Functional characterization of Cytoplasmic Polyadenylation Element Binding proteins in the developing and diseased brain

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"One of the ultimate challenges for biology is to understand the brain's processing of unconscious and conscious perception, emotion, and empathy"

Eric R. Kandel

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CONTENTS

ABE	BREVIA	ΓΙΟΝς	11
1	INTRO	DUCTION	.14
1.1	l Glio	omas	. 14
	1.1.1	Cellular origin of gliomas	. 14
	1.1.2	Histopathological and molecular classification of gliomas	.15
	1.1.3	Microenvironment of glioblastoma	18
	1.1.4	Implications for diagnosis and therapy	. 19
1.2	2 Trai	nslational control by CPEBs	20
	1.2.1	Principles of translation	20
	1.2.2	CPEBs	21
	1.2.3	Regulation of polyadenylation-induced translation	. 22
1.3	3 Role	e of CPEBs in germline and somatic cells	. 24
	1.3.1	CPEBs in cell cycle progression	. 24
	1.3.2	CPEBs in metabolism and senescence	.25
	1.3.3	CPEBs in cancer	.26
	1.3.4	CPEBs in the nervous system	. 29
1.4	4 Alte	erations regulating expression and activity of CPEBs	31
	1.4.1	DNA methylation	31
	1.4.2	Alternative splicing	. 33
	1.4.3	Phosphorylation	.35
2	AIM OI	THE STUDY	.37
3	MATER	NALS	.39
3.1	l Ant	ibodies	. 39
3.2	2 Cell	cultures	41
	3.2.1	Reagents	. 41

3.2	.2	Media composition	. 41
3.2	.3	Cell culture consumables	. 42
3.3	Che	emicals	. 43
3.4	Ext	raction of nucleic acids	. 43
3.5	Frag	gment analysis	. 44
3.5	.1	Reagents	. 44
3.5	.2	Primers	. 44
3.6	Imr	nunocytochemistry	. 46
3.6	.1	Reagents	. 46
3.6	.2	Buffers and solutions	. 46
3.7	Imr	nunohistochemistry	. 47
3.7	.1	Reagents	. 47
3.7	.2	CSA II staining system for immunohistochemistry	. 48
3.8	Lab	poratory equipment	. 48
3.9	Met	thylation	. 49
3.9	.1	Bisulfite conversion reagents	. 49
3.9	.2	Bisulfite-DNA amplification reagents	. 50
3.9	.3	Pyrosequencing reagents	. 50
3.9	.4	Primers	. 51
3.10	Patl	nways activity assay	. 51
3.11	Sen	ni-quantitative real time PCR	. 52
3.1	1.1	Reverse transcription reagents	. 52
3.1	1.2	TaqMan semi-quantitative real time PCR reagents	. 52
3.1	1.3	Primers and probes	. 52
3.12	Tra	nsfection	. 54
3.1	2.1	Reagents	. 54
3.1	2.2	Expression vectors	. 54

	3.13	We	stern blotting	54
	3.1.	3.1	Protein lysis buffer	54
	3.1.	3.2	BCA Protein Assay	55
	3.1.	3.3	SDS-PAGE and protein transfer	55
	3.1.	3.4	Blocking and antibody solutions	57
4	MI	ETH	DDS	
	4.1	Ani	mals	59
	4.2	Cel	l cultures	59
	4.2.	.1	Glioblastoma cell cultures	59
	4.2.	.2	HEK-293FT cell cultures	59
	4.2.	.3	Primary hippocampal cultures	60
	4.3	Hu	nan specimens	60
	4.4	Ext	raction of nucleic acids for methylation and fragment analysis studies	61
	4.5	Me	thylation of CPEB1-4 genes	61
	4.5	.1	Bisulfite conversion	61
	4.5	.2	Pyrosequencing	
	4.5	.3	Data analysis	
	4.6	Fra	gment analysis of CPEB1-4 alternative splice isoforms	
	4.6	.1	Fragment analysis	64
	4.6	.2	Data analysis	65
	4.7	Ger	neration of custom-made antibodies	65
	4.8	Imr	nunohistochemistry	66
	4.8	.1	Staining of paraformaldehyde fixed tissues	66
	4.8	1.1	Tissue preparation	66
	4.8	.1.3	Microscopy and data analysis	66
	4.8	.2	Staining of formalin fixed paraffin-embedded tissues	67
	4.8	.2.1	Generation of tissue microarrays	

4.	8.2.3	Microscopy and data analysis	68
4.9	Tra	nsfection and stimulation of cultured cells	68
4.	9.1	Transfection of cultured cells	68
4.	9.2	HEK-293FT cells stimulation with forskolin	69
4.10	Imr	nunocytochemistry	69
4.	10.1	Coating slides with poly-L-lysine	69
4.	10.2	Immunocytochemical staining	69
4.	10.3	Microscopy and data analysis	70
4.11	Ext	raction of RNA for semi-quantitative real time PCR	70
4.12	Sen	ni-quantitative real time PCR	70
4.	12.1	Reverse transcription	70
4.	12.2	Semi-quantitative real time PCR	71
4.	12.3	Data analysis	72
4.13	We	stern blotting	72
4.	13.1	Tissue and cell culture lysates	72
4.	13.2	SDS-PAGE and Western blotting	72
4.	13.3	Data analysis	73
4.14	Me	asurement of cellular proliferation and viability	73
4.	14.1	FACS samples preparation	73
4.	14.2	Proliferation and viability analysis	74
4.15	Me	asurement of cellular migration	74
4.	15.1	In vitro scratch assay	74
4.	15.2	Cell migration analysis	75
4.16	Car	ncer associated pathway activity assay	75
4.	16.1	Reverse transfection	75
4.	16.2	Dual-luciferase reporter assay	76
4.	16.3	Cancer associated pathways activity analysis	76

	4.17	Statistics	76
5	RE	SULTS	78
	5.1	Functional analysis of CPEB 1-4 in the pathogenesis of gliomas	78
	5.1.	1 Methylation of CPEB 1-4 genes in the 5'-CpG islands in gliomas	78
	5.1.	2 IDH1 mutation in gliomas	84
	5.1.	3 Expression profile of CPEB 1-4 in gliomas	85
	5.1.	4 <i>CPEB1</i> gene methylation and its influence on expression profile	92
	5.1.	5 Activity dependent expression of CPEB3 protein in gliomas	93
	5.1.	6 Alternative splice isoforms of CPEB1-4 in human gliomas	96
	5.2	Alterations of growth properties and cancer-associated parameters in glioblastoma- derived cells mediated by CPEBs	105
	5.2.	1 Expression profile of CPEB 1-4 in A172 cultured glioblastoma cells	105
	5.2.	2 Functional characterization of CPEB1 and CPEB2 protein overexpression in cultured A172 glioblastoma cells	107
	5.2.	3 Identification of cancer-associated signaling pathways altered by CPEB1 and CPEB2 proteins	111
	5.3	Expression profile of CPEB2 protein in mouse brain	114
	5.3.	1 Expression of CPEB1 and CPEB2 proteins in primary hippocampal cultures	114
	5.3.	2 Expression of CPEB2 protein in juvenile and adult mouse brain	115
	5.3.	3 Differential expression of CPEB2 in excitatory, inhibitory and	
		dopaminergic neurons	118
6	DIS	CUSSION	120
	6.1	The role of CPEBs in development and progression of glioma	120
	6.1.	1 Expression of CPEBs is heterogeneous in human glioma tissues	120
	6.1.	2 CPEB expression patterns associate with clinical prognosis of glioma patients	123
	6.1.	3 Methylation of CPEB1 gene does not correlate with silenced expression	124
	6.1.	4 Expression of CPEB3 and active CPEB3 protein is tissue specific	125
	6.1.	5 Alternative splicing determines the expression pattern and activity of CPEBs	126

6.2	6.2 The impact of CPEBs on growth properties and cancer-relevant parameters			
	in cultured glioblastoma cells	127		
6.2	2.1 CPEB3 protein shuttle between nucleus and cytoplasm	127		
6.2	2.2 Forced overexpression of CPEB1 and CPEB2 alters growth properties and car	ncer-		
ass	sociated parameters of glioblastoma cells	128		
6.2	2.3 Elevated expression of CPEB1 upregulates cancer-associated signaling pathw	ays 129		
6.3	Expression of CPEB2 in different cellular populations, brain regions, and			
	stages of development	130		
7 SU	UMMARY	133		
8 PE	ERSPECTIVE	135		
REFER	RENCES	137		
APPEN	NDIX I	148		

ABBREVIATIONS

°C	degree Celsius	JH-O	distilled water
α-tubulin	alpha tubulin		Dulbecco's modified eagle's medium
β-ΜΕ	β-mercaptoethanol	DMSO	dimethyl sulfoyide
μg	microgram	DNMT	DNA methyltransferases
μl	microlitre	dNTP	deovyribonucleotide phosphate
μΜ	micromolar		Dulhagaa's phosphate huffared saling
4EBP	eIF 4E-binding protein	DIDS	dithiethraitel
AII	diffuse astrocytoma, WHO II		doovuthumiding triphognhate
AAIII	anaplastic astrocytoma, WHO III		ethylene diamine tetra acetic acid
APS	ammonium persulfate	ECED	enhanced groep fluorescent protein
ATP	adenosine triphosphate		enhanced green hubrescent protein
BCA	bicinchonic acid		eukaryotic translation initiation factors 2
Bcl-2	B-cell lymphoma 2		eukaryotic translation initiation factors 5
bp	base pair	CIF4A	eukaryotic initiation factor 4A
BSA	bovine serum albumin	CIF4E	eukaryotic initiation factor 4E
	calcium-calmodulin dependent protein		eukaryotic initiation factor 4F
CaMKII	kinase II	EMT	enithelial to mean shumal transition
cAMP	cyclic adenosine monophosphate		ephrin recentor A 4
cDNA	complementary DNA	ЕрпА4 Ер	endonlogmia ratioulum
CNS	central nervous system	EK	
CPE	cytoplasmic polyadenylation element	EV	extracentular vesicle
0000	cytoplasmic polyadenylation element	FACS	fluorescence-activated cell sorting
CPEB	binding protein	FAM	futurescent amidite
CpG	cytosine-phosphate-guanine dinucleotide	FCS	fetal call serum
	cleavage and polyadenylation specificity	IW	forward primer
CPSF	factor	g CDM	gram
CSC	cancer stem cell	C CIM	glioblastoma multiforme, wHO IV
СТ	threshold cycle	G-CIMP	glioma-CpG Island methylator phenotype
	3,3'-diaminobenzidine-	GFAP	
DAB	tetrahydrochloride	GFP	green fluorescent protein
dATP	deoxyadenosine triphosphate	Gld2	germline development 2 poly(A)
dCTP	deoxycytidine triphosphate		NMDA recentor 24
$_{dd}H_2O$	double-distilled water	h	hour
dGTP	deoxyguanosine triphosphate	11	noui

HEK	human embryonic kidney cell	MEM	minimum essential media		
LEDEC	4-(2-hydroxyethyl)-1-	OS	overall survival		
NEPES	piperazineethanesulfonic acid	р	probe		
HIF-1a	hypoxia inducible factor-1a	PABP	poly(A)-binding protein		
HNF4	hepatocyte nuclear factor 4	PARN	poly(A) ribonuclease		
HRP	horse radish peroxidase		phycoerythrin- cleaved poly		
ICC	immunocytochemistry	PAKP	(ADP-ribose) polymerase, Asp214		
IDH	isocitrate dehydrogenase	PARV	parvalbumin		
IgG	immunoglobulin G	PBGD	porphobilinogen deaminase		
IHC	immunohistochemistry	PBS	phosphate buffered saline		
kDa	kilo Dalton	PCR	polymerase chain reaction		
КО	knockout	PDA	pancreatic ductal carcinomas		
1	liter	PFA	paraformaldehyde		
LAR	luciferase assay reagent	pGBM	primary glioblastoma multiforme		
LTP	long term potentiation	11112	Alexa fluor 647-phospho(Ser10) histone		
m7GpppG	7-methyl-guanosine	рннз	Н3		
М	molar milliampere		phosphatidylinositol-4,5-bisphosphate 3-		
mA		PI3K/AKt	kinase/ protein kinase B		
MAP2	microtubule associated protein 2	РКА	protein kinase A		
MEF	mouse embryonic fibroblast	PLB	passive lysis buffer		
min	minute	poly Q	polyglutamine		
miRNA	micro RNA	ps	pyrosequencing primer		
ml	milliliter	PSD	postsynaptic density protein		
mМ	millmolar	PVDF	polyvinylidene fluoride		
MMP7	matrix metallopeptidase 7	rev	reverse primer		
n	number	RIPA	RNA immunoprecipitation assay		
NDUEVO	ubiquinone oxidoreductase core subunit	RNP	ribonucleoprotein complex		
NDUF V2	V2	rpm	rotations per minute		
Neo	neomycin	RPS6K	ribosomal S6 kinase		
NEAA	non-essential amino acids	RRM	RNA recognition motif		
ng	nanogram	RT	room temperature		
NGS	normal goat serum		reverse transcription polymerase chain		
nM	nanomolar	KI-PCK	reaction		
NMDA	N-methyl-D-aspartate	sec	second		
NP-40	nonidet P-40	SEM	standard error of the mean		
NSC	neural stem cell	SDS	sodium dodecyl sulphate		
oct4	octamer-binding transcription factor 4	sGBM	secondary glioblastoma multiforme		

sqRT-PCR	semi quantitative real-time polymerase	UTR	untranslated region
	chain reaction	v	transcript variant
TBS-T	tris buffered saline with tween 20	V	volt
TEMED	tetramethyl ethylene diamine	WB	Western blot
TGFβ	transforming growth factor β	WHO	World Health Organization
tPA	tissue plasminogen activator	Znf	zinc finger
Tris	tris (hydroxymethyl) aminomethane	ZO-1	zona occludens-1

1 INTRODUCTION

1.1 Gliomas

1.1.1 Cellular origin of gliomas

The cellular origin of gliomas is a matter of investigation. Evidence from glioma mouse models and human clinical data indicate that gliomas are developing from neural stem cells (NSCs), glial progenitor cells or differentiated progeny. The cell of origin has the potential to initiate oncogenic mutations that drive gliomagenesis (Modrek et al., 2014). There are two working hypothesis postulating that cellular heterogeneity and propagation of gliomas is regulated by clonal or cancer stem cell (CSC) model (Reya et al., 2001). The hypothesis of clonal growth of cancer postulates that single cancer cell has the ability to proliferate, self-renew and acquire genetic mutations, thus creating clonally derived subpopulations within the tumor (Schonberg et al., 2014). The CSC model assumes that a population of cells in the tumor possesses stem cell-like properties, such as the ability to self-renew, differentiate and proliferate. CSCs give rise to the cells that after reaching a fully differentiated stage reach limited tumorigenic potential (Schonberg et al., 2014). According to both models, the CSC and the clonal model, glioma cells acquire genetic mutations, accounting for the cellular and genetic heterogeneity (Modrek et al., 2014).

Cells having the ability to form gliomas are widely spread throughout the Central Nerves System (CNS), mostly developing within the cerebral hemispheres (Zong et al., 2012). Some of the glioblastomas occur in the subventricular zone adjacent to the lateral ventricles, while others arise in the subcortical white matter (Bohman et al., 2010; Lim et al., 2007). Region-specific features of the brain microenvironment and properties of the local progenitor population may affect the tumor phenotype (Gibson et al., 2010; Johnson et al., 2010). Distinct cells of origin may give rise to the same pathological manifestations, but it is also possible that different genetic mutations might transform the same cell into

different types of gliomas (Zong et al., 2012). Hence, defining the key driver mutations and cell types giving rise to gliomas is a prerequisite to understand the cancers biology, develop prevention strategies and effective treatments (Modrek et al., 2014; Zong et al., 2012).

1.1.2 Histopathological and molecular classification of gliomas

Gliomas are classified based on histopathological features, with reference to their association with specific glial lineages (Vigneswaran et al., 2015). The principles of histological analysis include findings of nuclear atypia, proliferative activity, microvascular proliferation, and necrosis (Louis, 2006). According to the grading of the World Health Organization (WHO) malignant gliomas are divided into astrocytic, oligoastrocytic, oligodendroglial, and ependymal tumors (Louis et al., 2007). Astrocytomas have morphological similarities with normal and reactive astrocytes, and express the astrocytic marker GFAP (Rousseau et al., 2006). Oligodendrogliomas are related to cells of the oligodendrocyte lineage, specifically oligodendrocyte progenitor cells, which express the markers including Olig2, NG2 and PDGFR α (Riemenschneider et al., 2004; Rousseau et al., 2006; Shoshan et al., 1999; Zong et al., 2012).

Malignant astrocytomas are further classified on the basis of tumor grade into less aggressive, diffuse astrocytomas, defined as WHO grade II (AII), aggressive, anaplastic astrocytomas, defined as WHO grade III (AAIII), and the most aggressive glioblastomas (GBMs) defined as WHO grade IV (Fig. 1.1.2-1) (Zong et al., 2012). Among astrocytomas, GBMs are the most frequent brain malignancies in adults (Louis et al., 2007). Based on clinical presentation GBMs are further classified as primary (pGBM) or secondary (sGBM) (Kim et al., 2013). Secondary GBMs are progressing from lower-grade precursor lesions, whereas primary GBMs develop *de novo* and usually at the time of diagnosis present advanced tumor features. Secondary GBMs are found in younger patients with median diagnosis age of ~45 years and occur less frequently than pGBM, i.e. in ~5% of GBM cases. The median age of diagnosis of pGBM patients is ~60 years (Olar and Aldape, 2012; Vigneswaran et al., 2015).

Due to histopathological similarity, GBMs require an identification of genetic alterations to describe their molecular subtypes (Ohgaki et al., 2004; Phillips et al., 2006; Verhaak et al., 2010). Phenotyping at a genome-wide level expanded the classification of gliomas by further dividing morphologically identical tumors into four subtypes, including classical, proneural, neural, and mesenchymal tumors (Verhaak et al., 2010; Vigneswaran et al., 2015; Zong et al., 2012). Secondary GBMs are usually classified as proneural, whereas primary GBMs may be of any of the subtypes (Cohen et al., 2013).



Figure 1.1.2-1. Simplified classification of gliomas based on WHO Classification of Tumors of The Central Nervous System (Louis et al., 2007). Classification is based on findings of histopathological features of cancer cells, including nuclear atypia, proliferative activity, microvascular proliferation, and necrosis. Figure adapted from the WHO Classification of Tumours of The Central Nervous System System (Louis et al., 2007).

The new WHO Classification of Tumors of The Central Nervous System (Louis et al., 2016) introduces molecular and cytogenetic information to assist glioma classification (Fig. 1.1.2-2). Detection of mutations in isocitrate dehydrogenase (IDH) (Bleeker et al., 2009; Parsons et al., 2008) is currently a prerequisite for tumors classification. The wild-type IDH1 catalyzes the oxidative decarboxylation of isocitrate to α -ketoglutarate. Whereas the mutant protein, with arginine 132 altered to histidine, converts α -ketoglutarate to R(-)-2-

hydroxyglutarate (Dang et al., 2010; Jin et al., 2011). Accumulation of 2-hydroxyglutarate, which acts as an oncometabolite, inactivates oxygenases (histone demethylases and TET 5'-methylcytosine hydroxylases) and leads to significant modifications in methylation profile, length of telomeres and gene expression (Cohen et al., 2013; Garber, 2010). Histologic assessment supported by genetic and epigenetic analysis in consequence produces more accurate and reproducible diagnostic criteria (Vigneswaran et al., 2015).



Figure 1.1.2-2. The new WHO Classification of Tumors of The Central Nervous System (2016). In contrast to the old grading, the new classification is based not only on histopathological features, but also includes IDH status and other genetic alternations. The IDH1 refers to isocitrate dehydrogenase 1, ATRX to alpha-thalassemia/mental retardation syndrome X-linked, and NOS to not otherwise specified. Figure adapted from the WHO Classification of Tumours of The Central Nervous System (Louis et al., 2016).

1.1.3 Microenvironment of glioblastoma

Solid tumors exist as tightly connected entities depending on their cellular environment. Individual cells may adapt to the local environment, but also change the surrounding to accommodate their own needs. For this reason, a complex communication involving interaction between tumor cells and non-malignant neighboring cells is required (Godlewski et al., 2015). GBMs are predominantly composed of cells resembling immature glia (Zong et al. 2013). The heterogeneous GBM tissue consists of tumor cells, surrounding blood vessels, immune cells, and extracellular matrix. Additionally, its structure includes stem cell-like cells and parenchymal cells. Tumor-associated non-neoplastic parenchymal cells include vascular cells, microglia, peripheral immune cells, normal astrocytes and neural precursor cells that play an essential role in cell-cell communication. The vasculature supports GBM cells with nutrients, oxygen and provides a specialized niche for stem-cell like cells. Microglia contribute to the tumor mass and support cell invasion. Normal astrocytes not only can be transformed into reactive cells under the pressure of the environment, but also can secrete a number of factors that alter tumor biology (Fig. 1.1.3-1). Altogether, cytokines, growth factors, and chemokines released in extracellular vesicles (EV) may support tumor initiation, angiogenesis, proliferation, and invasion (Pollard et al., 2004; D'Asti et al., 2016), thus providing an additional level of complexity and network communication (Godlewski et al., 2014).



Figure 1.1.3-1. The microenvironment of glioblastoma. Tumor surrounding is composed of specialized cell types, which may support tumor growth or invasion. Different cell types from the tumor microenvironment communicate both locally and at distant ranges via the release and uptake of EVs. Such communication can contribute to tumor progression by transferring bioactive molecules. Figure from Godlewski et al., 2014.

1.1.4 Implications for diagnosis and therapy

Gliomas characterized by an astrocytic phenotype have a poorer prognosis than oligodendroglias of a corresponding grade (Ohgaki et al., 2004). Genetic alterations associated with good predictions, such as loss of heterozygosity of 1p19q (Barbashina et al., 2005), IDH1 mutation (Yan et al., 2009) and glioma-CpG island methylator phenotype (G-CIMP) (Noushmehr et al., 2010) are also more common in oligodendrogliomas and sGBMs (Zong et al., 2012). Among gliomas, the most common and lethal CNS tumors are GBMs. Despite surgical resection and aggressive treatment the prognosis for GBM patients is typically very poor. In the United States, the GBM incidence rate is 3.19/100000 people (Thakkar et al., 2014). The mean survival for GBM patient under currently available treatment is approximately 14.6 months from the time of diagnosis. The therapy commonly

includes surgical resection, radiotherapy and adjuvant temozolomide application (Stupp et al., 2005).

The failure of conventional approaches in curing GBM indicates the need for novel therapies, which target the cells of origin (Zong et al., 2012) or residual tumor cells (Wilson et al., 2014). Until now, molecule-targeted therapies, including inhibition of growth factor, and intracellular signaling pathways provided very limited or no therapeutic benefit. Similarly, immunotherapy or gene therapy, including cytotoxic gene therapies, or oncolytic viral vectors demonstrated minimal efficacy (Wilson et al., 2014). To understand the complex biology of GBMs, a variety of therapeutic approaches were undertaken but despite promising results in the preclinical phase, the therapies had limited or no effects in clinical trials (Wilson et al., 2014). Therefore, treatment of GBMs remains highly challenging.

1.2 Translational control by CPEBs

1.2.1 **Principles of translation**

Translation of mRNAs is tightly regulated in three major phases: initiation, elongation and termination (D'Ambrogio et al., 2013; Groppo and Richter, 2009). Regulation of translation efficiency is predominantly controlled at the initiation phase and involves eukaryotic translation initiation factors (eIFs) and other auxiliary proteins interacting with eIFs and with mRNAs. Initiation factors assemble on the 7-methyl-guanosine (m7GpppG) cap structure at the 5'end of RNAs (Sonenberg and Hinnebusch, 2009) and form the eIF4F complex. The complex is assembled with eIF4E, a cap binding factor, eIF4G, a scaffold protein interacting with eIF4E, eIF4A, an RNA helicase, and eIF3, a factor binding eIF4G. The integrated activity of eIFs recruits the 40S ribosomal subunit on the 5'end of the mRNA and allows formation of the pre-initiation complex (Dever, 2002).

Upon overexpression, one of the initiation factors, eIF4E elicits the preferential translation of mRNAs with long-structured 5'untranslated regions (UTRs) (D'Ambrogio et al., 2013) that usually encode growth factors and proto-oncogenes, like c-myc or cyclin D1 (Sonenberg, 1993). Knowing that eIF4E initiates the translation of proto-oncogenes

(Sonenberg, 1993), its excess is strongly correlated with cancer etiology (Lazaris-Karatzas et al., 1992; Ruggero et al., 2004). Proto-oncogenic activity of eIF4E is controlled by eIF4E-binding proteins (4EBPs). The 4EBPs impair association of the 40S subunit to the cap structure of mRNAs (Teleman et al., 2005) and counteract recruitment of eIF4G (Darnell and Richter, 2012). 4EBPs mimic eIF4G, bind the eIF4E, and as a result interrupt the eIF4E-eIF4G interaction, which downregulates the translation of many mRNAs (Richter and Sonenberg, 2005). Therefore, the key components of the translational machinery involved in the initiation step may either promote or suppress cancer formation. Translational control is additionally regulated by auxiliary mRNA-binding proteins. One of such group of factors is the family of CPEBs (D'Ambrogio et al., 2013).

