CHARACTERIZATION OF *C17ORF45* AS A NOVEL TARGET OF THE WNT/B-CATENIN PATHWAY

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ABSTRACT

CHARACTERIZATION OF *C17ORF45* AS A NOVEL TARGET OF THE WNT/B-CATENIN PATHWAY

β-catenin is a key component of Wnt pathway which plays an important role in tumorigenesis. In our previous study, candidate genes regulated by Wnt/β-catenin/TCF pathway were identified in hepatocellular carcinoma-derived Huh7 cells overexpressing hyperactive β-catenin (S33Y mutant). SAGE (Serial Analysis of Gene Expression) and genome wide microarray analysis were performed to compare global gene expression between Huh7 cells with high and low TCF activity. After comparison, *C17orf45* was found to be up-regulated in Huh7 cells with high β-catenin/TCF activity. In this study, *C17orf45* gene was further verified as a Wnt target based on lithium treatment, GFP/luciferase reporter, and ChIP assays. *C17orf45* is a novel gene that has not been studied before and its expression is up-regulated in several brain tumors according to sagereveal (a bioinformatic tool data mining gene expression data from tumor and normal samples using SAGE library data). Based on this prediction, to investigate the potential link between *C17orf45* and tumorigenesis; expression analysis was performed in human brain tumors and the growth effect on U373-MG cells was examined with proliferation assay. To investigate the subcellular localization of *C17orf45* protein, GFP fused constructs were used and the localization was found to be mitochondria. Identification of novel targets of Wnt signaling is important for better understanding of cancer pathophysiology and may serve as potential targets for drug therapies. New targets of this pathway will expand the cancer research area. To sum up, results of this study reveal a novel gene putatively implicated in tumorigenesis and the Wnt pathway.

ÖZET

WNT/B-KATENİN YOLAĞININ YENİ HEDEF GENİ OLARAK *C17ORF45*'İN KARAKTERİZE EDİLMESİ

Wnt yolağının bir elemanı olan β-katenin tümör oluşumunda önemli bir rol oynamaktadır. Önceki çalışmamızda β-katenin mutant formunu yüksek miktarda üreten karaciğer kanseri hücresi Huh7'de Wnt/β-katenin/TCF yolağı tarafından etkilenen aday genler tespit edilmişti. Gen anlatımının seri analizi (serial analysis of gene expression - SAGE) ve mikrodizin yongaları metodlarıyla TCF aktivitesi yüksek ve düşük Huh7 hücreleri arasında global gen anlatımı karşılaştırılması yapılmıştı. Bu karşılaştırma neticesinde Wnt/β-katenin/TCF4 yolağı yüksek olan hücrelerde *C17orf45* geninin mRNA seviyesinin arttığı bulunmuştur. Bu çalışmada *C17orf45* geni Wnt/β-katenin yolağının yeni hedef geni olarak tespit edilmiştir. Hücrelerin lityum klorür tuzuna maruz bırakılması, GFP/Lusiferaz raportör analizi ve immünoçöktürme deneylerinin bulguları baz alınarak *C17orf45* Wnt hedef geni olarak tanımlanmıştır. *C17orf45* geni ile daha önce hiç bir çalışma yapılmamış olup sagereveal biyoinformatik yazılımı ile çeşitli beyin kanserlerinde arttığı öngörülmektedir. Bu bağlamda *C17orf45* geninin olası tümör oluşumu ile ilgisini araştırmak için insan beyin dokularında gen anlatım analizi ve U373-MG astrositom hücre hattında hücre büyüme testi yapılmıştır. C17orf45 proteininin araştırmalarında başlangıç olarak hücre içi lokalizasyonuna bakılmıştır. GFP ile birleşik *C17orf45* proteini kullanılarak lokalizasyon mitokondri olarak tespit edilmiştir. Wnt sinyal yolağının yeni hedef genlerinin tespit edilmesi kanser patofizyolojisini anlamak adına önemlidir. Özet olarak bu çalışma Wnt yolağı tarafından hedeflenen ve tümör oluşumu ile ilgili olabilecek daha önce tanımlanmamış bir gen sunmaktadır.

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LIST OF SYMBOLS/ABBREVIATIONS

A	Adenine
C	Cytosine
G	Guanine
S	Serine
T	Thymine
Y	Tyrosine
μg	Microgram
μl	Microliter
μM	Micromolar
APC	Adenomatous polyposis coli gene product
APS	Ammonium peroxidisulfate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BPB	Bromophenol blue
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CO_2	Carbondioxide
DAPI	4 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNET	Disembryoblastic neuroepithelial tumor
dNTP	Deoxyribonucleotide
DPH	DEPC treated water
DTT	Dithiothreitol
DVL	Dishevelled

EDTA	Ethylenediaminetetraacetate
ERLB	EtBr RNA loading buffer
EtBr	Ethidium bromide
EtOH	Ethanol
FBS	Fetal bovine serum
Fz	Frizzled
GFP	Green flourescent protein
GSK3β	Glycogen synthase kinase 3 beta
HCC	Human hepatocellular carcinoma
hrs	Hours
kb	Kilobase
KCl	Potassium chloride
LB	Luria-Bertani
LEF	Lymphoid enhancer factor
LiCl	Lithium chloride
LRP	Low density lipoprotein receptor-related protein
mg	Milligram
MGC	Mammalian Gene Collection
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
min	Minute
mL	Milliliter
mm	Millimeter
MOPS	Morpholino propane sulfonic acid
mRNA	Messenger ribonucleic acid
MTS	Mitochondria Targeting Sequence
NaCl	Sodium chloride
NaOAc	Sodium Acetate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	Nanogram

nm	Nanometer
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonyl fluoride
Q-RT-PCR	Quantitative reverse transcriptase mediated PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAGE	Serial Analysis of Gene Expression
SD	Standard Deviation
SE	Standard Error
TAE	Tris-acetic acid EDTA
TCF	T cell factor
TSS	Transcriptional Start Site
UV	Ultraviolet
WNT	Wingless-type mouse mammary tumor virus (MMTV) integration site family member

1. INTRODUCTION

1.1. Wnt/β-catenin Signaling Pathway

Wnt/β-catenin signaling pathway has important roles in several cellular functions during embryonic development, maintenance of organs, cell proliferation and differentiation, adult tissue homeostasis [1–6], cell fate determination, and angiogenesis [7]. Wnt/β-catenin pathway regulates the stability of the transcription cofactor β-catenin and therefore β-catenin-dependent gene expression [1, 2]. Aberrant activation of Wnt/β-catenin signaling is associated with a broad spectrum of human malignancies, including cancer, osteoporosis, aging and degenerative disorders [2, 5, 8–13]. β-catenin is the key molecule of the Wnt/β-catenin signaling. A hallmark of Wnt/β-catenin pathway activation is the elevation of cytoplasmic β-catenin protein levels leading to its nuclear translocation [14–16]. Nuclear accumulation of β-catenin due to aberrant activation of Wnt/β-catenin signaling is reported in several tumors such as colorectal carcinomas [9], liver tumors [17], and breast cancer [18].

In the absence of Wnt ligand (Figure 1.1), cytoplasmic β-catenin protein is kept low with the action of a degradation complex assembled by the scaffolding protein Axin, the tumor supressor adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3β(GSK3β). CK1 [19] and GSK3β [20] sequentially phosphorylate the amino terminal region of β-catenin leading to β-catenin recognition by b-Trcp, an E3 ubiquitin ligase subunit, targeting β-catenin for proteasomal degradation [21]. This constitutive degradation of β-catenin prevents β-catenin from entering the nucleus and activating target gene expression.

The Wnt/β-catenin pathway can be activated (see Figure 1.1) when a canonical Wnt ligand binds to the seven-pass transmembrane Frizzled (Fz) receptor [22] and its co-receptor, low-density lipoprotein receptor-related protein 6 (LRP6) or LRP5. With the binding of the ligand, Dishevelled (Dvl) protein is recruited to the receptor complex [23] and this results in phosphorylation and activation of LRP5/6 and recruit-

ment of AXIN to the plasma membrane [24] . When AXIN is recruited to the plasma membrane, the formation of degradation complex is inhibited and thereby leading to stabilization of β-catenin which accumulates and penetrates into the nucleus to form a complex with a transcription factor of the TCF/LEF family and activates Wnt target genes.

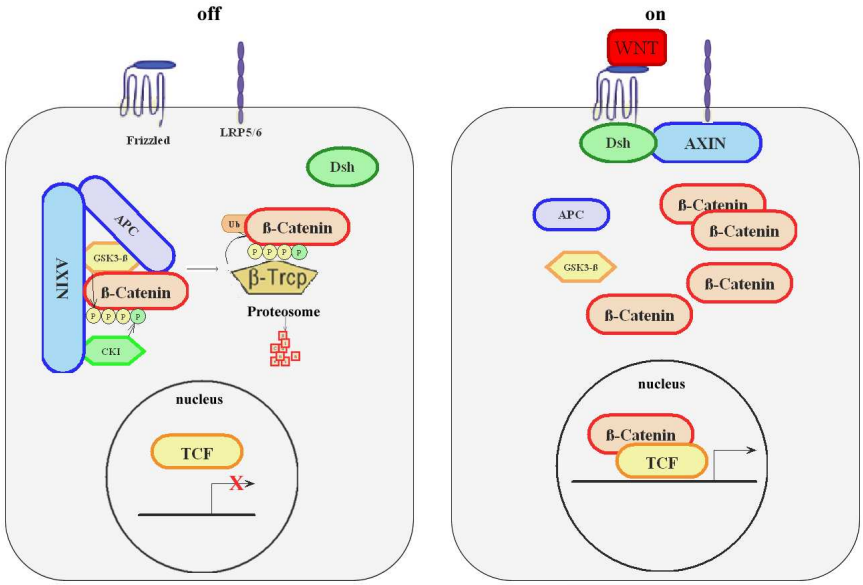

Figure 1.1. Overview of Wnt/β-catenin Signaling (Adapted and modified from MacDonald *et al.*,2009)

The increased stability of β-catenin upon Wnt stimulation leads to its nuclear translocation and activation of target genes. This process is regulated by the TCF/LEF transcription factor with the interaction of β-catenin. In the absence of the Wnt signal, TCF/LEF forms a complex with repressors such as Groucho protein [25] and binding to target DNA sequences is inhibited. Upon activation of the pathway, β-catenin binds to the amino terminus of TCF/LEF and promotes displacement of repressors and interaction of the β-catenin/TCF complex with target DNA sequences.

