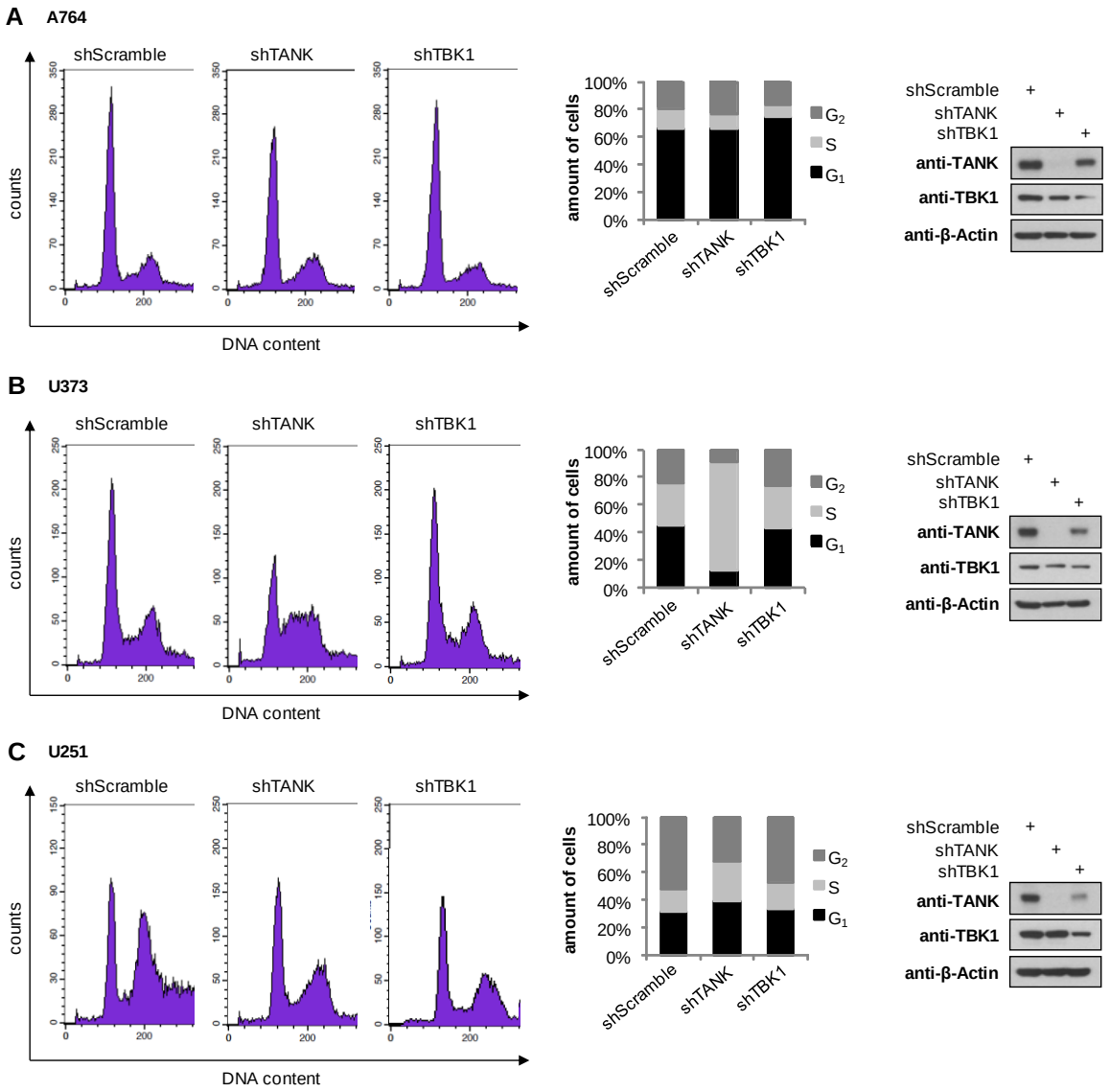


Fig. 3.7.: Cell cycle analysis of glioma cell lines after TANK or TBK1 knock-down. (A) TANK or TBK1 were knocked down as described previously in A764 cell line. After selection with puromycin, DNA content of the cells was measured by staining with propidium iodide and subsequent analysis by flow cytometry (left panel) using FACSCalibur. Amount of cells in certain phases of the cell cycle were quantified by ModFit software and depicted as stacked-bar graph (middle panel). Aliquots of cells were lysed and analyzed by Western blots for efficient knock-down (right panel). Those experiments were also done for U373 (B) as well as U251 (C) cell lines.

In summary, TANK is able to influence the cell cycle by inhibiting the progression from S to G_2 phase, but not all glioma cell lines show this altered cell cycle profile. In order to investigate the impact of the TANK or TBK1 knock-down on cell cycle progression, the kinetic parameters of cell cycle were examined. Therefore, knock-down cells were treated with vinblastine to block progression of the cell cycle in late G_2 phase and cell cycle profiles were analyzed by propidium iodide staining as described above after 10 or 24 hs (Fig. 3.8). Confirming the previous results, the knock-down of TANK caused an arrest in S phase in the U373 cell line, but did not alter the cell cycle profile in the A764 cell line. The

cell cycle profiles of both cell lines were also not altered upon silencing of TBK1. As the arrest in late G_2 phase was more pronounced in the U373 cell line compared with the A764 cell line, it is conclusive that the U373 cell lines progresses faster in the cell cycle (Fig. 3.4). This indirectly indicates that the U373 cell lines proliferates faster. The treatment of TANK-depleted cells with vinblastine did not result in further changes of the cell cycle profile in both investigated cell lines which indicates a stop of cell cycle progression. This also suggests a repressive effect on the cell proliferation by the knock-down of TANK. Additionally, TBK1 silencing also resulted in inhibited cell cycle progression after vinblastine treatment in the A764 cell line, but failed to lead to an arrest in late G_2 phase in the U373 cell line. So, the cell cycle progression and eventually the proliferation of the U373 cell line does not depend on TBK1. Finally, vinblastine has been described to induce apoptosis [413, 414], explaining sub G_1 peaks in both cell lines after treatment.

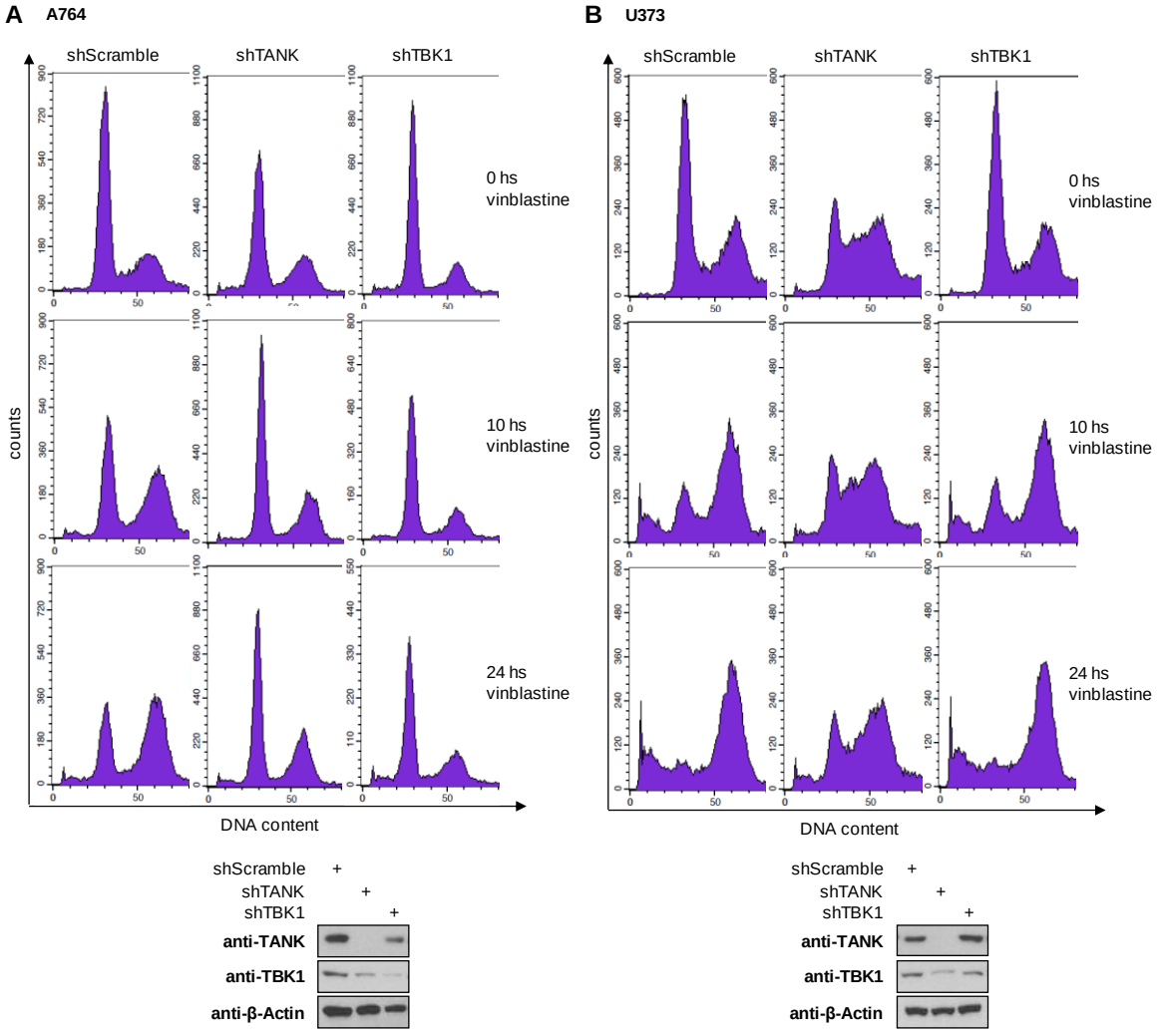


Fig. 3.8.: The influence of TANK and TBK1 on the cell cycle progression of glioma cell lines. (A) TANK or TBK1 were knocked down as described previously for the A764 cell line. After selection with puromycin, cells were treated with 0.3 µM vinblastine to arrest cells in M/ late G_2 phase of the cell cycle. Shifts in cell cycle

profiles to the G_2 phase were followed by measuring the DNA content of the cells by staining with propidium iodide and subsequent analysis by flow cytometry (upper panel) using FACSCalibur after 10 or 24 hs treatment with vinblastine. Aliquots of cells were lysed and analyzed by Western blot for efficient knock-down (lower panel). Those experiments were also done for U373 cell line (B).

Taken together, the ability of glioma cell lines to progress in cell cycle is affected by the depletion of TANK or TBK1. Interestingly, TANK knock-down can also lead to an S phase arrest is some glioma cell lines.

3.2.3. The role of the non-canonical IKK complex in migration

Formation of metastases and invasion into tissues are further important hallmarks of cancer [410]. Invasion is well described for GBM in particular. In order to evaluate the role of the non-canonical IKK complex in the migration of glioma cell lines, either TANK or TBK1 were silenced by shRNA as described before and wound-healing assays were performed (Fig. 3.9). For all investigated cell lines, control knock-down cells were found to close the scratch almost completely within 24 hs. However, the knock-down of TANK had a great impact on the migration ability of the analyzed cell lines. Except for U251, also TBK1 knock-down prevented the closure of the scratch. In summary, TANK but also TBK1 influence the migration ability of glioma cells.

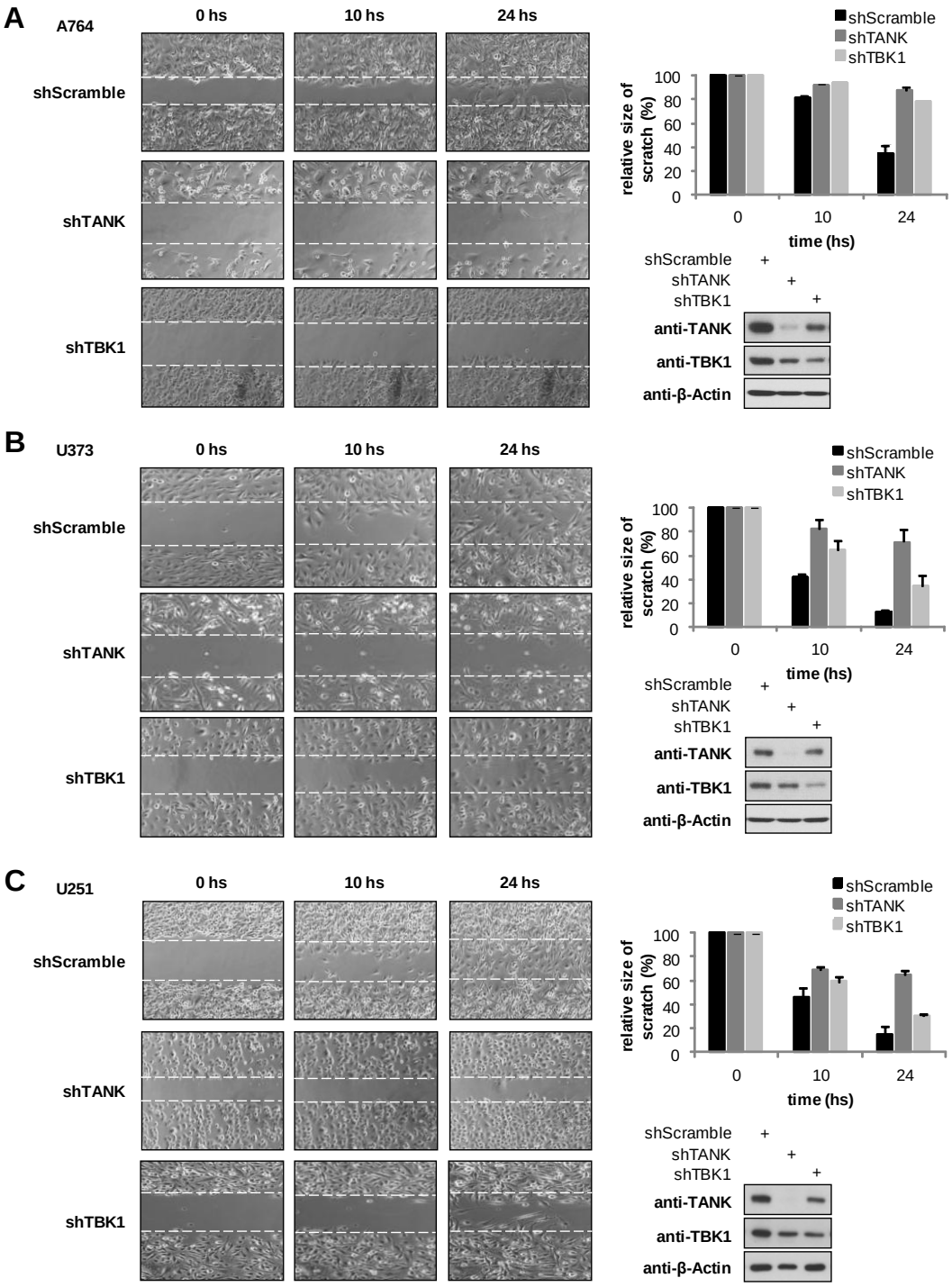


Fig. 3.9.: The influence of TANK and TBK1 on the migration ability of glioma cell lines. (A) Knock-down and control A764 cells were seeded at densities between 1,5-2x10^5 in a 6 well plate and allowed to grow to 80% confluency. To prevent proliferation cells were cultured in serum-free medium supplemented with 1 µg/ml aphidicolin over night before the assay was carried out (left panel). A scratch was gently made with a pipette tip. The cells were then washed two times with PBS and serum-free medium was added. Immediately, after 10 and after 24 hs a picture of the scratched area was taken with a NIKON Inverted Research Microscope Eclipse TE2000-Ea. The size of the scratch was quantified (upper right panel). Mean values ± SD of three independent experiments are shown. Aliquots of cells were lysed and analyzed by Western blot to ensure efficient knock-down (lower right panel). Experiment was also performed with U373 (B) and U251 (C) cell lines.