1.2.2 **CPEBs**

Cytoplasmic polyadenylation element binding proteins (CPEBs) are translational factors that associate with consensus sequences present in 3'UTRs of mRNAs and regulate their translation (Darnell and Richter, 2012; Richter, 2007). The family of CPEBs in vertebrates comprises four genes. Each member of the family consists of an N-terminal regulatory domain and a C-terminal RNA binding domain (Fig. 1.2.2-1) (Kaczmarczyk et al., 2016; Theis et al., 2003). Although all CPEBs have two RNA recognition motifs (RRM), and two zinc fingers (ZnF), vertebrate CPEB 2-4 are more closely related to each other, forming a subfamily (Huang et al., 2006; Mendez and Richter, 2001), whilst CPEB1, the founding member of the family, is evolutionarily most distinct. CPEBs were first discovered in Xenopus laevis oocytes, where they control meiosis (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). CPEB 2-4 display ~98% sequence similarity in RRMs and phosphorylation sites (Theis et al., 2003). In addition, these proteins possess common splicing patterns (Wang and Cooper, 2010), similar miRNA regulatory motifs (Morgan et al., 2010) and overlapping mRNA targets, between themselves and CPEB1 (Fernández-Miranda and Méndez, 2012; Igea and Méndez, 2010; Novoa et al., 2010; Theis et al., 2003; Turimella et al., 2015).



Figure 1.2.2-1. Structural relationships between CPEBs. CPEBs consist of an N-terminal regulatory domain and a C-terminal RNA binding domain containing two conserved RNA recognition motifs, two zinc-fingers, and poly Q regions corresponding to polyglutamine stretches. CPEBs with identically marked RRMs indicate strong similarity. Figure adapted from Ivshina et al., 2014.

1.2.3 Regulation of polyadenylation-induced translation

Cytoplasmic polyadenylation begins in the nucleus, where CPEB binds into uracil-rich (UUUUUAU, or similar) cytoplasmic polyadenylation elements (CPE) present in the 3'UTR of pre-mRNAs. Like most of the nuclear pre-mRNAs, the CPEB bound mRNAs have ~100 nucleotides long poly(A) tails (Lin et al., 2010). The cleavage and polyadenylation specificity factor (CPSF) associates with the AAUAAA sequence of mRNA and together with CPEB and Maskin shuttle to the cytoplasm (Lin et al., 2012). Following export, CPEBs recruit a number of molecules including symplekin, a scaffold protein, Gld2, a germline development 2 non-canonical poly(A) polymerase and PARN, a poly(A) ribonuclease to form the ribonucleoprotein (RNP) complex (Fig. 1.2.3-1) (Darnell and Richter, 2012; Kim and Richter, 2006).



Figure 1.2.3-1. Polyadenylation-induced translation regulated by CPEB1. Cytoplasmic polyadenylation begins in the nucleus, where CPEB1 binds to the CPE elements present in 3'UTR of pre-mRNA. CPSF associates with the AAUAAA nuclear pre-mRNA cleavage and polyadenylation site. CPEB1 recruits RNP complex molecules, including symplekin, Gld2 polymerase, PARN ribonuclease and Maskin containing 4EBP activity. PARN activity shortens the long poly(A) tails added to the mRNA in the nucleus. Whereas Maskin binds eIF4E at the eIF4G binding site and thus inhibits translation initiation. Figure from D'Ambrogio et al., 2013.

Both of the RNP complex enzymes, PARN deadenylase and Gld2 polymerase are catalytically active. When the robust PARN activity exceed the activity of Gld2 polymerase, it shortens the poly(A) tails on CPE-containing RNAs to 20-40 nucleotides, thereby locks mRNAs in a dormant state (Kim and Richter, 2006). Hormonal stimulation leads to Aurora kinase A activation and phosphorylation of CPEB1 on serine 174 (Mendez et al., 2000a; Sarkissian et al., 2004). These modifications result in expulsion of PARN deadenylase from the RNP complex (Kim and Richter, 2006). As a consequence, Gld2 catalyzes polyadenylation and poly(A)-binding protein (PABP) associates with the newly elongated poly(A) tail (Barnard et al., 2004; Kim and Richter, 2006, 2007). The length of the poly(A) tail is correlated with the ribosome density and association of PABPs. Changes in the RNP complex potentiate the assembly of the initiation complex at the 5'end of the mRNA at the expense of the Maskin-eIF4E interaction (Fig. 1.2.3-2) (Cao and Richter, 2002; Kim and Richter, 2007). Phosphorylation of Maskin allows for its dissociation from eIF4E and beginning or resuming translation (Cao et al., 2006). Cytoplasmic polyadenylation takes place in sequential waves and is synchronized with the partial

destruction of CPEB, the number and location of CPEs within a 3'UTR, and the presence of other RNA binding proteins (Ivshina et al., 2014).



Figure 1.2.3-2. Polyadenylation-induced translation regulated by CPEB1. The RNP complex remains dormant until cell stimulation. Stimulation activates Aurora kinase A, which further phosphorylates CPEB1. PARN is removed from the complex and that allows Gld2-mediated polyadenylation. PABP and eIF4G attach to the newly elongated poly(A) tail. eIF4G replaces Maskin from eIF4E and, through the eIF3 complex, shifts the 40S ribosomal subunit to the 5' end of the mRNA. Figure from D'Ambrogio et al., 2013

1.3 Role of CPEBs in germline and somatic cells

1.3.1 CPEBs in cell cycle progression

In vertebrates, meiotic cell divisions occur in the absence of transcription, but depend on translational control of mRNAs. Many of these mRNAs may undergo regulation by CPEBs (Novoa et al., 2010). In *Xenopus* oocytes, CPEBs mediate both, meiotic progression and mitotic divisions. For instance, entry into the M phase is guided by CPEB1 phosphorylation by Aurora kinase A, while switch from M to S phase is regulated by CPEB1 dephosphorylation catalyzed by protein phosphatase 2A (Cao et al., 2006; Groisman et al., 2002). As oocyte maturation is similar to the somatic cell cycle (Liu and Maller, 2005; Peng and Maller, 2010) apart from meiosis, CPEBs were detected in mitosis of mammalian cells (Giangarrà et al., 2015; Groisman et al., 2002; Novoa et al., 2010). Based on this

finding, cytoplasmic regulation of the poly(A) tails length is not only needed to compensate for the lack of transcription in cell divisions but is a general mechanism of controlling cell cycle progression (Novoa et al., 2010). In addition to CPEB1, CPEB2 and CPEB4 were recently found to be necessary in the phase-specific polyadenylation and translational activation in the mitotic cell cycle. Accordingly, CPEB1 is essential for entrance into prophase, CPEB2 for metaphase and CPEB4 for cytokinesis (Giangarrà et al., 2015).

1.3.2 **CPEBs in metabolism and senescence**

In contrast to normal cells that generate energy required for cellular processes by mitochondrial oxidative phosphorylation, cancer cells rely on anaerobic glycolysis (Vander Heiden et al., 2009). Human primary fibroblasts with reduced CPEB1 expression demonstrated lower mitochondria number, which resulted in decreased respiration rates. Interestingly, ATP content remained unchanged. To maintain constant ATP level, cells increased glycolysis while reducing oxygen consumption. This process, known as the Warburg effect, is characteristic for transformed cancer cells (Burns and Richter, 2008). Metabolic balance between mitochondrial respiration and glycolysis is mediated by mRNAs containing CPEs, such as *p53* and *c-myc* (Burns and Richter, 2008; Groisman et al., 2006). Therefore, knockout of CPEB1 in mouse embryonic fibroblasts (MEF) results in reduced polyadenylation and translation of p53 mRNA that subsequently renders cells immortal and escaped senescence (Groisman et al., 2006; Ivshina et al., 2014).

During increased oxidative stress CPEB1 and CPEB2 bind to the hypoxia-inducible factor-1 α (HIF-1 α) mRNA and regulate its expression (Hägele et al., 2009). HIF-1 α regulates homeostatic responses to oxidative stresses, by stimulating transcription of genes involved in angiogenesis, metabolism and cell survival. At normal oxygen concentration, HIF-1 α is continuously synthesized at a reduced level and undergoes proteasome-mediated degradation. This type of protein synthesis confirms that HIF-1 α mRNA remains ribosomeassociated, thereby promoting quick responses to stress. Under hypoxia or increased oxidative stress, the level of HIF-1 α is immediately increased (Chen and Huang, 2012; Chen et al., 2015). Thus, by regulating HIF-1 α (Chen et al., 2015) or p53 (Burns and Richter, 2008) CPEBs mediate important alterations in cellular metabolism.

1.3.3 CPEBs in cancer

CPEBs mediate control of cellular senescence, proliferation, and migration (Fernández-Miranda and Méndez, 2012; Jones et al., 2008), thus alterations in their expression are crucial for malignant transformation. Recently published meta-analysis shows the extensive correlation between the level of CPEB mRNA expression and human cancers (Fig. 1.3.3-1) (D'Ambrogio et al., 2013). CPEB expression is downregulated in many tumors, affecting the reproductive and digestive system, head and brain, including gliomas. Transcript expression level of CPEB1, CPEB2 and CPEB3 appears to be reduced in gliomas and only CPEB4 expression is upregulated in oligo-lineage tumors (D'Ambrogio et al., 2013).

Downregulation of CPEB1 was observed in several types of human tumors, including ovarian and gastric, as well as in breast-, myeloma- and colorectal cancer-derived cell lines (Caldeira et al., 2012; Hansen et al., 2009; Heller et al., 2008), where it was associated with the capacity of malignant cells to promote invasion and angiogenesis (Caldeira et al., 2012). Moreover, by mediating apical localization and translation of zona occludens-1 (ZO-1) mRNA, CPEB1 participates in maintaining the polarity of mammary epithelial cells. In the absence of CPEB1, a randomly distributed ZO-1 mRNA cause the loss of cell polarity (Nagaoka et al., 2012) that further leads to an epithelial-to-mesenchymal transition (EMT) (Nagaoka et al., 2016). This often results in cell dedifferentiation and metastasis (Tam and Weinberg, 2013). Therefore, deprivation of CPEB1 in mammary epithelial cells changes the gene expression profile and increases its metastatic potential (Nagaoka et al., 2016).



Figure 1.3.2-1. CPEB expression in cancers. Blue color indicates downregulation, while red color indicates upregulation of CPEB mRNA levels in cancer after comparison with reference samples. Figure adapted from the D'Ambrogio et al., 2013.

Thus far, CPEB2 was found to be involved in the progression of breast cancer. One study showed that CPEB2 downregulates *TWIST1*, a transcription factor that supports epithelial to mesenchymal transition, during oncogenesis but not in metastatic cells (Nairismägi et al., 2012). Moreover, CPEB2 regulates metastatic potential of human triple negative breast cancer (TNBC) cells (Johnson et al., 2015). The high metastatic potential of TNBC cells is especially correlated with increase in expression of CPEB2 isoform lacking the B-region. Downregulation of the CPEB2b induces cell death, whereas overexpression of the CPEB2b increases metastatic potential of TNBC cells (Johnson et al., 2015).

Cancer etiology is also mediated by CPEB4. Its expression is increased in GBMs and pancreatic ductal carcinomas (PDA). PDA tumors have particularly elevated translation of tissue plasminogen activator (tPA) mRNA that is regulated by CPEB4. tPA is absent in normal pancreas but overexpressed in most of PDA, where it supports tumor vascularization and cells proliferation, migration, and invasion. The expression of CPEB4 is absent in normal astrocytes, but abundant in high-grade gliomas. CPEB4 mediates tumor growth and vascularization in GBMs. Downregulation of CPEB4 levels results in reduction of tumor size, cellular proliferation and microvessel density (Ortiz-Zapater et al., 2011; Fernández-Mirandaa and Méndez, 2012). Other mRNAs linked to tumorigenesis regulated by CPEB4 include Smad3, B-cell lymphoma 2 (Bcl-2), and matrix metallopeptidase 7 (MMP7) (Ortiz-Zapater et al., 2011). Therefore CPEB4 seems to have a significant role in the development of tumors, and might be the element of a more general mechanism of carcinogenesis.

In summary, all CPEBs are involved in growth of cancer, but it is noteworthy that CPEB3 is not well investigated, yet. Furthermore, although CPEB isoform are structural very similar, they are functionally different (Ivshina et al., 2014).

1.3.4 **CPEBs in the nervous system**

Long-term memory formation takes place in the hippocampus and requires new protein synthesis to modulate synaptic plasticity (Kang and Schuman, 1996). Synaptic plasticity is the ability of synapses to undergo morphological and biochemical changes in response to stimulation (Kandel, 2001; Mayford et al., 2012; Richter and Klann, 2009; Sutton and Schuman, 2006). Then the newly synthesized proteins either constitute the synaptic tags or influence synaptic activity (Ivshina et al., 2014).

At the postsynaptic sites of hippocampal neurons reside mRNA molecules. Their translation might be initially repressed by CPEB binding, and activated in response to synaptic stimulation (Huang et al., 2002; Richter, 2001; Udagawa et al., 2012; Wu et al., 1998). CPEB1, Gld2, and Neuroguidin comprise a complex that regulates mRNA translation at synapses and, thereby, synaptic efficacy. Several important mRNAs undergo activity-dependent polyadenylation, including calcium-calmodulin protein kinase II (CaMKII), tissue plasminogen activator (tPA) or GluN2A (N-methyl-D-aspartate (NMDA) receptor subunit) that is crucial for synaptic plasticity (Du and Richter, 2005; Shin et al., 2005; Wu et al., 1998).

CPEB1 represses translation until glutamatergic activation initiates its phosphorylation by either Aurora kinase A (Huang et al., 2002; Mendez et al., 2000a) or CaMKII (Fig. 1.3.4-1) (Atkins et al., 2004, 2005). Upon stimulation, CPEB1 induces long-term potentiation, GluN2A mRNA translation (Udagawa et al., 2012) and the latter is inserted into the synaptic membrane as an NMDA receptor subunit (Swanger et al., 2013). The CPEB1 protein is responsible for dendritic transport of mRNAs. Therefore, CPEB1 associates with the motor proteins, kinesin and dynein and transport curtail mRNAs as CaMKII in into dendrites (Huang et al., 2003; Ivshina et al., 2014). The contribution of CPEB1 to local protein translation is confirmed by its localization in dendrites (Wu et al., 1998) and enrichment of phospho-CPEB1 in a fraction of postsynaptic density of neurons (Atkins et al., 2004, 2005; Darnell and Richter, 2012).

Knockout (KO) of CPEB1 activity in mouse models leads to defects in synaptic plasticity, learning, and memory (Alarcon et al., 2004; Berger-Sweeney et al., 2006; Darnell and

Richter, 2012). However, KO of another CPEB, CPEB3 results in enhancement of hippocampus-dependent learning. This process is possibly induced by expression of plasticity-regulating molecules, including PSD-95 and the NMDA receptor subunit GluA1, previously shown to be regulated by CPEB3 (Huang et al., 2006). KO of CPEB4 has no effect on hippocampal plasticity or on learning and memory (Ivshina et al., 2014; Tsai et al., 2013).



Figure 1.3.4-1. Impact of CPEB1 in neurons. The RNP complex containing CPEB1 is transported in dendrites along microtubules by the motor proteins - kinesin and dynein. Synapse stimulation and cytoplasmic polyadenylation initiate GluN2A, CaMKII and NDUFV2 activation. GluN2A encodes an NMDA receptor subunit, CaMKII, is associated with the postsynaptic density and has kinase activity while NDUFV2 is a part of mitochondrial electron transport chain that supplies ATP. Figure from Ivshina et al., 2014.

30

1.4 Alterations regulating expression and activity of CPEBs

1.4.1 **DNA methylation**

Heritable alterations in gene function that occur without modification in the DNA sequence are called epigenetic changes. To the major epigenetic mechanisms belong: DNA methylation, histone modifications, and RNA-mediated gene silencing (Sharma et al., 2010). Methylation is the most common covalent modification of DNA in eukaryotes that plays an important role in biological processes, including genomic imprinting (Wilkins, 2005), aging (Jung and Pfeifer, 2015) and cancerogenesis (Chen et al., 2014). DNA methylation occurs at cytosine residues, in the cytosine-phosphate-guanine (CpG) dinucleotides (Fig.1.4.1-1) (Weber et al., 2007). CpG dinucleotides are concentrated in the genome in the CpG-rich DNA fragments called CpG islands, which are clustered around gene regulatory regions (Weber et al., 2007; Yamada et al., 2004).



Figure 1.4.1-1. Cytosine methylation pathway. Methylation of DNA occurs at cytosine residues, in CpG dinucleotides. Upon a reaction catalyzed by the DNA methyltransferases, cytosine is converted to 5-methyl cytosine. As a result the methyl group donor, S-adenosylmethionine, is transformed into S-adenosylhomocytosine.

Methylation is catalyzed by DNA methyltransferases (DNMT), including DNMT1, DNMT3A and DNMT3B. DNMT1 is required to maintain the methylation status, while DNMT3A and DNMT3B are needed for *de novo* DNA methylation during the embryo formation process (Bernstein et al., 2007; Chen et al., 2014). Generally, methylation takes place during DNA replication. Following replication, DNMT1 (Goll and Bestor, 2005) complements the missing methylation on the newly synthesized strand. It allows

maintenance of DNA methylation patterns through many rounds of cell division (Zilberman and Henikoff, 2007). Methylation does not alter nucleotide sequences and does not affect the specificity of DNA base pairing (Chen et al., 2014).

In normal cells, regions of the gene promoter containing CpG islands are commonly not methylated, while coding regions are often methylated. This is to sustain the transcriptionally active euchromatin structure (Fig. 1.4.1-2). A reverse methylation pattern is observed in cancer. Cancerous cells undergo alterations in promoter methylation that result in abnormal gene expression and a malignant phenotype. Genomic hypomethylation of proto-oncogenes usually results in genome instability and their enhanced expression (Ehrlich, 2002). In contrast, local promoter hypermethylation results in functional silencing of tumor-associated genes. Enhanced methylation at the promoter region results in their inactivation by change in the open euchromatin conformation to a compact heterochromatin structure (Fig. 1.4.1-2) (Chen et al., 2014). Therefore, DNA hypermethylation suppresses the activity of genes transcription though blocking the binding of a transcription factor (Herman and Baylin, 2003; Watt and Molloy, 1988), or by recruiting methylation binding proteins that support inhibition of gene expression (Nan et al., 1998).



Figure 1.4.1-2. DNA methylation in normal and cancer cells. In normal cells, regions of the gene promoters containing CpG islands are commonly not methylated. This is to sustain the transcriptionally active euchromatin structure. Cancer development drives hypermethylation of many genes. Increased methylation at the promoters regions containing CpG islands results in their inactivation. Through changing of the open euchromatin conformation to a compact heterochromatin structure expression of these genes is silenced. On the illustration above, cytosine residues are marked with C, while 5-methyl cytosine residues are marked with M. Figure from Chen et al., 2014.

1.4.2 Alternative splicing

The process of removing introns and joining exons in pre-mRNA complexes is known as splicing. RNA splicing is controlled by splice sites, the specific sequences present at the intron-exon borders, and carried out by spliceosomes, the complexes that assemble around splice sites at introns and catalysis the splicing reaction (Wessagowit et al., 2005). Depending on the similarity to the canonical splicing consensus sequences, splice sites are considered to be strong or weak (Kornblihtt et al., 2013). The strong splice sites allow identification of consensus sequences that leads to the constitutive splicing, while the weak splice sites are suboptimal for consensus sequences and their use depends on the cellular context (Wessagowit et al., 2005). The proximity of competing weak and strong sites along the pre-mRNA allows the synthesis of numerous gene transcripts with different properties (Kornblihtt et al., 2013). Therefore, alternative splicing of pre-mRNA molecules forms an additional level of regulation that occurs between transcription and translation (Berget et al., 1977; Chow et al., 1977). This process tightly controls expression of multiple mRNA variants from a single gene and determines signal transduction and chromatin modification (Kornblihtt et al., 2013). However, in the cell itself this is not clearly separated because splicing and alternative splicing are combined with transcription, thus factors that regulate transcription also affect alternative splicing (Kornblihtt et al., 2013).

All four *CPEB* genes are subject to alternative splicing (Theis et al., 2003; Turimella et al., 2015; Wang and Cooper, 2009, 2010), however the biological importance of this phenomenon is not fully known. Alternative splicing leads to differences in the protein sequence, affecting further their function and altering where, and how CPEBs bind with their target mRNAs (Wang and Cooper, 2010). Alternative splicing of CPEBs is

particularly relevant in the context of cytoplasmic polyadenylation. This is because the alternatively spliced regions contain regulatory phosphorylation sites (Kaczmarczyk et al., 2016; Skubal et al., 2016; Theis et al., 2003; Turimella et al., 2015).

Regions of high similarity between CPEB 2-4 are found in the 8-aa B-region. Less similarity is observed in the 17-30-aa C-region. The hexamer and octamer sequences present in the RRMs, as well as the linkers between RRMs are similar, thus it is possible that CPEB 2-4 share the same regulatory mechanisms and target similar populations of RNAs (Fig. 1.4.2-1). Compared to CPEB 2-4, CPEB1 demonstrates significant differences within the described domains. This implies that CPEB1 may not only be involved in different mechanisms for RNA interaction, but also recognizes different targets (Wang and Cooper, 2010). CPEB1 harbors a site for alternative splicing in the RRM. For CPEB1, the full-length isoform (Gebauer and Richter, 1996) and the isoform with 5-aa deletion in RRM1 (Δ 5) (Wilczynska et al., 2005) have been described. The N-terminal regulatory domain of CPEB 2-4 harbor regions of alternative splicing and give rise to multiple splice isoforms. Alternative splicing of CPEB 2-4 results in either inclusion or removal of the Cand B-region and gives rise to four splice variants including a (full length), b (lacking the B-region), c (lacking the C-region), and d (lacking both regions) (Theis et al., 2003; Wang and Cooper, 2010). Recent studies report that CPEB2 contains and additional 3-aa long region that undergoes alternative splicing, however its function is unknown (Turimella et al., 2015).



Figure 1.4.2-1. Comparison of mouse CPEB 1-4 containing regions undergoing alternative splicing. The alternatively spliced 17-30-aa long regions are marked in blue, 8-aa in red, 9-aa in orange. The underlined sequences refer to RRMs. The RRMs regions labeled in grey are consensus hexamer and octamer sequences. Asterisks mark perfect matches, colons indicate substitutions with similar amino acids, and gaps represent substitutions with distinct amino acids. Figure from Wang and Cooper, 2010.

1.4.3 **Phosphorylation**

Phosphorylation has been previously shown to regulate the activity of CPEBs (Theis et al., 2003). In *Xenopus* oocytes, CPEBs undergo phosphorylation by Aurora kinase A on serine 174, which subsequently triggers cytoplasmic polyadenylation and translation (Kim and Richter, 2006; Mendez et al., 2000b). In mouse brain, upon NMDA receptor activation, CPEB1 is phosphorylated and activated by Aurora A or CaMKII kinase (Tay and Richter, 2001; Tay et al., 2003; Hodgmanet al., 2001; Kaczmarczyk et al, 2016). The structural and functional differences that influence CPEB phosphorylation are generated during alternative splicing (Wang and Cooper, 2010). The splice variants of CPEB 2-4 differ

between each other by the presence or absence of B- and C-regions (Fig. 1.4.3-1). Especially important is the B-region located upstream of the two serine residues, that harbors the kinase recognition sites for cyclic AMP-dependent protein kinase A (PKA), protein kinase B (PI3K/Akt), ribosomal S6 kinase (RPS6K) and CaMKII (Kaczmarczyk et al., 2016; Theis et al., 2003; Wang and Cooper, 2010).



Figure 1.4.3-1. Comparison of CPEB3 isoforms in mouse brain. The CPEB3 isoform differs by the presence or absence of the alternatively spliced B- and C-regions. Splicing results in expression of four isoforms, including CPEB3a (containing both regions), CPEB3b (lacking the B-region), CPEB3c (lacking the C-region), and CPEB3d (lacking both regions). Phosphorylated residues S419 and S420 are marked by an asterisk, the consensus phosphorylation sites for PKA and CaMKII kinase are labeled above the alignment. Figure from Kaczmarczyk et al., 2016.
2 AIM OF THE STUDY

The present study aimed at elucidating principles of translational regulation by CPEBs and their function in the healthy brain and in tumor tissue. Three main aspects were investigated in detail.

Expression of CPEBs in the context of human glioma pathogenesis recently became an object of intensive investigation, and first reports already pre-described CPEB expression patterns (Galardi et al., 2016; Hu et al., 2015). However, the question how CPEB expression is regulated in gliomas remains largely unanswered. Therefore, the first part of the present work aimed at determining CPEB expression patterns in high- and low-grade human gliomas and assessing a potential contribution of CPEBs to the survival of glioma patients. Selected mechanisms involved in the regulation of CPEBs, such as methylation, alternative splicing and phosphorylation were subjected to further analysis. DNA methylation was considered due to its role in transcriptional silencing of tumor suppressor genes (Baylin, 2005), while analysis of phosphorylation and alternative splicing were relevant in the context of cytoplasmic polyadenylation (Kaczmarczyk et al., 2016).