There are also feedback regulations by which the Wnt/β-catenin pathway can affect the expression of several wnt components expression such as AXIN2, Fz, LRP, and HSPG2. AXIN2 is a component of the degradation complex that enhances the degradation of β-catenin. Its gene expression is up-regulated upon pathway activation

[26]. Fz is the receptor that wnt ligands bind to, and Fz expression is decreased upon pathway activation [27].

Table 1.1. Human genetic diseases and mutation in Wnt signaling components (Adapted from Moon *et al.*, 2004).

Gene	**Disease**	**Reference(s)**
APC	Familiar Adenomatous Polyposis (FAP)	[28, 29]
	sporadic colorectal cancers	[30]
LRP5	Familial Exudative Vitreoretinopathy	[31, 32]
LRP6	early coronary disease	[33]
	Late onset Alzheimer	[34]
FZD4	Familial Exudative Vitreoretinopathy:	[35]
	Retinal angiogenesis	[32]
Norrin	Familial Exudative Vitreoretinopathy	[36]
WNT3	Tetra-Amelia	[37]
WNT4	Mullerian-duct regression and virilization	[38]
WNT4	SERKAL syndrome	[39]
WNT5B	Type II diabetes	[40]
WNT7A	Fuhrmann syndrome	[41]
WNT10A	Odonto-onycho-dermal dysplasia	[42]
WNT10B	Obesity	[43]
	Split-Hand/Foot Malformation	[44]
AXIN1	caudal duplication	[45]
TCF4	Type II diabetes	[46–48]
AXIN2	Tooth agenesis	[49]
	Colorectal cancer	[50]
WTX	Wilms tumor	[51, 52]

1.2. Wnt Signaling in Cancer and Human Diseases

Deregulation of Wnt/β-catenin pathway leading to a variety of genetic diseases may occur by mutations (see Table 1.1) in the pathway elements. Mutations in β-catenin and APC gene have been reported in human colon cancers and hepatocellular carcinomas [53]. APC was first isolated as a tumor suppressor gene in human colon cancer [54] and its loss of function contributes to sporadic and hereditary colorectal tumorigenesis [28]. β-catenin amino terminal mutant forms including the phosphorylation sites can activate Wnt target genes constitutively and are found in various cancers (see Table 1.2). Mutations in the *AXIN1* gene have been also reported in hepatocellular

carcinomas [55] and colorectal cancers with wild type β-catenin and APC forms. Mutations in the β-catenin gene (listed in Table 1.2) affecting the amino-terminal region of the protein make it resistant against 1the destruction complex. These mutations affect specific serine and threonine residues and amino acids adjacent to them. These residues are essential for both formation of the degradation complex and phosphorylation of β-catenin. Thus, these mutations abrogate the phosphorylation dependent ubiquitination and proteasomal degradation of β-catenin.

Table 1.2. β-catenin mutations are found in various human cancers (Adapted from Polakis, 2000)

Tissue	*Frequency	S29	Y30	L31	D32	S33	G34	I35	H36	S37	G38	A39	T40	T41	T42	A43	P44	S45	L46	S47	G48	K49	reference
colorectal	9/202						1							3				5					[56]
colorectal	2/92													1				1					[57]
colorectal-w/o APC mutation	7/58																						[58]
colorectal-w/o APC mutation	13/27					2	1							3				5					[59]
colorectal HNPCC	12/28				2		2			1				2				5					[60]
colorectal w/ MSI	13/53																	6					[61]
colorectal w/o MSI	0/27																						[61]
desmoid, sporadic	1/1													1									[62]
desmoid, sporadic	22/42													10				12					[63]
endometrial w/ MSI	3/9				2	1																	[61]
endometrial w/o MSI	10/20				3	1	2			3				1									[61]
gastric, Intestinal-type	7/26	2			5																		[64]
gastric, diffuse-type	0/17																						[64]
hepatocellular w/HCV	9/22				3	1				3				1				2					[65]
hepatocellular	12/35				1	1	2	1	1				1	2				2		1			[66]
hepatocellular	6/26				2		1			1								1					[67]
hepatocellular	14/75				5	1	1							1				4					[68]
hepatocellular	21/119				3	3	1	1		2				4				8					[69]
hepatoblastoma, sporadic	27/52				2		3			1				5									[70]
hepatoblastoma	12/18				2		1							1				1					[71]
kidney, Wilms' tumor	6/40													1				2					[72]
medulloblastoma, sporaic	3/67					2				1													[73]
melanoma	1/65																	1					
ovarian, endometriod	7/13				3	1				2				1									[74]
ovarian, endometriod	3/11									2				1									[75]
ovarian, endometriod	10/63					2	2			6													[76]
pancreatic tumors	0/111																						[77]
pilomatricoma	12/16				2	4	3			2				1									[78]
prostate cancer	5/104				1	2								1				1					[79]
thyroid, anaplastic	19/31					1			1	3	1		8	2	1	1	4	2	1	2			[80]
uterine endometrium	10/76					1				2				4				3					[81]

*Frequency is the number of tumors with mutations/total number of tumor.

1.3. *C17orf45* (MGC40157)

C17orf45 which resides on chromosome 17 was identified as a hypothetical protein coding gene in the NIH's (The National Institutes of Health) MGC (Mammalian Gene Collection) project, which aims to provide complete open reading frame (ORF) clones for every human gene [82]. Sequence data obtained in the frame work of this project full length clones are made publicly accessible in the GenBank nucleotide sequence database with specific accession numbers. Hypothetical protein products of a gene or whether a cDNA clone contains a complete coding sequence (CDS) is determined by a combination of statistical assessments and analyses [83] of nucleotide sequences. The reference sequences of the *C17orf45* transcript and its protein product was determined using the procedures indicated in Gerhard *et al.* [82]. The reference sequences of the transcript (NM152350.3) and protein sequence (AAH40159) belonging to *C17orf45* can be accessed in GenBank database with their accession no's as seen in Table 1.3.

Table 1.3. GenBank entries for *C17orf45* transcripts/clones in four primates

Gene ID	Organism	GenBank Entry	Protein Entry	Reference
125144	*Homo sapiens*	NM_152350.3	AAH40159	[82]
699779	*Macaca mulatta*	XR_010639.1	—	[84]
746682	*Pan troglodytes*	XR_022196.1	—	[85]
—	*Macaca fascicularis*	AB171949.1	BAE89012	[86]

In Serial Analysis of Gene Expression (SAGE) and microarray screens performed in our laboratory we found that *C17orf45* expression was upregulated in the Huh7 cell line (a hepatocellular carcinoma), which constitutively overexpresses β-catenin (Kavak *et al.*, unpublished data).

1.3.1. BLAST Search of Human Hypothetical C17orf45 Protein

In a BLAST search with the human predicted C17orf45 amino acid sequence (GenBank entry:AAH40159), no homologous protein sequence in another organism

except the protein with the accession number BAE89012 (see Table 1.3) was found. BAE89012 is described as "unnamed protein product" in *Macaca fascicularis* and it is the predicted protein product of a cDNA clone (AB171949) which was isolated in a macaque cDNA project [84] and highly similar to human *C17orf45* transcript (NM_152350.3). The amino acid sequence alignment of three hypothetical proteins, belonging to human, chimpanzee and macaque, is seen in Figure 1.2.

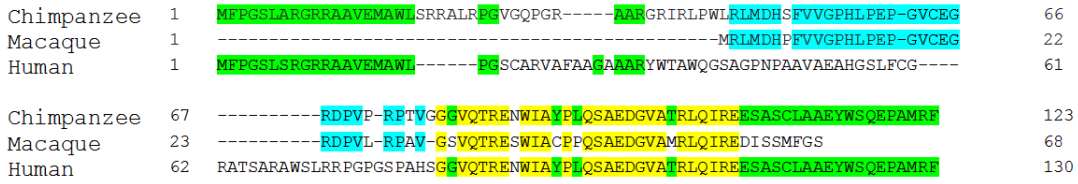

Figure 1.2. Amino acid sequence alignment of hypothetical human, chimpanzee and macaque C17orf45 proteins. The amino acid sequence of C17orf45 in chimpanzee was deduced from a putative open reading frame in *Pan troglodytes* mRNA with XR_022196.1 accession.

1.3.2. *C17orf45* is a snoRNA Host Gene

Small nucleolar RNAs (snoRNAs) are 60-150 nt long non-coding RNAs that have a cellular function of guiding modifications of nucleotides in rRNAs or spliceosomal RNAs [87]. The majority of human snoRNAs are encoded in introns of host genes [88]. *C17orf45* encodes three snoRNAs, U49B, U49A and HBII-135, in its introns (See Figure 1.3). These snoRNAs interestingly are more conserved than *C17orf45* exons in vertebrate species.

To confirm the Genome Browser alignment that the snoRNAs in the introns of C17orf45 are more conserved than the exons of C17orf45, the chromosomal region of *Mus musculus* genome in the alignment (Figure 1.2) was analyzed. This region corresponds to the mouse *2410006H16Rik* gene locus. 2410006H16Rik gene (GeneID: 69221) resides on chromosome 11 (62416392-62418311) of mouse genome. Three alignments were performed. Human C17orf45 protein (AAH40159) and mouse 2410006H16Rik

(Accession no: XP_001479778) were aligned and no conservation was observed (Figure 1.4a). Then the putative open reading frames in Refseq (NCBI Reference Sequence mRNA, Accession no: XM_001479267) of mouse *2410006H16Rik* gene were taken and the amino acid sequence alignment of the mouse putative proteins and human C17orf45 protein was performed. No homology was observed (data not shown). Lastly, the amino acid sequences deduced from putative open reading frame in genomic region of *2410006H16Rik* (NCBI Reference Sequence: NC_000077.5) and human C17orf45 gene (NCBI Reference Sequence: NC_000017.10) were aligned. Homology was not observed in amino acid sequences deduced from putative open reading frames in human C17orf45 and mouse 2410006H16Rik genomic sequences.