3.3. The influence of the non-canonical IKK complex on cell death induced by chemotherapeutic drugs

In the treatment of GBM a lot of chemotherapeutic drugs have been developed [415]. In this work, two commonly applied drugs were taken into consideration. Activated AraC is able to inhibit DNA replication by its incorporation into nascent DNA instead of CTP (cytidine triphosphate) [416]. This results in cell cycle arrest, reduced growth as well as apoptosis [417]. More often, TMZ is used in the treatment of GBM. TMZ is an alkylating agent which is able to damage the DNA, thereby leading to cell death [418].

Since TANK and TBK1 were influencing several hallmarks in cancer, it was interesting to investigate the role of the non-canonical IKK complex on drug sensitivity. For that purpose, the consequences of TANK or TBK1 knock-down on the sensitivity of tumor cells towards either AraC or TMZ was studied. First of all, the cytostatic function of both chemotherapeutic agents was validated by MTT assays (Fig. 3.10). Therefore, 11 glioma cell lines were plated out in 96-well plates and treated with either AraC or TMZ for three days, before the metabolic activity was measured. Compared to untreated control cells, all glioma cell lines were affected in their cell viability by both drugs to a similar extent. In line with the mechanisms behind the drugs, slowly growing cell lines such as A764 still reveal a higher cell viability compared to fast growing cell lines as for instance U251 (see also Fig. 3.4). This result illustrates the cytostatic function of both drugs for all examined cell lines.

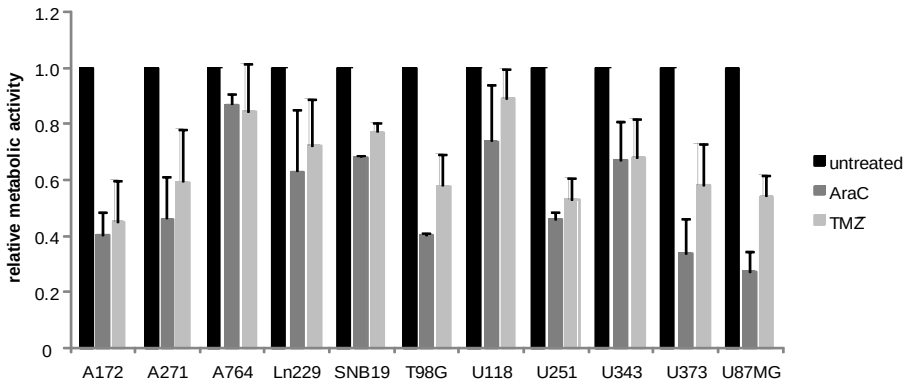


Fig. 3.10.: Analysis of the cell death induced by the chemotherapeutic drugs AraC and TMZ. All investigated cell lines were seeded at a density of $1x10^3$ cells, treated next day with 1 µM AraC or 100 µM TMZ. 72 h later, MTT assays were carried out according to the manufacturer's instructions. Mean values ± SD are shown of three independent experiments

Next, a potential contribution of TANK or TBK1 to the death-inducing effects of AraC and TMZ was investigated. Accordingly, either TANK or TBK1 were silenced by shRNA in A764, U251 or U373 cell lines and cells were treated with AraC or TMZ as described

above. Cell viability was again measured by MTT assays (Fig. 3.11). As shown afore, the treatment with either AraC or TMZ decreased cell viability for all investigated cell lines. In the same way, each knock-down was sufficient to diminish cell viability. While TANK knock-down further decreased the cell viability moderately in combination with AraC or TMZ in all cell lines, the effects of TBK1 silencing were not as pronounced. These experiments show that only the knock-down of TANK slightly increased drug induced cell death in the examined cell lines.

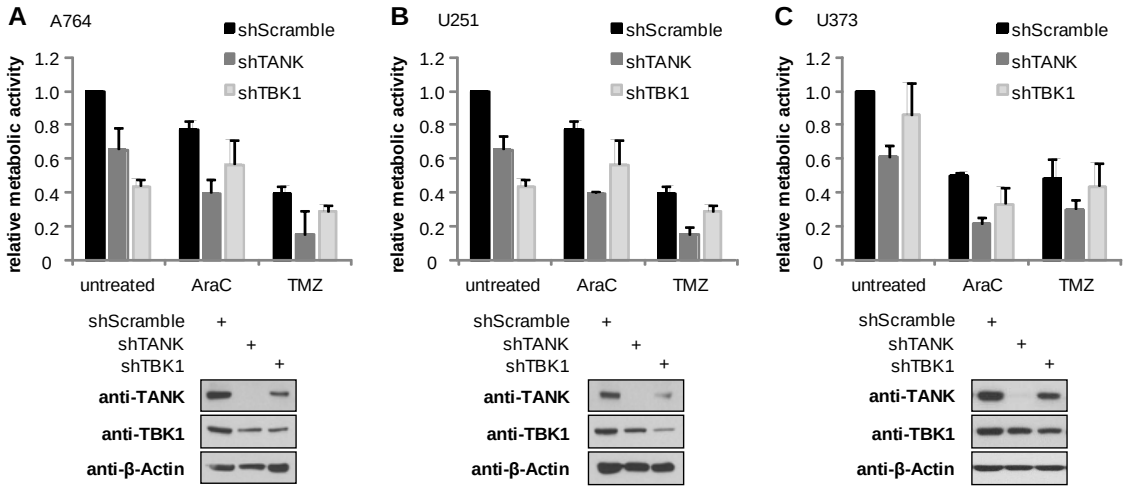


Fig. 3.11.: The influence of TANK and TBK1 on drug-induced cell death in glioma cell lines. (A) A764 were retrovirally infected with shRNA to knock-down either TANK or TBK1. Cells were seeded at a density of $1x10^3$ cells and treated the next day with 1 µM AraC or 100 µM TMZ. 72 hs later MTT assays were performed, mean values ± SD from three independent experiments are shown. Western blot of lysed cell aliquots proved sufficient knock-down of TANK or TBK1 (lower panels). Experiments were also performed for U251 (B) and U373 (C) cell lines.

3.4. The non-canonical IKK complex in Akt and ERK1/2 signaling pathways

Cell proliferation is a process influenced by many factors. On the one hand, intrinsic signaling leads to regulation of cell cycle and thereby controls the proliferation. On the other hand, transcription factors can induce expression and release of soluble factors such as growth factors and cytokines that influence the proliferation of surrounding cells. [419, 420]

This raises the question whether the effects of TANK or TBK1 depletion are caused by intrinsic signaling or by soluble factors secreted in the culture medium. To address this question, the effect of medium from knock-down cells on cell proliferation was measured in proliferation assays (Fig. 3.12). The previously described repressive effect of either the TANK or TBK1 knock-down on cell proliferation of different glioma cell lines was

confirmed. In contrast, medium taken from TANK- or TBK1-silenced A764 cells did not result in decreased proliferation of control A764 cells, but even led to slightly increased proliferation. The same was true for U373 cell line, suggesting that the medium from knock-down cells does not contain soluble factors negatively affecting cell proliferation. Moreover, the slightly increased proliferation in response to the medium from knock-down cells suggests the presence of pro-proliferative factors.

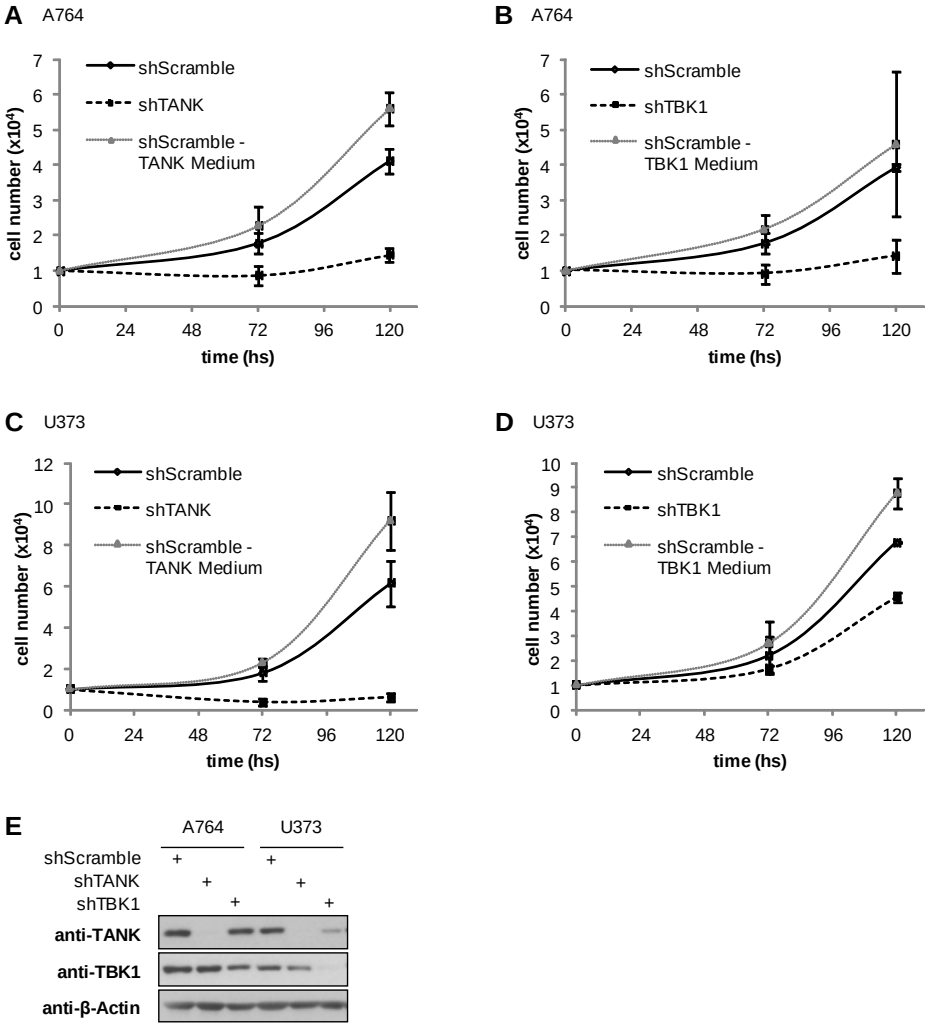
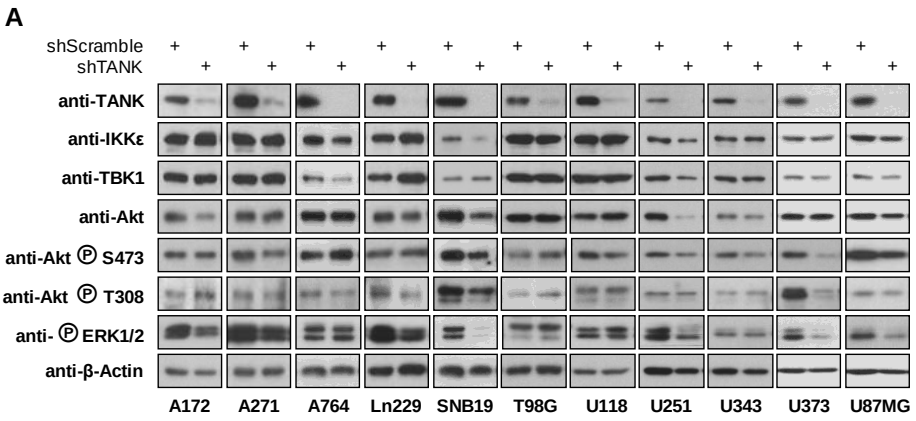


Fig. 3.12.: The influence of knock-down-induced spilled factors in the medium on the proliferation of control cells. (A) A764 cells were retrovirally infected with shRNA against TANK. After puromycin selection, proliferation assays were performed. Thereby, knock-down control cells were seeded in either fresh culture medium or in medium taken from cells with a TANK knock-down. Proliferation was measured as described previously. Mean values ± standard deviation of three independent experiments are shown. (B) Experiment was repeated as in (A) for A764 where TBK1 was silenced. Assay was also performed for U373 cell line with either TANK (C) or TBK1 (D) knock-down. (E) A part of the cells was lysed and further analyzed by Western blotting for efficient knock-down.

3.4.1. The effects of TANK on constitutive AKT and ERK1/2 signaling

Akt as well as ERK1/2 are known kinases involved in the regulation of proliferation [419, 420]. Since both of them are constitutively phosphorylated in glioma cell lines (Fig. 3.2.A), the influence of the non-canonical IKK complex on their activity was further analyzed. Therefore, the adaptor protein TANK was silenced in the glioma cell lines by retroviral infection with shTANK. Protein expression and phosphorylation of Akt and ERK1/2 were subsequently analyzed by Western blot (Fig. 3.13). The phosphorylation of Akt was altered at the two main activation sites S473 and T308 in a few cell lines. While the phosphorylation of S473 was reduced in 5 cell lines, T308 phosphorylation was alleviated in only 2 of 11 (Ln229 and U373) examined cell lines. The phosphorylation of ERK1/2 was reduced in 6 glioma cell lines. In three cell lines (SNB19, U251 and U373) the phosphorylation of Akt at S473 was correlated with the phosphorylation of ERK1/2. T308 phosphorylation of Akt correlated with ERK1/2 phosphorylation in those 2 cell lines (Ln229 and U373) that were shown to have reduced levels of T308 phosphorylation after silencing TANK.



B

Akt Ⓟ S473

Ⓟ ERK 1/2	↑	↓	±	total
↑	0	0	0	0
↓	0	3	3	6
±	0	2	3	5
total	0	5	6	11

Akt Ⓟ T308

Ⓟ ERK 1/2	↑	↓	±	total
↑	0	0	0	0
↓	0	2	4	6
±	0	0	5	5
total	0	2	9	11

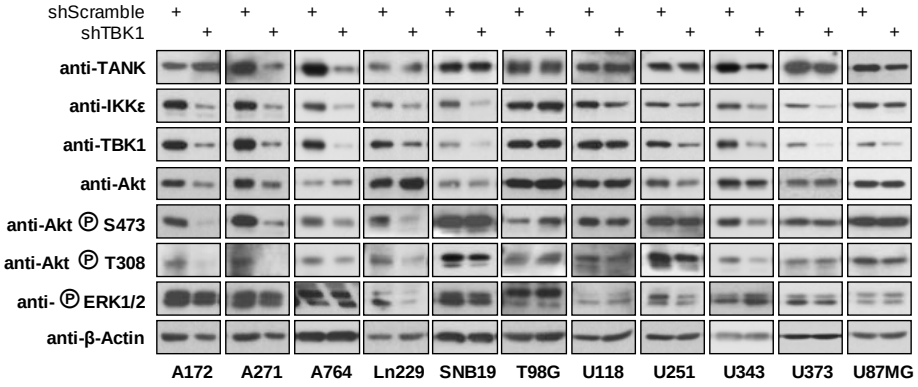
Fig. 3.13.: Analysis of the role of TANK on Akt and ERK1/2 phosphorylation in glioma cell lines. (A) TANK was silenced by shRNA as described before in 11 glioma cell lines. Cells were lysed in 1 x SDS sample buffer and protein expression as well as phosphorylation of Akt and ERK1/2 was analyzed by Western blot. (B) Results were summarized in tables showing the indicated correlations between Akt and ERK1/2 phosphorylation.