Although altered expression of CPEBs in gliomas unequivocally indicates the importance of translational regulation in the brain tumors, the exact impact of CPEB dysregulation on GBM cells is unclear. Therefore the second part of the present study aimed at investigating the relationship between CPEB activity, growth properties and cancer-relevant parameters in a simplified cell culture model. The answer to the question whether overexpression of CPEB1 and CPEB2 in cultured GBM cells is associated with the change of cancer-related signaling pathways may help identifying new putative CPEB targets. Likewise, inquiring the effect of potential CPEB1 and CPEB2 overexpression on migration, apoptosis and proliferation may provide important information about the characteristics of altered CPEB expression in GMB cells.

All members of CPEB family are expressed in the mouse hippocampus, a region crucial for memory formation (Theis et al., 2003). In this context, CPEB1 was implicated in regulation of the local protein synthesis (Theis et al., 2003; Udagawa et al., 2012). However, beyond a general description, not much is known about the function of other family members. The main objective of the final part of the study was to determine the CPEB2 expression profile in mouse brain. Additionally, the study intended to validate whether CPEB2 subcellular localization resembles CPEB1 expression in primary hippocampal cultures and whether CPEB2 expression varies between different cellular populations, brain regions, and stages of development.

3 MATERIALS

3.1 Antibodies

Table 3.1-1. Primary antibodies

antigen	host	type	concentration	application	company	catalogue no.
CPEB1	Rb	Р	1:100	ICC, IHC,	Eurogentec,	custom-made
				WB	Cologne	
CPEB2	Rb	Р	1:250 _{(ICC),}	ICC,	Eurogentec,	custom-made
			1:50 (IHC, WB)	IHC, WB	Cologne	
CPEB3	Rb	Р	1:100	ICC, IHC,	Abcam,	ab10833
				WB	Cambridge	
CPEB4	Rb	Р	1:250	ICC, IHC	Eurogentec,	custom-made
					Cologne	
GFP	Ch	Р	1:500	IHC	Abcam,	ab13970
					Cambridge	
MAP2	Ms	М	1:100	IHC	Sigma,	M4403
					Saint Louis	
PARP	Ms	М	1:5	FACS	BD Biosciences,	552933
					San Jose	
PARV	Ms	М	1:250	IHC	Millipore,	MAB1572
					Temecula	
РКА	Rb	Р	1:500	IHC	Abcam,	ab59218
					Cambridge	
рНН3	Rb	Р	1:20	FACS	Cell Signaling,	9716
					Danvers	
antigen	host	type	concentration	application	company	catalogue no.
phospho-	Rb	М	1:1000	IHC	Cell Signaling,	3361

CaMKII					Danvers	
phospho-	Rb	Р	1:50	ICC, IHC,	Eurogentec,	custom-made
CPEB3				WB	Cologne	
ТҮН	Ms	М	1:500	IHC	Millipore,	MAB318
					Temecula	

Ch: chicken; M: monoclonal antibody; Ms: mouse: P: polyclonal antibody; PARP: anti-cleaved poly(ADP-ribose) polymerase (*Asp214*); *PARV: anti-parvalbumin; pHH3: phospho-histone H3* (*Ser10*); *Rb: rabbit; TYH: anti-tyrosine hydroxylase*

Table 3.1-2. Custom primary antibodies

antigen	peptide sequence
CPEB1	RGIHDQLPDFQDSEETVT
CPEB2	LQLPAWGSDSLQDSWC
CPEB4	KPPSPWSSYQSPSPTP
phospho-CPEB3	RRGRSSLFPFED

Table 3.1-3.	Secondary	antibodies
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antigen	host	concentration	application	company	catalogue no.
Alexa 488	goat anti-	1:500	ICC	Invitrogen,	A11006
	mouse			Rockford	
Alexa 488	goat anti-	1:500	ICC	Invitrogen,	A11034
	rabbit			Rockford	
antigen	host	concentration	application	company	catalogue no.
			••	1 2	
				1 2	
Alexa 568	goat anti-	1:500	ICC	Invitrogen,	A11031
Alexa 568	goat anti- mouse	1:500	ICC	Invitrogen, Rockford	A11031
Alexa 568 Alexa 568	goat anti- mouse goat anti-	1:500 1:500	ICC	Invitrogen, Rockford Invitrogen,	A11031 A11011

Alexa 594	goat anti-	1:500	ICC	Invitrogen,	A11032
	mouse			Rockford	
Alexa 594	goat anti-	1:500	ICC	Invitrogen,	A11037
	rabbit			Rockford	
IgG HRP	goat anti-	1:10 000	WB	GE Healthcare,	31430
	mouse			Amersham	
IgG HRP	goat anti-	1:10 000	WB	GE Healthcare,	31460
	rabbit			Amersham	

3.2 Cell cultures

3.2.1 Reagents

Dulbecco's Modified Eagle Medium with 25 mM glucose (DMEM)	Gibco, Darmstadt
Dulbecco's Phosphate Buffered Saline (DPBS)	Gibco, Darmstadt
Fetal Calf Serum heat-inactivated (FCS)	Gibco, Darmstadt
L-Glutamine	Gibco, Darmstadt
MEM Non-Essential Amino Acids (NEAA)	Gibco, Darmstadt
MEM Sodium Pyruvate	Gibco, Darmstadt
Opti-MEM (Reduced Serum Medium)	Gibco, Darmstadt
Penicillin/Streptomycin	Gibco, Darmstadt
Trypsin-EDTA 0,05%	Gibco, Darmstadt

3.2.2 Media composition

MEF cell culture medium:

DMEM with 25 mM glucose	500 ml
FCS (heat-inactivated)	10%
L-Glutamine	6 mM
MEM Non-Essential Amino Acids	0.1 mM

MEM Sodium Pyruvate	1 mM
Penicillin/Streptomycin	1%

Trans-MEF cell culture medium:

DMEM with 25 mM glucose	500 ml
FCS (heat-inactivated)	2%
L-Glutamine	6 mM
MEM Non-Essential Amino Acids	0.1 mM
MEM Sodium Pyruvate	1 mM
Penicillin/Streptomycin	1%

Serum-starved MEF cell culture medium:

DMEM with 25 mM glucose	500 ml
FCS (heat-inactivated)	1%
L-Glutamine	6 mM
MEM Non-Essential Amino Acids	0.1 mM
MEM Sodium Pyruvate	1 mM
Penicillin/Streptomycin	1%

Opti-MEM with 5% FBS:

	Opti-MEM	500 ml
	FCS (heat-inactivated)	5%
Opti-MEM with 10% FBS a	and 1%NEAA:	
	Opti-MEM	500 ml
	FCS (heat-inactivated)	10%
	MEM Non-Essential Amino Acids	0.1 mM

3.2.3 Cell culture consumables

6-well plate	Thermo Fisher Scientific, Roskilde
24-well plate	Thermo Fisher Scientific, Roskilde

96-well plate	Thermo Fisher Scientific, Roskilde
50 ml, 25 cm ³ flask	Greiner Bio-One, Frickenhausen
2 ml serological pipette	Greiner Bio-One, Frickenhausen
5 ml serological pipette	Costar Stripette, Corning
10 ml serological pipette	Greiner Bio-One, Frickenhausen
25 ml serological pipette	Costar Stripette, Corning
1 ml syringe	BD Plastipak, Madrid
25 ml tube	Greiner Bio-One, Frickenhausen
50 ml tube	Greiner Bio-One, Frickenhausen
cell scraper	Sarstedt, Newton
glass pipettes	Carl Roth, Karlsruhe
hypodermic needle, 27G	Braun, Melsungen
microscopic slides	Thermo Fisher Scientific
pipettes	BRAND, Wertheim
pipette tips	Greiner Bio-One, Frickenhausen

3.3 Chemicals

Chemicals were purchased from AppliChem (Darmstadt), Carl Roth (Karlsruhe) and Sigma-Aldrich (Saint Louis). Cell culture media, antibiotics and serum were purchased from Gibco (Darmstadt). Primers were purchased from Biolegio (Nijmegen) and Eurogentec (Cologne).

3.4 Extraction of nucleic acids

RNeasy Mini Kit

Qiagen, Hilden

content:

Buffer RLT Buffer RW1 Buffer RPE RNase-Free Water

3.5 Fragment analysis

3.5.1 Reagents

_{dd} H ₂ O	Fresenius Kabi, Bad Homburg
50 mM MgCl ₂	Invitrogen, Carlsbad
10 mM dNTP	Invitrogen, Carlsbad
5x First-Strand Buffer	Invitrogen, Carlsbad
PlatinumTaq DNA Polymerase	Invitrogen, Carlsbad

3.5.2 **Primers**

Table 3.5.2-1. Primers for CPEB1-4 alternative isoforms identification

primer sequence	binding	predicted	genebank	detected	isoform
	position	length	association no.	length	

CPEB1

fw 5' GGATTGGTTAACACCTTCCGTGTTTTTGGC 3'

967 (v1)

751 (v2)

```
rev 5' AGGCCATCTGGGCTCAGCGGG 3'
```

1131 (v1)	176 bp (v1)	NM_030594	169 bp	Δ5
921 (v2)	191 bp (v2)	NM_001079533	182 bp	full-length

CPEB2

fw 5' AACTCCATCACTGACTCCAAAATCT 3'

1860

rev 5' CAAGCCATCATCTATTGGAAAGAGGGAAGA 3'

2206	375 bp (v4)	NM_001177382	375 bp	2a
2182	352 bp (v2)	NM_182485	351 bp	2b
2125	295 bp (v3)	NM_001177381	294 bp	2 c*
2116	286 bp (v5)	NM_001177383	286 bp	2c
2101	271 bp (v6)	NM_001177384	271 bp	2d*
2092	262 bp (v1)	NM_182646	262 bp	2d

primer sequence	binding	predicted	genebank	detected	isoform
	position	length	association no.	length	

CPEB3

fw 5' CAAAAAGCCCTTCTCCAGCAAC 3'

876

rev 5' TTCAGCTTTGTGAGGCCAGTCTA 3'

1478	604 bp (v1)	XM_006717715	600 bp	3a
1433	580 bp (v3)	XM_011539514	576 bp	3b
1388	535 bp	*	531 bp	3c
1364	511 bp	*	507 bp	3d

CPEB4

fw 5' CAGCTCTGCCTTTGCACCTAAAT 3'

1053

rev 5' GGCCATCATCCAAGAATCCATC 3'				
278 bp (v1)	NM_30627	277bp	4a	
255 bp	*	254bp	4b	
227 bp (v2)	NM_001308189	226bp	4c	
203 bp (v3)	NM_001308191	203bp	4d	
	GAATCCATC 3' 278 bp (v1) 255 bp 227 bp (v2) 203 bp (v3)	GAATCCATC 3' 278 bp (v1) NM_30627 255 bp * 227 bp (v2) NM_001308189 203 bp (v3) NM_001308191	GAATCCATC 3' 278 bp (v1) NM_30627 277bp 255 bp * 254bp 227 bp (v2) NM_001308189 226bp 203 bp (v3) NM_001308191 203bp	GAATCCATC 3' 278 bp (v1) NM_30627 277bp 4a 255 bp * 254bp 4b 227 bp (v2) NM_001308189 226bp 4c 203 bp (v3) NM_001308191 203bp 4d

"fw" and "rev" mark forward and reverse primers, * marks constructs based on a transcript variant alignment

3.6 Immunocytochemistry

3.6.1 Reagents

DAPI	Sigma, Saint Louis
Hoechst	Life Technologies, Carlsbad
Mounting Medium, PermaFluor	Thermo Scientific, Fremont
Normal Goat Serum	Abcam, Cambridge
Triton X-100	Carl Roth, Karlsruhe

3.6.2 **Buffers and solutions**

10xPBS (pH 7.4):

NaCl	80 g
KCl	2 g
Na ₂ HPO ₄	14.4 g
KH ₂ PO ₄	2.4 g
_d H ₂ O	11

0.01% NaN ₃ in 1xPBS (pH 7.4):		
	NaN ₃	0,1 g
	1xPBS (pH 7.4)	11
30% sucrose in 1xPBS (pH 7.4):		
	sucrose	30 g
	1xPBS (pH 7.4)	11

4% PFA:

PFA	4 g
$_{\rm d}{\rm H}_{2}{\rm O}$	90 ml
NaOH	1M
10xPBS (pH 7.4)	10 ml

poly-L-lysine:

poly-L-lysine	500 μ l of 2 mg/ml
_d H ₂ O	50 ml

3.7 Immunohistochemistry

3.7.1 Reagents

Cytoseal XYL medium	Thermo Scientific, Runcorn
Hematoxylin	Merck, Darmstadt
Permafluor	Thermo Scientific, Runcorn
PT Modul Buffer	Medac, Hamburg
Xylene	Merck, Darmstadt
100% ethanol	AppliChem, Darmstadt

90% ethanol70% ethanol

AppliChem, Darmstadt AppliChem, Darmstadt

3.7.2 CSA II staining system for immunohistochemistry

CSA II Biotin-free Tyramide Signal Amplification System *content:*

Dako, Carpinteria

TBS-T (Tris Buffered Saline- Tween) Peroxidase Block Protein Block Anti-Rabbit Immunoglobulins-HRP Amplification Reagent Anti-Fluorescein-HRP DAB Substrate Buffer Liquid DAB Chromogen

3.8 Laboratory equipment

3130 Genetic Analyzer	Applied Biosystems, Foster City
ABI 7900HT fast real time PCR system	Applied Biosystems, Darmstadt
Advanced Tissue Arrayer	Chemicon, Rolling Meadows
Axiophot	Carl Zeiss Microimaging GmbH, Göttingen
Axio Imager Z1 fluorescence microscope	Carl Zeiss Microimaging GmbH, Jena
BD FACS Canto II digital benchtop analyzer	BD Biosciences, Heidelberg
Centrifuge HERAEUS	Thermo Fisher Scientific, Schwerte
Centrifuges	Eppendorf GmbH, Wesseling
Fast Real-Time PCR System 7900HT	Applied Biosystems, Darmstadt
Gel electrophoresis chamber	Biorad, Munich
GeneGenome Syngene Bioimaging	Synaptics ltd. Cambridge
GeneTools System	Synaptics ltd. Cambridge
GloMax 96 Microplate Luminometer	Promega, Madison
Heat block	VWR International, Darmstadt

Lab Vision PT Modul Leica TCS confocal Mirax Slide Scanner Nanophotometer Pearl Novex Minicell WB module Olympus BX53 research microscope PCR machines (MyCycler thermal cycler) pH meter Pyromark Q24 instrument, Biotage Refrigerators (-80°C) Semi-automated IHC Stainer Shaker Shaking incubator (GFL) Shaking water bath Surgery equipment VWR benchtop centrifuge Thermoblock Vibratome, VT1000S Vortexer Mini-Protean 3 cell WB power supply Weigh balance

Thermo Scientific, Fremont Leica Microsystems, Wetzlar Carl Zeiss Microimaging GmbH, Jena Implen GmbH, München Invitrogen, Darmstadt Olympus, Tokyo Biorad, München Mettler Toledo, Giessen Qiagen, Hilden Thermo Fisher Scientific, Schwerte Tecan, Crailsheim, Heidolph Rotomax120, Schwabach Progen Scientific, London Memmert GmbH, Schwabach Fine Science Tools (F.S.T), Heidelberg VWR International, Darmstadt Biometra, Goettingen Leica, Nussloch VWR International, Darmstadt Biorad, Munich Biorad, Munich Sartorius group, Güottingen

3.9 Methylation

3.9.1 **Bisulfite conversion reagents**

EpiTect Fast DNA Bisulfite Kit content:

Bisulfite Solution DNA Protect Buffer Buffer BL Buffer BW Qiagen, Hilden

Buffer BD Buffer EB Carrier RNA

3.9.2 Bisulfite-DNA amplification reagents

ddH2O

Fresenius Kabi, Bad Homburg Qiagen, Hilden

PyroMark PCR Kit content: Master Mix 2x

CoralLoad Concentrate 10x

3.9.3 Pyrosequencing reagents

Streptavidin-Sepharose High Performance Beads PyroMark PCR Kit *content:*

> PyroMark Annealing Buffer PyroMark Binding Buffer, pH 7.6 PyroMark Denaturation Solution PyroMark Enzyme Mixture PyroMark Nucleotides (dATP, dCTP, dGTP, dTTP) PyroMark Substrate Mixture PyroMark Wash Buffer, pH 7.6

GE-Healthcare, Solingen Qiagen, Hilden

3.9.4 **Primers**

Table 3.9.4-1. Primers for amplification of bisulfite treated DNA

gene	primer sequence
CPEB1	fw 5' GGGGGTTAGAGATTTAAGTTTGAG 3' rev 5' ACTCCCATCCAAAAAAAAACCAATAATATCT 3'
CPEB2	fw 5' GGGGGTTATTAGTTTAAGTGAGAGTG 3' rev 5' TCCCCTACCCAAATTCACT 3'
CPEB3	fw 5' GGGGGTTATTAGTTTAAGTGAGAGTG 3' rev 5' ACCACCAACCCATCATAAC 3'
CPEB4	fw 5' GGGGAAAAGAGAGAGAGAAAGT 3' rev 5' ACTTCCTCTCCCCCATAA 3'

"fw" and "rev" mark forward and reverse primers

Table 3.9.4-2. Primers for pyrosequencing

gene	primer sequence		
CPEB1	ps 5' AAGAGGGTAAGATTTATAAG 3'		
CPEB2	ps 5' TGGGGGAGTGGGAGA 3'		
CPEB3	ps 5' CCAACCCATCATAACC 3'		
CPEB4	ps 5' GGTTTTAGTATTTTTAG 3'		

"ps" mark pyrosequencing primers

3.10 Pathways activity assay

Cignal 45-Pathway Reporter Array

SABioscience, Frederick

Dual-Luciferase Reporte	r Assay	Promega, Madison
content:		
PLB	5x Passive Lysis Buffer (PLB) dissolved in 4 volumes of $_{d}H_{2}O$	
LAR II	Luciferase Assay Substrate suspended in Luciferase Assay Buffer II	
Stop & Glo Reagent	200 μl of 50x Stop& Glo Su	abstrate dissolved in10 mL of Stop& Glo
	Buffer	

3.11 Semi-quantitative real time PCR

3.11.1 Reverse transcription reagents

5x First-Strand Buffer	Invitrogen, Carlsbad
0.1 M DTT	Invitrogen, Carlsbad
50 mM MgCl ₂	Invitrogen, Carlsbad
10 mM dNTP	Invitrogen, Carlsbad
250 ng Random Hexamer Primer	Invitrogen, Carlsbad
SuperScript III Reverse Transcriptase	Invitrogen, Carlsbad
_{dd} H ₂ O	Fresenius Kabi, Bad Homburg

3.11.2 TaqMan semi-quantitative real time PCR reagents

Applied Biosystems, Warrington
Eurogentec, Cologne
Eurogentec, Cologne
Eurogentec, Cologne
Fresenius Kabi, Bad Homburg

3.11.3 Primers and probes

Table 3.11.3-1. Primers and probes for semi-quantitative real time PCR

primers and probes sequences

binding position length

CPEB1; genebank association no: NM_030594		
fw 5' GCACCCAGGACTCAGATTCC 3'	335 (v1),	73 bp (v1),
	110 (v2)	
rev 5' CCCAGTGGGTTATGGAGCAT 3'	358 (v1),	73 bp (v2)
	134 (v2)	
p 5' CCCAGAGCAGCACACACTCGGTACTG 3'	358 (v1),	
	134 (v2)	
CPEB2; genebank association no: NM_001177382		
fw 5' TGCAGCAGAGGAACTCCTATAACC 3'	1631 (v1-v6)	81 bp (v1-v6)
rev 5' CCCAGCCACTGCTCTGATG 3'	1693 (v1-v6)	
p 5' CCAGCCTCTTCTGAAACAGTCTCCCTGG 3'	1659 (v1-v6)	
PBGD; genebank association no: NM_000190		
fw 5' GCTATGAAGGATGGGCAACT 3'	808 (v1),	
	756 (v2),	149 bp
	688 (v3),	(v1-v4)
	637 (v4)	
rev 5' GTGATGCCTACCAACTGTGG 3'	936 (v1),	
	886 (v2),	
	817 (v3),	
	766 (v4)	
p 5' TGCCCAGCATGAAGATGGCC 3'	906 (v1),	
	855 (v2),	
	786 (v3),	
	735 (v4)	

"fw" and "rev" mark forward and reverse primers, "p" probe and "v" transcript variant

3.12 Transfection

3.12.1 Reagents

Forskolin	Cell Signaling, Danvers
Lipofectamine 3000 Transfection Kit	Invitrogen, Carlsbad
Staurosporine S4400	Sigma, Saint Louis

3.12.2 Expression vectors

Table 3.12.2-1. Expression vectors

host	insert	vector	company
human	CPFB1	nCMV6Neo	OriGene Rockville
human	CPEB2	pCMV6Neo	OriGene, Rockville
human	-	pCMV6Neo	OriGene, Rockville
human	-	pmaxGFP	Lonza, Cologne
mouse	CPEB3a	pEGFP-N1	Clontech Laboratories, Heidelberg
mouse	CPEB3aKD	pEGFP-N1	Clontech Laboratories, Heidelberg
mouse	CPEB3b	pEGFP-N1	Clontech Laboratories, Heidelberg

CPEB3a-EGFP, CPEB3aKD-EGFP and CPEB3b-EGFP vectors were generated by Dr. Vamshidhar Vangoor (Institute of Cellular Neurosciences, University of Bonn), vector maps are provided in appendix I

3.13 Western blotting

3.13.1 Protein lysis buffer

Protein lysis buffer: modified RIPA buffer pH 7.5, stored at -20°C

Tris	6.05 g	50 mM
NaCl	8.76 g	150 mM
NP40	5 ml	0.5%
Sodium deoxycholate	5 g	0.5%
Triton X-100	10 ml	1%

Halt Protease Phosphatase Single Use Inhibitor Cocktail (Thermo Scientific, Rockford) Contents (except NP40 and triton) were dissolved in 800 ml $_{dd}H_2O$; pH was adjusted to 7.5; NP40 and Triton X-100 were added; volume was adjusted to 11; buffer was aliquoted 10 ml each and stored at -20°C. Modified RIPA lysis buffer was supplemented with Inhibitor Cocktail directly before use.

3.13.2 BCA Protein Assay

BCA Protein Assay Kit

Thermo Scientific, Rockford

content: Albumin Standard 2mg/mL BCA Reagent A BCA Reagent B

3.13.3 SDS-PAGE and protein transfer

Consumables:

MethanolAppliChem, DarmstadtPVDF membraneGE Healthcare Life Sciences, BuckinghamshireRoti Load 1 4x Sample bufferCarl Roth GmbH, KarlsruheWhatman paperWhatman International, Maidstone

Buffers and solutions:

Tris, 1.5 M, pH	8.8:	
	Tris	18.16 g/100 ml _d H ₂ O
Tris, 0.5 M, pH	6.8:	
	Tris	$6.05 \text{ g}/100 \text{ ml}_{d}\text{H}_2\text{O}$
10% APS:		
	APS	$0.1 \ g/1 \ ml \ _dH_2O$
10% SDS:		
	SDS	$10 \text{ g/100 ml}_{d}\text{H}_2\text{O}$
Resolving gel (I	10%):	
	_d H ₂ O	7.94 ml
	1.5 M Tris (pH 8.8)	5 ml
	10% SDS	0.2 ml
	Acrylamide	6.66 ml
	10% APS	0.2 ml
	TEMED	0.02 ml
Stacking gel (49	%):	
	_d H ₂ O	2.81 ml
	0.5 M Tris (pH6.8)	1.25 ml
	10% SDS	0.05 ml
	Acrylamide	0.83 ml
	10% APS	0.05 ml
	TEMED	0.005 ml
10x TBS-T, pH	7.4:	
	25 mM Tris	30.3 g
	150 mM NaCl	87.7 g

Running buffer:

10x Tris-Glycine-SDS buffer, pH 8.3:

25 mM Tris	30.3 g
192 mM Glycine	144 g
0.1% SDS	10 g
_d H ₂ O	11

Blotting buffer:

10x Tris-Glycine buffer, pH 8.3:

25 mM Tris	30.3 g
192 mM Glycine	144 g
_d H ₂ O	11

3.13.4 Blocking and antibody solutions

General blocking solution:

5% milk powder	0.5 g
1x TBS-T (pH 7.4)	10 ml
0.05% Tween-20	0.5 ml

Blocking solution for phospho-specific antibody:

3% BSA	0.3 g
1x TBS-T (pH 7.4)	10 ml

Solution for incubation with primary and secondary antibody:

2,5% milk powder	0.25 g
1x TBS-T (pH 7.4)	10 ml

Solution for incubation with phospho-specific primary antibody:

2% BSA	0.2 g
1x TBS-T (pH 7.4)	10 ml

4 METHODS

4.1 Animals

Maintenance and handling of animals was according to the local government regulations. Animals were housed in a 12 h/12 h dark-light cycle, food and water ad libitum. C57Bl6J wild-type mice were purchased from Charler River Laboratories (Sulzfeld). Thy1-GFP mice were provided by Prof. Valentin Stein (Institute of Physiology, University of Bonn).