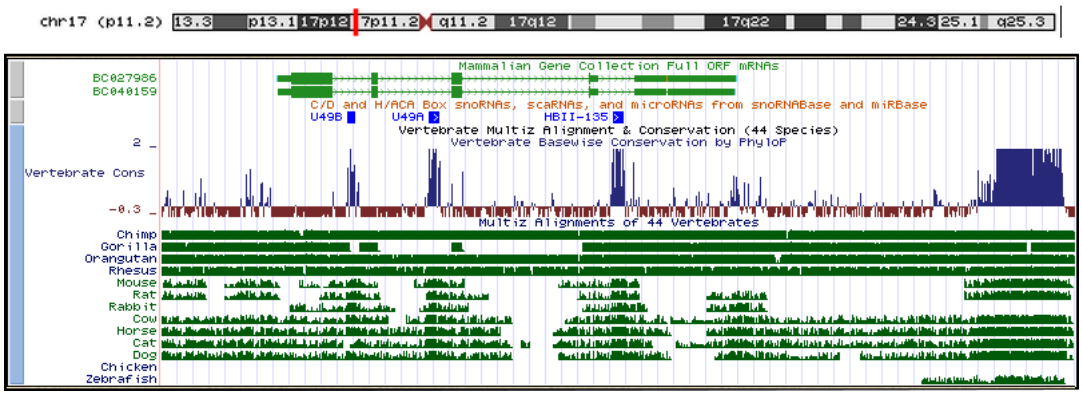

Figure 1.3. *C17orf45* annotation on the human UCSC Genome Browser [89]. snoRNAs (light blue)were encoded in the introns of *C17orf45*. Multiple alignments of vertebrate species (dark green) and conservation intensities in vertebrates (dark blue) show that sequences corresponding to snoRNAs are more conserved than those of the exons of the host gene.

1.3.3. *C17orf45* were Differentially Expressed in Several Tumors According to Sagereveal

Serial analysis of gene expression (SAGE) technique can be used to compare the global gene expression level in tumor samples with control samples. Sagereveal, a bioinformatic tool developed by Erşen Kavak (Kavak *et al.*, unpublished data), data

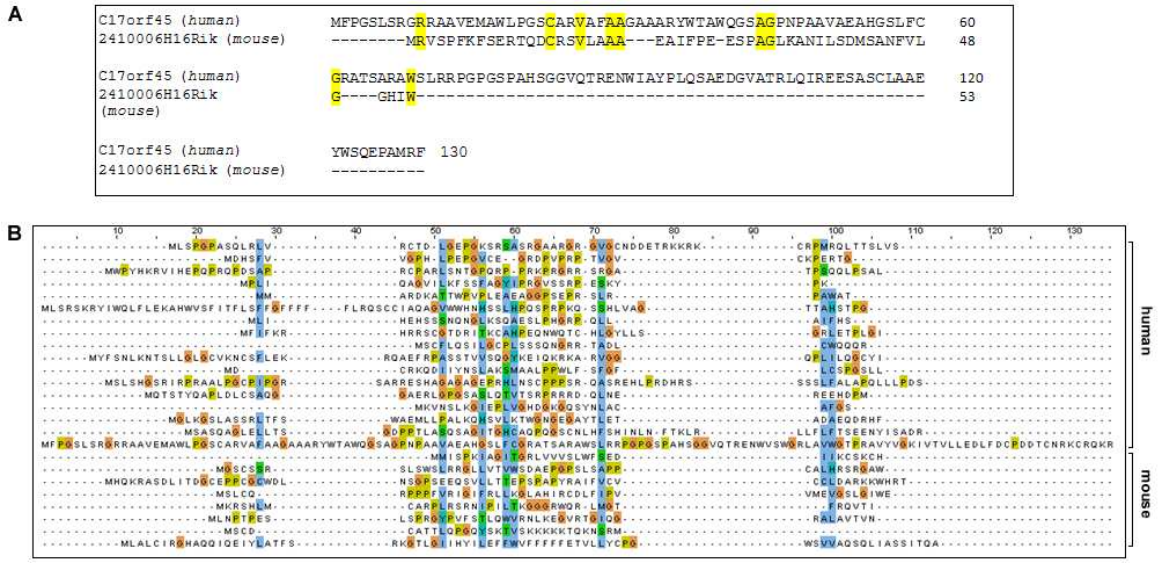

Figure 1.4. Amino acid sequence alignment of C17orf45 and mouse 2410006H16Rik (A) Refseq proteins and (B) peptides deduced from putative open reading frames in their genomic sequences.

mines publicly accessible SAGE cancer data and suggest candidate genes that were differentially expressed in a variety of tumor samples and potentially to be related with tumorigenesis (For the details of the program see ref [90]). *C17orf45* is one of the genes that were identified by sagereveal to be expressed differentially in human tumors shown in Table 1.4.

Table 1.4. Tumors in which *C17orf45* mRNA level change according to sagereveal.

SAGE Tag	**Tumor Type**	
	Increase	**Decrease**
AACTAATACT	MedullaBlastoma Thyroid Astrocytoma Grade2 Astrocytoma Grade3	Lung
CCCAACAAGA	Melanoma (skin)	
CTCAGCAGAT	MedullaBlastoma Astrocytoma Grade2 Astrocytoma Grade3	Lung

2. PURPOSE

The aim of this study was to verify and extend the role of *C17orf45* as a target in the Wnt pathway and its putative role in tumorigenesis based on SAGE and genome wide microarray screens performed in our laboratory.

3. MATERIALS

3.1. General Chemicals and Kits

All laboratory chemicals were analytical grade from Sigma (St. Louis, MO, USA) and Merck (Schucdarf, Germany), unless stated otherwise in the text. Tissue culture media and solutions were purchased from Gibco (Paisley, UK), Applichem (Darmstadt, Germany), and Biochrom AG (Berlin, Germany), unless stated otherwise in the text. In vitro transfection reagents, Exgen 500 and TurboFect were purchased from Fermentas (Burlington, Canada).

Qiagen Plasmid Purification Kits : mini, midi and maxi were used for plasmid purification from overnight grown *E.coli* cultures, RNeasy Mini Kit, for RNA Purification from cell lines, and RNeasy Lipid Tissue Kit, for RNA Purification from human brain tissues, were from Qiagen (Hilden, Germany). ImProm-II Reverse Transcription System from Promega (Madison, WI, USA) was used for cDNA synthesis prior to RT-PCR and QRT-PCR. Cell Titer 96 Aqueous One Solution Proliferation Assay was purchased from Promega (Madison, WI, USA). Light Cycler Fast Start DNA Master SYBR Green I kit was purchased from Roche (Basel, Switzerland) for quantitative real time PCR.

3.2. Enzymes

For molecular cloning purposes, Expand Long Template PCR System, which contains a high-fidelity DNA polymerase Tgo, was purchased from Roche Applied Biosciences (Indianapolis, USA). Restriction endonucleases, Calf Intestinal Alkaline Phosphatase and T4 DNA ligase were purchased from Promega (Madison, USA). Taq Polymerase was purchased from Fermentas (Burlington, Canada) together with the $MgCl_2$ (25mM) and the 10X reaction buffer. Trypsin (0.025 per cent, ready to use) was purchased from Gibco (Paisley, UK).

3.3. Nucleic Acids

DNA molecular weight markers and deoxyribonucleotides were purchased from Fermentas (Burlington, Canada).

3.3.1. Plasmids

pEGFP-N2 (Clontech, CA, USA), pcDNA3 (Invitrogen, CA, USA) plasmids were commercially obtained. Full length open reading frame *C17orf45* in pCMVS-ports6 (IRAT p970G1149D) was purchased from RZPD German Resource Center for Genome Research (Berlin, Germany). CS2+/β-catenin-4m (S33A, S37A, T41A, S45A quadruple-mutant) plasmid was kindly provided by Dr.Xi He, Harvard Medical School. MTS-dsRED plasmid was kindly provided by Dr.Mikhail F. Alexeyev (University of South Alabama, USA).

Table 3.1. Primers used in this study

Primer ID	**Sequence**	**Application**	**RE sites**
MGC40157_7F	AGAGAACTGGATTGCGTACC	Q-RT-PCR	—
MGC40157_8R	ATGTCCTCTGATACATAAGGCA	Q-RT-PCR	—
ACTB_1F	AAGATCAAGATCATTGCTCCTC	Q-RT-PCR	—
ACTB_2R	GGGTGTAACGCAACTAAGTC	Q-RT-PCR	—
GAPDH_1F	TTAGCACCCCTGGCCAAGG	Q-RT-PCR	—
GAPDH_2R	CTTACTCCTTGGAGGCCATG	Q-RT-PCR	—
HSRRN18S_1F	CTGAAACTTAAAGGAATTGACGGA	Q-RT-PCR	—
HSRRN18S_2R	GTTATCGGAATTAACCAGACAAATC	Q-RT-PCR	—
AXIN2_1F	TTATGCTTTGCACTACGTCCCTCCA	Q-RT-PCR	—
AXIN2_2R	CGCAACATGGTCAACCCTCAGAC	Q-RT-PCR	—
MGC40157_9Fp	TAAGCTAGCTCTCCATCTCTTTCCTGAC	ChIP	NheI
MGC40157_10Rp	ATGAAGCTTACAAGCATTTCCCAATCCC	ChIP	HindIII
MGC40157_11Fp	TAAGCTAGCTACACAAGTCTGTCACCTCC	cloning	NheI
MGC40157_12Rp	ATGAAGCTTGGGCATTCTGTGGGTACTG	cloning	HindIII
MGC40157_13Fp	TAAGCTAGCTGCCTTCCTAAGACATAAGAC	cloning	NheI
MGC40157_14Rp	CATGCTCGAGGCTCTTTATTTGCTCTTGCC	cloning	XhoI
MGC40157_29F	AAGAGCTCGAGCTTCTGTG	cloning	—
MGC40157_30R	GATCGAATTCAAACCTCATGGCAGGCTC	cloning	EcoRI
MGC40157_4F_cl	GATCAAGCTTATGTGGACAGCCTGGCAGGGCAGC	cloning	HindIII
MGC40157_20Rp	CAGACATGTGGATGGAGGTG	cloning	EcoRI

"F" and "R" in primer ID denote forward and reverse primer respectively.

3.3.2. Oligonucleotides

Primers used in polymerase chain reactions, sequencing and cloning were purchased from Harvard University MGH DNA Sequencing Core (Boston, USA). Primers that were used in this study are tabulated in Table 3.1.

3.4. Bacterial Strains

Bacterial strain used in this study was *E. coli* TOP10 (genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG).

3.5. Cell Culture Reagents and Cell Lines

Huh7, Hep3B, SNU182, Mahlavu and SNU449 (human hepatocellular carcinoma; kindly provided by Dr.Mehmet Öztürk) and U373MG (human astrocytoma kindly provided by Dr.P.-O. Couraud) cell lines were used. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), calcium and magnesium-free phosphate buffered saline (PBS), Penicillin/Streptomycin mixtures were commercially obtained from BIOCHROM AG (Berlin, Germany).