In summary, the repressive effect of TANK silencing on the phosphorylation of Akt and ERK1/2 kinases was found for about half of the examined cell lines.

3.4.2. The effects of TBK1 on constitutive AKT and ERK1/2 signaling

To measure the effect of the IKK-related kinase TBK1 on constitutive Akt and ERK1/2 phosphorylation, TBK1 was knocked down in the glioma cell lines by shRNAs. The analysis of protein extracts for expression and phosphorylation of proteins showed that silencing of TBK1 resulted not just in reduced protein levels of TBK1 but also decreased IKKε protein levels. (Fig. 3.14). Silencing of TBK1 led to reduced phosphorylation of Akt and ERK1/2 in about half of the investigated cell lines but did not increase the phosphorylation in any case. In A171, A271 and Ln299 cell lines S473 phosphorylation of Akt was correlated with ERK1/2 phosphorylation. The same three cell lines and the U251 cells additionally revealed a correlation between T308 phosphorylation of Akt and ERK1/2 phosphorylation.

A



B

Akt Ⓟ S473

Ⓟ ERK 1/2	↑	↓	±	total
↑	0	0	0	0
↓	0	3	2	5
±	1	2	3	6
total	1	5	5	11

Akt Ⓟ T308

Ⓟ ERK 1/2	↑	↓	±	total
↑	0	0	0	0
↓	0	4	0	4
±	0	3	4	7
total	0	7	4	11

Fig. 3.14.: Analysis of the role of TBK1 on Akt and ERK1/2 phosphorylation in glioma cell lines. (A) TBK1 was silenced by shRNA as described before in 11 glioma cell lines. Cells were lysed in 1 x SDS sample buffer and protein expression as well as phosphorylation of Akt and ERK1/2 was analyzed by Western blot. (B) Results were summarized in tables by correlation Akt and ERK1/2 phosphorylation.

Taken together, TBK1 positively regulates the kinases Akt and ERK1/2 in most of the glioma cell lines by inducing their phosphorylation and consequently their activation.

In order to verify the results obtained upon TBK1 silencing, a pharmacological approach was used to inhibit the IKK-related kinases IKKε and TBK1 by treating the cells with the small molecule inhibitor BX795. Subsequent analysis by Western blot is shown in figure 3.15.A and summarized in tables in 3.15.B. The analysis of the phosphorylation status in BX795 treated glioma cell lines revealed similar effects as for TBK1 depletion. Reduced phosphorylation of Akt at S473 was observed in 7 of 11 cell lines and T308 phosphorylation was alleviated in 8 of 11 cell lines. Also decreased phosphorylation of ERK1/2 was found in the majority of investigated cell lines. For five cell lines, the reduced phosphorylation of Akt at T308 and S473, respectively, was correlated with decreased ERK1/2 phosphorylation. In contrast, for two cell lines an increase in ERK1/2 phosphorylation was detected (A271 and U343) in response to TBK1 silencing.

While earlier results showed that the knock-down of TBK1 does not significantly change the sensitivity of cells towards chemotherapeutic drugs (Fig. 3.11), it was then interesting to investigate whether the activity of the IKK-related kinases has an impact on the response to chemotherapeutic drugs. Therefore, TBK1 as well as IKKε were inhibited using BX795. These cells were additionally treated with AraC or TMZ and cell viability was measured by MTT assays (Fig. 3.15.C-D). The treatment with BX795 alone resulted in decreased cell viability, also indirectly indicating a negative effect on the cell proliferation. The combination of BX795 and AraC treatments did not result in a further decrease of the cell viability compared to cells only treated with AraC. Similar findings were shown for the TMZ treatment. However, combined treatment of TMZ and BX795 in the cell lines T98G, U118 and U251 led to slightly further decreases of the cell viability compared to each compound alone. Interestingly, the knock-down of TBK1 in U251 cells had no additional effects on the drug-induced cell death, whereas the inhibition of both IKK-related kinases by BX795 resulted in a further decrease of the cell viability at least in the combination with TMZ.

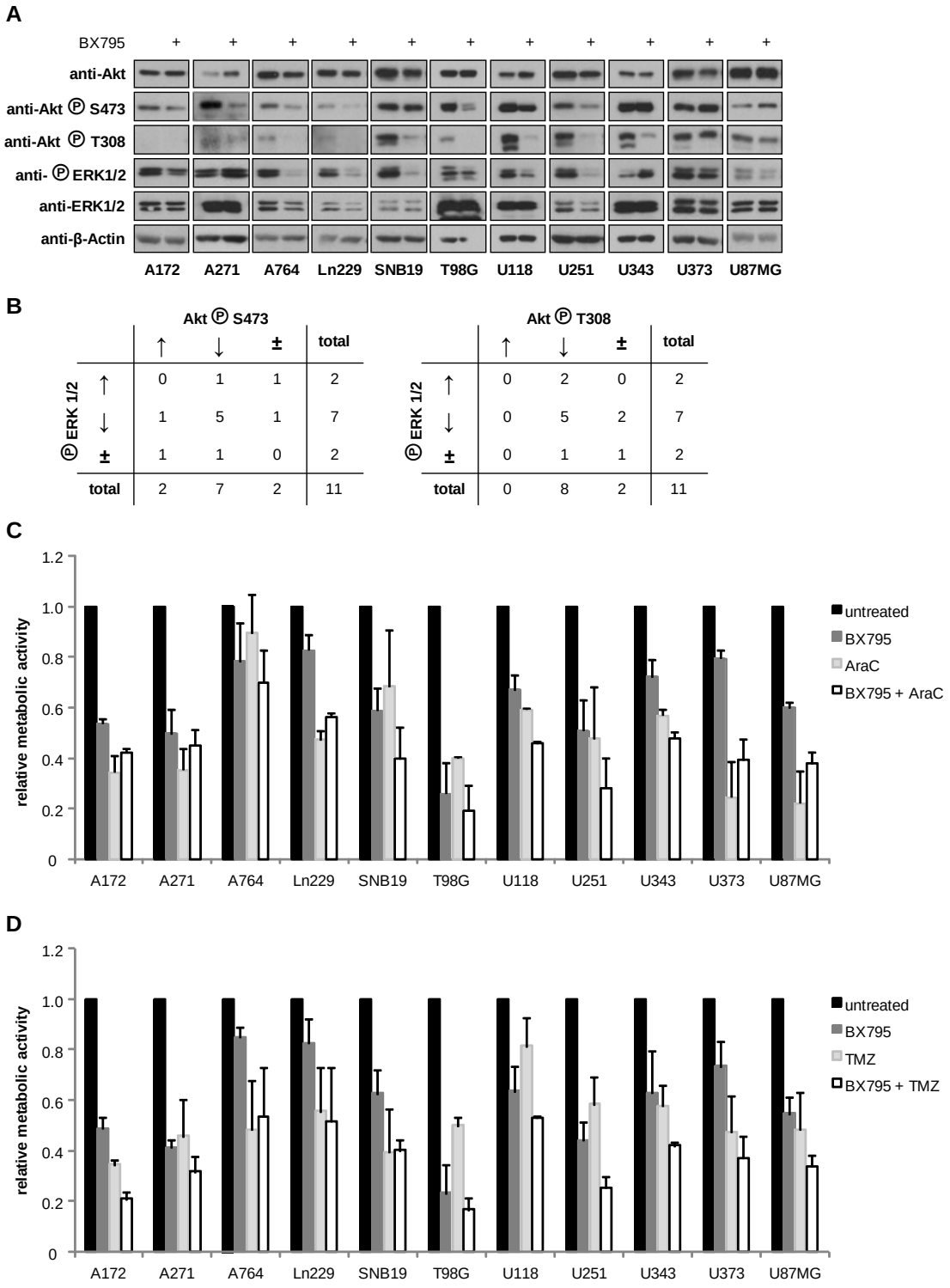


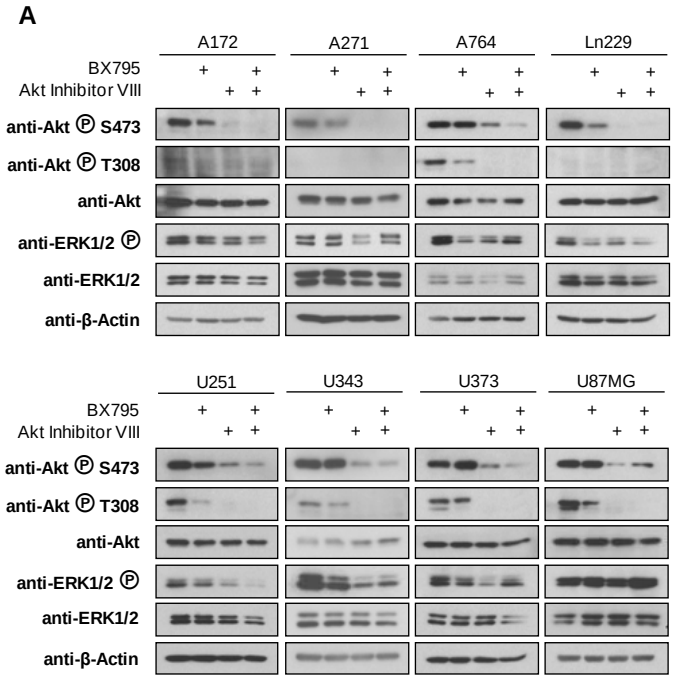
Fig. 3.15.: Analysis of the role of the IKK-related kinases on Akt and ERK1/2 phosphorylation and drug-induced cell death in glioma cell lines. (A) Glioma cell lines were treated 24 hs with 1 µM BX795, lysed in 1 x SDS sample buffer and analyzed by Western blot using indicated antibodies to detect phosphorylation of Akt and ERK1/2. (B) Results from (A) were summarized in tables showing the indicated correlations between Akt and ERK1/2 phosphorylation. (C) MTT Assays were performed to determine cell viability. Cells were seeded at a density of $1x10^3$ cells/well and treated the next day with either 1 µM BX795 and/or 1 µM AraC. 72 hs later cell viability was determined by MTT assays, mean values ± SD are shown from three independent experiments (D) The experiment was done as in (C) with the only difference that the glioma cells were treated with either 1 µM BX795 and/ or 100 µM TMZ.

In summary, TANK and TBK1 influence the phosphorylation of downstream kinases such as Akt and ERK1/2. The inhibition of the IKK-related kinases showed no or only weak effects on chemotherapeutic-induced cell death in glioma cell lines.

3.4.3. The interplay of signaling pathways in glioma cell lines

How can TANK and TBK1/IKKε regulate signaling to Akt and ERK1/2? To address this question, the signaling network was examined by combinatorial treatments with different kinase inhibitors. On the one hand, Akt inhibitor VIII was used to inhibit the phosphorylation of Akt at the two main phosphorylation sites that are crucial for proper activation of the kinase [421]. ERK1/2 phosphorylation was inhibited by U0126 which interferes with the activity of the direct upstream regulator MEK1/2 [422]. The TAK1 kinase which is involved in signaling pathways such as NF-kB and MAPK was specifically blocked with 5Z-7-oxozeaenol [423]. All inhibitors were applied either alone or together with BX795 to additionally block the non-canonical IKK complex.

Eight glioma cell lines were treated with either BX795 and Akt inhibitor VIII alone or in combination. Phosphorylation of Akt and ERK1/2 were determined by subsequent analysis by Western blot and are shown in figure 3.16.A. The consequences of the treatments are summarized in tables (Fig. 3.16.B). As expected, the treatment with Akt inhibitor VIII led to blocked phosphorylation of Akt at both crucial phosphorylation sites. In 5 of 8 cell lines also ERK1/2 phosphorylation was decreased (A271, A764, U251, U343 and U373). An additional treatment with BX795 had contradictory effects on the ERK1/2 phosphorylation. In some cell lines ERK1/2 phosphorylation was restored (U373), in others the reduced phosphorylation did not change further (A172, A764).



B

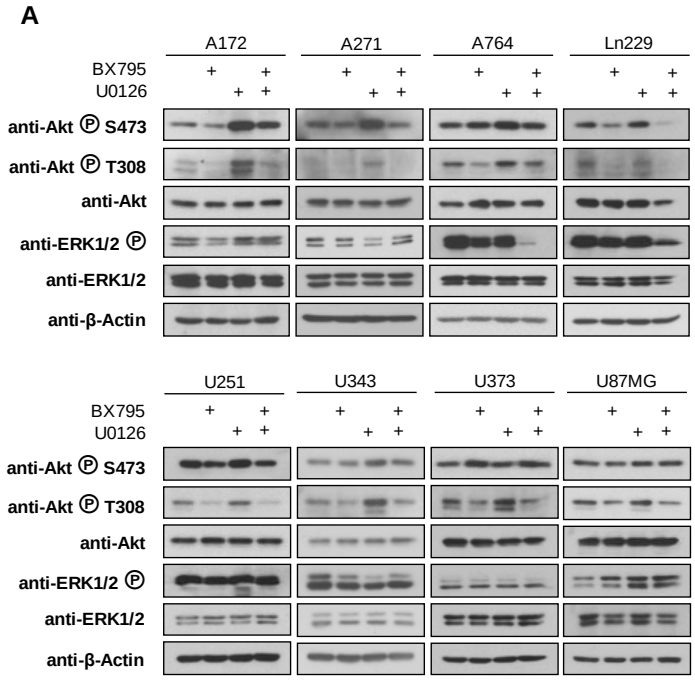
Ⓟ ERK 1/2 \ Akt Ⓟ S473	↑	↓	±	total
↑	0	0	0	0
↓	0	5	0	5
±	0	3	0	3
total	0	8	0	8

Ⓟ ERK 1/2 \ Akt Ⓟ T308	↑	↓	±	total
↑	0	0	0	0
↓	0	5	0	5
±	0	3	0	3
total	0	8	0	8

Fig. 3.16.: The effect of the Akt inhibitor VIII on the constitutive active signaling network in glioma cell lines. (A) Cells were treated for 24 hs with Akt inhibitor VIII (5 µM), BX795 (1 µM) or with a combination of both, lysed in 1 x SDS sample buffer and analyzed by Western blot using indicated antibodies to detect phosphorylation of Akt and ERK1/2. (B) Results from (A) were summarized in tables showing the correlations between Akt and ERK1/2 phosphorylation.