4.2 Cell cultures

4.2.1 Glioblastoma cell cultures

Human glioblastoma cell lines A172, A178, LN229, U373MG, U87MG and T98G were obtained from the Ludwig Institute for Cancer Research (San Diego). Individual cell lines identity was confirmed by STR DNA profiling of 15 loci and sex-determining marker amelogenin (Genetica DNA Laboratories, Cincinnati). Cells were grown in DMEM with 25 mM glucose (Gibco, Darmstadt) supplemented with 10% FCS (Gibco, Darmstadt), 1% penicillin/streptomycin (Gibco, Darmstadt), 200 mM L-glutamine (Gibco, Darmstadt) and 100 mM sodium pyruvate (Gibco, Darmstadt) at 37°C, 5% CO₂.

4.2.2 HEK-293FT cell cultures

Cultured HEK-293FT cells were grown in DMEM with 25 mM glucose supplemented with 10% FCS, 1% penicillin/streptomycin, 200 mM L-glutamine and 100 mM sodium pyruvate at 37°C, 5% CO₂.

4.2.3 Primary hippocampal cultures

Primary hippocampal cultures were provided by Prof. Susanne Schoch (Institute of Neuropathology, University of Bonn).

4.3 Human specimens

Human glioma specimens were received from 69 patients (26 females, 43 males) admitted to the University Hospital of Bonn (ethics volume no. FKZ. 01GS08187). Histological characterization performed by neuropathologists of the German Brain Tumor Reference Center in Bonn confirmed that each specimen used further for extraction of nucleic acids and immunohistochemical staining consisted of at least 80% tumor cells. To avoid a possible tumor infiltration, human normal cerebellar tissues were used as a control. Tumors were graded according to the World Health Organization (WHO) classification of tumors of the central nervous system (Louis et al., 2007) into the following groups: diffuse astrocytoma (AII), anaplastic astrocytoma (AAIII), primary glioblastoma multiforme (pGBM), and secondary glioblastoma multiforme (sGBM) (Fig. 1.1.2-1). All patients agreed to preform molecular analysis on their samples. Specimens were treated in an anonymous manner approved by the ethics committee at the University of Bonn.

Table 4.3-1. Investigated human tissues

diagnosis	WHO grade	no. of patients	
diffuse astrocytoma	II	11	
anaplastic astrocytoma	III	22	
primary glioblastoma multiforme	IV	28	
secondary glioblastoma multiforme	IV	8	

4.4 Extraction of nucleic acids for methylation and fragment analysis studies

Extraction of DNA and RNA for methylation and fragment analysis was carried out using ultracentrifugation of homogenized tumor tissue through a CsCl gradient. Extraction was performed by Johannes Freihoff (Institute of Neuropathology, University of Bonn) according to instructions described before by Ichimura et al. (1996).

4.5 Methylation of CPEB1-4 genes

4.5.1 **Bisulfite conversion**

DNA methylation occurs on cytosine residues in regions with a high content of CpG dinucleotides known as CpG islands. The methylation status of CpG islands of CPEB1-4 genes was determined by bisulfite conversion according to instructions provided in the EpiTect Fast DNA Bisulfite Conversion Kit (Qiagen, Hilden). The bisulfite reaction consists of steps necessary for DNA denaturation, subsequent sulfonation and cytosine deamination. Isolated DNA samples were incubated with high bisulfite salt concentrations at high temperature and low pH to convert unmethylated cytosine residues into uracyl. Methylated cytosines remained unchanged. Afterwards converted single-stranded DNA was purified on MinElute DNA columns. Membrane-bound DNA was washed with 500 μ l of washing buffer and desulfonated by incubation for 15 min with 500 μ l of desulfonation buffer. Next, DNA was washed again with 500 μ l of elution buffer. Finally, bisulfite converted DNA was amplified in PCR reaction and sequenced.

Table 4.5.1-1. Analyzed samples

no. of glioblastoma cell lines:	
no. of reference tissues:	
no. of glioma specimens:	
AAIII	12
SGBM	10
pGBM	41

Table 4.5.1-2. Bisulfite conversion reaction and program

reagent	amount			
DNA template	5 µl	denaturation	95°C	5 min
_{dd} H ₂ O	15 µl	incubation	60°C	10 min
bisulfate solution	85 µl	denaturation	95°C	5 min
DNA protection solution	35 µl	incubation	60°C	10 min
total volume:	140 µl	hold	20°C	∞

Table 4.5.1-3. Bisulfite-DNA amplification PCR reaction and program

reagent	amount				
bisulfite - DNA	3 µl	initial	95°C	15 min	
master mix	12.5 µl	denaturation	95 C	15 11111	
coral load	2.5 μl	denaturation	94°C	30 sec	
forward primer	1.5 µl	annealing	56°C	30 sec	45x
reverse primer	1.5 µl	extension	72°C	30 sec	
$_{dd}H_2O$	4 µl	final extension	72°C	10 min	1
total volume:	25 µl				

4.5.2 **Pyrosequencing**

Pyrosequencing of the PCR products containing methylation sites was performed using pyrosequencing primers and the PyroMarkGold Reagents (Qiagen, Hilden). 20 µl of each bisulfite converted PCR product was pipetted into fresh 96-well reaction plates, complemented by 20 µl mastermix and placed on a shaker to maintain dispersion of beads. The mastermix composed of 1.5 µl streptavidin-sepharose beads and 18.5 µl binding buffer. Afterwards, 1 µl of pyrosequencing primers mixed with 24 µl of annealing buffer was pipetted into the fresh pyrosequencing annealing plate. With the use of a vacuum prep tool all DNA-beads samples were uniformly sucked from the plate. Attached samples were transferred into the 70% ethanol, denaturation solution, washing buffer and beads were released into the already prepared annealing plate. The pyrosequencing plate was incubated at 80°C for 2 min, cooled down and placed in the sequencing Pyromark Q24 instrument (Qiagen, Hilden). Additional cartridge containing PyroMark enzyme, substrate and nucleotides were attached to the sequencing instrument. Bisulfite conversion and pyrosequencing were performed by Jennifer Hammes (Institute of Neuropathology, University of Bonn).

4.5.3 Data analysis

Pyrogram outputs were analyzed using the PyroMark Q24 software (Qiagen, Hilden). Values obtained by CpG-islands pyrosequencing of individual tumor and normal brain sample were averaged and compared between each other. Normal brain tissues of agematched patients were considered as controls. As a cut-off level for methylation the three fold the standard deviation of mean methylation of normal brain samples was chosen. Ultimately, values obtained by pyrosequencing were imported into MultiExperiment Viewer and visualized as a heat map.

4.6 Fragment analysis of CPEB1-4 alternative splice isoforms

4.6.1 Fragment analysis

Identification of CPEB1-4 alternative splice variants was performed using RT-PCR products with primers spanning previously defined splice variants. CPEB1-4 cDNA was amplified by Platinum Taq DNA Polymerase and CPEB primers. One of each primer pairs was labeled with a specific fluorescent dye allowing sensitive detection and sizing of the PCR products. CPEB splice forms were estimated by electrophoretic separation of fluorescently labeled PCR products. Fragment sequencing was performed on thin capillaries allowing for distinction of 1bp differences.

Table 4.6.1-1. Analyzed samples

no. of glioblastoma cell lines:		
no. of reference tissues:		
no. of glioma specimens:		
AII	1	
AAIII	12	
SGBM	8	
pGBM	37	

Table 4.6.1-2. PlatinumTaq reaction and PCR program

reagent	amount				
_{dd} H ₂ O	13.7 µl	initial	05°C	15 min	
MgCl2	0.8 µl	denaturation	95 C	15 11111	
dNTP	0.4 µl	denaturation	95°C	45 sec	
5x First-Strand Buffer	2 µl	annealing	54°C	45 sec	40x
forward primer	1 µl	extension	72°C	45 sec	
reverse primer	1 µl	final extension	72°C	10 min	1
PlatinumTaq DNA polymerase	0.1 µl				
cDNA template in _{dd} H2O	1 µl				
total volume:	20 µl				

4.6.2 Data analysis

RT-PCR products were generated in separate PCR reactions optimized for the individual CPEB and diluted to adjust optimal signal strength. Next, fluorescently labeled PCR products were pooled and loaded for each individual tumor specimen or cell line. Detection and separation of PCR products was performed on an automated DNA sequencing machine 3130 (Applied Biosystems, Foster City). Electropherogarams of the individual samples were analyzed using GeneMapper v3.7 software by reading out the length of the RT-PCR product given in base pairs and the signal intensity expressed in relative fluorescent unit, which was proportional to the amount of generated PCR product. The fragment analysis was performed by Jennifer Hammes (Institute of Neuropathology, University of Bonn).

4.7 Generation of custom-made antibodies

Custom-made rabbit polyclonal antibodies were raised against CPEB1, CPEB2, CPEB4 and the phosphorylated form of CPEB3a/c (phospho-CPEB3). Peptide antigens (Kaczmarczyk et al., 2016; Turimella et al., 2015) were based on antigenic index (Jameson and Wolf, 1988), hydrophobicity (Kyte and Doolittle, 1982) and surface probability (Emini et al., 1985) and generated by Eurogentec GmbH (Cologne, Germany). As a carrier protein the keyhole limpet hemocyanin was used. For every CPEB two specific pathogen-free (SPF) rabbits were injected 4 times with 200 µg of each peptide, in 2-week intervals. Three months after the first immunization animals were sacrificed, bled and antibodies were purified by affinity purification. The phospho-CPEB3 antibody was directed against a region surrounding the S419 and S420 serine residues. The RRGRSSLFPFEDC peptide was chosen for immunization and cross-affinity purification of antibody. Purified antibodies were directed to mouse as well as human CPEBs. Their specificity was tested by peptide competition assay and immunoblot analysis (Kaczmarczyk et al., 2016; Turimella et al., 2015). Antibodies bound specifically and did not cross-react with other CPEBs.

4.8 Immunohistochemistry

4.8.1 Staining of paraformaldehyde fixed tissues

4.8.1.1 **Tissue preparation**

Juvenile (p12) or adult (p90) C57Bl6J mice and juvenile (p12) Thy1-GFP mice (Feng et al., 2000) were perfused via the vascular system using 4% paraformaldehyde (PFA). Next, isolated brains were fixed for 24 h in 4% PFA at 4°C and transferred to phosphate buffered saline (PBS). Coronal sections of 40 μ m thicknesses were prepared on a VT1000S vibratome (Leica, Nussloch) and stored in 0.01% NaN₃ in PBS at 4°C.

4.8.1.2 Immunohistochemical staining

Brain sections were washed three times for 10 min with PBS and blocked for 2 h in 10% of normal goat serum (NGS), 0.4% Triton X-100 in PBS. Following blocking, sections were incubated with the custom-made CPEB2 antibody– (1:50) and GFP– (1:500), MAP2– (1:100), PARV– (1:250) or TYH– (1:500) antibodies in 5% of NGS, 0.1% Triton X-100 in PBS for 48 h at 4°C. Afterwards, sections were washed with PBS and incubated for 90 min with secondary Alexa Fluor antibody– (1:500). Cell nuclei were stained with Hoechst for 10 min. Then sections were washed with PBS and mounted in Permafluor (Thermo Scientific, Runcorn).

4.8.1.3 Microscopy and data analysis

Images were taken with an Axio Imager Z1 fluorescence microscope (Carl Zeiss GmbH, Jena). For quantitative assessment of CPEB2 expression in excitatory, inhibitory, and dopaminergic neurons, GFP-parvalbumin-tyrosine hydroxylase-positive cells colocalizing with CPEB2 were counted (7-5 slices from three animals per type of neurons).

4.8.2 Staining of formalin fixed paraffin-embedded tissues

4.8.2.1 Generation of tissue microarrays

The tissue microarray technique allows for simultaneous analysis of multiple histological specimens assembled on one glass slide. Here, dissected glioma tissues were fixed with 4% PFA for 24–48 h at 4°C and embedded in a paraffin block. Microarrays were prepared by taking core needle biopsies from separate paraffin tissue blocks and re-embedding these tissues in an arrayed master block on Advanced Tissue Arrayer (Chemicon, Rolling Meadows). Afterwards, 4 µm thick tissue sections were prepared in the Lab Vision PT Modul (Thermo Scientific, Fremont) followed by pretreatment for 20 min at 99°C in PT Modul Buffer (Medac, Hamburg) and a 20 min cool-down phase at room temperature. Tissue microarrays were prepared by Brigitte Söndgen (Institute of Neuropathology, University of Bonn).

Table 4.8.2.1-1. Analyzed glioma samples

no. of specimens:		69
A	II	11
A	AIII	22
S	GBM	8
p	GBM	28

4.8.2.2 Immunohistochemical staining

Immunohistochemical staining was performed with a CSA II Biotin-free Tyramide Signal Amplification System (Dako, Carpinteria). Endogenous peroxidase activity forming high non-specific background staining was eliminated by pretreatment of tissue with hydrogen peroxide. Five min peroxidase blocking was followed by 60 min protein block prior to incubation of primary antibody. Next, specimens were incubated overnight at 4°C with the following antibodies: GFAP- (1:100), CPEB1- (1:100), CPEB2- (1:250), CPEB3-

(1:100), phospho-CPEB3– (1:50), CPEB4– (1:250), phospho- CaMKII– (1:1000) and PKA– (1:500). Afterwards sections were treated for 1h with poly-HRP-goat anti-mouse or anti-rabbit secondary antibody. Generated signal was enhanced with amplification reagent, anti-fluorescein-HRP and 3,3'-diaminobenzidine (DAB), 15 min each. Cell nuclei were stained with hematoxylin.

4.8.2.3 Microscopy and data analysis

Histological evaluation was performed by microscopic observation and digital scan of stained specimens. Stainings were observed with an Olympus BX53 research microscope (Olympus, Tokyo) and scanned by a Mirax Slide Scanner (Carl Zeiss GmbH, Jena). Tissues were categorized as positive or negative for the individual antibody by Dr. Gerritt Gielen (Institute of Neuropathology, University of Bonn). Specimens classified as a positive were further divided into three intensity groups: weak, intermediate and strong.

4.9 Transfection and stimulation of cultured cells

4.9.1 Transfection of cultured cells

Cells were seeded in 6-well plates, grown in complete mouse embryonic fibroblast (MEF) culture medium and transfected when ~75% confluency was obtained. One hour prior to transfection MEF medium was replaced by trans-MEF cell culture medium with reduced serum (2%). The transfection reaction was prepared in 250 μ l of opti-MEM with 2 μ g of expression plasmid and 3 μ l of lipofectamine reagent. Transfected cells were incubated for 5h at 37°C, 5% CO₂ and trans-MEF medium was replaced by fresh MEF medium. Afterwards cells were incubated for another 48 h and collected for RNA and protein analysis.

4.9.2 HEK-293FT cells stimulation with forskolin

In order to test phosphorylation of CPEB3 protein, HEK-293FT cells were transiently transfected with CPEB3a-EGFP, CPEB3KD-EGFP and CPEB3b-EGFP constructs. 24 h post transfection cells were stimulated for 1 h with 200 μ M forskolin, which increases the intracellular cAMP concentration and activates cAMP-dependent protein kinase (PKA). Control cells were treated with the same amount of dimethyl sulfoxide (DMSO). Following forskolin treatment cells were washed with ice-cold PBS and collected for protein analysis.

4.10 Immunocytochemistry

4.10.1 Coating slides with poly-L-lysine

Glass coverslips were coated with poly-L-lysine to enhance cell attachment. An aliquot of 500 μ l poly-L-lysine (2 mg/ml) was diluted in 50 ml _{dd}H₂O and incubated with coverslips for 2h at 37° C. Afterwards coverslips were washed with _{dd}H₂O and dried.

4.10.2 Immunocytochemical staining

Glioblastoma A172 cells were seeded and grown on glass coverslips until obtaining approximately 80% of confluency. Next, cells were washed with PBS and fixed for 20 min with 4% sucrose in 4% PFA. Cell membranes were permeabilized with 0.5% Triton X-100 and blocked for 60 min with 10% NGS and 0.5% Triton X-100 at room temperature. Following blocking of unspecific binding, cells were stained overnight at 4°C with polyclonal antibodies directed to CPEB1-4. Primary antibodies were diluted in PBS supplemented with 5% NGS and 0.1% Triton X-100 at the following dilutions: CPEB1– (1:100), CPEB2– (1:250), CPEB3– (1:100), phospho-CPEB3– (1:50), CPEB4– (1:250). Afterwards, cells were washed with PBS and incubated for 60 min with Alexa secondary antibodies (Invitrogen, Rockford). Counterstaining was performed with DAPI dye (Sigma,

Saint Louis). Coverslips were mounted using the PermaFluor mounting medium (Thermo Scientific, Fremont).

4.10.3 Microscopy and data analysis

Images were taken with the Zeiss Axio Imager Z1 fluorescence microscope. For qualitative estimation of endogenous CPEB1-4 and phospho-CPEB3 expression, human glioblastoma A172 cells stained with individual CPEBs were compared to their expression pattern in human glioma tissues.

4.11 Extraction of RNA for semi-quantitative real time PCR

Isolation of RNA from glioblastoma cultured cells was carried out using the RNeasy Mini Kit (Qiagen, Hilden). Cells transfected with the CPEB1- pCMV6Neo, CPEB2- pCMV6Neo or pCMV6Neo vector were washed with ice-cold PBS and directly lysed with 350 μ l of RLT buffer (Qiagen, Hilden) supplemented with 1% β -mercaptoethanol (β -ME). Samples were frozen in -20°C or the isolation procedure was immediately continued following the manufacturer's protocol.

4.12 Semi-quantitative real time PCR

4.12.1 Reverse transcription

1 μ g of template RNA was reversely transcribed with SuperScript III reverse transcriptase. Isolated RNAs were mixed with 10 mM dNTPs, 50 mM MgCl₂, 0.1M DTT and 250 ng of random hexamers in a total of 20 μ l reaction volume and transcribed to cDNA by reverse transcription PCR.

reagent	amount			
dNTP	1 μl	annealing	25 ° C	10 min
Random Hexamer Primer	1 µl	extension	42 ° C	50 min
5x First-Strand Buffer	2 µl	reverse		
MgCl ₂	2 µl	transcriptase	70 ° C	15 min
DTT	2 µl	inactivation		
SuperScript III reverse transcriptase	0.25 µl	hold	4 ° C	∞
$_{dd}H_2O$	1.75 µl			
1 μ g/ μ L RNA template in $_{dd}$ H ₂ O	10 µl			
total volume:	20 µl			

Table 4.12.1-1. Reverse transcription reaction and PCR program

4.12.2 Semi-quantitative real time PCR

Semi-quantitative assessment was achieved by amplification of 1 μ l cDNA with 900 nM of CPEB and PBGD primers, 100 nM of fluorogenic Taqman probes and TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington). Probes consisted of a 6-FAM fluorophore attached to the 5'-end of oligonucleotide and 3'-end attached the TAMRA quencher. The final TaqMan reaction volume was 12.5 μ l.

reagent	amount				
TaqMan Universal PCR Master	6.25 μl	UNG activation	50°C	2 min	
Mix		initial	95°C	10 min	
TaqMan Assay forward primer	1.125 µl	denaturation			
TaqMan Assay reverse primers	1.125 µl	denaturation	95°C	15 sec	
TaqMan Assay probe	0.25 µl	annealing and	60°C	60 sec	40x
_{dd} H ₂ O	2.75 μl	extension	00 0		
template cDNA	1 µl				ļ
total volume:	12.5 µl	UNG (Uracil-N glycosylase)			

Table 4.12.2-1. TaqMan sqRT-PCR reaction and PCR program

4.12.3 Data analysis

The critical threshold cycle (CT) value for each reaction was determined using SDS 5.0 Software (Applied Biosystems, Warrington). Normalization was performed using the housekeeping porphobilinogen deaminase (PBGD) gene as a reference against the expression of *CPEB* genes transcripts. The transcript level of CPEB1 and PBGD genes was calculated using the following equation: $X_{CPEB}/X_{PBGD} = 2^{CT PBGD - CT CPEB}$, where X is the respective input copy numbers and CT is the threshold cycle numbers for CPEB1 and PBGD (Seifert and Steinhäuser, 2007).

4.13 Western blotting

4.13.1 Tissue and cell culture lysates

Tissue and culture cells were lysate with modified RIPA buffer supplemented with a phosphatase and protease inhibitor cocktail. Tissue was homogenized in a 1.5 ml tube with a pestle, whereas cells were scraped in cold lysis buffer. Samples were disrupted with a 27G needle, then sonicated for 10 min and incubated for 30 min on ice. Afterwards cells were centrifuged for 15 min at 4°C, 14.000 g and their supernatants were collected for the further analysis. Total protein content was estimated by a BCA protein assay. For this purpose 25 μ l of protein standard (20-2000 μ g/ml range; Thermo Scientific, Rockford) and 5 μ l of lysate mixed with 20 μ l _{dd}H₂O was pipetted into a 96-well plate. The plate was gently shaken for 25 min at 37°C. Colorimetric detection and quantitation of total protein concentration was performed with an OptiMax Tunable Microplate Reader (Molecular Devices, Sunnyvale).

4.13.2 SDS-PAGE and Western blotting

30 µg of protein samples were mixed with denaturing sample buffer and heated for 5 min at 95°C. Next proteins were separated according to their molecular weight by SDS-PAGE (30
min at 80 V, 0.04 mA followed by 1.5h at 220 V, 0.06 mA) and blotted (2h at 100 V) on a PVDF membrane (GE Healthcare Life Sciences, Buckinghamshire). Afterwards, membranes with transferred non-phosphorylated proteins were blocked for 1h in 5% milk powder in T-BST and membranes with transferred phosphorylated proteins in 3% BSA in T-BST. Then membranes were incubated overnight with primary antibodies suspended in 2.5% milk powder (non-phosphorylated antibodies) or 2% BSA (phosphorylated antibodies) in T-BST. The next day membranes were washed with TBS-T buffer, incubated for 1h with horseradish peroxidase (HRP) secondary antibody in 2.5% milk powder in T-BST and visualized.

4.13.3 Data analysis

HRP activity was detected with Supersignal West Dura Substrate (Thermo Scientific, Rockford). Generated chemiluminescence was measured with the Gene Genome digital documentation system (Synoptics, Cambridge). Densitometry was performed with GeneTools System (Synoptics, Cambridge) and tested for significant differences.

4.14 Measurement of cellular proliferation and viability

4.14.1 FACS samples preparation

The dis-regulation of CPEB expression observed in glioma was the reason to study their influence on growth properties and apoptotic activity in *in vitro* conditions. Glioblastoma cells were transiently transfected with CPEB1-pCMV6Neo, CPEB2-pCMV6Neo or control pCMV6/Neo vector and subsequently their expression was monitored by sqRT-PCR and Western blot. Here, cells were additionally co-transfected with pmaxGFP to allow detection of CPEB-GFP positive cells by fluorescence-activated cell sorting (FACS). 24 h post transfection MEF medium was replaced with serum-starved MEF medium to induce apoptosis. The next day attached and detached cells of the flask were collected, washed with ice-cold PBS and transferred to the FACS tubes (polystyrene, non-pyrogenic). Cells

were fixed for 10 min in 4% PFA, permeabilized for 5 min in 0.5% Triton X-100 and stained. Proliferating cells were labeled with an Alexa Fluor 647-labeled antibody against phospho-(ser10)-histone H3 (pHH3; 1:20), whereas apoptotic cells were stained with a phycoerythrin-labeled antibody recognizing cleaved poly(ADP-ribose) polymerase (PARP; 1:5). Incubation with antibody for 30 min at room temperature was followed by 30 min DAPI staining (10 μ g/ml) at 37°C.

4.14.2 **Proliferation and viability analysis**

Samples were analyzed with the use of BD FACS Canto II digital benchtop analyzer (BD Biosciences, Heidelberg). In each experiment at least 100000 GFP- CPEB1-pCMV6/Neo, GFP- CPEB2-pCMV6/Neo, GFP- pCMV6/Neo or pmaxGFP positive single cells were analyzed. Cells transfected with GFP- pCMV6/Neo or pmaxGFP vector were considered as reference samples. Non-transfected cells labeled only with pHH3 antibody or stimulated for 24 h with 1 μ M staurosporine and stained with PARP antibody were used as a control to monitor proliferation and apoptosis, respectively.

4.15 Measurement of cellular migration

4.15.1 In vitro scratch assay

Migration of cultured glioblastoma cells transiently overexpressing CPEB1 and CPEB2 proteins was tested by an *in vitro* wound healing assay. A reproducible size scratch was created by the tip of a glass pipet on a confluent cell monolayer in the center of culture dish. Cell motility was monitored for 24 h.

4.15.2 Cell migration analysis

The motility of CEPB overexpressing cells was measured by the area covered by cells over time. 24 h after the scratch cells migrated into the center of the wound, which was imaged and quantified.