3.6. Solutions and Media

3.6.1. DNA Gel Electrophoresis

50X Tris-acetic acid EDTA (TAE)	2M Tris-acetate 50mM ethylenediaminetetraacetic acid pH 8.5
TE Buffer	10mM TrisHCl 1mM EDTA, pH 8.0
Ethidium bromide (EtBr)	10 mg/ml
10X Tris Base EDTA (TBE)	108 g Tris base 55 g Boric acid 9.3 g EDTA Distilled water up to 1 L
Loading buffer	For 10ml: 2.4 ml dH2O 0.1 ml 1M Tris-HCl, pH 7.6 0.3 ml 1 per cent Bromophenol Blue (BPB) 6 ml 100 per cent glycerol 1.2 ml 0.5M EDTA

3.6.2. RNA Gel Electrophoresis Buffers

Diethylpyrocarbonate treated	1 per cent (v/v) Diethylpyrocarbonate
10X Morpholino Propane Sulfonic Acid (MOPS)	41.8 g MOPS 20ml 0.5M EDTA 16.8ml 3M NaOAc DEPC treated water upto 1L. pH 7.00
EtBr RNA loading buffer	0.72 ml formamide 0.16 ml 10X MOPS 0.26 ml formaldehyde 0.18 ml DEPC treated water (DPH) 0.1 ml 80 per cent glycerol 0.08 ml Bromophenol blue 50 μg EtBr

3.6.3. Microbiological Media and Antibiotics

Luria-Bertani medium (LB)	10 g tryptophan 5 g yeast extract 10 g NaCl Distilled water up to 1 L, autoclaved
Ampicillin stock	10 g tryptophan 5 g yeast extract 5 g NaCl 15 g Agar Distilled water up to 1 L, autoclaved

Kanamycin stock	50 mg/ml in distilled water Sterilized by filtration and stored at $-20°C$ 50 μg/ml (working concentration)
SOC	20 g tryptone 5 g yeast Extract 2 ml of 5M NaCl. 2.5 ml of 1M KCl. 10 ml of 1M $MgCl_2$ 10 ml of 1M MgSO4 20 ml of 1M glucose Distilled water up to 1L Sterilized by filtration and stored at $-20°C$

3.7. Equipment

Autoclave	Midas 55, Prior Clave, UK
Balances	DTBH 210, Sartorius, GERMANY
	Electronic Balance VA 124, Gec Avery, UK
Carbon dioxide tank	2091, Habaş, TURKEY
Cell culture incubator	Hepa Class 100, Thermo, USA
Centrifuges	Ultracentrifuge J2MC, Beckman Coulter, USA
	Mini Centrifuge 17307-05, Cole Parmer, USA
	Centrifuge 5415R, Eppendorf, USA
	Centrifuge, Allegra X-22, Beckman Coulter, USA
Deepfreezers	−20°C, Arçelik, TURKEY
	−70°C Freezer, Harris, UK
	−86°C ULT Freezer, ThermoForma, USA
Documentation System	Gel Doc XR System, Bio-Doc, ITALY
Flow Cytometer	FACSCalibur , Becton Dickinson, USA
Heat blocks	DRI-Block DB-2A, Techne, UK
Hemocytometer	Improved Neubauer, Weber Scientific International Ltd, UK
Laminal flow cabinet	Labcaire BH18, UK
Luminometer	Fluoroskan Ascent FL, Thermo Electron, USA.
Magnetic Stirrers	M221 Elektro-mag, TURKEY
	Clifton Hotplate Magnetic Stirrer, HS31, UK
Micropipettes	Finnpipette, Thermo, USA
Microplate Reader	680, Biorad, USA
Microscopes	Inverted Microscope, CKX41, Olympus, JAPAN
	Fluoroscence Microscope, Observer.Z1, Zeiss, GERMANY
Microwave oven	M1733N, Samsung, MALAYSIA

pH meter	WTW, GERMANY
Pipettor	Pipetus-akku,Hirschmann
	Laborger$\ddot{a}te, GERMANY$
Power Supply	Biorad, USA
Real Time PCR	LightCycler 1.5,
	Roche Diagnostics, SWITZERLAND
Refrigerators	2082C, Arçelik, TURKEY
	4030T, Arçelik, TURKEY
Shakers	VIB Orbital Shaker, InterMed, DENMARK
	Lab-Line Universal Oscillating Shaker, USA
Software	Quantity One, Bio-Rad, ITALY
	Light Cycler 4.0 Analysis Software,
	Roche Diagnostics, SWITZERLAND
	CellQuest Becton Dickinson, USA
Spectrophotometer	Agilent 8453, USA
	NanoDrop 1000, USA
Thermocyclers	Gene Amp. PCR System 2700,
	Applied Biosystems, USA
Vacuum pump	KNF Neuberger, USA
Vortex	Vortexmixer VM20, Chiltern Scientific, UK
Water baths	TE-10A, Techne, UK
Water purification	WA-TECH, GERMANY

4. METHODS

4.1. Preparation of Chemically Competent Cells

5ml of LB medium supplemented with 25 μg/ml of streptomycin was inoculated with a 100 μl aliquot of E. coli strain TOP10 glycerol stock and grown overnight at 37°C while shaking at 200rpm. Then, 25ml LB was inoculated with 250μl of the overnight culture. Cells were grown until optical density at 590nm reached 0.4-0.6. Cells were centrifuged at 4000g for 10 min at 4°C. The pellet was resuspended in 12.5ml of ice-cold sterile 50mM $CaCl_2$ and incubated on ice for 30 min. Cells were centrifuged again (4000g for 10 min at 4°C) and the pellet was resuspended in 2.5ml ice-cold sterile 50mM $CaCl_2$. 50-200 μL of this preparation was used for transformations. For long term storage at -80°C, glycerol was added to 10 per cent final concentration and cells were flash-frozen in liquid nitrogen.

4.2. Transformation of the Chemically Competent TOP10

A vial of competent cells was thawed on ice for 15min and 10-50 ng plasmid was added. After incubation on ice for 10-30 min, the vial was placed in 42°C heat-block for 1 min, and then immediately on ice for 2 min. 500 μl SOC or LB medium was added onto cells. Cells were incubated for 1 hr at 37°C with vigorous shaking (200rpm). After 1hour incubation, 100μl of the cell suspension was spread on antibiotic-containing plates and cells were grown overnight at 37°C, in an inverted position.

4.3. Plasmid Purification

All plasmid purifications were carried out with Qiagen plasmid kits, (mini, midi and maxi) according to the manufacturer's protocols. Plasmids that would be used in transfections were purified with midi or endotoxin free maxi gravity flow kits to yield transfection grade plasmids. Quality of plasmids was checked by spectrophotometric measurements and agarose gel electrophoresis. OD260/280 was between 1.8-2.00.

4.4. Plasmids and Molecular Cloning

4.4.1. Restriction Enzyme Digestion of DNA

Restriction enzyme digestions were performed in 20μl reaction volumes with the appropriate reaction buffer and condition according to manufacturer's recommendations.

4.4.2. Ligation

Ligation reactions were carried out in 10 μl reaction volume using 100ng plasmid and appropriate amount of insert by taking the molar insert to vector ratio into account.

4.4.3. Promoter Plasmids

5'-upstream fragments of C17orf45 genes were amplified from human genomic DNA (isolated from peripheral blood) using the primers listed in Table 3.1. The PCR fragments were cloned into Firefly luciferase reporter vector pGL3-basic (Promega) via NheI and HindIII.

4.5. Total RNA Agarose Gel Electrophoresis

Total RNA was fractionated by 1 per cent agarose gels containing formaldehyde to disrupt hydrogen bonds. 0.4g agarose was dissolved in 35ml DEPC treated water and after cooling to about 60°C, 4ml 10x MOPS buffer and 1.2ml 37 per cent formaldehyde was added to the mixture. 1μg of RNA was mixed with 20μl of ERLB. Samples were kept at 95°C for 2min, spinned and chilled on ice for at least 1min. Gels were run in 1X MOPS buffer.

4.6. Cell Culture Techniques

4.6.1. Growth Conditions of Cells

HCC derived cell line Huh7, Mahlavu, and Hep3B and human astrocytoma U373-MG cells were grown in DMEM containing 10 per cent FBS, 1 per cent penicillin/streptomycin and 1 per cent nonessential amino acids. SNU182 and SNU449 HCC cells were grown in RPMI medium containing 10 per cent FBS, 1 per cent penicillin/streptomycin and 1 per cent nonessential amino acids. All cells were incubated in 5 per cent CO_2 incubator at 37°C. Media were kept at 4°C and warmed to 37°C in a sterile water bath before use.

4.6.2. Passaging

The cells were passaged before reaching confluence. The growth medium was aspirated and the cells were washed once with 1X calcium and phosphate-free PBS. In order to remove the monolayer cells from the surface, trypsin (0.025 per cent, ready to use) was added to the petri dish and cells were incubated at 37°C for 3-5min. 10 volumes of fresh medium was added to inactivate trypsin and the suspension was pipetted gently to disperse the cells. The cells were transferred to fresh petri dishes in a 1:10 ratio for standard passaging.

4.6.3. Thawing

One vial of frozen cell line was taken from -80°C freezer and thawed under 40°C (at most) running tap water. Immediately after cells are thawed, they were transferred in a falcon tube and 4ml fresh medium was added. Cells were precipitated at 500g for 5min. The pellet was resuspended in an adequate amount of medium and transferred to a fresh petri dish.

4.6.4. Cryopreservation

Cells were harvested by trypsinization and neutralized with 10 volumes of growth medium. The cells were counted in a haemocytometer and precipitated at 1500 rpm for 5 min. The pellet was suspended in freezing medium (5 per cent DMSO, 10 per cent FBS and 85 per cent DMEM). 1.5 ml of this solution was placed into 2 ml screw capped-cryotubes. The tubes were left at -20°C for 2hours and then transferred to -80°C freezer. Next day, main stocks were transferred into the liquid nitrogen storage tank. The number of cells frozen in a single vial was between 1-3 millions.

4.6.5. Extraction of Total RNA From Tissue Culture Cells

RNA extraction from 6 and 12 well tissue culture plates was done according to the manufacturer's protocol (Qiagen RNAeasy Mini Kit).