Next, the consequences of the MEK inhibitor U0126 on the phosphorylation of Akt and ERK1/2 were examined. The experiment settings were as described previously and results are shown in figure 3.17.A. Furthermore, the phosphorylation of Akt was correlated with the ERK1/2 phosphorylation (Fig. 3.17.B). Most of the analyzed glioma cell lines did not respond to the inhibitor U0126 with reduced phosphorylation of ERK1/2. Only the cell lines A271 and U343 exhibited alleviated levels of ERK1/2 phosphorylation upon the treatment with U0126. Interestingly, most of the glioma cell lines (5 of 8) showed increased phosphorylation of Akt at S473 and 6 of 8 cell lines revealed higher phosphorylation levels at residue T308 when MEK1/2 were inhibited. The combined treatment with U0126 and BX795 did not induce further changes in the phosphorylation of ERK1/2, except for two cell lines. In A764 and Ln229 each treatment alone was not

efficient to decrease ERK1/2 phosphorylation considerably, whereas the combination of both resulted in extensive reduction thereof, indicating two independent signaling axis that are able to compensate each other.



B

Ⓟ ERK 1/2 \ Akt Ⓟ S473	↑	↓	±	total
↑	0	0	0	0
↓	2	0	0	2
±	3	0	3	6
total	5	0	3	8

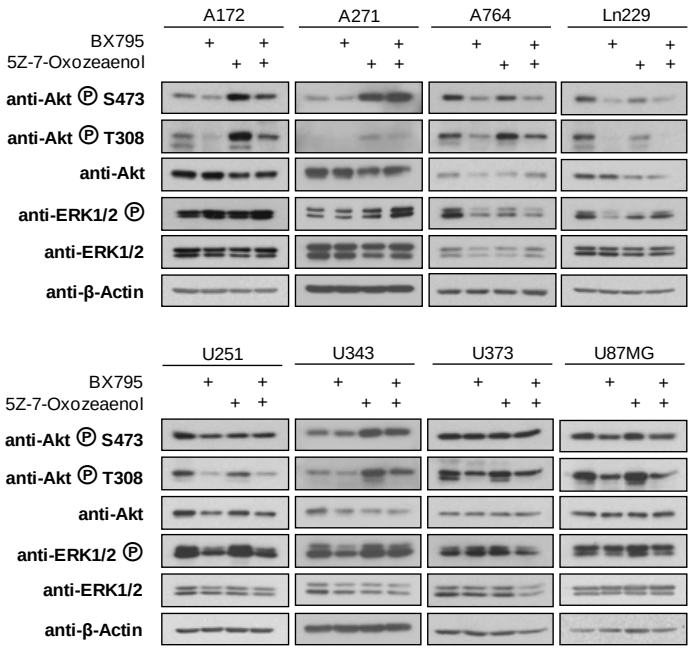
Ⓟ ERK 1/2 \ Akt Ⓟ T308	↑	↓	±	total
↑	0	0	0	0
↓	2	0	0	2
±	4	0	2	6
total	6	0	2	8

Fig. 3.17.: The effect of the MEK1/2 inhibitor on the constitutive active signaling network in glioma cell lines. (A) Glioma cell lines were treated 24 hs with 5 µM U0126, 1 µM BX795 or in combination of both, lysed in 1 x SDS sample buffer and analyze by Western blot using indicated antibodies to detect phosphorylation of Akt and ERK1/2. (B) Results from (A) were summarized in tables showing the correlations between Akt and ERK1/2 phosphorylation.

Furthermore, the effects of TAK1 inhibition on the signaling network was analyzed as described above. Eight cell lines were treated either alone with 5Z-7-oxozeaenol or BX795, or with the combination of both. Cell lysates were analyzed by Western blot for phosphorylation of Akt and ERK1/2 (Fig. 3.18.A) and summarized in figure 3.18.B. The TAK1 inhibitor was sufficient to increase Akt phosphorylation at T308 and S473 in about half of the investigated cell lines. In contrast, the phosphorylation of ERK1/2 was not altered in most of the cell lines. Only A764 and Ln229 exhibited reduced ERK1/2

phosphorylation. A correlation between Akt and ERK1/2 phosphorylation after the treatment with 5Z-7-oxozeaenol was not detected. The combined treatment of the glioma cells with 5Z-7-oxozeaenol and BX795 also had no further impact in most of the cell lines. However, in A271 cells an increased ERK1/2 phosphorylation after combined treatment with BX795 and 5Z-7-oxozeaenol was detected, whereas treatment with both inhibitors decreased ERK1/2 phosphorylation in U373 cell line.

A



B

Akt Ⓟ S473

Ⓟ ERK 1/2	↑	↓	±	total
↑	0	0	0	0
↓	0	1	1	2
±	3	0	3	6
total	3	1	4	8

Akt Ⓟ T308

Ⓟ ERK 1/2	↑	↓	±	total
↑	0	0	0	0
↓	1	1	0	2
±	3	0	3	6
total	4	1	3	8

Fig. 3.18.: The effect of the TAK1 on the constitutive active signaling network in glioma cell lines.. (A) Cell lines were treated 24 hs with 1 µM 5Z-7-oxozeaenol, 1 µM BX795 or a combination of both, lysed in 1 x SDS sample buffer and analyzed by Western blot using indicated antibodies to detect phosphorylation of Akt and ERK1/2. (B) Results from (A) were summarized in tables showing correlations between Akt and ERK1/2 phosphorylation.

Taken together, the treatment experiments revealed new insights in the complex signaling network in GBM. On the one hand, the repression of the IKK-related kinases showed a negative effect on the phosphorylation of the kinases Akt and ERK1/2 in most of the cell lines which suggests a signaling path leading through the non-canonical IKK complex to activate proliferation and survival signals in glioblastomas. On the other hand, the

MEK1/2-induced repression of the Akt phosphorylation indicates a possible negative feedback loop on the Akt-induced phosphorylation of ERK1/2. Finally, the loss of ERK1/2 phosphorylation after inhibiting the TBK1/IKKε together with MEK1/2 lead to the idea of a MEK-independent ERK1/2 activation at least in some glioma cell lines.

3.5. The effect of TANK and TBK1 on the pro-inflammatory microenvironment in glioma cell lines

The link between inflammation and cancer is well documented [424]. GBM has been described to be associated with a pro-inflammatory microenvironment [425, 426]. Since the non-canonical IKK complex is involved in inflammatory signaling by modulating NF-κB and IRF transcription factors, the impact of TANK and TBK1 on these processes was studied. Therefore, in two cell lines (U373 and U251) either TANK or TBK1 were silenced as described before. Western blot analyses were performed to reveal the phosphorylation of NF-κB and IRF transcription factors, and mRNA levels of certain pro-inflammatory cytokines were measured by semi-quantitative real-time PCR (Fig. 3.19). The depletion of TANK resulted in reduced phosphorylation of IRF3 in U373 cells. In contrast, the phosphorylation of IRF3 was in general much lesser in this cell line compared to U87MG, where IRF3 phosphorylation was slightly increased upon TANK silencing. The constitutive phosphorylation of p65 at S536 was higher in the U87MG cell line compared to U373. Silencing of TBK1 markedly impaired p65 phosphorylation in U87MG cells. Additionally, real time PCR analysis of mRNA levels of pro-inflammatory cytokines was carried out (Fig. 3.19.B) in both cell lines. On the one hand, the U373 cell line was found to express increasing amounts of *TNFα* and *Vcam1* mRNA after silencing TANK. On the other hand, the knock-down of TBK1 led to a slightly increased expression of *IL-6* mRNA in U373 cells. In contrast, the knock-down of either TANK or TBK1 in U87MG cells generally had a more repressive effect on the mRNA levels of TNFα, Vcam1 and IL-6. Just the knock-down of TANK did not lead to changes in the TNFα mRNA levels in U87MG cells. In this cell line, the strongest effect was found for the IL-6 expression in response to a TBK1 knock-down.

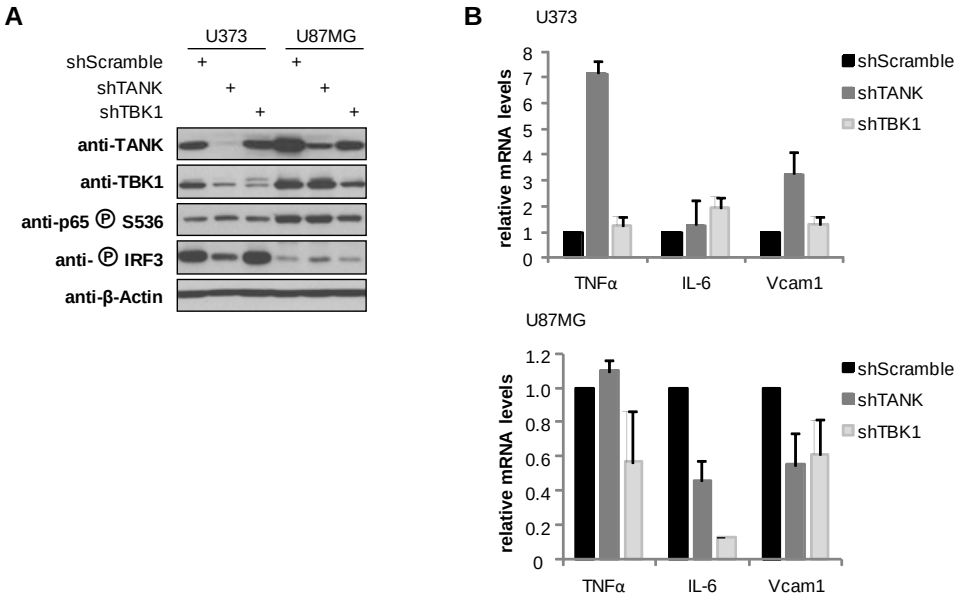


Fig. 3.19.: Analysis of the effect of TANK and TBK1 on the pro-inflammatory microenvironment in glioma cell lines. (A) U373 and U87MG cells were used to silence TANK or TBK1 as described above. One fraction of the knock-down cells was lysed in 1 x SDS sample buffer and subsequently analyzed by Western blot using indicated antibodies. (B) In parallel, another fraction of cells was used to measure mRNA levels of pro-inflammatory cytokines. Total RNA was isolated and transcribed into cDNA using Oligo(dT) primers. Real time PCR was carried out using specific primers for TNF, IL-6 and Vcam. Mean values ± SD of three independent experiments are shown.

3.6. The influence of TANK protein levels on signal output

Adaptor proteins such as TANK usually do not facilitate intrinsic enzyme activity but serve as bridges for proteins to ensure proper signaling. Nevertheless, a well balanced expression of such adaptors is necessary to form functional protein complexes. [427] In order to address the importance of stoichiometric amounts of TANK, U373 cells were infected with viruses either leading to the shRNA-mediated downregulation of TANK or allowing the over-expression of the adapter protein. Both silencing and over-expression of TANK resulted in reduced phosphorylation of IRF3, a downstream target of the non-canonical IKK complex (Fig. 3.20.A), whereas the phosphorylation of p65 was not altered as shown previously (Fig. 3.19.A). Additionally, the functional consequences of TANK expression were examined in U251 cells. TANK was either silenced or over-expressed and cell proliferation was analyzed (Fig. 3.20.B). Both over-expression and silencing revealed the same effects and led to decreased proliferation in the U251 cell line. Taken together, glioma cell lines depend on a stoichiometric expression of the adaptor protein TANK to mediate signal output from the non-canonical IKK complex. Too low as well as too high TANK levels presumably interfere with the correct formation of the complex and interrupt appropriate signaling.

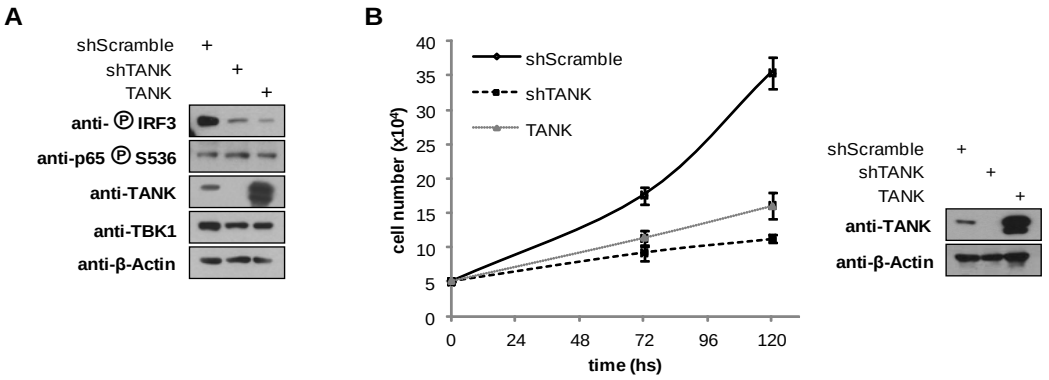


Fig. 3.20.: Analysis of the functional consequences of the dysregulation of TANK in glioma cell lines(A) U373 cell lines were infected in order to over-express or silence TANK. Cells were lysed in 1 x SDS sample buffer and protein expression as well as phosphorylation of IRF3 and p65 were determined by Western blot. (B) U251 cells were also infected to induce TANK over-expression or silencing and were seeded at a density of $5x10^4$. Number of cells was determined with FACSCalibur 72 and 120 hs after seeding. Mean values ± SD of three independent experiments are shown. The right part shows a control Western blot ensuring reduced or increased protein expression.

3.7. The role of the non-canonical IKK complex in different cancer types

The non-canonical IKK complex is expressed in several tissues [72, 74]. As the data thus far indicate a role of TANK and TBK1 for the proliferation of GBM cells, it was interesting whether they may also regulate proliferation of other cancer types.

In order to address this question, several cell lines derived from different types of cancer were taken into consideration. The HeLa cell line is a prominent cancer cell line used in research and derived from cervix carcinoma. MCF7 is a breast cancer cell line, HCT116 a colon cancer cell line and A549 cells are derived from a lung adenocarcinoma.

Either TANK or TBK1 were silenced in those four cell lines as described previously to investigate their impact on signaling and cell function (Fig. 3.21). Western blot analysis revealed great differences in TANK expression among different cancer types, as it also occurred within several glioma cell lines (Fig. 3.2.A). Whereas HCT116 expressed high levels of TANK, A549 cells contained only little amounts of this adapter protein. Akt and ERK1/2 were expressed and constitutively phosphorylated in all of these cancer cell lines, although to a different extent. In contrast to glioma cell lines, their phosphorylation was not changed after silencing of TANK or TBK1, raising the possibility that TANK and TBK1 do not affect ERK1/2 and Akt signaling in all cancer types. Proliferation assays were performed with MCF7, HCT116 and A549 cell lines to analyze possible functional consequences of silencing TANK or TBK1 (Fig. 3.21.B-D). MCF7 breast cancer cells

showed a decreased proliferation upon reduced TANK or TBK1 levels, further supporting the idea, that the non-canonical IKK complex is involved in the regulation of proliferation in breast cancer cells [128]. In contrast, proliferation of colon colorectal cancer and lung adenocarcinoma cell lines was only weakly decreased upon silencing of either TANK or TBK1.