4.16 Cancer associated pathway activity assay

4.16.1 Reverse transfection

A pathway reporter array allowed for simultaneous analysis of 45 cancer-associated signaling pathways by screening their activities. Quantitative assessment was possible upon overexpression of CPEB1-pCMV6/Neo, CPEB2-pCMV6/Neo and control pCMV6/Neo plasmids in cultured cells and its reverse transfection with reporter constructs from array plates. Every construct was assembled of a constitutively expressed *Renilla* reporter and an inducible transcription factor responsive firefly luciferase reporter. Positive control was composed of a mixture of a constitutively expressed firefly luciferase and Renilla luciferase GFP constructs. Negative control was composed of non-inducible firefly luciferase reporter and constitutively expressed Renilla reporter. Reverse transfection was initiated by activation of reporter constructs from array plates by adding 50 µl of Opti-MEM. For each transfection reaction 0.3 µl lipofectamine in 50 µl of Opti-MEM was used. The mixture was added onto the plate, gently mixed and incubated for 20 min. A172 cells transiently expressing CPEB1-pCMV6/Neo, CPEB2-pCMV6/Neo and control pCMV6/Neo plasmids were washed with PBS without Ca^{2+} and Mg^{2+} , trypsinized, suspended in Opti-MEM with 5% FBS and counted. 15.000 cells per well in 50 µl of Opti-MEM with 10% FBS and 1% NEAA was added into each well and incubated for 24 h at 37°C. Afterwards the medium was replaced with MEF growth medium, incubated for another 24 h at 37°C and signals were detected by a dual-luciferase assay.

4.16.2 **Dual-luciferase reporter assay**

A dual luciferase assay was performed 48 h after reverse transfection. Grown medium was removed from cultures cells, cells were rinsed with PBS and 10 μ l of PLB was added into each well of Cignal 45-Pathway Reporter Array plates. After 15 min of gentle shaking at room temperature firefly (*Photinus pyralis*) and *Renilla (Renilla reniformis*) luciferases activities were measured sequentially from each well. First the firefly luciferase reporter was measured by adding 50 μ l of Luciferase Assay Reagent II to generate a stabilized luminescent signal. After quantification the firefly luminescence, reaction was quenched, and the *Renilla* luciferase reaction was initiated by 50 μ l of Stop & Glo Reagent added to the same well. *Renilla* luciferase signal decayed slowly over the course of the measurement.

4.16.3 Cancer associated pathways activity analysis

Firefly and *Renilla* luciferase activities were analyzed using GloMax 96 Microplate Luminometer. Results were calculated by dividing the normalized luciferase activity of each pathway reporter reverse transfected into A172 cells overexpressing CPEB1-pCMV6/Neo or CPEB2-pCMV6/Neo plasmid by the normalized activity of pathway reporter transfected with the control pCMV6/Neo vector. Reporters showing minimum two-fold change of relative luciferase units were considered as up- or downregulated. Experiments repeated three times in duplicates were further tested for statistical significance using OrigeneLab Corporation software (Northampton, MA, USA).

4.17 Statistics

In chapters 5.1.1, 5.1.4 and 5.2.2, the subsequent statistical analysis was conducted. Each data set was tested for Gaussian distribution by the Shapiro-Wilk-test. Next, for the comparison of two independent variables the normally distributed data was further examined with the F-test for equivalence of variances. Accordingly, Student's t-Test

without or with Welsh correction was performed. For comparison of more than two independent variables one-way ANOVA with post-hoc Tukey-test was applied. Therefore, the data were tested for equal variances by the Levene-test and based on the result Welsh-correction was performed. Non-normal distributed data were analyzed with non-parametric tests. Therefore, data sets with two independent variables were tested with Mann-Whitney U test and sets with three or more independent variables with the Kruskal-Wallis-ANOVA and post-hoc Bonferoni-corrected Mann-Whitney U test. Data are represented as mean \pm SEM. In chapter 5.1.2, *IDH1* mutation was correlated with CPEB1 and CPEB3 methylation by Fisher's two-sided exact test. While, in chapter 5.1.3.2, clinical course of glioma patients was correlated with CPEB expression data by Kaplan-Meier survival analysis.

5 RESULTS

5.1 Functional analysis of CPEB 1-4 in the pathogenesis of gliomas

5.1.1 Methylation of CPEB 1-4 genes in the 5'-CpG islands in gliomas

Methylation of DNA belongs to the heritable alternations in gene function, occurring without a change in the DNA sequence. Such epigenetic reaction may have inhibitory effects on gene expression. It neither alters nucleotide sequences nor affects the specificity of DNA base pairing. Differential methylation hybridization is a commonly used technique to identify changes in methylation patterns observed in cancers. Here, the methylation status of CPEB1-4 genes was determined by bisulfide treatment and pyrosequencing of glioma (n=63), normal brain (n=3-6) and cultured glioblastoma samples (n=5) (Fig. 5.1.1-1). De novo hypermethylation in the investigated CpG-islands was detected in CPEB1 (Fig. 5.1.1-1) and the gene was identified as a target for epigenetic inactivation in gliomas. In normal brain specimens of age-matched patients only trace methylation of up to 16% was observed (Fig. 5.1.1-2). The methylation cut-off was set to three-fold the standard deviation of the mean of normal brain samples (n=6; threshold methylation of 13.12%). CPEB1 methylation was detected in most of the AAIII (9/11; mean methylation of $62.31 \pm 2.93\%$), and sGBM specimens (10/10; mean methylation of $55.40 \pm 8.79\%$) that developed following malignant progression of lower-grade precursor lesions (Fig. 5.1.1-1). Furthermore, all examined glioblastoma cell lines showed hypermethylation of CPEB1 (mean methylation of $72.7 \pm 4.89\%$, data not shown). In contrast to *CPEB1*, methylation of CPEB3 was less abundant (mean methylation of $10.19 \pm 0.43\%$). In the cohort of investigated samples only a few pGBM cases showed an increased methylation up to 19.09% (Fig. 5.1.1-1). CPEB2 and CPEB4 did not demonstrate methylation of CpG-islands in any of the investigated tumors. This is indicated by a blue color on the heat map, which corresponds to the lack of methylation (Fig. 5.1.1-1).

CPEB1



	CPEB2											
0.0											20.0)
CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10	CpG11	mean	
												T1968 - pGBM T2466 - pGBM T2864 - pGBM T2864 - pGBM T2526 - AAIII T2526 - AAIII T2526 - AAIII T2526 - pGBM T3527 - pGBM T3527 - sGBM T3527 - sGBM T3527 - pGBM T3031 - pGBM T3031 - pGBM T3031 - pGBM T3031 - pGBM T3031 - pGBM T3032 - pGBM T3032 - pGBM T3032 - pGBM T3032 - pGBM T311 - pGBM T3075 - sGBM T3075 - sGBM T3075 - sGBM T3076 - pGBM T311 - pGBM T263 - pGBM T263 - pGBM T263 - pGBM T264 - pGBM T2007 - sGBM T264 - pGBM T265 - pGBM T2070 - sGBM T2070 - pGBM T2070 - pGBM T2771 - AAIII T3070 - pGBM T2771 - AAIII T2881 - pGBM T2771 - AAIII T2881 - pGBM T2771 - AAIII T2897 - AAIII T1289 - pGBM T172 - pGBM T172 - pGBM T172 - pGBM T1220 - pGBM T2270 - AAIII T1220 - pGBM T1220 - pGBM T2270 - AAIII T1220 - pGBM T2270 - pGBM T2270 - AAIII T1220 - pGBM T2270 - pGBM





Figure 5.1.1-1.



Figure 5.1.1-2.

Figure 5.1.1-1. Methylation profile of *CPEB1-4* **genes in glioma and normal brain tissues**. Bars above the heat maps indicate the methylation in % (range 0-50 for CPEB1; range 0-20 for CPEB2-4). Columns show investigated CpG dinucleotides, while rows present the individual glioma and control tissue samples. Blue

color corresponds to the lack of methylation, while red indicates increase in methylation of investigated tumors.

Figure 5.1.1-2. Pyrosequencing analysis of the CpG island in the 5'-region of *CPEB1* gene in sGBM, pGBM and normal brain tissue. The pyrogram of a sGBM (A) shows a strong methylation of up to 83% of the CpG positions. The pyrograms of a pGBM (B) and the normal brain tissue (C) show methylation of the investigated region up to 16%.

5.1.2 **IDH1 mutation in gliomas**

Mutation of *IDH1* gene occurs frequently in low-grade and secondary high-grade gliomas. *IDH* mutations drive increased methylation of DNA and are associated with improved prognosis for patients (Cohen et al., 2013). In the investigated cohort of gliomas, 1/1 AII, 9/11 AAIII, 7/10 sGBM and 4/41 pGBM revealed mutation in the *IDH1* gene. Secondary GBM tumors with mutated *IDH1* (n=7) demonstrated an average methylation of 69.37 \pm 6.78%. Surprisingly, few pGBM samples with mutated *IDH1* (n=4) also disclosed a significant increase of *CPEB1* methylation (mean 73.53 \pm 4.26%). Secondary GBMs without *IDH1* mutation (n=3) and pGBMs with wild type *IDH1* (n=37) showed an average methylation of 21.81 \pm 8.93% and 19.84 \pm 2.74% in the investigated region of *CPEB1* (Tab. 5.2.2-1). The described pattern indicates that *IDH1* mutation, are tightly linked to the *CPEB1* methylation status. *CPEB1* belongs to the methylation targets affected by the glioma associated CpG island methylator phenotype (G-CIMP) in *IDH1* mutant gliomas. In contrast to *CPEB1*, no correlation was observed between *CPEB3* methylation and *IDH1* mutation.

Table 5.1.2-1. Mutation of IDH1 and methylation of CPEB1 in human glioma specimens.

 $mean \pm SEM$

IDH1 mutation	no. of investigated samples	no. of samples with IDH1 mutation	CPEB1 methylation [%]	no. of samples without IDH1 mutation	CPEB1 methylation [%]
AII	1	1	73.11	0	_
AAII	11	9	62.31±2.93	2	9.50
sGBM	10	7	69.37 ± 6.78	3	21.81 ± 8.93
рGBM	41	4	73.53 ± 4.26	37	$19.84 \pm 2.74\%$

5.1.3 Expression profile of CPEB 1-4 in gliomas

5.1.3.1 Expression of CPEB 1-4 in human glioma specimens

Histological characterization of CPEBs was the next step to understand their role in human brain cancer. Glioma specimens assembled on tissue microarrays were stained with custom CPEB antibodies and evaluated by microscopic observation and neuropathologic assessment (Fig. 5.1.3.1-1; Fig. 5.1.3.1-2; Tab. 5.1.3.1-1). All of the investigated specimens were stained with GFAP antibody to confirm their affiliation to the group of astrocytomas. CPEB1 was detected in the infiltration areas of tumor cells in normal brain tissue. However, the majority of cells in the tumor center, in the areas of angiogenesis and necrosis showed no CPEB1 expression (Fig. 5.1.3.1-1). Strong immunoreactivity against CPEB1 was present only in few (2/61) glioma tissues (Tab. 5.1.3.1-1). Downregulation of CPEB1 protein was observed with a rising grade of glioma malignancy (Fig. 5.1.3.1-2). The vast majority of astrocytoma specimens revealed a positive staining for CPEB1 (26/29: 8/8 AAII and 18/21 AAIII). 23/32 glioblastoma (6/7 sGBM and 17/25 pGBM) samples contained CPEB1 positive cells (Tab. 5.1.3.1-1). CPEB2 was present in reactive astrocytes of normal brain tissue and in endothelial cells of vessels residing within the tumor tissue (Fig. 5.1.3.1-1). Positive staining was revealed in most of the studied glioma specimens (8/9 AAII; 18/20 AAIII; 7/8 sGBM; 18/25 pGBM) (Tab. 5.1.3.1-1). Among CPEBs in gliomas, CPEB3 occurred to be the most abundant and widespread in the cytoplasm and processes of astrocytic tumor cells (Fig. 5.1.3.1-1). Strong immunoreactivity against CPEB3 was detected in 8/10 AAII, 19/20 AAIII, 7/7 sGBM and 23/24 pGBM (Tab. 5.1.3.1-1). Enhanced CPEB3 signal was associated with an increased grade of malignancy. The opposite trend was observed with a phosphospecific CPEB3 antibody. Here, phosphorylation of CPEB3 protein was observed mainly in low-grade gliomas (7/8 AAII; 17/20 AAIII) and distinctly reduced in glioblastomas (10/26 pGBM) (Tab. 5.1.3.1-1). Analogous to CPEB1, strong expression of CPEB4 was found in only few tumors (10/62) (Tab. 5.1.3.1-1). Proteins were distributed in tumor cell bodies and processes (Fig. 5.1.3.1-1). In conclusion, above experiments revealed a distinctive and differential expression pattern of individual CPEBs that could be correlated with the glioma malignancy grade.



Figure 5.1.3.1-1.



Figure 5.1.3.1-2.

Figure 5.1.3.1-1. Immunohistochemical analysis of CPEB1-4 protein expression in gliomas. Staining of AII (CPEB3) and AA III (CPEB1, CPEB2, and CPEB4) tissues with CPEB1, CPEB2, CPEB3 and CPEB4 antibodies. The brown areas represent CPEB protein deposits while the blue regions correspond to the cell nuclei.

Figure 5.1.3.1-2. Evaluation of CPEB1-4 and phospho-CPEB3 protein expression in glioma tissues. A: CPEB1, B: CPEB2, C: CPEB3, D: phospho-CPEB3, E: CPEB4: Light blue graph sections represent the lack of CPEB expression, while blue and dark blue sections correspond to the positive CPEB staining divided subsequently to intensity groups: weak, intermediate, and strong.

Table 5.1.3.1-1. Quantification of CPEB1-4 and phospho-CPEB3 expression in human glioma specimens.Table contains number of not accessible and investigated specimens assembled on tissue microarrays.

CPEB1 expression	no. of not accessible samples	not no. of no. o ble investigated negatives samples samp		no. of positive samples- weak intensity	no. of positive samples- intermediate intensity	no. of positive samples- strong intensity	
AII	2	Q	0	2	1	1	
	1	0 21	2	2	4	1	
AAII «CDM	1	21	1	8	2	1	
SGDM = CDM	1	25	1	4	2	0	
рбвм	3	25	8	12	5	0	
sum:	8	61	12	27	20	2	
percentage:		100%	19.67%	44.26%	32.79%	3.28%	
CPEB2 expression							
AII	2	9	1	2	2	4	
AAII	2	20	2	9	4	5	
sGBM	0	8	1	1	4	2	
pGBM	3	25	7	10	6	2	
sum:	7	62	11	22	16	13	
percentage:		100%	17.74%	35.48%	25.81%	20.97%	
CPEB3 expression							
AII	1	10	2	3	3	2	
AAII	2	20	1	8	9	2	
sGBM	1	7	0	0	3	4	
pGBM	4	24	1	6	12	5	
sum:	8	61	4	17	27	13	
percentage:		100%	6.56%	27.87%	44.26%	21.31%	

phospho-CPEB3 expression	no. of not accessible samples	no. of no. of investigated negative samples samples		no. of positive samples- weak intensity	no. of positive samples- intermediate intensity	no. of positive samples- strong intensity
411	3	8	1	2	3	2
	2	20	2	10	5	2
AAII -CDM	2	20	5	10	0	1
SGBM	0	8	1	/	0	0
pGBM	2	26	16	10	0	0
sum:	7	62	21	29	9	3
percentage:		100%	33.87%	46.77%	14.52%	4.84%
CPEB4 expression						
AII	1	10	0	6	3	1
AAII	2	20	0	12	5	3
sGBM	1	7	1	1	4	1
pGBM	3	25	4	11	5	5
- sum:	7	62	5	30	17	10
percentage:		100%	8.06%	48.39%	27.42%	16.13%

5.1.3.2 Correlation of CPEB 1-4 expression with clinical prognosis of glioma patients

Clinical course of glioma patients was correlated with CPEB expression data by Kaplan-Meier survival analysis. Overall survival was determined as a time between surgery of primary tumor and death of the patient. When the expression of CPEB1 protein in all investigated samples (n=61) was compared with patients survival no correlation was detected (Fig. 5.1.3.2-1A). Nevertheless, if the studied group was narrowed to only low-grade astrocytoma samples (n=29), the Kaplan-Meier curve suggested a positive correlation between CPEB1 expression and longer patient survival (Fig. 5.1.3.2-1B). The remaining pGBM (17/25) and sGBM (6/7) specimens expressing CPEB1 protein did not reveal any significant relation with the life expectancy of patients. Despite a robust expression, neither CPEB2-3 nor CPEB4 showed correlation with survival of glioma patients (Fig. 5.1.3.2-1C, D, G). CPEB3 activation, as determined by phosphorylation was significantly correlated with longer patient survival in all investigated tumor samples (n=62) (Fig. 5.1.3.2-1E). Interestingly, in the analysis focused on low-grade astrocytomas (n=28), which are considered precursor lesions for sGBM and show a molecular pathology distinct from pGBM, a significant correlation between CPEB3 activity and prolonged patient survival

was observed (Fig. 5.1.3.2-1F). It indicates that phospho-CPEB3 protein might be a novel biological marker for a better prognosis of low-grade glioma patients. However, it may be also considered as a marker of glioma patients independently of histological entity or grading.





Figure 5.1.3.2-1. Correlations between expression of CPEB 1-4 and survival of glioma patients. A: Kaplan- Meier survival analysis showed no correlation between CPEB1 expression and longer survival in all investigated glioma patients B: Positive correlation between CPEB1 expression and patient survival was observed in a subpopulation of AAII and AAIII. C, D, G: No correlation was observed between CPEB2, CPEB3 and CPEB4 expression and patient survival. E: Significant correlation between phospho-CPEB3 expression and survival data was observed in all investigated glioma samples. F: Detail studies on AAII and AAIII (n=28) as well as primary (n=26) and secondary (n=8) GBM samples indicated that phospho-CPEB3 expression when compared to survival reach statistical significance only in the group of AAII and AAIII.

5.1.4 *CPEB1* gene methylation and its influence on expression profile.

The current study on expression of CPEBs in gliomas revealed an interesting dependency between tumor malignancy grade and the change in their expression level. In parallel to the increasing grade of glioma, expression of CPEB1 protein decreased (Fig. 5.1.3.1-2; Tab. 5.1.3.1-1). The observed effect was verified by sqRT-PCR transcript analysis in glioma (n=25) and reference normal brain (n=6) samples. Investigated specimens contained significantly reduced transcript level, as compared to the control tissue. In particular, CPEB1 expression was decreased by 37% in AAIII (n=6), 6 % and 9 % in sGBM (n=3) and pGBM (n=16) (Fig. 5.1.4-1A), respectively. Afterwards, the same set of specimens was analyzed for *CPEB1* gene methylation. A significant increase in methylation was detected in AAIII and sGBM, but not in pGBM, when compared to the control brain samples (Fig. 5.1.4-1B). As hypermethylation of DNA is considered to have an inhibitory effect on gene expression, the correlation between methylation and expression profile of CPEB1 was examined performing Pearson's correlation analysis (data not shown). However, despite the



strong trend indicating such dependence, *CPEB1* methylation and expression were not correlated.

Figure 5.1.4-1. Comparison of *CPEB1* methylation and expression in glioma and reference samples. A: Significant decrease of CPEB1 transcript was observed with rising grade of tumor malignancy in AAIII, sGBM and pGBM samples. B: Significant increase in *CPEB1* methylation was observed in AAIII and sGBM, but not in pGBM samples.

5.1.5 Activity dependent expression of CPEB3 protein in gliomas

Evaluation of CPEB3 and the phospho-CPEB3 protein uncovered their differential expression patterns in human glioma specimens. In parallel to the rising grade of glioma malignancy, expression of CPEB3 increased, while expression of phospho-CPEB3 was reduced (Fig. 5.1.3.1-2C, D). To confirm the specificity of phospho-CPEB3 antibody, the splice variants of CPEB3a, CPEB3aKD (CPEB3a kinase dead) or CPEB3b were cloned into the pEGFP-N1 expression vector and overexpressed in HEK-293FT cells (Fig. 5.1.5-1). Transfection of CPEB3 plasmids was followed by cell stimulation with 200 μ M forskolin, which increases the intracellular cAMP concentration and activates PKA. Afterwards, expression of CPEB3 variants was quantified by Western blot analysis. The full-length CPEB3a isoform was detected by both, CPEB3 and phospho-CPEB3 antibody. The effect of forskolin stimulation was observed as an increased intensity bands detected

by phosphospecific CPEB3 antibody in CPEB3a overexpressing cells. The CPEB3aKD variant contained two serines (S419; S420) in the phosphorylation site, which were mutated to alanine. Therefore, despite forskolin treatment, no phosphorylation was observed. Similarly, CPEB3b isoform lacking the B-region localized next to putative phosphorylation sites for PKA, CaMKII and RPS6K did not reveal any phosphorylation (Fig. 5.1.5-1). This finding confirmed that only the CPEB3 isoform comprising both, phosphorylation sites and the B-region, could be effectively activated by phosphorylation.

Next, in order to understand activity dependent CPEB3 expression, CPEB3, phospho-CPEB3 and kinases indicated before as putative CPEB3 activators, CaMKII and PKA, were further examined by immunostaining (Kaczmarczyk et al., 2016; Theis et al., 2003). Two groups of tumor samples were investigated. The first group of low-grade astrocytomas (n=6; AII and AAIII) was characterized by strong CPEB3 and phospho-CPEB3 expression. The second group contained primary GBMs samples (n=7) positive for CPEB3, but negative for phospho-CPEB3 antibody (Fig 5.1.5-2). Robust expression of PKA and phospho-CaMKII was detected in low-grade astrocytomas, which also contained active CPEB3 protein (Fig 5.1.6-1). However, despite the kinases activity in high-grade gliomas, the expression of phosphorylated CPEB3 protein was reduced (Fig 5.1.5-2). Therefore, lack of active kinases cannot explain the loss of CPEB3 phosphorylation in high-grade gliomas.



Figure 5.1.5-1.



Figure 5.1.5-2.

Figure 5.1.5-1. Immunoblot presenting phosphorylation of CPEB3a-EGFP, CPEB3aKD-EGFP and CPEB3b-EGFP isoforms in HEK-293FT cells. Overexpressed CPEB3-EGFP protein was stimulated with forskolin to activate PKA. Immunoreactivity against CPEB3 antigene was detected in all investigated splice isoforms, while immunoreactivity against phospho-CPEB3 antigene was observed only in the CPEB3a variant.

Figure 5.1.5-2. Immunohistochemical analysis of altered CPEB3 activity in low-grade astrocytoma and primary GBM specimens. In the upper row of the figure cell nuclei (violet) and cytoplasm (pink) were stained by hematoxylin and eosin, respectively. The following lower rows represent brown areas of respective: GFAP; CPEB3; phospho-CPEB3; phospho-CaMKII; PKA antibody staining and dark blue areas corresponding to cell nuclei stained by hematoxylin.

5.1.6 Alternative splice isoforms of CPEB1-4 in human gliomas

The presence of multiple alternative splice isoforms of CPEB paralogs uncovered the complexity of their regulatory functions. Here, alteration in the abundance of CPEB1-4 splice variants was studied in primary glioma tissues (n=58), glioblastoma cell lines (n=5), and normal brain tissues (n=4) (Tab. 5.1.6-1). The optimized RT-PCR approach allowed for the quantitative assessment of CPEB variants by electrophoretic separation of fluorescently labeled PCR products. Differences between CPEB splice variants depend on presence or absence of respective B- and C-regions. Of particular importance is the B-region adjacent to the two serine residues (S419; S420), because it is responsible for phosphorylation and further activation of CPEBs (Fig. 5.1.6-1).

In the present study, a significant change in expression of alternative transcripts was observed between tumor and reference brain tissues. Most of the investigated AAIII specimens contained the same alternative transcripts, while GBMs lost several of the CPEB2-4 splice variants as compared to normal brain samples (Fig. 5.1.6-2). In the reference tissue the following alternative variants of CPEBs were the most abundant: CPEB1 Δ 5 (169bp); CPEB2a (375bp; full-length isoform), CPEB2c (286bp; lack of C- and E-region); CPEB3a (600bp; full-length isoform), CPEB3b (576b; lack of B-region), CPEB3c (531bp; lack of C-region), CPEB3d (507bp; lack of B- and C-region); CPEB4a (277bp; full-length isoform of CPEB4), CPEB4b (254bp; lack of B-region), CPEB4c

(226bp; lack of C-region), CPEB4d (203bp; lack of B- and C-region) (Fig. 5.1.6-2). Interestingly, the abundance of CPEB1 seemed not to be altered in gliomas. In all except two pGBM samples, which revealed the 182bp CPEB1 splice variant, corresponding to the full-length isoform, the only found CPEB1 transcript was 169bp CPEB1 Δ 5 (Fig. 5.1.6-2; Tab. 5.1.6-1). The abundance of the full-length isoforms of CPEB2-4 appeared to be reduced and instead the b/d variants, lacking the B-region were expressed. Consequently 20/37 pGBM and 5/8 sGBM samples were deprived of CPEB2a, 36/37 pGBM and 8/8 sGBM samples were deprived of CPEB3a and 30/37 pGBM and 6/8 sGBM were deprived of CPEB4a as compared to AAIII and reference tissues (Tab. 5.1.6-1). Furthermore, 11/37pGBM and 3/8 sGBM samples were devoid of the CPEB4c variant also containing the B-region (Tab. 5.1.6-1). Additionally, some of the detected CPEB2 isoforms contained the E-region (Tab. 5.1.6-1; Fig. 5.1.6-1), which was previously described in mouse CPEB2 (Turimella et al., 2015).