4.6.6. Transient Transfection of Cells

Transfections were carried out in 6, 12 or 24 well plates with *in vitro* polyethylenimine transfection reagent, Exgen500 and Turbofect (Fermentas). Optimization for transfection efficiency was done with the GFP encoding pEGFP-N2 (Clonetech) mammalian expression vector. The amount of plasmid and corresponding amount of transfection reagent was determined according to the manufacturer's protocol. Cells were seeded the day before transfection to obtain 50-60 per cent confluency at the day of transfection. On the next day the medium was exchanged with certain amount of antibiotic-free medium if transfection reagent was Exgen. If Turbofect was used, the medium was changed with certain amount of growth medium with antibiotic. 3-4hrs after transfection media were replaced with fresh growth medium.

4.7. Luciferase Reporter Assay

500ng pGL3-luciferase reporter plasmid including promoter of interest and 25ng of pRL-TK (internal control, Renilla luciferase) plasmid were used per well of 12-well

plate. 1μg of empty plasmid as control or 1μg of β-catenin or 1μg of TCF4 plasmid were used for each transfection. Transfection is done by using Exgen or Turbofect transfection reagents (Fermentas) according to the manufacturer's instructions. 48hrs after transfection cells were collected and harvested using 200μl 1X PLB (Passive Lysis Buffer) provided by the Dual-Glo Luciferase Assay System (Promega) per sample. The lysates were vortexed and spun at 16,000g for 5min at 4°C, to pellet the cell debris. 100μl of the cleared lysates were mixed with Firefly luciferase substrate reagent and after 10min incubation at room temperature (in the dark), measurements were taken using a fluorometer (Fluoroskan Ascent FL, Thermo Electron). Next, 100μl of Renilla luciferase substrate reagent (StopGlo) that also quenches the Firefly luciferase luminescence was added and after 10min incubation at room temperature (in the dark), measurements were taken. Luminescence reads were 1-2.5 seconds (integration time). Firefly luciferase readings were normalized to Renilla luciferase readings.

4.8. GFP Reporter Assay

400.000-500.000 cells were seeded in 6-well before transfection. Huh7 cells were transfected with 200ng GFP reporter plasmid under the control of C17orf45 promoter using Turbofect transfection reagent (Fermentas). For overexpression β-catenin or TCF4 plasmids were used. 24hrs after transfection cells were trypsinized and precipitated at 500g for 5min. Then cell pellets were washed with 1X PBS and resuspended in 500μl PBS. GFP fluorescence is detected by FL1 detector in the flow cytometry machine (FACScalibur, Bekson Disckison, USA). Before analysis, voltage settings for forward scatter, sight scatter and FL1 detectors were done. By using CellQuest software two-parameter dot-plot of Forward Scatter (FSC) vs. Side Scatter (SSC) and a single-parameter FL1 (used as GFP detection) histogram with linear x-axis to illustrate relative promoter activity or GFP expression were plotted. In dot-plot of Forward Scatter (FSC) vs. Side Scatter (SSC) living cells were determined during analysis of positive control cell (cells transfected with GFP plasmid). The region on the dot-plot graph where GFP positive events fit mostly is gated as the position of the cells that were analyzed. In the histogram graph (showing GFP signal and counts) gated events data were monitored. The threshold was selected (the point at which a signal will be

accepted as a positive event) according to the negative control (untransfected cell). The statistics of the histogram graphs were analyzed using the same software. Mean fluorescence intensity of GFP positive events were taken as reference to compare the promoter activity of each sample.

4.9. Lithium Treatment Assay

300.000-500.000 cells were seeded into 6-well plates. Next day, lithium chloride or sodium chloride was added to the medium of the samples to a final concentration of 25mM. For RNA isolation 48 hrs after addition of lithium and sodium salts 350μl of RLT lysis buffer provided by RNeasy Mini Kit (QIAGEN) was used to lyse the cells following aspiration and washing steps. Genomic DNA was sheared with a 23G syringe needle before proceeding with RNA isolation.

4.10. Chromatin Immunoprecipitation(ChIP) Assay

ChIP was performed on HCC cells, Huh7, Hep3B and SNU182 using the Epigentek ChIP kit (catalog P-2002). 500.000-1.000.000 HCC cells were used for immunoprecipitation. Cells were trypsinized and washed once with PBS. Cells were crosslinked in 9ml growth medium including 1 per cent formaldehyde. To inhibit the crosslinking, 1ml of 2M glycine were added and incubated for 5 min. Following the lysis steps of the membrane and nucleus according to manufacturer's protocol, sonication was performed to shear genomic DNA. The cells were sonicated five times for 30 seconds at power setting 5 with 50 per cent power efficiency with 30 seconds interval. Other than sonication, manufacturer's instructions were followed. 2.5 μg of mouse monoclonal anti-β-catenin antibody (BD Bioscience, Catalog 610154), 1μg of anti-RNA polymerase II and 1μg of mouse normal IgG antibody from the kit as controls. PCR was performed from purified DNA fragments by using Fermentas Taq polymerase, starting with an initial denaturation at 94°C for 5 minutes, followed by 46 cycles of 30 seconds at 94°C, 1 minute at 54°C, 1 minute at 72°C and finally 5 minutes of 72°C.

4.11. Proliferation Assay

CyQUANT NF Cell Proliferation Assay Kit (Invitrogen) were used for the proliferation assay. Cells were transfected with empty pcDNA3 and pcDNA3 including *C17orf45*. 24 hrs after transfection cells were trypsinized, counted and seeded in 96well plates (1000-1500 per well) and the first reading was taken next day (∼48hrs after transfection). The assay is based on measurement of cellular DNA content via fluorescent dye binding. Fluorescence readings were taken for 8 days with Fluoroskan Ascent (Thermo Electron, USA). The medium was changed every 3-4 days.

4.12. Semi-Quantitative and Quantitative Reverse Transcriptase Mediated PCR (RT and QRT-PCR)

4.12.1. cDNA synthesis

cDNA synthesis was performed with ImPromII Reverse Transcription System Kit (Promega) according to manufacturer's protocol. 1μg total RNA was used for each reaction. At the end of the reaction cDNA is diluted to a final volume of 100μl.

4.12.2. Primer Design

Primer design for the Q-RT-PCR was done with the program, Perl Primer (`http://perlprimer.sourceforge.net/`). Primers were designed to encompass an intron to see any genomic contamination, and the amplicon size was usually between 100-300bp.

4.12.3. Semi-Quantitative Polymerase Chain Reaction

1-2 per cent of cDNA synthesized from 1μg total RNA was used as template in PCR reactions. The optimum cycle number for each gene was determined by changing the cycle number in the range of 23-30.

4.12.4. Quantitative Polymerase Chain Reaction

Real Time PCR was done with the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche; Basel, Switzerland) according to the manufacturer's protocol. Standard curves were constructed for each primer pair and cDNA sample in order to calculate the efficiency. Results were analysed with Light Cycler 4.0 Analysis Software (Roche; Basel, Switzerland). Reaction starts with an initial denaturation step at 95°C for 10min. Amplification cycle consists three steps; a denaturation step at 95°C for 10sec, an annealing step at 57°C for 5sec and an elongation step at 72°C for 10sec. 45 cycles are followed by melting curve step.

5. RESULTS

5.1. SAGE and Microarray Combined Screens Reveal that *C17orf45* mRNA Level Increased in Huh7 Over-expressing Mutant β-catenin

In order to identify novel genes regulated by the Wnt/β-catenin/TCF4 pathway, we previously compared transcriptomes of high and low TCF activity Huh7 cells by using SAGE (Serial Analysis of Gene Expression) and genome wide microarray analysis [90]. After comparing global gene expression between Huh7 cells with high and low TCF activity, *C17orf45* was found to be up-regulated in Huh7 cells with high TCF activity by 2.5 fold according to SAGE (Figure 5.1a) and by 1.65 and 1.29 folds according to 2 different Affymetrix probe sets in microarray screen (Figure 5.1b). SAGE and microarray data were verified by Q-RT-PCR, using the same RNA used for the screens (Figure 5.1c).

(A)

SAGE Tag	SAGE Fold
AACTAATACT	2,5

(B)

Affymetrix Tag	Fold
225065_x_at	1.65
227963_at	1.29
236913_at	1.12

(C)

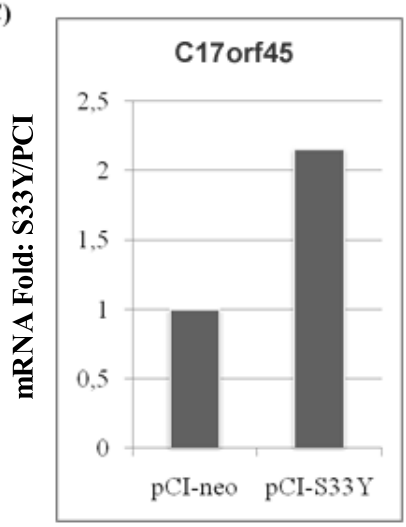

Figure 5.1. Increase of *C17orf45* mRNA level upon stable overexpression of S33Y-β-catenin in Huh7 cells as detected by (A) SAGE screen and (B) genome wide Affymetrix microarray screen performed by Kavak *et al.*,(unpublished data). (C) Confirmation of SAGE and microarray data by Q-RT-PCR. β-actin and GAPDH were used for normalization. pCI-Neo, control empty vector, does not encode β-catenin. S33Y is the mutant form of β-catenin.

5.2. LiCl Mediated Inhibition of GSK3-β Leads to Increased *C17orf45* Transcription

Lithium ion is a well known inhibitor for GSK3-β [91] which is a key molecule in the degradation complex leading to phosphorylation of β-catenin and initiation of its degradation. Lithium salts such as lithium chloride have been widely used to mimic the activation of the Wnt/β-catenin pathway [92]. In order to support the hypothesis that hyperactivation of the Wnt/β-catenin pathway increases the expression of *C17orf45*, hepatocellular carcinoma cell lines Huh7, Hep3B, Mahlavu and SNU449, and human astrocytoma cell line U373-MG were treated with 25mM LiCl for 48 hours. mRNA level changes in lithium treated cells were compared to NaCl treated control cells by Q-RT-PCR (Figure 5.2). In all lithium treatments, *AXIN2* which is a well established Wnt/β-catenin target [93] was used as positive control (Figure 5.2).