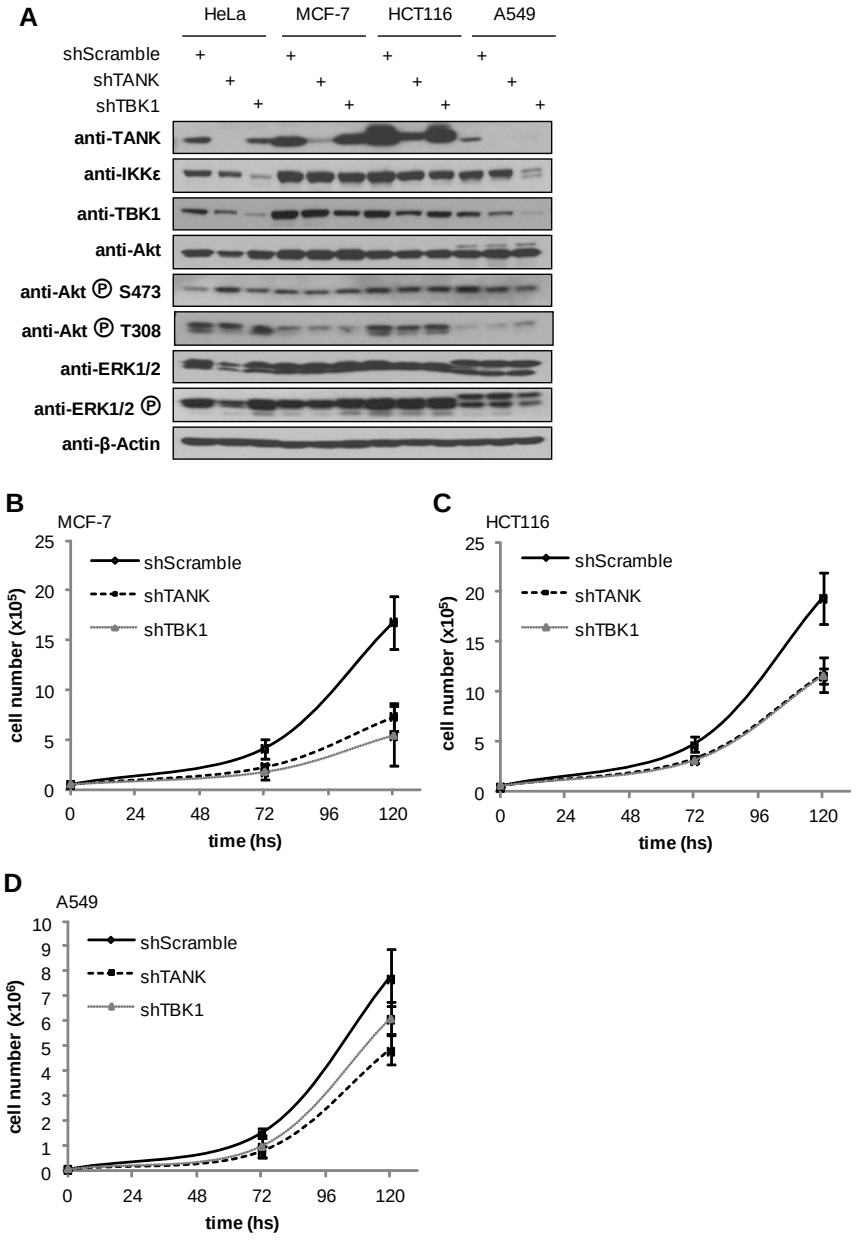


Fig. 3.21.: Analysis of the role of the non-canonical IKK complex in other cancer types. (A) Either TANK or TBK1 were silenced in HeLa, MCF7, HCT116 and A549 cells by infecting with shRNA as described before. After puromycin selection cells were lysed in 1 x SDS sample buffer and subsequently analyzed for protein expression and phosphorylation by Western blot using the indicated antibodies. (B) Proliferation assays with control cells and cells silenced for TANK or TBK1 were performed with MCF7 cells by seeding selected cells at a density of 5 x 10^4 cells. The number of cells was determined with FACSCalibur 72 and 120 hs after seeding. Mean values ± SD are shown. Experiments were also carried out for HCT116 (C) and A549 (D) cell line.

4. DISCUSSION

4.1. The expression of TANK is dysregulated in glioblastoma multiforme

Glioblastoma multiforme is one of the most lethal brain tumors [13]. Here, many signaling pathways such as Akt and the Ras-Raf-MEK-ERK cascade have been discovered to be dysregulated leading to tumorigenesis [28]. To identify other molecular factors involved in gliomagenesis this study focused on the adaptor protein TANK, usually involved in TBK1 and IKKε mediated IRF transcription factor activation after bacterial or viral infection [67], and its role in glioblastomas.

The analysis of patient tissue samples as well as several glioma cell lines revealed a highly varying expression of the adaptor protein TANK (figures 3.1-2). This great variation is a feature that can also be observed for other proteins expressed in GBM. Mineo and colleagues investigated the expression of HER2 (epidermal growth factor receptor) in 57 glioma tissues and found a fluctuation of HER2 expression in different GBM population samples which is caused by inter-individual variations and depends on the tumor types [428]. The clinical picture of GBM is chameleonic and exhibits a variable appearance in morphology and histology as Bailey and Cushing reported when they characterized GBM for the first time [1]. Of note, protein as well as mRNA levels were different between primary patient tissue and glioma cell lines (figure 3.1-2). GBM tissue is characterized by necrotic areas [27] and hyperplastic blood vessels [429]. In fact, glioblastoma is one of the most vascularized tumors [430]. Thus, the presence of distinct cell subpopulations could contort the expression levels of TANK, TBK1 and IKKε measured in patient tissue samples. Furthermore, post-surgical treatment and freezing of patient tissues could technically influence the amount of detectable protein and especially RNA levels and lead to contortion of the findings between patient tissue samples and glioma cell lines. Isolated cells from cancer tissues are immortalized and able to proliferate in culture but still reflect their genetic and molecular characteristics [431].

Additionally, the findings of this study indicate an over-expression of TANK in many GBM cases (figure 3.1). A constitutive expression of TANK has been documented by Conti *et al.* in glioma patient tissues [432]. In general, there are several mechanisms which could lead to this over-expression. Gene amplification, epigenetic changes, increased

transcription, higher mRNA stability as well as greater stability of the proteins are possible alterations which could cause exaggerated TANK protein levels.

Several numerical and structural changes of chromosomes appear in GBM. Besides gains and losses of chromosomes, also breakpoints in chromosomes have been discovered [433-435]. Rogatto and Casartelli identified a region in chromosome 2 where 6 out of 12 human primary gliomas exhibit a specific recurrent chromosome break – 2q24-2q32 [436]. Interestingly, the *Tank* gene is located in this region. The break in chromosomes can lead to translocation of the genetic information behind a more active promoter or can even result in a duplication of the genetic information, both possible explanations for increased levels of TANK. However, Crespo *et al.* characterized the genome of 46 patients suffering from GBM and did not find gene rearrangements in the TANK-encoding region [437].

The transcriptional rate of the *Tank* gene has been assessed using ChIP technique revealing distinct intensities for transcription of TANK in different glioma cell lines (figure 3.3). The transcription of the *Tank* gene has shown to be induced by the transcription factor SOX11 (SRY-related HMG-box) [438]. However, the role of this transcription factor in tumorigenesis remains unclear, since on the one hand, it has been found to be over-expressed in glioma tissue [439], but on the other hand, it was shown to induce differentiation [440, 441] thereby preventing tumorigenesis [442]. In order to investigate ongoing transcription at the *Tank* gene by an alternative approach, a nuclear-run-on-assay could be performed including a control cell line such as non-transformed human fibroblasts to conclude on changes of *Tank* gene transcription. Furthermore, it would be of interest to focus on the transcription factor SOX11, and if it is dysregulated in glioma cell lines.

The mRNA stability of TANK was considerably higher in glioma cell lines with lower transcriptional activity (figure 3.3), indicating that TANK protein levels are also controlled by post-transcriptional processes. Hao and Baltimore found the expression of pro-inflammatory molecules to be regulated by transcriptional control as well as mRNA stability [443]. Generally, the mRNA half-life is determined by *cis* and *trans* elements regulating either the decay of the mRNA or its stabilization. Important c*is*-acting elements that influence mRNA stability can be found in the 5'UTR and 3'UTR as well as in the coding sequence of mRNAs. Examples for *cis* elements are the poly(A)-tail, miRNA binding sites or AU-rich elements (ARE). AREs are found in the 3'UTR of many mRNAs with short half-lives such as transcription factors and cytokines, and were shown to target those mRNAs for rapid deadenylation and decay [444-446]. Additionally, decapping of the mRNA induces its subsequent degradation by exoribonucleases [447]. The influence of

cis-regulatory elements on the stability of mRNA in GBM has been addressed by Tsukamoto *et al.* who investigated the stability of GLUT1 mRNA. Here, the destabilization of the mRNA by instability determinants in the 3'UTR lead to under-expression of the glucose transporter [448]. The mRNA of human TANK contains 7 AUUUA pentamers in the 3'UTR (http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi) which are a classical ARE feature. AREs are able to recruit several *trans*-acting factors such as the RNA binding protein HuR (human antigen R) [449]. The recruitment of this *trans* regulatory factor is known to stabilize mRNAs by protecting the mRNA body from degradative enzymes [450]. Bolognani *et al.* reported HuR to be over-expressed in glioma cancer tissues in correlation with the tumor grade. Higher levels of HuR have been found in higher grade gliomas [451]. Furthermore, Filippova and colleagues found HuR to be important for growth and survival of glioma cell lines [452]. In this context, over-expressed HuR could lead to an increased mRNA stability of TANK and in turn contribute to elevated protein levels of TANK in GBM. The investigation of other possible mechanisms regulating mRNA stability in GBM such as impaired binding of destabilizing factors will be an interesting challenge for the future.

The protein stabilities of TANK and TBK1 were also taken into consideration. Here, all investigated glioma cell lines exhibit high stabilities for both proteins, but the protein stability of the adaptor protein TANK was higher compared to the kinase TBK1 (figure 3.3). This is in accordance with the findings of Wu *et al.* who identified protein kinases to be less stable than other protein classes upon the release of their stabilizing binding partner [453]. Considering that a high expression of functional proteins does not consequently lead to their activation, it would be interesting to investigate the constitutive activation of the non-canonical IKK complex in GBM by a kinase activity assay. Finally, it would be of interest to determine post-translational modifications that are known to be involved in the activation of the non-canonical IKK complex such as phosphorylation or ubiquitination.

The measured mRNA levels for TANK in different glioma cell lines were not always in accordance with the observed protein levels. This discrepancy is in line with the findings of Vogt *et al.* who observed a similar phenomenon when investigating the *TERT* gene (telomerase reverse transcriptase) amplification. This amplification was suggested to lead to enhanced mRNA and consequently protein levels, but Vogt *et al.* just found a little correlation between mRNA and protein levels [454].

4.2. The non-canonical IKK complex influences cellular functions in glioma cell lines

TANK has been found to be dysregulated in GBM. In order to address the consequences of this dysregulation, cellular processes such as proliferation and migration were investigated. TANK and TBK1 were identified to play an important role in mediating proliferation (figure 3.5). Among the three components of the non-canonical IKK complex, only the two kinases have been linked to proliferation so far. Qin and Cheng reported an anti-proliferative effect of IKKε knock-down in breast cancer cell lines [128]. Similarily, IKKε knock-down also led to decreased proliferation in glioma cell lines [455]. Since IKKε and TBK1 are structurally and functionally similar, it is likely that also TBK1 fulfills a role in promoting proliferation in cancer. For instance, Kim *et al.* described a decrease in proliferation after TBK1 knock-down in hepatocellular carcinoma cells [456]. The activity of both IKK-related kinases has further been shown to depend on an adaptor molecule such as TANK [80, 457], thus, its absence results in a decrease of IKKε and TBK1 activity and in turn in reduced proliferation. In order to investigate the reason for this anti-proliferative effect of the non-canonical IKK complex in gliomas, cell cycle analyses were performed. A vinblastine induced cell cycle arrest led to delayed or even rarely detectable accumulation of cells in G_2 phase after silencing TANK and in some cell lines also for TBK1 silencing (figure 3.8). This confirms the inhibitory effects of the knock-down on proliferation. Furthermore, the knock-down of TANK or TBK1 in glioma cell lines led to a slightly increased proportion of cells in G_1 phase in some cell lines (figure 3.7). This is in line with the findings of Kittler *et al.* who reported the TANK knock-down to induce G_0/G_1 arrest in a genome-scale RNAi profiling of HeLa cells [458]. Additionally, IKKε knock-down experiments in breast cancer lines and glioma cell lines have been found to induce a cell cyle arrest in G_0/G_1 phase which resulted in decreased proliferation [128, 455]. This arrest in G_0/G_1 has been associated with a decrease in NF-κB activation, thus leading to a reduced expression of cyclin D1 [455]. Furthermore, the phosphorylation of ERα by IKKε has been shown to induce the transcriptional expression of cyclin D1 [131] which has been demonstrated to be over-expressed in various cancers and to promote G_1/S transition [459, 460]. In contrast to IKKε, the role of TBK1 in cancer cell proliferation is not that clear. The proliferation ability of some breast cancer cell lines seems to depend on TBK1 [127]. However, the suppression of TBK1 in cancer cell lines, which depend on oncogenic KRas expression, rather induces apoptosis than a decrease in proliferation. Here, TBK1 activates anti-apoptotic NF-κB signals [461].

Interestingly, one of the investigated glioma cell lines accumulated in S phase after silencing TANK (figures 3.7-8). A defect in the transition from S to G_2 phase has been

reported for cells over-expressing p21. The binding of p21 to PCNA (proliferating cell nuclear antigen) results in the inhibition of DNA synthesis [462-464]. The expression and activity of p21 can be modulated by Akt and ERK1/2 kinases [235, 310], both known to crosstalk and to be dysregulated in GBM. The role of the non-canonical IKK complex in this crosstalk of Ras-Raf-MEK-ERK and PI3K-Akt signaling pathways will be discussed later.