Differences between splice isoforms were further reflected by properties of the expressed proteins, because only CPEB a/c variants containing the B-region were subject to phosphorylation. As most of the splice variants missing the B-region were expressed in high-grade gliomas, alternative splicing seemed to play an important role in tumor progression.



Figure 5.1.6-1.



Figure 5.1.6-2.

Figure 5.1.5-1. Scheme of CPEB1-4 splice variants detected by fragment analysis in normal brain and glioma samples. Individual panels contain labeled B, C or E-regions and phosphorylation sites of respective CPEB variants.

Figure 5.1.6-2. Relative abundance of respective splice variants of the four CPEB genes revealed by the **RT-PCR fragment analysis.** Values of the x-axis correspond to the fragment length of the RT-PCR product given in base pairs. The y-axis defines signal intensity (relative fluorescent units) which is proportional to the amount of generated PCR product. The size of the RT-PCR product generated by fragment analysis does not describe the actual length of the respective splice product, but length of fragments detected with primers spanning previously defined splice variants.

Table 5.1.6-1. The RT-PCR products of specific splice variants of the CPEB1-4 genes detected in AII; AAIII, pGBM, sGBM, normal brain tissues and GBM cultured cells. The upper values above the table correspond to the RT-PCR product (size in base pairs) generated by fragment analysis. The following alternative variants of CPEBs were detected: CPEB1Δ5 (169bp), CPEB1 (182bp); CPEB2d (262bp; lack of B, C, E-region) CPEB2d* (271bp; lack of B, C-region), CPEB2c (286bp; lack of C, E-region), CPEB2c* (294bp; lack of C-region), CPEB2b (351bp; lack of B-region), CPEB2a (375bp; full-length isoform); CPEB3d (507bp; lack of B, C-region), CPEB3c (531bp; lack of C-region), CPEB3b (576b; lack of B-region), CPEB3a (600bp; full-length isoform); CPEB4d (203bp; lack of B, C-region), CPEB4c (226bp; lack of Cregion), CPEB4b (254bp; lack of B-region), CPEB4a (277bp; full-length isoform of CPEB4).

Α

DiagnoseDD-T-NummerCPEB1608 by182 by508 byAAIII237726683AAIII227521376AAIII2774233806AAIII2774233806AAIII277431813AAIII228924278AAIII342354168AAIII354522815AAIII354632681AAIII354632681AAIII354832681AAIII354832681PGEM7138072PGEM32227337PGEM32227337PGEM32227337PGEM32325658PGEM32825658PGEM131130762PGEM216918711PGEM21693273PGEM21693273PGEM216918711PGEM216918711PGEM2169PGEM2169PGEM2169PGEM2169PGEM2169PGEM3007PGEM3011PGEM3131PGEM3141PGEM3262PGEM3132PGEM3141PGEM3263PGEM3264PGEM3274PGEM3264PGEM3274PGEM3263PGEM3274PGEM3264PGEM3274PGEM	Detected sp	lice isoforms:		CPEB1 Δ5	CPEB1		
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pcBM 1619 20905 pGBM 1619 2010 pGBM 2010 34031 pGBM 2104 23173 pGBM 2169 18711 pGBM 2304 25799 pGBM 2481 27551 pGBM 2494 30091 pGBM 2486 26420 pGBM 2494 30031 pGBM 2757 20864 pGBM 284 21244 pGBM 2884 21244 pGBM 3031 37154 pGBM 3032 26770 pGBM 3036 7859 pGBM 3070 16581 1909 pGBM 33747 36070 GBM sark 3555 18439 2160 Normal Brain NBrontal 17581 783 Normal Brain NBcocipital 14549 4461 SGBM 177 24962 3412	pGBIVI *CDM	1311		30762			
pcBM 1968 20143 pGBM 2104 34031 pGBM 2104 23173 pGBM 2169 18711 pGBM 2481 27551 pGBM 2486 26420 pGBM 2486 26420 pGBM 2486 26420 pGBM 2486 20034 pGBM 2854 30034 pGBM 2854 30034 pGBM 2854 30034 pGBM 3031 37154 pGBM 3032 26770 pGBM 3032 26770 pGBM 3031 30871 pGBM 3070 16581 1909 pGBM 3747 36070 GBM sark 2555 pGBM 3747 36070 GBM sark 2555 QBM sark 2555 20061 GBM sark 2643 Normal Brain NBrontal 17681 17833 Normal Brain	pGBIVI *CDM	1619		20905			
DeBM 2010 34031 pGBM 2104 23173 pGBM 2169 18711 pGBM 2304 25799 pGBM 2481 27551 pGBM 2486 26420 pGBM 2484 30091 pGBM 2484 30091 pGBM 2484 30034 pGBM 2854 30034 pGBM 2854 30034 pGBM 2854 30034 pGBM 3007 52052 pGBM 3032 26770 pGBM 3032 26770 pGBM 3032 26770 pGBM 3031 37154 pGBM 3070 16581 1909 pGBM 3747 36070 GBM Sark 2555 20061 68M sark GBM Sark 3555 18439 2160 Normal Brain NBrontal 1783 Normal Brain NBrontal 14549 Normal Brain NBrontal 24962 <tr< td=""><td>PGBIVI *CDM</td><td>1968</td><td></td><td>20145</td><td></td><td></td><td></td></tr<>	PGBIVI *CDM	1968		20145			
DeBM 2104 21173 pGBM 2169 18711 pGBM 2304 25799 pGBM 2486 26420 pGBM 2494 30091 pGBM 2735 43101 pGBM 2757 20864 pGBM 2854 30034 pGBM 2884 21244 pGBM 2896 19339 pGBM 3007 52052 pGBM 3031 37154 pGBM 3032 26770 pGBM 3032 26770 pGBM 30331 37154 pGBM 3032 26770 pGBM 3070 15581 pGBM 3070 16581 pGBM 3070 21366 GBM sark 2557 21366 GBM sark 2555 20061 GBM sark 3555 18439 PGBM 17581 1864 Normal Brain NBremporal 24862 All 1418 26306 pGBM 1329 3412 SGBM 176 24982 All 1418 26306 pGBM 1214 24982	pGBIVI *CDM	2010		34031			
pGBM 2169 16711 pGBM 2481 25799 pGBM 2486 26420 pGBM 2486 26420 pGBM 2757 20864 pGBM 2757 20864 pGBM 2854 30034 pGBM 2884 21244 pGBM 2896 19339 pGBM 3007 52052 pGBM 3031 37154 pGBM 3032 26770 pGBM 3032 26770 pGBM 3033 37154 pGBM 3032 26770 pGBM 3031 37154 pGBM 3070 16581 pGBM 3513 30871 pGBM 3573 21366 GBM sark 2555 20061 GBM sark 3555 18439 Normal Brain NBroital 17581 Normal Brain NBraitell 44549 Normal Brain NBraitell 3412 GBM 1214 24982	PGBIVI	2104		23173			
pGBM 2304 27551 pGBM 2486 26420 pGBM 2486 26420 pGBM 2494 30091 pGBM 2735 43101 pGBM 2735 20864 pGBM 2757 20864 pGBM 2854 30034 pGBM 2884 21244 pGBM 3031 37154 pGBM 3032 26770 pGBM 3066 7859 pGBM 3066 7859 pGBM 3070 16581 1909 pGBM 3066 7859 pGBM 3070 16581 1909 pGBM 3066 7859 pGBM 3070 16581 1909 pGBM 3747 36070 6070 GBM sark 255 20061 608 GBM sark 3555 18439 2160 Normal Brain NBrontal 114549 14549 Normal Brain NBrontal 14549 1464 <t< td=""><td>pGBIVI</td><td>2109</td><td></td><td>25700</td><td></td><td></td><td></td></t<>	pGBIVI	2109		25700			
pGBM 2461 27331 pGBM 2494 2630 pGBM 2494 30091 pGBM 2735 43101 pGBM 2757 20864 pGBM 2854 30034 pGBM 2896 19339 pGBM 3007 52052 pGBM 3031 37154 pGBM 3032 26770 pGBM 3066 7859 pGBM 3070 16581 pGBM 3747 36070 GBM sark 2555 20061 GBM sark 2555 20061 GBM sark 3555 18439 Ormal Brain NBcocipital 17581 Normal Brain NBcocipital 17581 Normal Brain NBcocipital 14649 Normal Brain NBcocipital 14549 SGBM 677 18769 3412 SGBM 1214 24962 3412 SGBM 1329 <td< td=""><td>pGBM pGBM</td><td>2304</td><td></td><td>23755</td><td></td><td></td><td></td></td<>	pGBM pGBM	2304		23755			
pGBM 2400 2001 pGBM 2735 43101 pGBM 2757 20864 pGBM 2854 30034 pGBM 2854 30034 pGBM 2884 21244 pGBM 2896 19339 pGBM 3007 52052 pGBM 3031 37154 pGBM 3032 26770 pGBM 3070 16581 1909 pGBM 3070 16581 1909 pGBM 3070 16581 1909 pGBM 3717 36070 GBM safk 2655 GBM safk 2555 20061 GBM safk 3555 GBM safk 2555 18439 2160 Normal Brain NBcocipital 14589 14549 Normal Brain NBcocipital 14549 1864 Normal Brain NBcocipital 14549 1464 Normal Brain NBcocipital 24982 3412 All 1418 26306 3412	nGBM	2481		26420			
pGBM 2434 30034 pGBM 2757 20864 pGBM 2854 30034 pGBM 2884 21244 pGBM 2896 19339 pGBM 3007 52052 pGBM 3007 52052 pGBM 3007 52052 pGBM 3031 37154 pGBM 3032 26770 pGBM 3066 7859 pGBM 3070 16581 pGBM 3513 30871 pGBM 3747 36070 GBM sark 2655 20061 GBM sark 3555 18439 2160 Normal Brain NBrontal 17581 Normal Brain NBrontal 14549 Normal Brain NBraifetal 14549 Normal Brain NBraifetal 14549 Normal Brain NBraifetal 3412 GBM 1214 24982 3412 GBM 1214 24461 3606 GBM 1214 24461<	nGBM	2480		30091			
pCBM 2757 20864 pCBM 2854 30034 pCBM 2884 21244 pCBM 2896 19339 pCBM 3007 52052 pCBM 3031 37154 pCBM 3032 26770 pCBM 3032 26770 pCBM 3066 7859 pCBM 3070 16581 1909 pGBM 3747 36070 GBM 0igo 2655 20061 GBM sark 2527 21366 GBM sark 2555 18439 2160 Normal Brain NBfortal 17581 Normal Brain NBfortal 1864 Normal Brain NBtemporal 24982 All 1418 26306 pGBM 1214 24461 3412 sGBM 677 24154 sGBM 1329 39085 sGBM 1329 39085 sGBM 1430 24163 sGBM 2007 43109 sGBM	nGBM	2735		43101			
pGBM 2854 30034 pGBM 2884 21244 pGBM 2896 19339 pGBM 3007 52052 pGBM 3031 37154 pGBM 3032 26770 pGBM 3032 26770 pGBM 3066 7859 pGBM 3070 16581 1909 pGBM 3513 30871 1909 pGBM isark 2643 17833 17833 GBM sark 2643 17833 17833 GBM sark 255 18439 2160 1864 Normal Brain NBtorntal 1459 1864 Normal Brain NBternporal 24982 3412 All 1418 26306 3412 1864 sGBM 677 18769 3412 3412	pGBM	2757		20864			
DGBM 2884 21244 pGBM 2896 19339 pGBM 3007 52052 pGBM 3031 37154 pGBM 3032 26770 pGBM 3032 26770 pGBM 3066 7859 pGBM 3070 16581 1909 pGBM 3513 30871 pGBM 3513 30871 pGBM 3527 21366 GBM sark 2527 21366 GBM sark 3555 18439 2160 Normal Brain NBfortal 17581 Normal Brain NBbccipital 14584 Normal Brain NBbccipital 14584 Normal Brain NBtemporal 24882 All 1418 26306 pGBM 677 18769 3412 SGBM 1214 24461 SGBM 1329 39085 36GM SGBM 1224 24461 3412 SGBM 2007 43109 3412	pGBM	2854		30034			
J J J JGBM 2896 19339 19339 JGBM 3007 52052 19339 JGBM 3031 37154 1909 JGBM 3032 26770 1968 JGBM 3066 7859 1909 JGBM 3070 16581 1909 JGBM 3513 30871 1909 JGBM 3747 36070 16581 JGBM 3747 36070 16581 GBM oligo 2655 20061 1668 GBM sark 2527 21366 17833 GBM sark 3555 18439 2160 Normal Brain NBcoccipital 14589 1864 Normal Brain NBtemporal 24982 1844 Normal Brain NBtemporal 24982 3412 All 1418 26306 1844 JGBM 1214 24461 3412 SGBM 1329 39085 </td <td>pGBM</td> <td>2884</td> <td></td> <td>21244</td> <td></td> <td></td> <td></td>	pGBM	2884		21244			
J J J pGBM 3007 52052 pGBM 3031 37154 pGBM 3032 26770 pGBM 3066 7859 pGBM 3070 16581 1909 pGBM 3513 30871 909 pGBM 3747 36070 GBM GBM asita 3627 21366 1909 GBM sark 2563 20061 16581 GBM sark 2563 20061 16581 GBM sark 2555 20061 1664 Normal Brain NBcocipital 14549 1864 Normal Brain NBcocipital 14549 1864 Normal Brain NBtemporal 24982 1864 All 1418 26306 1864 pGBM LÜ 820 38785 1864 pGBM 1214 24461 3412 sGBM 1329 39085 3412 sGBM 1342 <td>pGBM</td> <td>2896</td> <td></td> <td>19339</td> <td></td> <td></td> <td></td>	pGBM	2896		19339			
DGBM 3031 37154 pGBM 3032 26770 pGBM 3066 7859 pGBM 3070 16581 pGBM 3513 30871 pGBM 3513 30871 pGBM 3747 36070 GBM age 2655 20061 GBM age 3527 21366 GBM sark 2655 20061 GBM sark 3555 18439 2160 Normal Brain NBrontal 17581 Normal Brain NBparietal 1864 Normal Brain NBparietal 1864 Normal Brain NBtemporal 24982 All 1418 26306 pGBM LÜ 820 38785 pGBM LÜ 820 38785 pGBM 1214 24461 39085 sGBM 1214 24461 sGBM 1944 16999 sGBM 2007 43109 sGBM 2727 20646 sGBM 3475 17974 G	pGBM	3007		52052			
j GBM 3032 26770 pGBM 3066 7859 pGBM 3070 16581 1909 pGBM 3513 30871 pGBM 3747 36070 GBM 0igo 2655 20061 GBM sark 2643 17833 GBM sark 2643 17833 GBM sark 3555 18439 2160 Normal Brain NBforotal 17581 Normal Brain NBrotral 14549 Normal Brain NBtemporal 24982 All 1418 26306 pGBM LÜ 820 38785 pGBM Sark 176 24154 sGBM 677 18769 3412 sGBM 1329 39085 sGBM sGBM 1329 39085 sGBM 3412 sGBM 2007 43109 sGBM 568 sGBM 21727 20646 SGBM 3475 sGBM 3475 17974 58776 GBM cells LN428	pGBM	3031		37154			
pGBM 3066 7859 pGBM 3070 16581 1909 pGBM 3513 30871 1909 pGBM 3747 36070 16581 1909 pGBM 3747 36070 2061 16581 1909 GBM oligo 2655 20061 1843 17833 17833 17833 17833 1864 Normal Brain NBfortal 17581 17681 1864 1864 Normal Brain NBtemporal 24982 1864 1864 1864 Normal Brain NBtemporal 24982 38785 1864 1864 SGBM 1714 24154 3412 3412 3412 sGBM 1329 39085 3412 3412 3613 1656 177	pGBM	3032		26770			
pGBM 3070 16581 1909 pGBM 3513 30871	pGBM	3066		7859			
pGBM 3513 30871 pGBM 3747 36070 GBM oligo 2655 20061 GBM RZ 3527 21366 GBM sark 2643 17833 GBM sark 3555 18439 2160 Normal Brain NBcocipital 17581 Normal Brain NBcocipital 1448 Normal Brain NBcocipital 1448 Normal Brain NBtemporal 24982 All 1418 26306 pGBM LÜ 820 38785 pGBMsark 176 24154 sGBM 677 18769 sGBM 1214 24461 sGBM 1329 39085 sGBM 1430 24163 sGBM 1430 24163 sGBM 2727 20646 sGBM 2727 20646 sGBM 3475 17974 GBM cells 1N229 20089 GBM cells L	pGBM	3070		16581	1909		
pGBM 3747 36070 GBM oligo 2655 20061 GBM RZ 3527 21366 GBM sark 2563 17833 GBM sark 3555 18439 2160 Normal Brain NBfontal 17581 Normal Brain NBcocipital 14549 Normal Brain NBcocipital 14549 Normal Brain NBenporal 24982 All 1418 26306 pGBM LÜ 820 38785 pGBMsark 176 24154 sGBM 677 18769 sGBM 1214 24461 sGBM 1329 39085 sGBM 1430 24163 sGBM 2727 20646 sGBM 2727 20646 sGBM 2172 38776 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells LN428 27441 GBM cells	pGBM	3513		30871			
GBM oligo 2655 20061 GBM RZ 3527 21366 GBM sark 2643 17833 GBM sark 3555 18439 2160 Normal Brain NBcrotal 17581 Normal Brain NBcrotal 14549 Normal Brain NBcroital 14549 Normal Brain NBparietal 1864 Normal Brain NBtemporal 24982 All 1418 26306 pGBMSark 176 24154 sGBM 677 18769 3412 sGBM 1214 24461 3686 sGBM 1329 39085 3412 sGBM 1329 39085 3412 sGBM 1329 39085 3412 sGBM 1329 39085 36BM sGBM 2007 43109 3412 sGBM 2007 43109 36B sGBM 2007 38776 GBM cells <td< td=""><td>pGBM</td><td>3747</td><td></td><td>36070</td><td></td><td></td><td></td></td<>	pGBM	3747		36070			
GBM RZ 3527 21366 GBM sark 2643 17833 GBM sark 3555 18439 2160 Normal Brain NBforotal 17581 Normal Brain NBcocipital 14549 Normal Brain NBcocipital 1864 Normal Brain NBtemporal 24982 All 1418 26306 pGBMSark 176 24154 SGBM 677 24154 SGBM 1214 24461 SGBM 1329 39085 SGBM 1329 39085 SGBM 1944 16999 SGBM 2007 43109 SGBM 2727 20646 SGBM 3475 17974 GBM cells A172 38776 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells LN428 27441	GBM oligo	2655		20061			
GBM sark 2643 17833 GBM sark 3555 18439 2160 Normal Brain NBfrontal 17581 Normal Brain NBcocipital 1459 Normal Brain NBcocipital 1459 Normal Brain NBtemporal 24982 All 1418 26306 pGBMSark 176 24154 sGBM 677 18769 3412 sGBM 1214 24461 356BM sGBM 1329 39085 36BM sGBM 1944 16999 36BM sGBM 2727 20646 36BM sGBM 3475 17974 38776 GBM cells 1N229 20089 36BM cells 1N229 GBM cells LN428 27441 GBM cells LN428	GBM RZ	3527		21366			
GBM sark 3555 18439 2160 Normal Brain NBfrontal 17581 Normal Brain NBpocipital 17581 Normal Brain NBpocipital 1459 Normal Brain NBpocipital 1864 Normal Brain NBpocipital 24982 All 1418 26306 pGBMLÜ 820 38785 pGBMsark 176 24154 sGBM 677 18769 3412 sGBM 1214 24461 3688 sGBM 1329 39085 3668M sGBM 1340 24163 368M sGBM 1430 24163 368M sGBM 2007 43109 368M sGBM 2727 20646 368M sGBM 3475 17974 GBM cells A172 38776 GBM cells LN428 27441 GBM cells LN428 27441 GBM cells LN428	GBM sark	2643		17833			
Normal Brain NBfrontal 17581 Normal Brain NBoccipital 14549 Normal Brain NBoccipital 14549 Normal Brain NBoparietal 1864 Normal Brain NBtemporal 24982 All 1418 26306 pGBM LÜ 820 38785 pGBMsark 176 24154 sGBM 677 18769 sGBM 1214 24461 sGBM 1329 39085 sGBM 1430 24163 sGBM 1944 16999 sGBM 2007 43109 sGBM 2727 20646 sGBM 3475 17974 GBM cells A172 38776 GBM cells LN428 27441 GBM cells LN428 27441	GBM sark	3555		18439	2160		
Normal Brain NBocipital 14549 Normal Brain NBparietal 1864 Normal Brain NBtemporal 24982 All 1418 26306 pGBM LÜ 820 38785 pGBM LÜ 820 38785 pGBM LÜ 820 38785 pGBM 176 24154 4 sGBM 677 18769 3412 sGBM 1214 24461 5 sGBM 1329 39085 5 sGBM 1430 24163 5 sGBM 1430 24163 5 sGBM 2007 43109 5 sGBM 2007 43109 5 sGBM 2727 20646 5 sGBM 3475 17974 5 GBM cells LN229 20089 5 GBM cells LN428 27441 5 GBM cells TN428 27441 GBM cells	Normal Brain	NBfrontal		17581			
Normal Brain NBparietal 1864 Normal Brain NBtemporal 24982 All 1418 26306 pGBM LÜ 820 38785 pGBMSark 176 24154 sGBM 677 18769 sGBM 1214 24461 sGBM 1329 39085 sGBM 1430 24163 sGBM 1944 16999 sGBM 2007 43109 sGBM 2007 43109 sGBM 2007 43109 sGBM 21727 20646 sGBM 3475 17974 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells LN428 27441	Normal Brain	NBoccipital		14549			
Normal Brain NBtemporal 24982 All 1418 26306 pGBM LÜ 820 38785 pGBMsark 176 24154 sGBM 677 18769 3412 sGBM 1214 24461 3686 sGBM 1329 39085 368M sGBM 1430 24163 368M sGBM 1329 39085 368M sGBM 1329 39085 368M sGBM 2007 43109 368M sGBM 2727 20646 38776 sGBM cells 1N229 20089 38776 GBM cells LN428 27441 GBM cells LN428 GBM cells LN428 27441 3426	Normal Brain	NBparietal					1864
Ali 1418 26306 pGBMLÜ 820 38785 pGBMsark 176 24154 sGBM 677 18769 3412 sGBM 1214 24461 sGBM 1329 39085 sGBM 1430 24163 sGBM 1944 16999 sGBM 2007 43109 sGBM 2727 20646 sGBM 3475 17974 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells LN428 27441	Normal Brain	NBtemporal		24982			
pGBM LU 820 38785 pGBMsark 176 24154 sGBM 677 18769 3412 sGBM 1214 24461 3412 sGBM 1329 39085 36BM sGBM 1430 24163 36BM sGBM 1944 16999 36BM sGBM 2727 20646 36BM sGBM 3475 17974 38776 GBM cells LN229 20089 36BM cells LN428 GBM cells LN428 27441 GBM cells LN428	All	1418		26306			
pGBMsark 176 24154 sGBM 677 18769 3412 sGBM 1214 24461 568 sGBM 1329 39085 568M 1342 sGBM 1430 24163 568M 1944 16999 sGBM 2007 43109 568M 2727 20646 sGBM 2727 20646 568M 3475 17974 GBM cells LN229 20089 6BM cells LN228 27441 GBM cells LN428 27441 6BM cells 1896 21426	pGBM LU	820		38785			
SUBM 677 18769 3412 SGBM 1214 24461 39085 SGBM 1329 39085 39085 SGBM 1430 24163 368 SGBM 1944 16999 36BM 2007 43109 SGBM 2007 43109 36BM 2727 20646 SGBM 3475 17974 38776 38776 38776 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells 18986 23426 3472 34741	pGBMsark	176		24154			
SUBM 1214 24461 SGBM 1329 39085 SGBM 1430 24163 SGBM 1944 16999 SGBM 2007 43109 SGBM 2727 20646 SGBM 3475 17974 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells T986 23426	sGBM	677		18769		3412	
SUBM 1329 39085 SGBM 1430 24163 SGBM 1944 16999 SGBM 2007 43109 SGBM 2727 20646 SGBM 3475 17974 GBM cells A172 38776 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells T986 23426	sGBM	1214		24461			
SUBM 1430 24163 SGBM 1944 16999 SGBM 2007 43109 SGBM 2727 20646 SGBM 3475 17974 GBM cells A172 38776 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells T986 23426	SGBM	1329		39085			
SUBM 1944 16999 sGBM 2007 43109 sGBM 2727 20646 sGBM 3475 17974 GBM cells A172 38776 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells T986 23426	SGBM	1430		24163			
SCDIM 2007 43109 SGBM 2727 20646 SGBM 3475 17974 GBM cells A172 38776 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells T986 23426	SGBM	1944		16999			
SCDIM 2/2/ 20b4b SGBM 3475 17974 GBM cells A172 38776 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells T986 23426	SGBM	2007		43109			
SCODM 34/5 1/9/4 GBM cells A172 38776 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells T986 23426	SGDIVI	2/2/		20646			
GBM cells A1/2 38/76 GBM cells LN229 20089 GBM cells LN428 27441 GBM cells T986 23426	SGBIM	34/5		1/9/4			
GBM cells LN229 20089 GBM cells LN428 27441 GBM cells T986 2326	GBIVI CEIIS	A1/2		38776			
GBM cells T98G 23426	GDIVI CEIIS	LIN229		20089			
130U /34/b	GBIVI CEIIS	LIN428		27441			
GRM cells 11178 49217	GBIVI CEIIS	1986		2342b 48217			

more abundant in normal brain tissue and reduced or lacking in tumors more abundant in glioma tissue and reduced or lacking in normal brain tissues