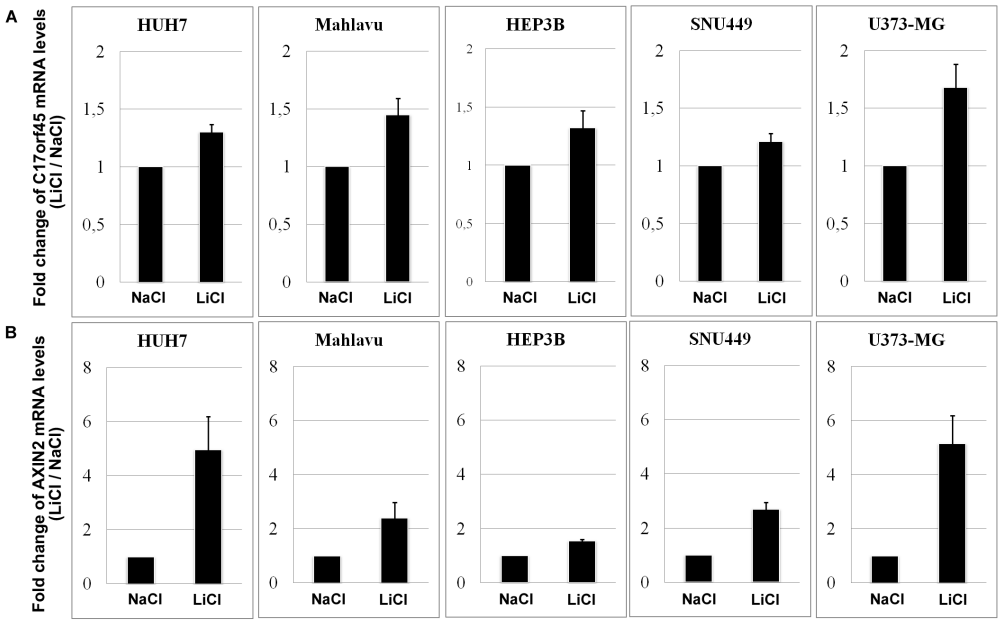

Figure 5.2. Lithium treatment leads to increase in (A) *C17orf45* and (B) *AXIN2* mRNA levels in four HCC and U373-MG cells. Cells were treated with 25mM LiCl for 48hrs with NaCl as a control and mRNA levels were analyzed by Q-RT-PCR. ACTB (B-actin) and GAPDH were used as internal controls for normalization (data not shown). Error bars represent standard deviation. Each graph is a representative of at least two independent experiments.

C17orf45 mRNA level increased significantly ($p<0.05$) after 48hrs of lithium treatment in SNU449 cells (∼1.3-fold, Figure 5.2). *C17orf45* mRNA fold changes in lithium treated Huh7, Mahlavu, Hep3B, and U373MG cells were not significant but were consistent in at least two independent experiments. Thus, the lithium treatment assay supports the hypothesis that *C17orf45* is a target of the Wnt/β-catenin pathway.

5.3. *C17orf45* Promoter is Regulated by the Wnt/β-catenin Pathway (Luciferase and GFP Reporter Assays)

Luciferase and GFP reporter assays were performed to analyze the activity of *C17orf45* promoter. For this purpose, two upstream fragments of *C17orf45* promoter were cloned into the pGL3 promoterless plasmid (See Section 4.4.3) and tested to assess their basal activity without stimulation in Huh7 cells (Figure 5.3). The location of the upstream fragments was indicated in Figure 5.3 as positions [-2500 -1538] and [-1600 +81] with respect to the transcription start site. In all luciferase experiments the reporter plasmid including the promoter fragment (-1600 +81) was used, because as it is seen in Figure 5.3 luminescence signal from the cells transfected with this construct is very high compared to cells transfected with promoterless pGL3 which is counted as background signal. The construct including the fragment (-2500 -1538) did not give a signal above background. Therefore, the construct of the promoter fragment (-1600 +81) was used for further analyses.

To test the effect of Wnt/β-Catenin pathway activation on *C17orf45* promoter activity, in all luciferase reporter assays unless stated otherwise in the text, β-catenin with four point mutations (S33A, S37A, T41A, S45A) leading to a degradation resistant form, TCF4 and N-terminal deleted form of TCF4 (ΔN-TCF4) were used for overexpression study in Huh7 cells. In all experiments and samples, cells were also co-transfected with internal control plasmid pRL-TK to be used for normalization to eliminate variations in transfection efficiencies and cell number between samples.

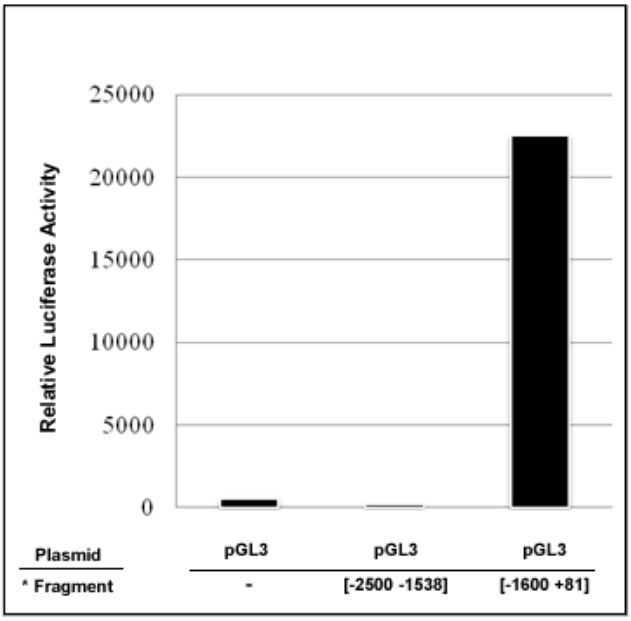

Figure 5.3. Basal promoter activity of two different *C17orf45* promoter fragments. Cells transfected with pGL3/[-1600 +81] produces higher luminescence signal than cells with promoterless pGL3. *Fragments reside on the upstream location of *C17orf45* gene indicated by the positions [-2500 -1538] and [-1600 +81] with respect to transcription site. Luciferase reading was normalized to Renilla luciferase activity used as internal control.

5.3.1. TK (Thymidine Kinase) Promoter is Activated by Over-expression of β-catenin and TCF4

To minimize variations such as transfection efficiency, cell viability and cell lysis between samples the Renilla luciferase encoding plasmid pRL-TK is commonly used as normalization control. In our experiments TK promoter activity is also altered by over-expression of β-catenin, TCF4 and ΔN-TCF4. The results from eight independent experiments (Figure 5.4b) show that over-expression of β-catenin leads to enhanced Renilla luciferase reporter gene expression in all experiments under the control of TK promoter by an average of $\sim$2-fold ($p < 0.005$, student's t-test) with respect to the control sample. Co-overexpression of TCF4 and β-catenin increased the TK promoter activity in all experiments by an average of $\sim$8-fold ($p < 0.005$, student's t-test). When ΔN-TCF4 was used instead of TCF4 in co-overexpression, increase in promoter activity was suppressed. ΔN-TCF4 lacks the N-terminus which corresponds to β-

catenin-binding region and competes with the endogenous TCF4 on binding to TCF regulatory elements in the target gene promoters. Thus, on promoter bound by ΔN-TCF4 a functional β-catenin/TCF4 complex cannot be formed.

5.3.2. *C17orf45* Promoter Activity is Regulated by β-catenin and TCF4

When TK promoter activity is not considered, according to non-normalized data from eight independent experiments (Figure 5.4) performed in Huh7 cells transfected with β-catenin and co-transfected with β-catenin and TCF4, the promoter activity of *C17orf45* increased by an average of ∼2-fold ($p < 0.005$, student's t-test) and ∼7-fold ($p < 0.01$, student's t-test) respectively. Using ΔN-TCF4 instead of TCF4 suppresses the increase that resulted from co-overexpression of β-catenin and TCF4 in all experiments (8/8) and the increase resulting from over-expression of β-catenin in 3 experiments (3/7).

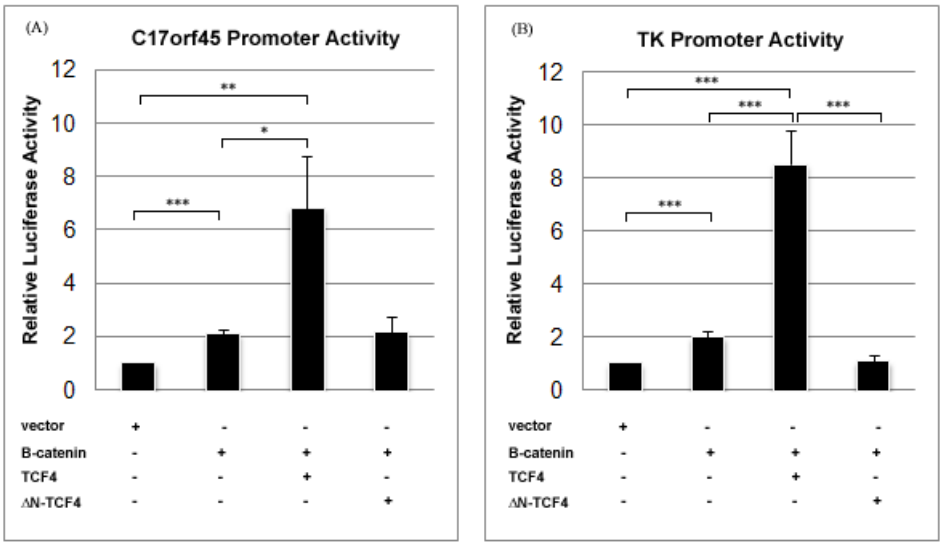

Figure 5.4. β-catenin and Tcf4 regulate (A) *C17orf45* and (B) TK (thymidine kinase) promoter activities in Huh7 cells without considering the normalization by TK promoter activity. Cells were transfected with promoter pGL3/*C17orf45* [-1600 +81] plasmids. Cells were stimulated by overexpression of β-catenin and TCF4. Each graph is a representative of eight independent experiments. Error bars represent standard error (* = $p<0.05$, ** = $p<0.01$ and *** = $p<0.005$, Student's t-test).

Even though there is not internal normalization used in the luciferase assays, it is likely to support our hypothesis that C17orf45 is a Wnt target.

5.3.3. GFP Reporter Assay

Considering lack of internal normalization in our luciferase assays, we wanted to verify the regulation of the *C17orf45* promoter by the Wnt/β-catenin pathway in another experimental system with an appropriate internal control. Flow cytometric analysis of the cells transfected with GFP (Green fluorescent protein) reporter plasmids including the promoter of *C17orf45* was performed. Fluorescence intensities of GFP expressed under the control of *C17orf45* promoter was used to compare the promoter activity between samples.