Invasion is a multifactorial process involving interaction of cells with neighboring cells and the ECM (extra-cellular matrix) as well as biochemical processes leading to active cell movement [465]. In contrast to invasion which is defined as three dimensional movement of cells within a matrix, migration describes a two dimensional and directed motion which is necessary for invasion [466]. In the present study, the influence of TANK or TBK1 knock-down regarding migration was investigated and revealed a great role of the non-canonical IKK complex for the migration ability of glioma cells (figure 3.9.). Because effects of TANK silencing exceeded those of TBK1 silencing, it is likely that IKKε additionally plays a great role for cell migration of glioma cell lines. This is in accordance with the literature where inhibited migration upon IKKε silencing in glioma and breast cancer cell lines, respectively, has been described [128, 455]. Other components of the NF-κB signaling network are also associated with migration in cancer cells. Very recently, IKKα and IKKβ-induced NF-κB activation was identified to modulate migration in HNSCC (head and neck cancer) probably by affecting c-Jun [467]. Furthermore, both canonical IKKs are involved in NF-κB-mediated MMP9 expression (matrix metalloproteinase 9) which is necessary for migration [468]. MMP9 is a member of the MMP family which is responsible for the breakdown of the ECM [469]. Serving as type IV collagenase, MMP9 secretion results in degradation of collagen of the ECM thereby providing space for invading cells [465]. Moreover, Akt kinase, that also can be directly phosphorylated by IKKε and TBK1, was identified to modulate MMP9 expression in cancer cells by inducing NF-κB activation [470]. The role of hyperactive Akt in migration has further been investigated by Zhang and colleagues. They reported that Notch1 is dysregulated in glioma which leads to an activation of Akt. Akt in turn increases the β-catenin activity which leads to the expression of genes that are necessary for the epithelial-to-mesenchymal transition [471, 472]. Furthermore, increased β-catenin leads to a disruption of the cadherin-catenin complex and decreases the intercellular contact which promotes migration. One of the cadherins, that can be bound by β-catenin, is the transmembrane cell adhesion protein E-cadherin [473]. The role of E-cadherin in migration still seems to be controversial. On the one hand, Lewis-Tuffin *et al.* found E-cadherin to be over-expressed in one third of glioma cases which leads to exaggerated growth, migration and

invasiveness [474]. On the other hand, reduced levels of the tumor supressor E-cadherin were reported to be associated with increased migration by disrupting intercellular contacts [475, 476]. The supression of E-cadherin in gliomas can be caused by the transcription factors ZEB1 [477] or ZEB2 (Zinc finger E-box-binding homeobox 1 and 2) [478] which results in increased migration. Edwards and colleagues investigated the relation between E-cadherin, ZEB transcription factors and NF-κB in glioblastoma and demonstrated that the activation of NF-κB can lead to the binding of ZEB1 to the E-cadherin promotor which results in the supression of E-cadherin and subsequently in enhanced migration [477]. Qi and colleagues showed an increased expression of ZEB2 in glioma which they found to be responsible for migration by the repression of E-cadherin [478]. In future, it would be of interest to further address the role of the non-canonical IKK complex in invasion by either performing a transwell invasion assay to measure invasion *in vitro* or a spheroid confrontation assay in order to determine cell invasion into tissue.

4.3. The importance of stoichiometric TANK expression

Even though adaptor proteins such as TANK do not hold any catalytic function, their expression is necessary for signaling [427]. Thereby, the amount of the expressed adaptor protein can influence the fidelity of signaling as depicted in figure 4.1.

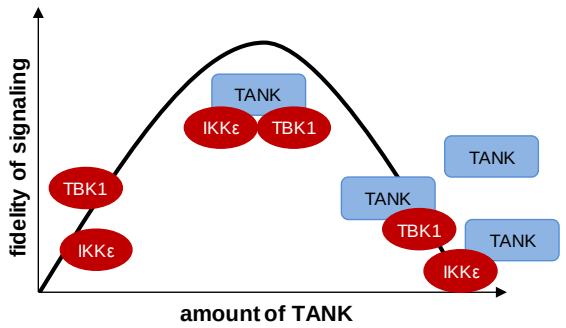


Fig. 4.1: Model of the consequences of over- or under-expression of adaptor proteins such as TANK. The optimal amount of adaptor proteins is necessary for a high fidelity of the signal outcome. For instance, too high as well as too low levels of TANK lead to a disruption of the complex resulting in decreased downstream signaling.

In accordance with this model, stoichiometric shifts within the complex by either silencing or substantial over-expression of TANK consequently resulted in reduced IRF activation as well as reduced proliferation in glioma cell lines (figure 3.20). In general, the observed phenomenon of decreased signal fidelity of a functional complex by over- and under-expression of its scaffold proteins has been described by Burack and Shaw using the example of scaffold proteins in the MAPK signaling pathway [479]. Silencing of the scaffold protein MP1 (MEK partner 1) has been shown to reduce ERK activation in breast cancer cells [480]. Schaeffer *et al.* found, that the over-expression of the scaffold protein MP1 resulted in enhanced signaling output only when ERK1 kinase was additionally over-

expressed [481]. Based on those findings, Burack and Shaw pointed out the importance of near-stoichiometric levels of the scaffold and the ligand for a functional complex and proper signaling [479].

The idea that TANK over-expression leads to a decrease in the signal fidelity of the non-canonical IKK complex seems to be in contrast to the observation that TANK over-expression results in promotion of proliferation and migration in GBM. As Goncalves and colleagues argued, the TANK adaptor protein competes with SINTBAD and NAP1 for the binding of the IKK-related kinases [80]. Those other possible adaptors are as well expressed in brain tissues [482, 483] and form alternative complexes with TBK1 and IKKε which have similar functions in the kinase activation [67, 80]. TANK has been shown to have the lowest affinity for TBK1- and IKKε-binding compared to SINTBAD and NAP1 [80]. So, the more TANK is expressed in glioma cells, the more likely is the binding of TANK to both IKK-related kinases, until a certain maximum is reached and the complex cannot form properly. Furthermore, IKKε has been identified to be over-expressed in gliomas and to be responsible for increased proliferation and migration [455]. This suggests that TANK might assemble an alternative complex with an IKKε homodimer which is involved in gliomagenesis. This would also be in accordance with results in this study which demonstrate greater effects of TANK knock-down than of TBK1 silencing on cellular functions such as proliferation and migration. TBK1 has not been described to be over-expressed in GBM but in other cancer types such as lung, colon and breast cancer [69, 79].

Further experiments investigating the role of NAP1 and SINTBAD in glioma cell lines by measuring their expression and influence on cellular functions will help to estimate the role of the non-canonical IKK complex in GBM. In this context, it would be interesting to address the affinity of the three adaptor proteins for TBK1- and IKKε-binding in glioma cell lines.

4.4. The non-canonical IKK complex is integrated in a complex network of signaling pathways

4.4.1. The influence of TANK and TBK1 on the proliferation of glioma cell lines is regulated by intrinsic signaling

In order to identify mechanisms by which the non-canonical IKK complex facilitates its function on proliferation, this study first determined whether the observed effects on

proliferation resulted from their influence on other signaling pathways or were an indirect consequence of secreted soluble factors. The treatment of control cells with supernatant of knock-down cells did not result in reduced proliferation, indicating that the influence of the non-canonical IKK complex on the proliferation of glioma cells is not a consequence of secreted factors but of altered intrinsic signaling (figure 3.12). In contrast, the cells treated with the supernatant showed a slightly increased proliferation. This could be caused by pro-proliferative factors in the extracellular environment such as growth factors and cytokines as IL-11, IL-6 and TNFα [426, 484, 485]. For instance, IL-6 has been shown by Qiu *et al.* to be over-expressed in glioma stem cells [486]. Furthermore, Kudo and colleagues reported high IL-6 expression in glioma cell lines to positively regulate proliferation by the activation of the JAK-STAT signaling pathway [487].

Predominantly, the decreased proliferation of glioma cell lines after silencing components of the non-canonical IKK complex seems to derive from changes in the intracellular signaling network. Since proliferation and migration in cancer cells often have been found to depend on Akt or ERK1/2 signaling [377, 398], it was interesting to assess, if and how the non-canonical IKK complex could be integrated into the signaling network described in 1.5.

4.4.2. TANK and TBK1 influence the phosphorylation of Akt and ERK1/2 in glioma cell lines

Within the present study, endogenous levels of Akt and ERK1/2 proteins as well as their phosphorylation status were determined and their constitutive activation was revealed. Interestingly, the analyses of the glioma cell lines disclosed an inverse phosphorylation pattern of Akt T308 and ERK1/2, meaning that if Akt is highly phosphorylated, ERK1/2 phosphorylation is decreased and in turn, if ERK1/2 is highly phosphorylated, Akt phosphorylation is decreased. The repressive effect of activated Akt on ERK1/2 activation has been shown by Mabuchi *et al.* in ovarian cancer cells as well as by Lee and colleagues in prostate cancer cell lines [372, 488]. Akt phosphorylates and inactivates Raf-1, thereby impairing ERK1/2 activation [372]. Taken together, Akt seems to have a suppressive effect on the phosphorylation of ERK1/2 in glioma cell lines.

But what is the role of the non-canonical IKK complex in the phosphorylation of Akt and ERK1/2? Within the scope of this study, silencing of either TANK or TBK1 has been shown to reduce Akt as well as ERK1/2 phosphorylation (figures 3.13-14). Furthermore, the inhibition of the non-canonical IKK complex by BX795 confirmed this effects as summarized in figure 4.2.A. This is the first time the non-canonical IKK complex is linked to ERK1/2 signaling. Recently, Akt has been demonstrated to act downstream of TBK1

and IKKε [125, 136, 137]. Moreover, Akt is known to act upstream of ERK1/2 through the activation or inhibition of the upstream kinase Raf [372, 374]. Some substances have been shown to decrease the phosphorylation of Akt and ERK1/2 simultaneously in different cell lines [489-491]. Chai *et al.* reported Sorafenib, a multikinase inhibitor known to target Raf [492], to induce apoptosis in neuroblastoma cell lines by the down-regulation of Akt and ERK1/2 phosphorylation [490]. Also Fei *et al.* showed decreased ERK1/2 phosphorylation in response to Perifosine [489] which is a Akt kinase inhibitor [493]. Furthermore, Ellert-Miklaszewska and colleagues described a repressive effect on Akt and ERK1/2 phosphorylation in a rat glioma cell line after the treatment with Cannabinoids which resulted in cell death and decreased proliferation [491]. Those effects can be explained by the crosstalk among the MAPKs and the influence of Akt kinase on this crosstalk. Activated Akt has been shown to block the activity of JNK [224, 494]. In turn, JNK has been found to have a repressive effect on ERK1/2 within the MAPK crosstalk [495, 496]. So, if Akt is inhibited, JNK activity is exaggerated and the repressive effect of JNK on ERK1/2 is increased which results in reduced phosphorylation of ERK1/2. Another possible connection between the non-canonical IKK complex, Akt and ERK1/2 might lead through GSK3α. Gulen and colleagues found IKKε to inactivate GSK3α by phosphorylation at S21 in response to IL-1. Furthermore, GSK3α inhibits Akt by the phosphorylation within the substrate binding site at T312 [497]. Wang *et al.* identified GSK3 as a negative regulator of ERK1/2 phosphorylation in colon cancer cells in a PKC-dependent manner [498]. Thus, the knock-down of IKKε would lead to increased GSK3 activity and consequently reduced Akt as well as ERK1/2 phosphorylation. Finally, it is also possible, that TBK1/IKKε directly (independent of Akt) phosphorylate ERK1/2 or possibly the upstream kinase MEK1/2. But so far, there is no evidence for such an interaction in literature. Also database researches did not reveal phosphorylation motifs in ERK1/2 or MEK1/2 that could be target sites for TBK1 or IKKε.

The negative correlation between Akt T308 and ERK1/2 phosphorylation in glioma cells shifted to a positive correlation upon silencing/inhibiting of the non-canonical IKK complex. In general, T308 is the major residue that needs to be phosphorylated to activate Akt, but full activation is known to additionally require S473 phosphorylation [153]. Both residues have been demonstrated to be phosphorylated by many kinases in response to different stimuli [247]. Moreover, the phosphorylation of tyrosine residues of Akt have been identified to be essential for its biological function [499]. This suggests the possibility that certain phosphorylation patterns determine whether Akt has a supportive or repressive effect on ERK1/2 phosphorylation. This distinct regulation of protein function by post-transcriptional modification patterns, termed *barcode hypothesis*, has been described for

p53 [500]. p53 can be induced by a wide range of stress stimuli and finally lead to either apoptosis or DNA repair [238, 501]. The phosphorylation of p53 at certain sites thereby influences the cellular outcome. For instance, the phosphorylation of one residue can lead to changes in the subcellular localization and thereby results in altered function. The phosphorylation of p53 at S315 has been associated with the inhibition of p53 by its export to the cytosol and its degradation [500, 502]. p53 phosphorylation at S315 is additionally known to induce the binding of p53 to E2F1, which leads to its retention in the nucleus and to p53-dependent gene expression [503]. The term *barcode hypothesis* has already been adopted for other proteins such as p65. Moreno *et al.* found that the combination of post-translational modifications of p65 is important to direct its transcriptional activity in a target specific fashion [95].

Finally, combined inhibition of Akt and the non-canonical IKK complex did not have any further effect on the ERK1/2 phosphorylation, suggesting the IKK-related kinases and Akt to act upstream of ERK1/2 in one signaling path.

4.4.3. The role of MEK1/2 and TAK1 in TANK/TBK1/IKKε-mediated ERK1/2 phosphorylation

The dependence of Akt-induced ERK1/2 phosphorylation on MEK1/2 has been investigated by MEK1/2 inhibition (summarized in figure 4.2.B). However, the treatment with U0126 failed to reduce ERK1/2 phosphorylation in most glioma cell lines (figure 3.17). On the one hand, Yip-Schneider and colleagues reported the development of a reversible resistance to U0126 within 24 hs in liver cancer cells as a consequence of feedback mechanisms to the Ras-Raf-MEK pathway [504]. Thus, it is possible that the investigated glioma cell lines also developed a resistance against the inhibitor U0126 since they were also treated for 24 hs. On the other hand, the observed ERK1/2 phosphorylation upon MEK1/2 inhibition could indicate the presence of an MEK1/2-independent pathway to activate ERK1/2. The treatment with MEK inhibitor U0126 was also insufficient to block Akt phosphorylation, indicating that Akt acts upstream of ERK1/2 in another signaling path (figure 3.17). Nevertheless, Akt phosphorylation was increased upon U0126 treatment suggesting the existence of a feedback loop from MEK1/2 or ERK1/2 to Akt. The paradox phenomenon of increased Akt phosphorylation after the treatment with MEK inhibitors has also been observed by Normanno *et al*. in breast cancer cell lines, and was found to result in proliferation and survival [505]. However, the underlying mechanisms still remain unclear and need to be investigated. A combined inhibition of MEK1/2 and the IKK-related kinases resulted in the abolishment of ERK1/2 phosphorylation in some glioma cell lines (figure 3.17.). This result further supports the

idea of an alternative ERK1/2 activating pathway, which is independent of MEK1/2, to ensure proliferation in GBM. In future it would be of interest to identify the players of this pathway. In this context, Grammer and Blenis found the PI3K-Akt pathway as well as PKC to be linked to an MEK1/2-independent ERK1/2 activation [506]. However, so far there is no evidence for a direct phosphorylation of ERK1/2 by either PKC or Akt. Taken together, these findings support the idea of escape signaling mechanisms in GBM to maintain rapid proliferation. The simultaneous inhibition of participating pathways thereby could improve the treatment of cancer.