В

Detected splice isoforms:					CPEB2d	CPEB2d*	CPEB2c	CPEB2c*	CPEB2b	CPEB2a
Diagnose	ID-T-Nummer	CPEB2	170 bp	203 bp	262 bp	271 bp	286 bp	294 bp	351 bp	375 bp
AAIII	2377				19243	3816	21200		7022	8456
AAIII	2526				15307		28051	4055		4453
AAIII	2725				13604	2454	17950	2852	3193	4612
AAIII	2744				7681		12972			4560
AAIII	2771				8959	1071	13648	1525	1776	2961
AAIII	2897				9514		34779	4853	2798	16075
AAIII	2899				5384		24686	4201		8263
AAIII	3423				9284		23739	2808		6835
AAIII	3545				19103		31827	3676	4930	10797
AAIII	3546				9080		34540	6921		7906
AAIII	3548				12417		23140	2490	3353	7882
AAIII	4045				3934		57221	4731		9744
pGBM	71				15391	2816	5999		1907	
pGBM	72				23004	3795	11148		5366	
pGBM	132				8845		9613		1354	
pGBM	172				16565		15177			5853
pGBM	327				19305		5559		2000	
pGBM	328				27750		20949			6870
pGBM	625				28744	3612	10825		7319	3753
pGBM	862				17776	2447	6002		1722	
pGBM	1010				15453	2242	9012	1193	3549	2778
pGBM	1311				39092	4376	12895		5148	
pGBM	1619				20667		14643			
pGBM	1968				22959		18904		6162	
pGBM	2010				21862	2844	16371	2312	7560	7230
pGBM	2104				20673	2917	32529	4586		4254
pGBM	2169				33190	4799	10029		4174	
pGBM	2304				20938	3913	8495		2218	
pGBM	2481				21328		23116			6976
pGBM	2486				23707		19784	3520		
pGBM	2494				10869		12771		2577	3845
pGBM	2735				30423	5149	16458		11587	8431
pGBM	2757				20331	3224	11469		5863	3850
pGBM	2854				16473	3507	31545	6160		4135
pGBM	2884				22520	3867	11169		3910	2412
pGBM	2896				18772	3692	21197			5594
pGBM	3007				35081	6034	25479	4176	7502	7393
pGBM	3031		422	567						
pGBM	3032				23106	4075	21910	3737	5109	5755
pGBM	3066				7374		4665		966	
pGBM	3070				11779		9861			
pGBM	3513				15812	2648	13211	1889	3364	3997
pGBM	3747				3935		35837	4589		9197
GBM oligo	2655				5707		16105	3979		
GBMRZ	3527				19454	3971	16649	3240	2610	2390
GBM sark	2643				15336		9016			
GBM sark	3555				15783	1412	8804		4047	2729
Normal Brain	NBfrontal						19221	2402		9947
Normal Brain	NBoccipital						17043	2654		8193
Normal Brain	NBparietal						27201	3259		15137
Normal Brain	NBtemporal						9943			5267
All	1418				10382		27422	3413		9416
pGBM LÜ	820				18678		17925			
pGBMsark	176				13646		6486			
sGBM	677				22717	4536	16365		6843	5106
sGBM	1214				14151		22018			
sGBM	1329				25454	4341	13992	2448	3946	3066
sGBM	1430				8732		13920			2573
sGBM	1944				2784		1408			
sGBM	2007				25122		13291		5809	4136
sGBM	2727				13130		13388	2144		
sGBM	3475				9065		25518	4897		6035
GBM cells	A172				31735	3767	4119		3787	
GBM cells	LN229				22869	5466	1544		2998	
GBM cells	LN428				32980	3384	8621			
GBM cells	T98G				43905				3575	
GBM cells	11178				48671					

more abundant in normal brain tissue and reduced or lacking in tumors more abundant in glioma tissue and reduced or lacking in normal brain tissues

С

Detected spl	ice isoforms:											CPEB3d	CPEB3c		CPEB3b	CPEB3a
Diagnose	ID-T-Nummer	CPEB3	100 bp	104 bp	122 bp	135 bp	143 bp	182 bp	203 bp	254 bp	452 bp	507 bp	531 bp	547 bp	576 bp	600 bp
AAIII	2377											14978			7471	
AAIII	2526											16481			5174	
AAIII	2725											8565			5827	
AAIII	2744									1336		8779			3911	
AAIII	2771											11567				
AAIII	2897											10303	3297		7329	6800
AAIII	2899											9728			5401	1662
AAIII	3423											8332			7130	3319
AAIII	3545											10263	1937		6631	5312
AAIII	3546											10090			7658	
AAIII	3548				2244		950					14927			5418	1641
AAIII	4045						2809		967	1267		6986				
pGBM	71				975							2644				
pGBM	72					890						12224			3767	
pGBM	132											2960				
pGBM	172														5473	
pGBM	327										4332					
pGBM	328											11773			3715	
pGBM	625				3411				1331			9924			4181	
pGBM	862				2902							4307				
pGBM	1010		2482									3189			3926	
pGBM	1311				1697							5664			4930	
pGBM	1619											8481			2164	
pGBM	1968								1309			12571			3929	
pGBM	2010				1832							11890		4069	5785	
pGBM	2104				1684							9475			5370	
pGBM	2169		2010		2775				2038	1369		7456				
pGBM	2304				3064							6016			5035	
pGBM	2481											4655			1948	
pGBM	2486											13216			5885	
pGBM	2494											7194			4258	
pGBM	2735				3702							19438			6404	
pGBM	2757											7269			3233	
pGBM	2854				2848							11950			4230	
pGBM	2884				906						2270	3166			2572	
PGBM	2896				2215				1700			8291	2000		35/3	40.42
pGBM	3007				3215				1202		1204	12085	2090		0410 EE21	4942
pGBM	3032					1/199			1/25		1354	11285	2410		5442	
pGBM	3066				027	1400			1433			5000			5442	
pGBM	3070				28/13							2016				
pGBM	3513				2045	1274			058			7020			5/03	
nGBM	3747					12/4			530	1060		13025			5380	
GBM oligo	2655									1000		6427			5669	
GBM RZ	3527											14338			8036	
GBM sark	2643					1052						2988			0000	
GBM sark	3555							1790				7409			4267	
Normal Brain	NBfrontal											2786			3347	6109
Normal Brain	NBoccipital											2747	1313		3014	4169
Normal Brain	NBparietal											2224			4410	8886
Normal Brain	NBtemporal											2423				4662
All	1418											10694			3442	
pGBM LÜ	820					781						14046			9726	
pGBMsark	176											1821			994	
sGBM	677				1794							16129			5345	
sGBM	1214											10861			4527	
sGBM	1329											5077			3081	
sGBM	1430											2564			1991	
sGBM	1944															
sGBM	2007				1156							12615			5049	
sGBM	2727											3751			2626	
sGBM	3475											12062			4703	
GBM cells	A172		4055		4301							8985			4284	
GBM cells	LN229											2498			2219	
GBM cells	LN428											8673			5298	
GBM cells	T98G		1685		1524											
GBM cells	U178		9331	1442	10162				1469			9216			3498	

more abundant in normal brain tissue and reduced or lacking in tumors

more abundant in glioma tissue and reduced or lacking in normal brain tissues

D

Detected sp	lice isoforms:					CPEB4d	CPEB4c	CPEB4b	CPEB4a			
Diagnose	ID-T-Nummer	CPEB4	115 bp	142 bp	182 bp	203 bp	226 bp	254 bp	277 bp	306 bp	507 bp	547 bp
AAIII	2377					21149		21655				
AAIII	2526					19619		19174			1675	
AAIII	2725					26046		20165				
AAIII	2744					21718		25345				
AAIII	2771					16637		12207		7133		
AAIII	2897					26150	5221	22566	4984			
AAIII	2899					19046	1503	15869	1628			
AAIII	3423					9538		8597	1103			
AAIII	3545					19463		25298				
AAIII	3546					22665		20903				
AAIII	3548					21383		22958	2407		2125	
AAIII	4045					26882		32885	2864			
pGBM	71					7320		4185				
pGBM	72					15172		15587				
pGBM	132					6518		5536				
pGBM	172					17460	4028	9620	6182			
pGBM	327					12086		8460				
pGBM	328					25485		21644				
pGBM	625				2634	30452		11702				
pGBM	862			3500		15071		5445				
pGBM	1010					22305		14188				
pGBM	1311					22087		17696				
pGBM	1619					14804		15511				
pGBM	1968					33990		34922				
pGBM	2010					22114		21939				10977
pGBM	2104					20095		12555	2397			
pGBM	2169					49763		37272				
pGBM	2304					16788		13189				
pGBM	2481					14181		14563				
pGBM	2486				1526	25177		22191			1216	
pGBM	2494					13383		10394	2237			
pGBM	2735					22615		30332			2482	
pGBM	2757		1010			13014		16528				
pGBIVI = CDM	2854		1810			27502		1//5/				
PGBIVI	2884					9538		11158	2257			
pGBIVI	2690					21592		22122	2557			
pGBM	3031					35312		22502				
pGBM	3032					37208		25352				
pGBM	3066					8825		7801				
pGBM	3070					9167		6748				
pGBM	3513					21270	2580	19710	3011			
pGBM	3747					21631		25574				
GBM oligo	2655					11793		9233	2176			
GBMRZ	3527					22106		22278				
GBM sark	2643					18032		12070				
GBM sark	3555				4261	11334		9271	5636			
Normal Brain	NBfrontal					9860	6940	6026	6231			
Normal Brain	NBoccipital					8343	8381	5602	7068			
Normal Brain	NBparietal					20994	14305	11481	10482			
Normal Brain	NBtemporal					9343	7008	5371	5927			
All	1418					19617		20652				
pGBMLU	820					20257		13442			1764	
pGBMsark	176					9898		5532				
SGBM	677					17046		1/065			1621	
SGBM	1214					16456	2/22	14162	2/1/			
SGBIM	1329					6/42	2555	4//8	2452	1		
SGBIVI	1430					15947	2555	9824	2453			
SGBIVI	1944					89/1		3/4/				
SGBIVI	2007					12050		10792				
*GBM	2/2/					11656		16520				
GBM calle	5475 ∆177					20800		12024				
GBM cells	N172					14344		10688				
GBM cells	1 N478					15692		12416				
GBM cells	T98G					27548		7879				
GBM cells	U178					38061		17132				

more abundant in normal brain tissue and reduced or lacking in tumors more abundant in glioma tissue and reduced or lacking in normal brain tissues

5.2 Alterations of growth properties and cancer-associated parameters in glioblastoma-derived cells mediated by CPEBs

5.2.1 Expression profile of CPEB 1-4 in A172 cultured glioblastoma cells

Altered expression of CPEBs in high- and low-grade gliomas indicated their role in the translational regulation in cancer. CPEB protein activity was studied in human glioma specimens and described in the previous section (see chapter 5.1.3.1). The current investigation addressed a potential influence of CPEBs on glioblastoma cells *in vitro*. With this intention, endogenous CPEB expression was monitored in human A172 glioblastoma culture (Fig. 5.2.1-1). Among CPEB paralogs, the most prominent expression was observed in CPEB 2-4. Proteins were detected as a punctate staining in the cytoplasm of investigated cells. Expression of CPEB1 and phospho-CPEB3 appeared to be reduced. Although all CPEBs are able shuttle to the nucleus (Kan et al., 2010), here, only phospho-CPEB3 and CPEB3 were observed in cell nuclei.



Figure 5.2.1-1. Endogenous expression of CPEB1-4 and phospho-CPEB3 proteins in human A172 glioblastoma culture cells. Starting from the left, the panels show staining of individual CPEBs, DAPI and their merged staining. Scale bars represent 25 μm.

5.2.2 Functional characterization of CPEB1 and CPEB2 protein overexpression in cultured A172 glioblastoma cells

5.2.2.1 Overexpression of CPEB1 and CPEB2 proteins

The contribution of CPEBs to the molecular pathogenesis of glioblastoma was investigated by overexpression of CPEB1 (n=4) and CPEB2 (n=7) proteins in glioblastoma-derived cells (Fig. 5.2.2.1-1). Next, A172 cultures transiently overexpressing CPEB1 or CPEB2 protein were investigated with regard to proliferation, apoptotic activity and migration.



Figure 5.2.2.1-1. Overexpression of CPEB1 and CPEB2 proteins in A172 glioblastoma culture. A, C: Representative immunoblot showing overexpression of CPEB1, CPEB2 proteins and tubulin as an internal control. B, D: Analysis of the immunoblots of CPEB1-pCMV/Neo (n=4) and CPEB2-pCMV/Neo (n=7) transfected cells in comparison to reference pCMV6/Neo vector.

5.2.2.2 Alteration of proliferation and apoptotic activity of glioblastoma cells mediated by CPEB1 and CPEB2 proteins

The influence of CPEB overexpression on proliferation and apoptosis of cultured A172 cells was identified by flow cytometric analysis. In each experiment 100000 GFP-CPEB1-pCMV6/Neo, GFP-CPEB2-pCMV6/Neo or control GFP-pCMV6/Neo transfected cells were analyzed. Apoptotic, cPARP+ cells were labeled by antibodies against poly(ADP-ribose) polymerase (Asp214), while proliferating, pHH3+ cells were detected by antibodies against Ser10-phosphorylated histone H3. Overexpression of CPEB1 resulted in 1.75-fold growth of the apoptotic cellular population. In contrast, overexpression of CPEB2 resulted in a decrease in apoptosis (to 70%). Proliferation appeared to be not affected by CPEB overexpression (Fig. 5.2.2.2-1).




Figure 5.2.2.2-1. Flow cytometric analyses performed in A172 glioblastoma cells overexpressing CPEB1 or CPEB2 protein. Proliferating cells (pHH3+) were detected by antibodies against Ser10-phosphorylated histone H3, whereas apoptotic cells (cPARP+) by antibodies against poly(ADP-ribose) polymerase (Asp214). In each experiment at least 100000 GFP- CPEB1-pCMV6/Neo, GFP- CPEB2-pCMV6/Neo, GFPpCMV6/Neo or GFP positive cells were analyzed. Cells transfected with GFP- pCMV6/Neo or GFP vector were considered as reference. Non-transfected cells labeled with pHH3 or stimulated with 1 μM staurosporine and stained with PARP antibody were considered as a proliferation and apoptosis control.

5.2.2.3 Influence of CPEB1 and CPEB2 overexpression on glioblastoma cells migration

The impact of CPEBs on cell migration was determined by overexpression of CPEB1 or CPEB2 protein in cultured glioblastoma cells followed by an *in vitro* scratch assay (Fig. 5.2.2.3-1A). Since FACS analysis (see Fig. 5.2.2.2-1) did not reveal any effect of increased CPEB expression on proliferation, the presence of cells in the wounded area was associated with their enhanced migratory capability. The change in the area covered by migrating cells over the time was raised up to 30% and 20% by elevated CPEB1 and CPEB2 protein levels, respectively (Fig. 5.2.2.3-1B).





Figure 5.2.2.3-1. Influence of CPEB1 and CPEB2 overexpression on migration of cultured A172 glioblastoma cells A: Motility of CPEB1 and CPEB2 overexpressing cells examined by an *in vitro* wound healing assay. Scale bars represent 100 μm. B: Quantification of the change in the cell-covered area over time (24h) upon elevated expression of CPEB1 (n=4) and CPEB2 (n=4) proteins. Error bars indicate standard error of the mean.

5.2.3 Identification of cancer-associated signaling pathways altered by CPEB1 and CPEB2 proteins

Changes in cancer-associated signaling pathways induced by CPEBs were investigated by a cell-based pathway reporter array. Quantitative evaluation was possible upon overexpression of CPEB1 and CPEB2 in cultured cells and its reverse transfection with reporters of transcription factors present at the array plate. Increased expression of CPEB1 revealed a significant upregulation of estrogen, hedgehog, HNF4 and TGF β pathways (Fig. 5.2.3-1A). In contrast, elevated expression of CPEB2 protein resulted in upregulation of c-myc, oct4, PI3K/Akt and TGF β cascades (Fig. 5.2.3-1B). Since overexpression of CPEB1 proteins led to enhancement of TGF β activity, it might indicate that the detected cascade is a novel target for translational regulation by CPEBs









Results



Figure 5.2.3-1. Cancer-associated signaling pathways altered by CPEB1 and CPEB2 proteins. A: Changes in cancer-associated signaling pathways monitored by cell-based reporter assays (n=3) revealed a significant upregulation of estrogen, hedgehog, HNF4 and TGF β upon CPEB1 overexpression. Error bars indicate SEM. B: Upregulation of myc, oct4, PI3K/Akt and TGF β signaling pathways upon CPEB2 overexpression (n=2; statistical quantification was not performed).

5.3 Expression profile of CPEB2 protein in mouse brain

5.3.1 Expression of CPEB1 and CPEB2 proteins in primary hippocampal cultures

In neurons, proteins involved in synaptic plasticity are predominantly derived from RNA molecules localized in dendrites. In response to synaptic stimulation, CPE elements present in the 3'UTR of dendritic mRNAs and CPE binding proteins promote translation induced by cytoplasmic polyadenylation. CPEBs also facilitate transport of mRNAs to dendrites. Localization of CPEB1 in cultured neurons has been observed in both, dendrites and cell bodies (Huang et al., 2003). Here, the expression of CPEB1 and CPEB2 was examined in rat primary hippocampal cultures. Neurons were co-stained with antibodies against CPEB1, CPEB2 and neuronal marker MAP2. Subcellular localization of both CPEBs overlapped in cultured neurons. Punctate distribution of expressed CPEB2 was observed in dendrites and the soma, similar to CPEB1 (Fig. 5.3.1-1).



Figure 5.3.1-1. Endogenous expression of CPEB1 and CPEB2 in primary hippocampal neurons. 7 days in vitro hippocampal cultures prepared from Wistar rats were stained with CPEB1 (green), CPEB2 (green), MAP2 (red) antibodies and Hoechst counterstain (blue). Scale bars represent 25 μm.

5.3.2 Expression of CPEB2 protein in juvenile and adult mouse brain

Expression pattern of CPEB2 protein in juvenile (p12) and adult (p90) C57Bl6J mouse brain was determined with custom made CPEB2 antibody directed to mouse and human. Antibodies specifically recognized CPEB2a and CPEB2a* splice variants containing the Band C-region and did not cross-react with other CPEBs (Turimella et al., 2015). Expression of CPEB2 was explored in neurons of different brain areas by immunostaining. Coronal sections from corresponding age groups were stained with antibodies against CPEB2 and the neuronal marker MAP2. The vast majority of MAP2 positive neurons in the CA1 region of the hippocampus, striatum radiatum, thalamus, cortex, and cerebellum displayed immunoreactivity against CPEB2 (Fig. 5.3.2-1). In individual cells of juvenile and adult animals, CPEB2 was located in the cytoplasm and processes. Dendritic localization of CPEB2 seemed to be more prominent in adult animals (Fig. 5.3.2-1B).





Figure 5.3.2-1.

Figure 5.3.2-1. Examination of CPEB2a and CPEB2a* expression in (A) juvenile (p12) and (B) adult (p90) C57Bl6J mice brains. Representative confocal images of CPEB2 (green), MAP2 (red), and Hoechst (blue) staining in the hippocampal CA1 region, striatum radiatum (STR), thalamus (TH), cortex (CTX), and cerebellum (CB). Scale bars represent 50 µm.

5.3.3 Differential expression of CPEB2 in excitatory, inhibitory and dopaminergic neurons

Differential expression of CPEB2 in excitatory, inhibitory, and dopaminergic neurons was assessed by immunostaining. 42.26±15.9% (n=3) of the GFP positive excitatory neurons in the hippocampus and dentate gyrus demonstrated CPEB2 immunoreactivity in Thy1-GFP mice (Fig. 5.3.3-1.). Further, in wild-type C57Bl6J mice, 93.6±9% (n=3) of the hippocampal inhibitory neurons and 62.36±9% (n=3) of the midbrain dopaminergic neurons demonstrated CPEB2 immunoreactivity (Fig. 5.3.3-1.). CPEB2 protein occurred to be heterogeneously expressed among investigated types of neurons and its strongest expression was observed in parvalbumin positive inhibitory neurons.



Figure 5.3.3-1. Endogenous expression of CPEB2 in excitatory, inhibitory, and dopaminergic neurons. Staining of GFP (green) and CPEB2 (red) or parvalbumin (PARV; red), tyrosine hydroxylase (TYH; red), and CPEB2 (green) was combined with Hoechst counterstain (blue). Scale bars represent 50 µm.

6 DISCUSSION

Understanding the molecular pathophysiology of tumors is fundamental to identify novel therapeutic and diagnostic factors. Thus far, cancer-related genes were abundantly investigated by transcript-based microarrays (van den Boom et al., 2003; Phillips et al., 2006; Rickman et al., 2001). In addition to these analyses, a recent study emphasizes the importance of post-transcriptional modification regulating gene expression changes under physiological conditions, but also giving rise to the pathology of tumors (Ortiz-Zapater et al., 2012). Translational control is particularly relevant in aggressive and therapy resistant malignancies. Despite reports endorsing the role of CPEBs in polyadenylation-induced translation in gliomas (Hu et al., 2015; Kochanek and Wells, 2013; Ortiz-Zapater et al., 2012), our knowledge of CPEB properties and their physiological relevance is incomplete. Thus far, the main research effort was focused on characterization of CPEB1. However, other members of the CPEB family mediate equally important processes, including synaptic plasticity and cancer formation (Ivshina et al., 2014). Furthermore, regardless of structural similarities, CPEBs are functionally non redundant (Ivshina et al., 2014). Therefore we chose to carefully examine the functional aspects of the CPEB isoforms in the developing and diseased brain.

6.1 The role of CPEBs in development and progression of glioma

6.1.1 Expression of CPEBs is heterogeneous in human glioma tissues

To examine functions of each of the CPEB subtypes in human glioma tissue, the present study focused on the expression patterns by comparing glioma tissues of different WHO grades. For this purpose, the abundance and localization of CPEBs was determined by immunohistochemistry. The vast majority of CPEB1 expression was detected in the infiltration areas of tumor cells into normal brain tissue. Cells in the tumor center, in the

areas of necrosis and angiogenesis, lacked CPEB1 (Fig. 5.1.3.1-1). It is generally known that tumor cells may adapt to the local environment or change the surrounding towards their own benefit (Godlewski et al., 2014). The fact that CPEB1 expression was reduced in GBMs may suggest a proto-oncogenic adaptation. Interestingly, expression of CPEB1 was downregulated with WHO grade, reaching the lowest level in GBMs (Fig. 5.1.3.1-2A; Tab. 5.1.3.1-1). A similar expression course was shown in the recent study of Galardi et al. (2016), where CPEB1 mRNA level was maximum in normal brain and significantly reduced in cancer tissues. In line with these findings, CPEB1 was indicated to be a putative tumor suppressor (D'Ambrogio et al., 2013). Abundant evidence form ovarian, melanoma, and gastric cancers and cell lines derived from breast, myeloma and colorectal cancer (Caldeira et al., 2012; Hansen et al., 2009; Heller et al., 2008; Nairismägi et al., 2012; Shoshan et al., 1999) report reduction in CPEB1 transcript and suggest a potential tumor repressing role. Furthermore, downregulation of CPEB1 in tumor cells disturbs the mitotic regulation of poly(A) tails, pre-mRNA alternative polyadenylation site selection, and inhibits cell proliferation (Bava et al., 2013; Giangarrà et al., 2015b; Novoa et al., 2010). In contrast, reduction of CPEB1 in primary fibroblasts is associated with bypass of senescence (Burns and Richter, 2008; Fernández-Miranda and Méndez, 2012) and the ability of tumor cells to enhance invasion, angiogenesis and to increase resistance to nutritional stress (Grudzien-Nogalska et al., 2014). Also knockout of CPEB1 induces epithelial-tomesenchymal transition (Grudzien-Nogalska et al., 2014), a process that is associated with increased cancer progression and metastatic potential (Kalluri and Weinberg, 2009). From these results it can be concluded that expression of CPEB1 is reduced in various cancers in order to support tumor development and progression.

To a certain extent, the expression pattern of CPEB2 was similar to CPEB1. Prevalent CPEB2 immunoreactivity was observed in less aggressive AII and AAIII, while pGBM specimens were negative or showed very weak CPEB2 staining (Fig. 5.1.3.1-2B; Tab. 5.1.3.1-1). CPEB2 was found in reactive astrocytes and in endothelial tumors cells, suggesting that protein synthesis takes place in the close proximity of blood vessels during tumor formation (Fig. 5.1.3.1-1). One of the possible explanations for CPEB2 expression in endothelial cells may be association of CPEB2 with stress conditions. It was shown that increased oxidative stress provokes CPEB2 binding to HIF-1 α mRNA and regulates its

expression (Hägele et al., 2009). In turn, HIF-1 α controls homeostatic responses to oxidative stress by stimulating transcription of genes involved in angiogenesis, metabolism and cell survival (Chen et al., 2015). However, further research is needed to explain the role of CPEB2 in hypoxia and vascularization.