Overexpression of β-catenin and co-overexpression of β-catenin with TCF4 increased the promoter activity of *C17orf45* by $\sim$1.2-fold and $\sim$1.6-fold respectively in terms of relative GFP mean fluorescence intensity compared to empty vector control (Figure 5.5). When GFP reporter and non-normalized luciferase reporter assays are taken together, these findings indicate that the *C17orf45* promoter is regulated by β-catenin and TCF4.

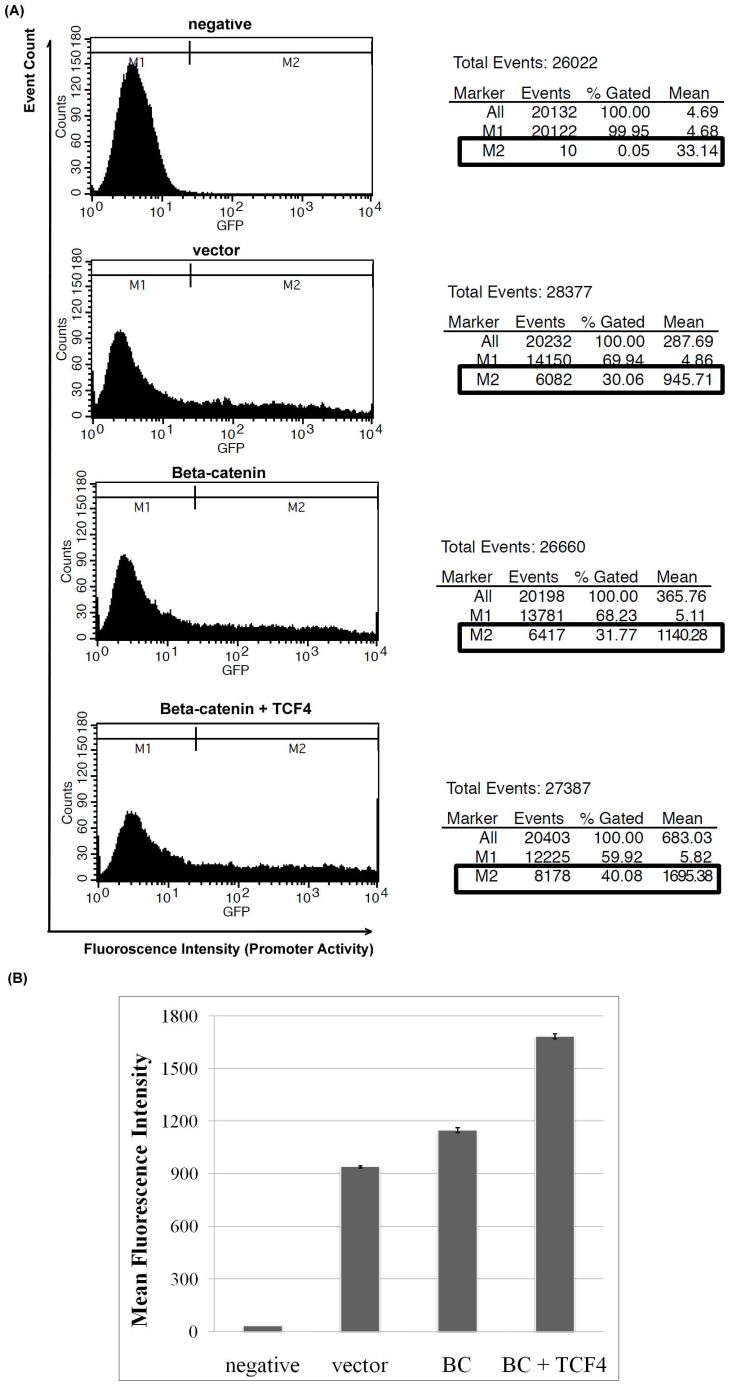

Figure 5.5. Flow cytometry analysis of GFP reporter expression under the control of the *C17orf45* promoter.(A) Histogram plots of Huh7 cells transfected with GFP reporter plasmid carrying the *C17orf45* promoter. Reporter expression was analyzed ∼24 hrs after transfection. GFP fluorescence (x-axis) and count of events (cells) (y-axis) (B) The mean GFP fluorescence intensities of the gated (M2: GFP positive cell population gated with respect to cell population in negative control) cell populations. Each graph is representative of two independent experiments. BC denotes β-catenin.

5.4. β-catenin Interacts with *C17orf45* Promoter (ChIP Assay)

Chromatin immunoprecipitation assay (ChIP) was performed on HCC cells Huh7, SNU182 and Hep3B to obtain evidence for the proposed β-catenin *C17orf45* promoter interaction. The *C17orf45* promoter fragment was detected after PCR analysis of immunoprecipitated genomic DNA by anti-β-catenin antibody (Figure 5.6). RNA polymerase II and monoclonal anti-mouse-IgG antibodies were used as positive and negative controls respectively.

PCR analysis of the immunoprecipitated DNA (Figure 5.6) revealed that anti-β-catenin and anti-RNA Polymerase II antibody, but not the normal mouse IgG, bind to *C17orf45* promoter in Huh7, SNU182 and Hep3B.

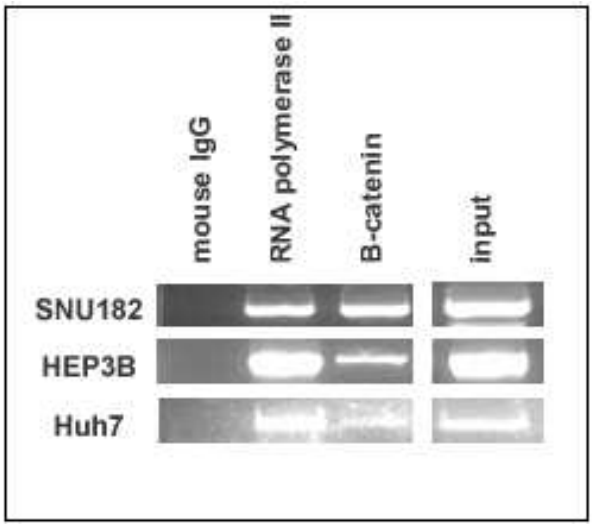

Figure 5.6. PCR analysis of immunoprecipitated genomic DNA shows that β-catenin interacts with *C17orf45* promoter in SNU182, Hep3B and Huh7 cells. Chromatin immunoprecipitation assay was performed with monoclonal anti-β-catenin, anti-RNA polymerase II and anti-mouse-IgG antibodies. Mouse IgG and RNA polymerase II were used as negative and positive controls, respectively. Input sample which was used as a positive control of the PCR analysis was isolated from pre-immunoprecipitation lysate.

5.5. Subcellular Localization of *C17orf45*

To determine subcellular localization of C17orf45 GFP fusion protein was prepared and transfected into Huh7 cells. The 130 amino acid long C17orf45 protein coding region without its stop codon was amplified via PCR and directionally cloned into the N-terminus of GFP coding region in the same translation frame of pEGFP-N2 plasmid (explained in Chapter 4). Huh7 cells were transfected with plasmids coding for GFP fused C17orf45 protein. 24hrs after transfection it was observed that GFP fused protein did not localize in the nucleus (no co-localization of DAPI and GFP, see Figure 5.7a). As the next step, a mitochondrial marker was used to determine if it localized to mitochondria. Fluorescent MTS-dsRED (mitochondria targeted sequence-dsRED) protein was used as a mitochondrial marker. Cells were co-transfected with plasmids coding for C17orf45-GFP and MTS-dsRED. 24hrs after transfection, co-localization of MTS-dsRED and C17orf45-GFP was observed (Figure 5.7b). Thus, C17orf45 GFP fused protein was found to be localized in mitochondria. The amino acid sequence of C17orf45 was analyzed in order to find a mitochondria localization signal TargetP 1.1 Server [94]. The sequence corresponding to the first 36 amino acid was predicted as a signal peptide using this program. To determine if this signal peptide is necessary for localization to mitochondria we generated a new GFP fusion construct lacking this signal peptide. After co-transfection of cells with the new GFP fusion construct and MTS-dsRED, the truncated form of C17orf45 did no co-localize with the mitochondria marker (Figure 5.7). Therefore, we conclude that C17orf45 protein contains a mitochondria localization signal that is required for localization to mitochondria.