Similar to the MEK inhibition, the treatment of glioma cell lines with a TAK1 inhibitor revealed an increased Akt phosphorylation in most cell lines as summarized in figure 4.2.D. This increased Akt phosphorylation did not correlate with ERK1/2 phosphorylation which was found not to be altered. Whereas TAK1 has been shown to activate p38 and JNK MAP kinases, there is no evidence for ERK1/2 activation by TAK1 [507]. The repressive effect of TAK1 on the phosphorylation of Akt is in contrast with the findings of Lee *et al.* who reported TAK1 to activate Akt in response to LPS in a PI3K-dependent manner in pre-B cells [507]. Furthermore, the existence of a TAK1-MEK-Akt pathway involved in survival has been claimed by Gingery and colleagues in osteoclasts in response to TGF-β [508]. Since the combination of TAK1 inhibitor with BX795 did not show any further effects on the phosphorylation of Akt and ERK1/2 compared to the treatments only with BX795 or 5Z-7-oxozeaenol, it is likely that there is no crosstalk between the non-canonical IKK complex and TAK1 in glioma cell lines (figure 3.18). In order to confirm the role of TAK1 in Akt and ERK1/2 signaling in glioma cell lines, further investigations need to be done. Silencing of TAK1 followed by the measurement of Akt and ERK1/2 phosphorylation as well as the measurement of TBK1/IKKε activity could reveal better insights into the signaling crosstalk. Furthermore, it would also be interesting to assess the role of TAK1 in cellular functions in glioma cell lines by investigating proliferation, migration and apoptosis after silencing TAK1.

The following figure summarizes the findings of this work (A-D) as well as the discussed crosstalk between the non-canonical IKK complex, Akt and ERK1/2 (E) and thereby shows a presumable model of signaling that occurs in GBM.

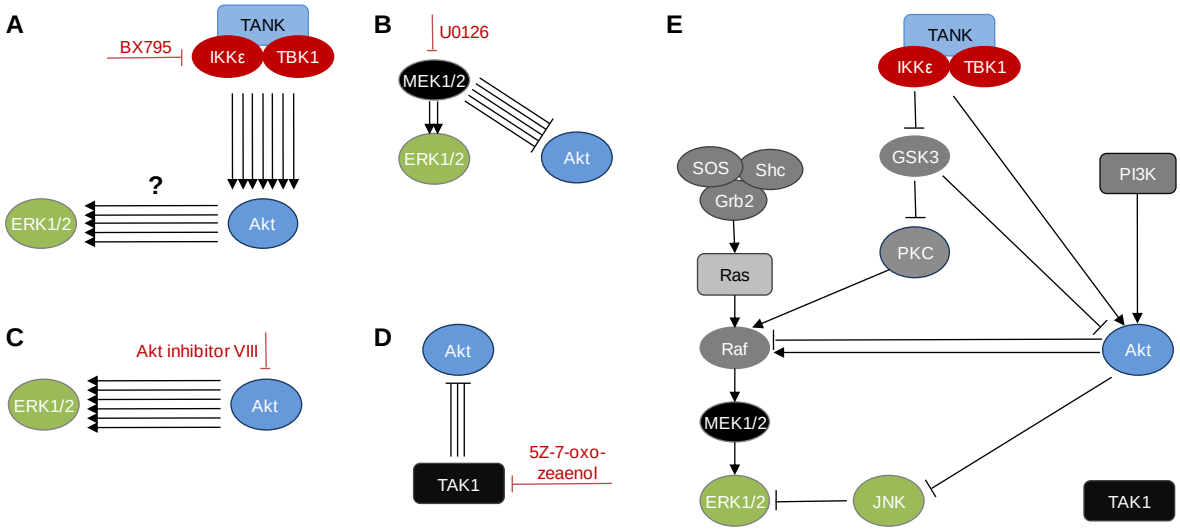


Fig. 4.2: Presumable signaling network regulating Akt and ERK1/2 in glioma cell lines. The effects of the indicated inhibitors on Akt and ERK1/2 phosphorylation in glioma cell lines are depicted in subfigures A to D. Here, the amount of arrows is representing the amount of glioma cell lines where the effect was detected. (E) The model of the signaling network in GBM how it could be explained by the literature. The non-canonical IKK complex has been identified to activate Akt. Akt in turn has distinct functions on ERK1/2 activation. Whereas ERK1/2 phosphorylation is repressed by Akt phosphorylated at T308 in steady-state conditions, the inhibition of Akt phosphorylation at both activation sites likewise resulted in reduced ERK1/2 phosphorylation. Underlying mechanisms are not clear so far but could depend on the crosstalk of MAP kinases with each other and Akt or on upstream GSK3, which in turn represses the activation of Raf-MEK-ERK by inhibiting PKC as well as Akt. Furthermore, MEK1/2 and TAK1 have been discovered in the present study to repress Akt activation independently of the non-canonical IKK complex. Abbreviations: SOS; Son of Sevenless; Shc: SHC-transforming protein; Grb2: growth factor receptor-bound protein 2; Ras: Rat sarcoma; MEK: MAP2K; ERK: extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinases; PKC: protein kinase C;.GSK3: glycogen synthase kinase 3; TANK: TRAF family member-associated NF-κB activator; IKKε: IκB kinase epsilon; TBK1: TANK binding kinase 1; PI3K: phosphatidylinositide 3-kinases; TAK1: TGF-β-activating kinase.

Of note, the proposed model is solely based on an approach using specific inhibitors, so it has to be validated by other techniques such as silencing experiments and kinase assays as well as by the determination of the cellular outcome such as proliferation and survival. Nevertheless, first hints disclose a network of signaling pathways in glioma involving ERK1/2, Akt and the non-canonical IKK complex to promote proliferation.

4.4.4. The role of the non-canonical IKK complex in other cancer cell lines

The influence of the non-canonical IKK complex on proliferation of cancer cell lines other than glioblastoma revealed a minor effect on HCT116 and A549 proliferation. In contrast, a profound decrease in proliferation has been observed in MCF-7 cell line after silencing either TANK or TBK1 (figure 3.21). Several groups found IKKε to induce transformation and further increase proliferation in some cancer types. Especially breast cancer is known to be dependent on IKKε-mediated transformation [127, 509]. Guo *et al.* showed this effect for breast cancer cell lines including MCF-7 [137]. Also Akt has been shown to

promote tumorigenic transformation in about 50% of breast cancer cases [510]. Boehm *et al.* showed in an integrative genomic approach, that IKKε is able to substitute Akt in cellular transformation [127] which would explain the influence of the non-canonical IKK complex on MCF-7 proliferation without changes in the Akt phosphorylation. Furthermore, Akt2 has been reported to act upstream of IKKε in breast cancer cell lines by influencing its expression [511]. Additionally, Kim *et al.* demonstrated that basal Akt phosphorylation was not altered after silencing of TBK1 in lung cancer cells [512]. In summary, this work did not show any influence of TANK or TBK1 silencing on the phosphorylation of Akt and ERK1/2 (figure 3.21), indicating that the described crosstalk of the non-canonical IKK complex, Akt and ERK1/2 is exclusively present in GBM.

4.5. TANK and TBK1 regulate inflammatory signaling cascades in glioblastoma multiforme

The importance of inflammation in cancer and especially GBM has been well described [425, 426]. The role of the non-canonical IKK complex in pro-inflammatory signaling in glioma cell lines was investigated. Thereby, a heterogeneous picture concerning the phosphorylation of IRF3 and p65 and the expression of pro-inflammatory cytokines was revealed (figure 3.19). A constitutive activation of NF-κB in GBM has been described by Raychaudhuri *et al.* as well as Tsunoda and colleagues [513, 514]. In many different types of cancers an aberrant NF-κB activation also leads to an increase in the expression of pro-inflammatory cytokines [119]. The mechanisms underlying the constitutive activation of NF-κB are still not clearly understood. In general, the constitutive phosphorylation and degradation of IκBα, which has been reported for instance in melanomas, results in nuclear translocation of the NF-κB dimer [515]. Furthermore, deletions or loss-of-function mutations of IκBα could induce this constitutive activation of NF-κB [516]. Such a deletion of *NFKBIA* has been found in glioblastomas [50]. Besides NF-κB, also other signaling cascades are involved in the expression of pro-inflammatory cytokines as for instance IL-6. Here, dysregulated MAPK signaling has been found to be important. Yeung and colleagues identified p38 to be responsible for the over-expression of IL-6 in GBM cell lines. However, the underlying mechanism remain unclear [517]. Although, Zauberman *et al.* found the transcription factor STAT3 to be activated by p38 which results in increased IL-6 expression [518].

The pro-inflammatory transcription factor IRF3 is known as a tumor suppressor and consequently lowly expressed in GBM [519]. Furthermore, IRF3 expression suppresses

migration and proliferation of glioma cells [519]. In general, the non-canonical IKK complex is able to influence NF-κB and IRF3 transcription factors by modulating their phosphorylation. It still needs to be investigated in detail how the complex cytokine network is affected by the over-expression of the complex in glioma cell lines. Within the scope of this study, a controversial picture of the influence of TANK and TBK1 on the inflammatory signaling in GBM became apparent.

4.6. The role of the non-canonical IKK complex in the treatment with chemotherapeutic drugs

As GBM is one of the most aggressive brain tumors with a bad prognosis, great effort has been done during the last decades to develop new treatment possibilities. At present, eight open studies for glioblastoma are in phase III of clinical studies (http://clinicaltrial.gov). So far, only TMZ arose as standard-of-care in the chemotherapeutic treatment. The cell death-inducing feature of TMZ has been confirmed in this study. Furthermore, also physiological concentrations of AraC have been found to induce cell death in glioma cell lines (figure 3.10). AraC has been reported to induce phosphorylation of the checkpoint kinase Chk1, but has no activating effect on the ATM/Chk2 pathway [520, 521]. This results in cell cycle arrest, reduced growth as well as apoptosis [417].

The influence of the non-canonical IKK complex on AraC and TMZ-induced cell death has been investigated. Thereby, an additional effect of TBK1 has been found which was even more pronounced for TANK (figure 3.11 and 3.15). This suggests, that IKKε seems to be the kinase mainly responsible for the additional effect of the non-canonical IKK complex on the TMZ or AraC treatment, respectively. Indeed, IKKε has been shown to be involved in anti-apoptotic functions in response to DNA damage. Renner *et al.* identified IKKε to be activated in response to DNA damage induced by Etoposide which results in the prevention of cell death. Here, the phosphorylation of p65 at S468 by IKKε induced the expression of p21 and MDM2 [96]. Moreover, Guan and colleagues demonstrated an anti-apoptotic state in glioma cells treated with UV light or Adriamycin (Doxorubicin) which was caused by IKKε-induced NF-κB signaling and subsequent Bcl-2 expression. In this context, the knock-down of IKKε further increased the cell death induced by the stimuli [522]. This is in line with the results of the present study and further underlines the anti-apoptotic function of the non-canonical IKK complex. Additionally, NF-κB might be involved in the pro-apoptotic function of TMZ and AraC. Caparoli *et al*. reported TMZ to

induce ATR activation and subsequent Akt phosphorylation which results in pro-apoptotic NF-κB signaling [523]. Akt kinase has also been shown to induce apoptosis in response to DNA damage mediated by ATM [524]. Furthermore, ATM has been identified to be activated in response to TMZ and to interact with NEMO which leads to pro-apoptotic NF-κB signaling [525, 526]. Taken together, cell death induced by TMZ could be mediated by NF-κB as well as suppressed by IKKε also depending on NF-κB signaling. In general, NF-κB is known to support both pro- and anti-apoptotic signaling in response to DNA damage. The balance of pro-and anti-apoptotic signals depends on cell type and stimulus [527, 528]. In order to receive a better impression of the role of the non-canonical IKK complex in the induction of apoptosis, the measurement of apoptosis by Annexin V/FITC staining after silencing components of the complex would be an interesting approach. The additional treatment of glioma cells with chemotherapeutic drugs such as TMZ or AraC will thereby verify the shown effect of the non-canonical IKK complex on the drug-induced cell death. Additionally, the identification of involved DNA damage sensors and signaling pathways would provide a better insight into the role of the non-canonical IKK complex in drug-induced apoptosis.

In future, the development of new chemotherapeutic drugs for the treatment of GBM will be of great importance. This study revealed new insights in the complex signaling network in GBM. The non-canonical IKK complex as well as Akt signaling were identified to play a role in ERK1/2 phosphorylation. The activation of alternative pathways provides the cell with mechanisms to escape chemotherapeutic drugs. Thus, targeting several players in the network simultaneously could be an interesting approach for drug discovery studies. Treatments with special respect to genetic and molecular changes will play a crucial role to keep up with the diversity and multiform appearance of GBM and to avoid the development of drug resistances by escape mechanisms.

5. SUMMARY

Glioblastoma multiforme is one of the most common and lethal brain tumors. Many genetic and molecular changes have been described to occur in this type of brain cancer. Within this work, the role of the non-canonical IKK complex in glioblastoma multiforme was investigated. The non-canonical IKK complex is composed of the IKK-related kinases TBK1 and IKKε as well as adaptor proteins such as TANK, and is usually involved in the defense against viral and bacterial pathogens by the induction of type I interferon expression. This work shows, that TANK is dysregulated and over-expressed in a wide range of GBM patient tissue samples as well as glioma cell lines. Several mechanisms have been found to be responsible for the greatly varying protein levels of TANK. Various experimental approaches showed, that differences in the *de novo* transcription of the *Tank* gene and differential stabilities of TANK mRNAs account for the variances in TANK protein levels in glioma cell lines. The influence of members of the non-canonical IKK complex on cellular functions of glioma cell lines was then investigated after the knock-down of TANK or TBK1. Proliferation assays as well as MTT assays revealed a markedly reduced proliferation rate of some glioma cell lines after silencing of TANK or TBK1. The proliferation of other glioma cells was dependent on either TANK or TBK1. The proliferation of the minority of glioma cell lines was not affected by the knock-down of TANK or TBK1. Additionally, the ability of glioma cells to progress in cell cycle was found to be reduced upon TANK or TBK1 knock-down in those glioma cell lines where the knock-down caused a reduced proliferation ability. The migration ability was examined by wound-healing assays. These experiments showed that cells, which depend on TANK or TBK1 for proliferation, also need these proteins for cell migration. Usually those cellular functions are regulated by signaling pathways such as the Ras-Raf-MEK-ERK or PI3K-Akt signaling pathways. Therefore, the involvement of the non-canonical IKK complex in those signaling cascades was determined in this work. Akt as well as ERK1/2 have been shown to be constitutively phosphorylated and activated in some primary tissue samples of GBM or astrocytoma grade III patients as well as most of the glioma cell lines. The knock-down of either TANK or TBK1 as well as the inhibition of both IKK-related kinases (TBK1 and IKKε) by BX795 had a repressive effect on those phosphorylations in some of the glioma cell lines. Thus, an alternative signaling path for the activation of proliferation and survival signals in glioblastoma multiforme was identified which is dependent on the cell line. A pharmacological approach was applied to study the complex signaling network in detail and revealed a negative feedback loop from MEK1/2 to Akt as well as a MEK-independent signaling pathway to activate ERK. Whether the MEK-independent TBK1/IKKε-Akt

signaling axis leads to the phosphorylation and activation of ERK directly or indirectly still needs to be clarified in the future. However, this TBK1/IKKε-Akt-ERK pathway represents an alternative pathway to activate proliferation and survival signals in cancer, thus providing new opportunities in the discovery of treatments of glioblastoma multiforme.