In the investigated cohort of gliomas, CPEB3 was the most abundantly expressed family member (Fig. 5.1.3.1-1). The present study provides the first analysis of CPEB3 in human glioma specimens. In contrast to CPEB1 and CPEB2, its immunoreactivity increased with progressing tumor grade, and achieved the strongest intensity in GBMs (Fig. 5.1.3.1-2C; Tab. 5.1.3.1-1). At the same time, the activity of the protein, determined by its phosphorylation, decreased with WHO grade (Fig. 5.1.3.1-2D; Tab. 5.1.3.1-1). Therefore, despite the strong CPEB3 expression, phospho-CPEB3 in GBMs was distinctly reduced. This suggests that a substantial part of CPEB3 protein in GBM specimens does not undergo phosphorylation and indicates phosphorylation to be the biologically significant mechanism regulating CPEB3 activity in gliomas.

In contrast to the other CPEBs, strong expression of CPEB4 was solely observed in a few of the investigated glioma specimens (Fig. 5.1.3.1-2E; Tab. 5.1.3.1-1), with a prominent immunoreactivity in cell processes (Fig. 5.1.3.1-1). This localization of CPEB4 indicates its involvement in local protein synthesis, which was shown to be particularly important for synaptic plasticity (Kandel, 2001; Zukin et al., 2009), but may also contribute to cancerogenesis. CPEB4 may be implicated in the syntheses of proteins involved in cell-cell communication and tumor-stromal interactions (Ortiz-Zapater et al., 2012), thus indirectly in cancer progression. Indeed, downregulation of CPEB4 was associated with tumor development (Giangarrà et al., 2015). Cells overexpressing CPEB4 showed an advantage for tissue colonization and invasion (Fernández-Miranda and Méndez, 2012), while its downregulation resulted in reduction of cellular proliferation in the GBMs and PDAs (Ortiz-Zapater et al., 2012). Knockdown of CPEB4 contributed to a decrease of tumor size and vascularization, indicating its impact on changes in cell proliferation (Ortiz-Zapater et al., 2012). In the present study the expression pattern of CPEB4 could not be associated with the WHO grade. However Hu et al. (2015) reported CPEB4 to enhance tumor progression in human high-grade gliomas. Considering that high CPEB4 expression

supports tumorigenic properties, this isoform might be involved in local protein synthesis and mediate cancer progression.

Altogether, these observations are in agreement with the fact that CPEB expression is heterogeneous in human gliomas. Destabilization of the normal function of a wide range of transcripts regulated by CPEB favors tumor growth. Therefore, CPEB-dependent translational control may be considered as a mechanism having a significant role during glioma formation and progression.

6.1.2 CPEB expression patterns associate with clinical prognosis of glioma patients

Since in the present study a decrease in CPEB1 and phoshop-CPEB3 was observed with increasing tumor grade, the potential prognostic value of CPEB expression in human glioma specimens was assessed by Kaplan-Meier survival analysis. CPEB expression profiles compared with the clinical data revealed an interesting dependency between CPEB1, phospho-CPEB3 and life expectancy. A pronounced association of CPEB1 expression with prolonged survival was observed in AII and AAIII, but not in GBM specimens (Fig. 5.1.3.2-1A, B). Though it is necessary to keep in mind that low-grade astrocytoma patients usually progress better, recent reports support a link between reduced CPEB1 expression and longer survival (Galardi et al., 2016; Yin et al., 2014). Despite the robust expression of CPEB 2-4, none of the proteins showed correlation with patient survival (Fig. 5.1.3.2-1C, D, G). However, Hu et al. (2015) reported that a high CPEB4 expression profile was associated with shorter survival of GBM patients. Interestingly, there was a difference in the survival of patients with and without phospho-CPEB3. A significant correlation between CPEB3 activity and survival was observed in less aggressive low-grade astrocytomas considered to be sGBM precursors (Fig. 5.1.3.2-1E), but not in pGBMs (Fig. 5.1.3.2-1F). In order to ascertain an association between survival and phospho-CPEB3 expression in GBMs, an increased number of specimens should be retested. In general, the data presented here support the hypothesis that CPEBs may serve as a sensitive prognostic factor for glioma patients.

6.1.3 Methylation of *CPEB1* gene does not correlate with silenced expression

Hypermethylation of DNA is a frequent condition in cancers (Chen et al., 2014), resulting in stable transcriptional silencing of the associated genes (Jones and Baylin, 2007). This is due to the methyl groups that attach to the CpG islands localized in the promoter region, which impede the binding of transcription factors (Chen et al., 2014). The finding that CPEB1 expression was gradually decreasing with tumor progression, suggested increased DNA methylation (Curradi et al., 2002). To test this hypothesis genome wide DNA methylation analysis was performed. Methylation of *CPEB* genes was formerly studied in tumors (Caldeira et al., 2012; Heller et al., 2008), but hypermethylation in gliomas was never reported. Among the investigated genes, *CPEB1* and *CPEB3* were methylated (Fig. 5.1.1-1), however only *CPEB1* reached prominent hypermethylation level. *CPEB1* methylation was additionally associated with mutation in the *IDH* gene (Fig. 5.1.2-1). Recent studies proved that *IDH* mutations, not only drive an increased promoter methylation, but actually entail hypermethylation (Christensen et al., 2013).

In the present study a significant reduction in CPEB1 expression on a transcript level was detected in AAIII and sGBM specimens containing increased methylation and *IDH1* mutation (Fig. 5.1.1-1). Although this observed trend was indicating a link between the high CPEB1 methylation and silenced expression it turned out not to be correlated. Therefore, the observed DNA methylation only partially may be involved in transcriptional repression of CPEB1. Interestingly, pGBM specimens, with wild type *IDH1*, where CPEB1 methylation was not detected also revealed reduced expression of CPEB1 transcript (Fig. 5.1.1-1). This allows concluding that CPEB1 expression in pGBM samples was reduced independently of the methylation status. Consequently, other epigenetic and non-epigenetic mechanisms play a role in transcription silencing. For instance microRNAs may contribute to blocking translation or promoting degradation of the transcripts (Burns et al., 2011; Morgan et al., 2010), and explain the reduction of CPEB1 expression. Although in the present study DNA methylation turned out not to be responsible for CPEB1 downregulation, promising results from clinical trials with DNA methylation inhibitors as

well as histone deacetylase inhibitors were recently reported, meaning that epigenetic approaches have a potential in cancer therapy (Chen et al., 2014).

6.1.4 Expression of CPEB3 and active CPEB3 protein is tissue specific

The difference between expression levels of CPEB3 and phosphorylated CPEB3 gives a solid foundation to further examine kinases regulating CPEB function (Fig. 5.1.5-2). PKA and CaMKII investigated here are known to activate CPEBs phosphorylation that further triggers polyadenylation-induced translation (Kaczmarczyk et al., 2016; Theis et al., 2003). A recent study demonstrated that phosphorylation of CPEB3 depends on the presence of the alternatively spliced B-region, and can be detected by phospho-specific CPEB3 antibody (Kaczmarczyk et al., 2016). The used antibody allowed for recognition of only one splice form of the protein containing a phosphorylation consensus sequence, namely the B-region adjacent to two serine (S419, S420) residues. The specificity of the phospho-CPEB3 antibody was confirmed in cultured cells transfected with full-length CPEB3a (containing the B-region), CPEB3aKD (S419, S420 mutated to A419, A420), and CPEB3b (lacking the B-region) (Fig. 5.1.5-1), where only full-length CPEB3a was subject for phosphorylation.

Since the alternatively spliced B-region and the overlapping phosphorylation site are conserved between CPEB 2-4, this site is strongly engaged in regulatory functions of CPEBs (Kaczmarczyk et al., 2016). The AII specimens analyzed here were characterized by robust expression of CPEB3 and phospho-CPEB3 (Fig. 5.1.5-2, *right column*), while pGBM specimens showed strong immunoreactivity against CPEB3 (Fig. 5.1.5-2, *left column*). Altogether, CPEB3, PKA and CaMKII kinases were abundantly expressed in both of the investigated groups, while phospho-CPEB3 protein was only present in AII (Fig. 5.1.5-2). Despite the abundant CPEB3 expression and strong activity of kinases initiating polyadenylation, no significant phosphorylation of CPEB3 was observed in GBMs. This strongly suggests the lack of functional regions in the expressed CPEB3 and blockade of presumed targets expression.

125

6.1.5 Alternative splicing determines the expression pattern and activity of CPEBs

As expression analysis of PKA and CaMKII kinases revealed no change in their immunoreactivity between low- and high-grade gliomas the next step was directed towards the investigation of alternative splicing of CPEBs. In eukaryotes, alternative splicing determines tissue differentiation, controls transcriptional and post- transcriptional mechanisms, and their role in cancer (Kornblihtt et al., 2013). Alternative splicing of CPEBs is particularly relevant in the context of cytoplasmic polyadenylation, because the splice variants of CPEB 2-4 differ in their regulation by phosphorylation due to presence or lack of respective consensus sites (Fig. 1.4.3-1; Fig. 5.1.6-1) (Kaczmarczyk et al., 2016; Theis et al., 2003).

CPEBs are involved in the regulation of synaptic plasticity, and multiple splice variants of CPEB paralogs were observed in a mouse brain (Kaczmarczyk et al., 2016; Theis et al., 2003; Turimella et al., 2015). In hippocampal pyramidal neurons, only CPEB2 splice variants containing the B-region, such as CPEB2 a/c were expressed. Two of the detected isoforms, CPEB2 a*/c*, contained an additional 9-nt E-region (Turimella et al., 2015). Furthermore, changes in the abundance of CPEB3 splice variants were induced by neuronal stimulation. As in the case of CPEB2, increased neuronal activity led to expression of CPEB3 isoforms containing the B-region (Kaczmarczyk et al., 2016). In cancers, alternative splicing of CPEB2 turned out to be a key driving mechanism in human TNBC metastasis (Johnson et al., 2015). The high metastatic potential of TNBC cells was associated with acquiring of anoikis resistance (AnR) and increase in expression of the Bregion-lacking isoform. Downregulation of the CPEB2b re-sensitized AnR cells to detachment and induced cell death, whereas overexpression of CPEB2b in TNBC cells generated the AnR and increased their metastatic potential (Johnson et al., 2015). In human glioma specimens, profiling of alternative splice variants revealed that in comparison to normal brain and low-grade astrocytomas, most of the GBMs lacked the full-length CPEBs (CPEB2a, CPEB3a, CPEB4a) (Fig. 5.1.6-2; Tab. 5.1.6-1B, C, D). Furthermore, the majority of GBM samples did not express the CPEB4c variant also containing the B-region (Tab. 5.1.6-1D). Instead, isoforms lacking the B-region (CPEB3b, CPEB3d, CPEB4b,

CPEB4d) were predominantly expressed (Fig. 5.1.6-2; Tab. 5.1.6-1C, D). Interestingly, the recently identified E-region was detected in CPEB2 c^*/d^* splice variants, but so far its function has not been addressed. Generally this observation indicated the loss of CPEB 2-4 activity in high-grade gliomas. Alternative splicing appeared to be cell type-specific and depended on the grade of tumor malignancy.

Summarizing, in physiological conditions, the B-region-containing splice variants are abundantly expressed (Kaczmarczyk et al., 2016; Turimella et al., 2015). In contrast, TNBC (Johnson et al., 2015) and GBM tumors show enforced expression of isoforms lacking the B-region. However, it is unknown whether similarly to breast cancers, splicing in GMBs is related to their metastasis potential. Certainly, binding of CPEB 2-4 to the target transcripts without the possibility to further activate their translation, might cause their translationalarrest and in consequence silence mRNAs expression. Importantly, a similar pattern was observed for CPEB1. Mutant CPEB1 protein lacking regions of phosphorylation could not be activated, therefore kept the bound mRNAs in translational arrest (Kochanek and Wells, 2013). Thus far, the question how alternative splicing of CPEBs in gliomas is regulated remains open. The finding that CPEB3 in GBMs is expressed as a form that cannot undergo activation suggests its contribution to changes in protein expression of high-grade gliomas. Therefore, CPEB3 may be considered as an attractive therapeutic target.

6.2 The impact of CPEBs on growth properties and cancer-relevant parameters in cultured glioblastoma cells

6.2.1 CPEB3 protein shuttle between nucleus and cytoplasm

The reduction of CPEB1 expression observed in human GBM specimens prompted us to determine whether reversing this process results in acquisition of tumor suppressor properties by GBM cells. Likewise, overexpression of CPEB2 aimed to prove whether enhanced protein levels impact growth properties and cancer-relevant parameters. Therefore, the A172 GBM line was selected as it expressed CPEB1 and CPEB2 (Fig. 5.2.1-1) similar to human glioma specimens (Fig. 5.1.3.1-1).

127

Similarly to the investigated glioma tissues, endogenous CPEB levels in the cell line were heterogeneous. Weak cytoplasmic expression of CPEB1 was contrasted with abundantly expressed CPEB 2-4 (Fig. 5.2.1-1). Intriguingly, phospho-CPEB3 and partially CPEB3 were observed in the cell nuclei (Fig. 5.2.1-1). However, as this was not the case in the investigated human gliomas the observed pattern was assumed as a culture artefact.

6.2.2 Forced overexpression of CPEB1 and CPEB2 alters growth properties and cancer-associated parameters of glioblastoma cells

CPEBs regulate important cellular processes such as astrocyte migration (Jones et al., 2008), or MEF growth and senescence (Fernández-Miranda and Méndez, 2012). Recently CPEBs were found to be expressed in gliomas (Galardi et al., 2016; Ortiz-Zapater et al., 2012). Therefore it is important to identify whether their altered expression evoke changes in growth properties or cancer-associated parameters of GBM cells. The high levels of CPEB1 turned out to be cytotoxic for cultured GBMs. Overexpression of CPEB1 resulted in meagre growth of apoptotic cell population. In contrast, overexpression of CPEB2 slightly decreased apoptotic activity of GBM cells, suggesting that higher CPEB2 level may possibly result in loss of its tumorigenic properties. Alterations in cellular proliferation of CPEB1 and CPEB2 were not observed (Fig. 5.2.2.2-1). Overexpression of CPEB1 was recently reported to reduce proliferation and infiltration of GBM cells (Galardi et al., 2016; Yin et al., 2014). Accordingly, CPEB1 was directly engaged in regulation of p27^{Kip1} expression (Galardi et al., 2016). However, despite CPEB1 reduction, Nagaoka et al., (2016) found no effect on cell proliferation in the investigated mouse mammary epithelial cells. Likewise, knockout of CPEB1 in MEFs resulted in no difference in cell cycle progression. Instead, MEFs as well as primary human cells escaped senescence. This process was shown to be mediated by reduced expression of CPEB1-dependent mRNAs, such *p53* and *c-myc* (Burns and Richter, 2008; Groisman et al., 2006).

Enhanced migration of transformed astrocytes leads to spread of tumors and a high recurrence rate (Stupp et al., 2005). This process not only involves changes in the cytoskeleton, but also depends on the synthesis of new proteins (Jones et al., 2008). New

protein synthesis was shown to be especially important in the migration of astrocytes into a site of the injury, and the process was precisely regulated by CPEB1-mediated synthesis of β -catenin in the leading edge of invading cells (Jones et al., 2008). In the present study, forced overexpression of CPEB1 and CPEB2 also enhanced the cell motility (Fig. 5.2.2.3-1). Therefore, it is highly possible that migration of cultured GBM cells is regulated by CPEB-dependent synthesis of β -catenin. Additionally, based on findings from FACS analysis, the wound healing was speeded up rather by elevated cell motility than by increased proliferation.

One of the possible explanations for enhanced apoptotic activity and migration by CPEB1 might be that the faster migration generates rapid changes in cytoskeleton. These morphological alterations on one hand may contribute to increased cell motility, but may also raise a chance of mistakes turning GBM cells towards an apoptotic pathway. In contrast, decreased apoptotic activity and enhanced migration through CPEB2 may indicate that CPEB2 overexpressing cells are losing their cancerous properties.

6.2.3 Elevated expression of CPEB1 upregulates cancer-associated signaling pathways

Altered expression of CPEBs in human glioma specimens may entail changes in associated signaling pathways. Therefore, cultured GBM cells overexpressing CPEBs were seeded on multi-pathway assay plates that allowed for identification of CPEB-mediated changes in cancer-relevant cascades. Overexpression of CPEB1 enhanced the activity of estrogen, hedgehog, HNF4 and TGF β (Fig. 5.2.3-1A). Overexpression of CPEB2 could not be quantitatively asset because the experiments number was too low (Fig. 5.2.3-1B). Among the upregulated pathways, TGF β appeared to be the most prominent. Depending on the cellular context, TGF β may result in tumor-suppressing or tumor-promoting activity (Derynck et al., 2001; Siegel and Massagué, 2003). In order to bypass a cell cycle inhibition, a majority of the cancers accumulate mutations in this pathway. TGF β may act as an oncogenic factor due to enhancement of cell proliferation, invasion, angiogenesis

and inhibition of immune response (Massagué, 2008; Seoane, 2006; Wesolowska et al., 2008; Zhang et al., 2012).

An association between CPEB1 and TGF β pathway was independently confirmed by Nagaoka et al. (2016). They found that mouse mammary epithelial cells deprived of CPEB1 and treated with TGF β show enhanced epithelial-to-mesenchymal transition and metastatic potential. Thus far, the mechanism triggering CPEB1-mediated TGF β activation is unclear. However, TGF β downstream targets, including Smad 2-4 were abundantly studied in gliomas (Bruna et al., 2007). High TGF β -Smad activity was predominantly present in aggressive gliomas and related to the poor patient prognosis (Bruna et al., 2007). Therefore, inhibitors of the TGF β pathway results in anti-tumor properties, such as reduction of cell viability and invasion (Kaminska et al., 2013). Inhibitor of the TGF β receptor I kinase (galunisertib, LY2157299) that downregulates the phosphorylation of Smad2 and selectively block TGF β pathway is currently investigated with a temozolomidebased radio-chemotherapy in newly diagnosed GBM patients. Summing up, CPEBs are involved in regulation of the TGF β signaling cascade. However, further studies are needed to fully explain the interaction between CPEB1-mediated translational control and the activation of the TGF β pathway.

6.3 Expression of CPEB2 in different cellular populations, brain regions, and stages of development

Expression of CPEB1 was well described in the hippocampal neurons (Theis et al., 2003), therefore it served as a reference for the present study of CPEB2. Both CPEB paralogs demonstrated analogous subcellular localization and immunoreactivity in primary hippocampal neurons (Fig. 5.3.1-1). Expression of CPEB2 was present in most of the neurons throughout the investigated mouse brain regions (Fig. 5.3.2-1), including majority of excitatory, inhibitory and dopaminergic cells (Fig. 5.3.3-1). This indicated a role of CPEB2 in physiology of the juvenile and adult brain. In addition, localization of CPEB2 in cell bodies and dendrites, pointed at its role in control of local protein synthesis. This raised

the question of which neuronal mRNAs are regulated by CPEB2. Indeed, CPEB2 may regulate CaMKII, the kinase engaged in LTP (Atkins et al., 2005) and β -catenin, which is involved in neuronal morphogenesis (Yu and Malenka, 2003). Additionally, Turimella et al. (2015) identified EphA4 as a novel CPEB2 target. EphA4 not only participates in the neuron–glia crosstalk (Carmona et al., 2009), but also controls LTP at excitatory synapses (Filosa et al., 2009). Overall, this indicates involvement of CPEB2 in synaptic plasticity and local protein synthesis in the hippocampal neurons of developing and adult brain.

7 SUMMARY

Gliomas are the most common primary brain tumors with aggressive progression and devastating prognosis. Therefore identification of new therapeutic and diagnostic factors is necessary to improve the dramatic situation of glioma patients. Thus far, cancer-related genes were thoroughly analyzed by transcript-based microarrays (van den Boom et al., 2003; Phillips et al., 2006; Rickman et al., 2001). However, recent research shed light on the importance of post-transcriptional modifications of mRNAs that alter gene expression under physiological conditions, but also gives rise to the pathology of gliomas.

The main goal of the present study was to investigate CPEB expression in human glioma specimens. CPEBs are auxiliary regulators associating with consensus sequences present in 3'UTRs of mRNAs, which activate or repress their translation. Via this mechanism CPEBs regulate essential cellular processes, such as development (Groisman et al., 2002; Novoa et al., 2010), memories formation (Theis et al., 2003) and progression of cancer (Ortiz-Zapater et al., 2012). In the current work aberrant CPEB expression was found to be a frequent phenomenon in both, low- and high-grade gliomas. Decreased CPEB1 expression was associated with the rising grade of tumor malignancy, suggesting it being a putative tumor suppressor. One of the mechanisms potentially underlying transcriptional silencing of cancer-related genes might be DNA methylation. However, despite hypermethylation of the *CPEB1* gene, DNA methylation proved not to be directly responsible for its downregulation in gliomas. Thus, the underlying mechanism remains elusive. Abundant expression of CPEB 2-4 was detected in numerous human glioma specimens. CPEB2 expression in endothelial tumor cells suggested that CPEB2-mediated protein synthesis takes place in the close proximity of blood vessels within tumor tissue. On the other hand, CPEB4 expression appeared to support tumorigenic properties by its putative entanglement into local protein synthesis in transformed cells. Intriguingly, only CPEB3 expression was correlated positively with tumor progression. Phosphorylation of CPEB3 within the alternatively spliced region was negatively correlated with tumor malignancy. The loss of CPEB3 activity in high-grade gliomas is likely caused by the expression of alternatively spliced variants. This suggests that a substantial part of the CPEB3 in GBM specimens does not undergo phosphorylation and indicates phosphorylation to be a biologically important mechanism regulating CPEB3 activity. Furthermore, a significant correlation between CPEB3 activity and survival was observed in less aggressive low-grade astrocytomas considered to be sGBM precursors. Consequently, CPEB3 may be considered as an attractive therapeutic target in gliomas.

To further investigate the relationship between CPEB activity, growth properties and cancer-relevant parameters an *in vitro* overexpression study was performed. This revealed a striking link between CPEB1, enhanced apoptotic activity and enhanced migration. One explanation could be that through rapid changes in the cytoskeleton of migrating cell the chance of mistakes rises, turning GBM cells towards an apoptotic pathway. On the other hand, CPEB2 overexpression decreased apoptotic activity, enhanced migration and by this additionally strengthened the cancerous properties of the cells. Importantly, CPEBs were also found to regulate various cancer pathways, including the TGF- β signaling cascade. However, further studies are needed to fully understand the interaction between CPEBs translational control and the pathophysiology of cultured GMB cells and human gliomas.

Finally, the study revealed that CPEB2 is expressed in different cellular populations, brain regions, and stages of development, which indicates that this protein plays an important role in regulation of local protein synthesis, synaptic plasticity, and neuronal morphogenesis.

The present study does not only increase our understanding of the function of CPEBs but also shows the importance of post-transcriptional modifications of mRNAs as a pathophysiological mechanism in gliomas and potentially other cancers. Therefore, these results may serve as a valuable basis for the identification of new therapeutic and diagnostic factors in cancer treatment.

8 PERSPECTIVE

Aberrant expression of CPEBs in human gliomas indicates the importance of posttranscriptional regulation in cancer cells. However, to fully understand the role of CPEBs in this relatively young research filed further investigations are required.

As increasing evidence confirms the implication of CPEBs in multiple pathological processes, most likely CPEB-dependent translation is a more general event in tumorigenesis. Therefore, particular attention should be paid to metastatic brain tumors, namely cancers beginning in lungs, breast, melanoma, colon, and kidney, and subsequently invade the brain. Further investigations of CPEB expression in different kinds of cancer may be performed by tissue microarrays or meta-analysis.

Knowing that CPEBs control the translation of various genes, the identification of target transcripts should be a major future task to understand their role in the complex tumor environment. Experiments with the objective of identifying mRNAs bound by CPEBs in normal versus cancer cells might involve RNA immunoprecipitation followed by DNA chip microarray analysis. As an alternative method, crosslinking and immunoprecipitation of RNA binding proteins followed by cloning and deep sequencing of the attached RNAs might be used.

Having in mind that solely CPEB splice variants containing the B-region may undergo activation, the variants lacking B-region may arrest cytoplasmic polyadenylation and translation of bound mRNAs. Therefore, it ought to be examined which mRNAs are silenced with the help of this mechanism. The answer may be provided by evaluation of the elongation of poly(A) tails of bound mRNAs in normal and cancer cells. Polyadenylation may be investigated by a whole-transcriptome approach, such as deep sequencing of mRNAs with poly(A) tails.

Finally, it ought to be considered whether CPEBs might be involved in therapy. For this purpose, a straightforward approach of animal models might be used. Xenografts or

syngeneic animal models reflecting conditions observed in human GBMs might be valuable for personalized therapy of brain tumor patients. This might be particularly important for patients who suffered a relapse or whose tumor continues to grow after standard treatment. Moreover, this might be the key to test CPEB function *in vivo* and to translate the latest findings on the molecular causes of brain tumors, their risk valuation and new therapeutic methods into innovative treatment concepts.

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APPENDIX I

pCMV6-Neo vector map



pEGFP-N1 vector map