6. ZUSAMMENFASSUNG

Das Glioblastom zählt nachwievor zu einem der häufigsten und tödlichsten Hirntumoren. Bisher konnten einige genetischen und molekularen Veränderungen in diesem Tumor identifiziert werden, die für diese Tumorart charakteristisch sind. Ziel der vorliegenden Arbeit war es, die Rolle des nicht kanonischen IKK Komplex im Glioblastom zu charakterisieren. Der nicht kanonische IKK Komplex besteht aus den beiden Kinasen TBK1 und IKKε, die durch ein Adaptorprotein wie beispielsweise TANK zusammengehalten werden. In erster Linie ist der Signalweg über diesen Komplex für die Reaktion auf virale oder bakterielle Pathogene verantwortlich, und führt in dieser Folge zur Ausschüttung von Typ I Interferonen. Im Rahmen dieser Arbeit konnte gezeigt werden, dass die Expression des Adaptorproteins TANK in einer Vielzahl von Patienten und Gliomzelllinien dereguliert und erhöht ist. Dabei wurden eine unterschiedliche de novo Transkription und mRNA Stabilität als mögliche Ursachen für die schwankenden Proteinlevel von TANK in den Gliomzelllinien gefunden. Weiterhin wurde die Rolle des nicht kanonischen IKK Komplexes auf zelluläre Funktionen der Gliomzelllinien untersucht, indem das Adaptorprotein TANK sowie die Kinase TBK1 retroviral herunter reguliert wurden. Dabei verdeutlichten Proliferationsstudien und MTT-Tests eine deutlich reduzierte Proliferationsfähigkeit in einigen Gliomzelllinien. In anderen Gliomzelllinien war die Proliferation abhängig von TANK oder TBK1, und in einigen wenigen Zelllinien hatten beide Proteine keinen Einfluss auf die Proliferation. Auch der Zellzyklusverlauf war in den Gliomzelllinien vermindert, in denen der Knock-down von TANK oder TBK1 zu einer reduzierten Proliferation führte. Des Weiteren wurde die Abhängigkeit der Zellmigration von TANK und TBK1 mittels Wound-healing Experimente in Gliomzelllinien untersucht und zeigte, dass in einigen Zelllinien auch diese TANK und TBK1 benötigt. Grundsätzlich werden Zellfunktionen durch verschiedene Signalwege wie beispielsweise den Ras-Raf-MEK-ERK- oder den PI3K-Akt-Signalweg reguliert. Deshalb wurde der Einfluss des nicht kanonischen IKK Komplexes auf diese Signalwege untersucht. Zunächst wurde festgestellt, dass sowohl Akt als auch ERK1/2 in einigen Gliomen und den meisten Gliomzelllinien konstitutiv phosphoryliert und damit aktiviert sind. Der Knock-down von TANK bzw. TBK1 wie auch die Inhibierung beider Kinasen (IKKε und TBK1) durch BX795 führte in einigen Gliomzelllinien zu einer Reduzierung dieser Phosphorylierungen. Damit konnte ein alternativer Signalweg aufgezeigt werden, der zu einer Aktivierung von Proliferations- und Überlebenssignalen in einigen Gliomzelllinien führt. Um einen genaueren Einblick in die komplexe Vernetzung der beteiligten Signalwege zu bekommen, wurde ein pharmakologischer Ansatz gewählt. Hierbei wurden eine negative

Rückkopplung von MEK1/2 zu Akt gefunden sowie ein ERK1/2-aktivierender Signalweg, der unabhängig von MEK1/2 ist. Inwiefern dieser TBK1/IKKε-Akt-Signalweg direkt oder indirekt zur Phosphorylierung und Aktivierung von ERK1/2 führt, muss noch in weiterführenden Experimenten untersucht werden. Dennoch stellt der Signalweg eine Alternative zur Aktivierung von Proliferations- und Überlebenssignalen in Krebszellen dar, und liefert damit neue Möglichkeiten für die Entwicklung von Therapien zur Behandlung des Glioblastoms.

7. SUPPLEMENTARY FIGURES

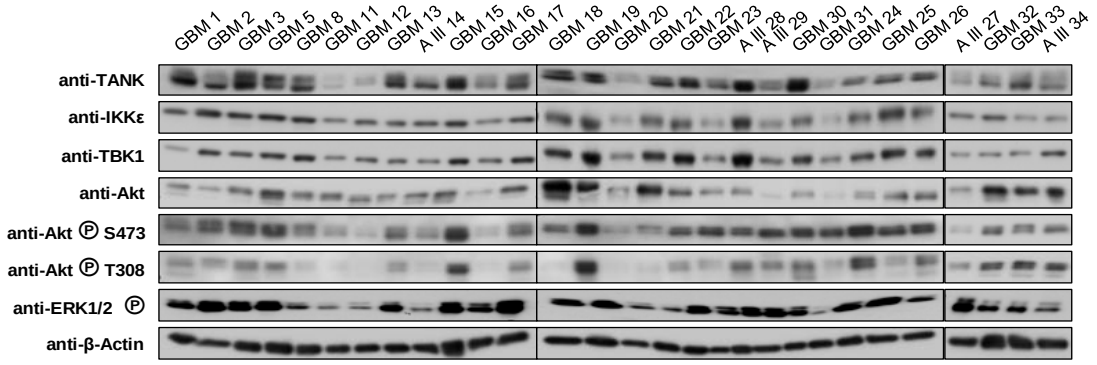


Fig. S1.: Analysis of the protein levels of members of the non-canonical IKK complex primary patient tissue samples. Patient tissue samples of normal brain (NB), glioblastoma multiforme (GBM) and astrocytoma grade III (A III) were kindly provided by Prof. Dr. Pagenstecher. Proteins were extracted from patient tissue by homogenizing of the material in NP40 lysis buffer and using a Turrax. Endogenous protein expression was then analyzed by Western blot using the indicated antibodies for endogenous proteins. A comparison of GBM/ A III with normal brain tissue is shown in figure 3.1.A.

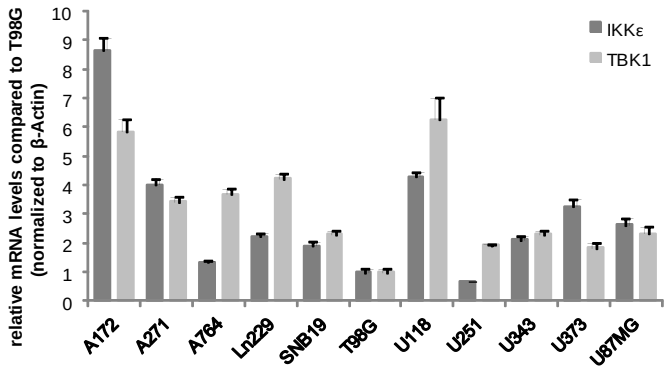
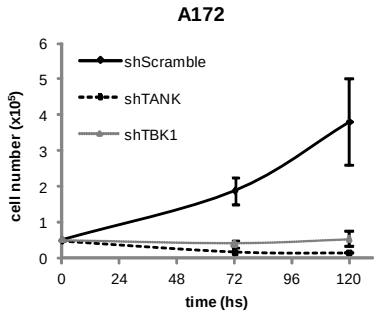


Fig. S2.: Analysis of the mRNA levels of the IKK-related kinases in glioma cell lines. Total RNA was extracted and translated into cDNA using Oligo(dT) primers. The amount of mRNA was further semi-quantitatively analyzed by real-time PCR using specific primers for either TBK1 or IKKε. Expression levels of T98G were set as 1 after normalization to the housekeeping gene *β-Actin*. Error bars express the standard deviation of three independent experiments performed in triplicates.

A



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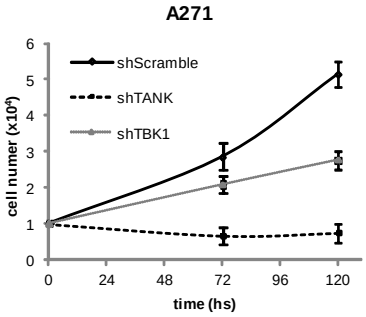

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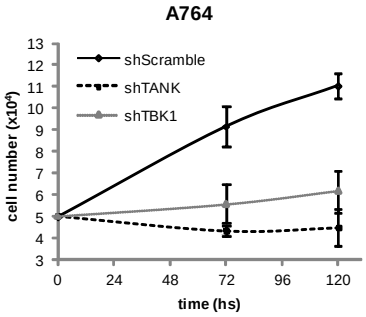

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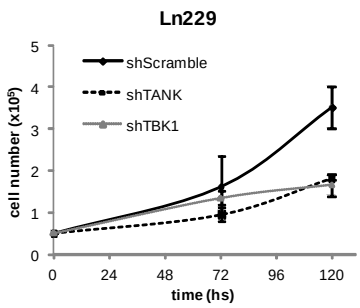

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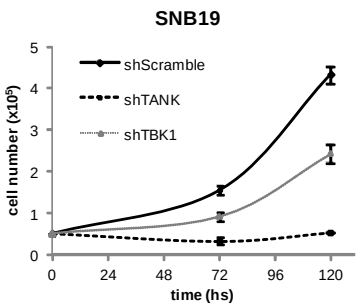

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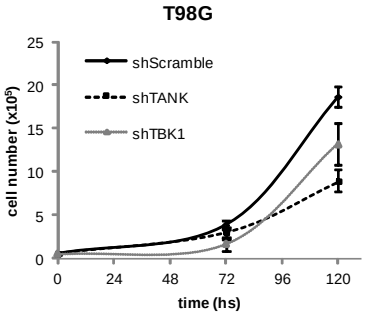

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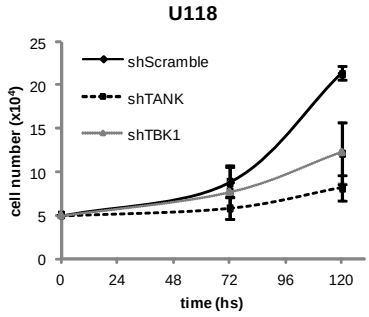

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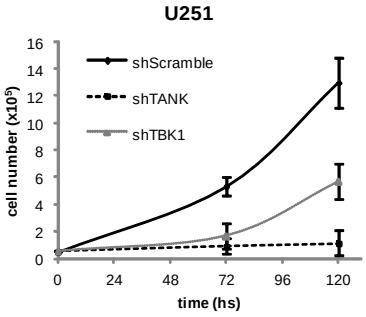

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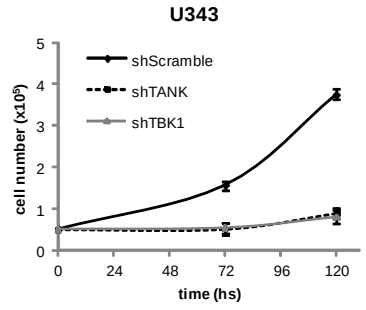

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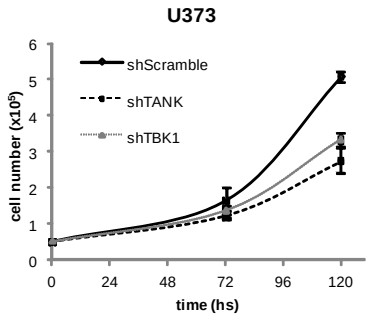

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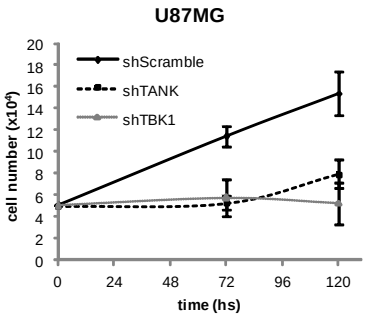

L

Cell Line	Influence of TANK on proliferation	Influence of TBK1 on proliferation
A172	++	++
A271	+	++
A764	++	+
Ln229	+	+
SNB19	++	+
T98G	nd	nd
U118	+	+
U251	++	+
U343	++	++
U373	+	+
U87MG	++	++

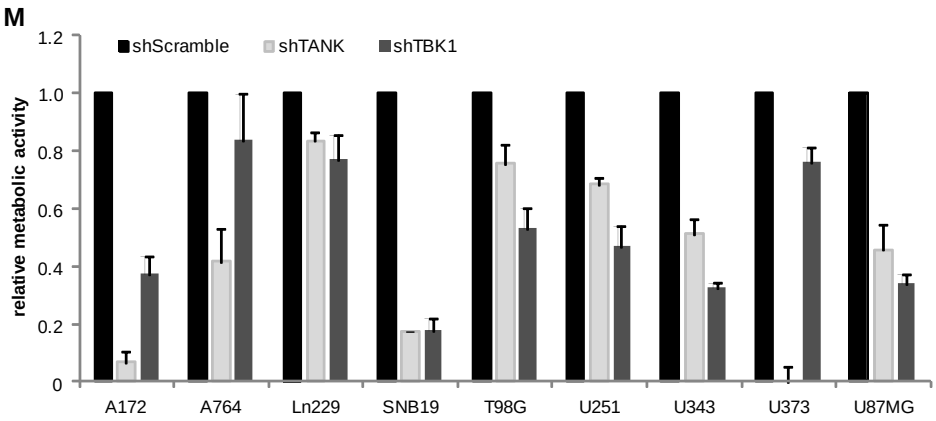

Fig. S3: The role of TANK and TBK1 in the proliferation ability of glioma cell lines. (A) A172 cells were retrovirally transduced to express shRNAs leading to a knock-down of TANK, TBK1 or a scrambled shRNA as a control. Transduced cells were selected with puromycin and used to determine cell proliferation. Proliferation was measured by seeding cells at a defined density, followed by determination of cell numbers after 72 and 120 hs using a FACSCalibur. Error bars show standard deviations from three independent experiments. (B-K) Experiments of (A) were performed for indicated cell lines cells. (L) Results from proliferation assays from all cell lines are summarized. Symbols indicate strength of influence on proliferation: - no, + low, ++ great influence and nd not detectable. (M) MTT assay was performed by seeding knock-down cells at a density of $1x10^3$ cells/well, followed by further growth for 4 days and performance of the MTT assay according to the manufacturer's instructions. Relative metabolic activity of control cells was arbitrarily set as 1, error bars show standard deviations of three independent experiments.

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EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre: Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichen Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei der von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissen-schaftlicher Praxis“ niedergelegt sind, eingehalten.

Gießen, den 30.10.2013 _____

PUBLICATIONS

Project-related publications

Stellzig J, Chariot A, Shostak K, Göktuna SI, Renner F, Acker T, Pagenstecher A, Schmitz ML: Deregulated expression of TANK in glioblastomas triggers protumorigenic ERK and AKT signaling pathways. Accepted in Oncogenesis in October 2013

General publications

Handschick K, Beuerlein K, Jurida L, Bartkuhn M, Müller H, Soelch J, Weber A, Dittrich-Breiholz O, Schneider H, Scharfe M, Jarek M, Stellzig J, Schmitz ML, Kracht M: Cyclin-dependent kinase 6 is a chromatin-bound cofactor for nuclear factor kappa b (NF-κB)-dependent gene expression. Submitted to Molecular Cell in October 2013

Grünwald S, Stellzig J, Adam IV, Weber K, Binger S, Boll M, Knorr E, Twyman RM, Vilcinskas A, Wenzel U. (2013) Longevity in the red flour beetle *Tribolium castaneum* is enhanced by broccoli and depends on *nrf-2, jnk-1* and *foxo-1* homologous genes. *Genes Nutr*. 2013 Jan 16. [Epub ahead of print]