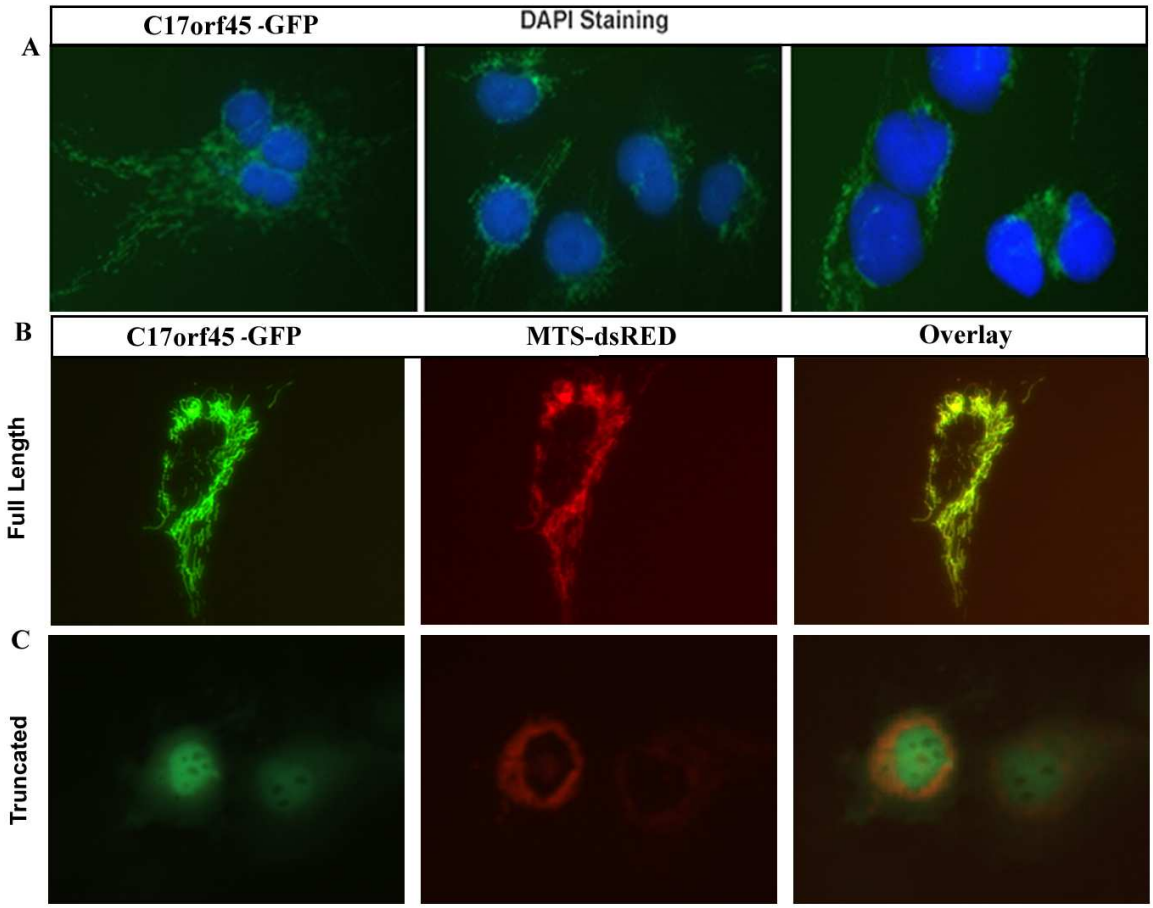

Figure 5.7. Subcellular localization of GFP fused C17orf45 protein. (A) Huh7 cells transfected with plasmid coding for C17orf45::GFP fused protein. 24hrs after transfection transfected cells were stained with DAPI and observed under a fluorescence microscope. (B) To determine the subcellular localization of the fused protein a mitochondria marker MTS-dsRED (mitochondria targeting sequence-dsRED) fluorescent protein was used. 24hrs after co-transfection of Huh7 cells with plasmids coding for C17orf45-GFP and MTS-dsRED, cells were observed. (C) Subcellular localization of Δ-N-C17orf45-GFP. The predicted mitochondria localization signal in C17orf45 protein was removed and an N-terminally truncated form of C17orf45-GFP protein was generated.

5.6. Expression of *C17orf45* in Human Brain Tumors

According to sagereveal finding (See section 1.3.3.), *C17orf45* is differentially expressed in human tumors as listed in Table 1.4. Based on this prediction, analysis of *C17orf45* mRNA expression level in human brain tumors was performed by Q-RT-PCR (Figure 5.8). mRNA expression level was normalized using 18S mRNA level. *C17orf45* mRNA expression is upregulated by ∼1.3 to ∼7.6-fold in all tumor types which were examined except one embryonic tumor; dysembryoplastic neuroepithelial tumor (DNET) tissue.

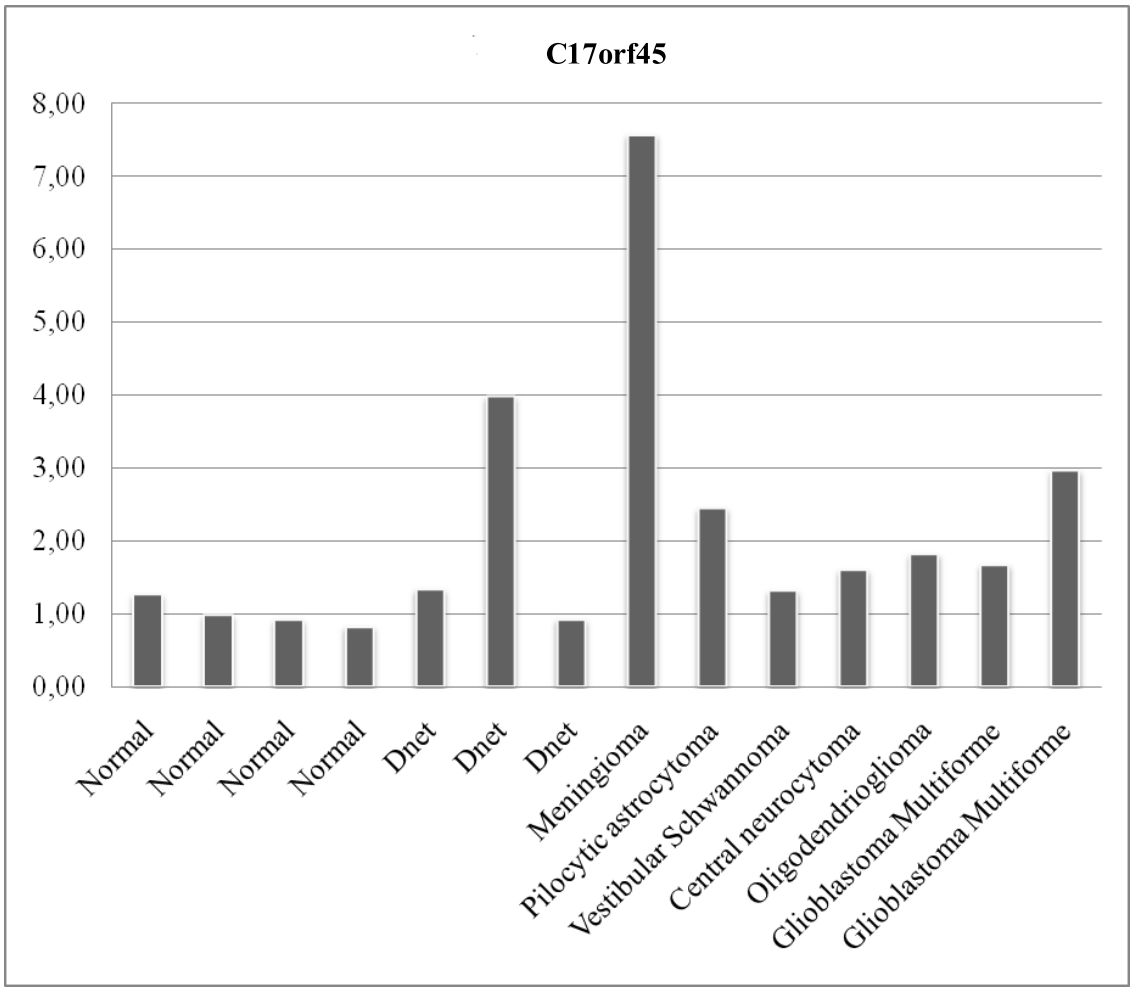

Figure 5.8. mRNA expression level of *C17orf45* in several human brain tumors. The Graph represents three Q-RT-PCR analyses of the same cDNA series including indicated tumor samples. DNET: dysembryoplastic neuroepithelial tumor

5.6.1. Expression of *C17orf45* in Human Meningioma Tumors

After the finding of ∼7.6-fold increase in *C17orf45* mRNA level in meningioma sample of human brain tumor cDNA series, two more meningioma samples were examined (Figure 5.9). Expression analysis in four normal and three meningioma samples shows that *C17orf45* mRNA level is upregulated in meningioma tumors significantly ($p < 0.05$, student's t-test).

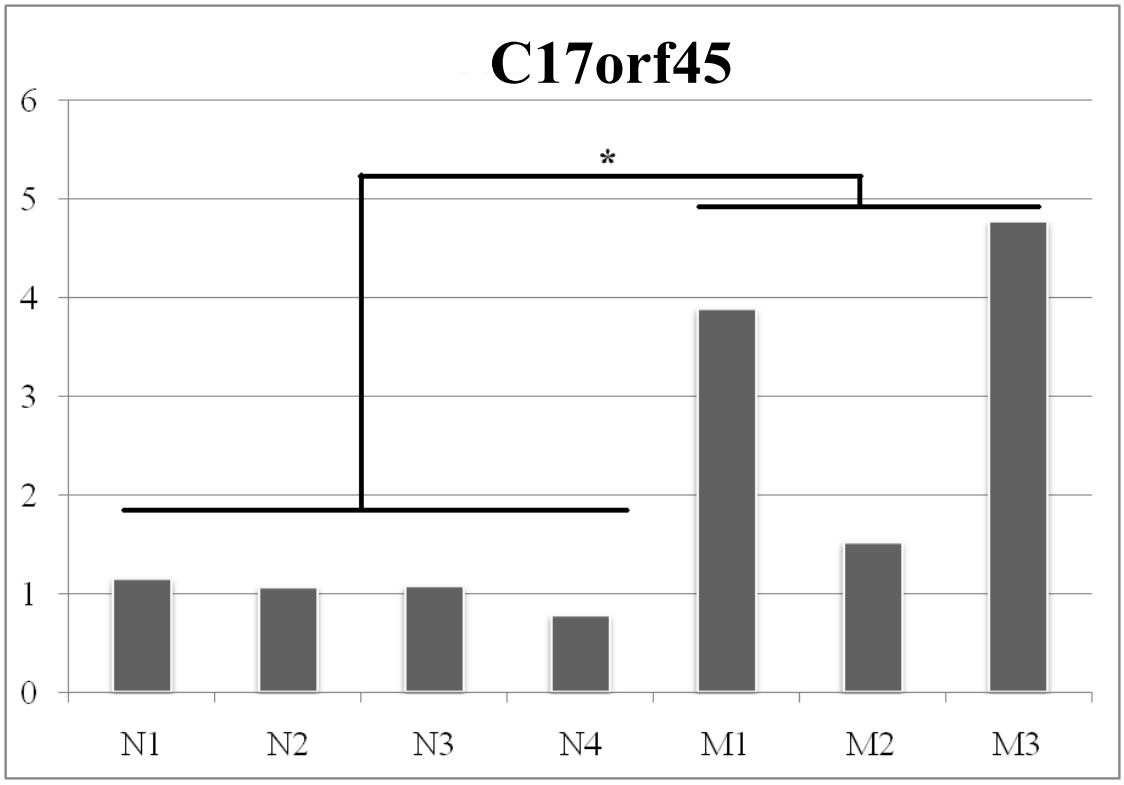

Figure 5.9. mRNA expression level of *C17orf45* in meningioma samples. *C17orf45* expression increased significantly in meningioma tumor (n=3) with respect to normal tissues (n=4). N denotes normal and M meningioma (* = $p<0.05$, Student's t-test).

5.7. Over-expression of *C17orf45* Inhibits Proliferation of Human Astrocytoma U373-MG Cells

In order to investigate the potential link between *C17orf45* and tumorigenesis, the effect of *C17orf45* on cell growth was examined by transient transfection of human astrocytoma cell lines U373-MG with a full length cDNA clone of *C17orf45* in pcDNA3. Proliferation assay was performed to examine the effect of *C17orf45* on cell growth. Over-expression of *C17orf45* inhibits the proliferation of U373-MG cells compared to the cells transfected with empty pcDNA3 plasmid (Figure 5.10.

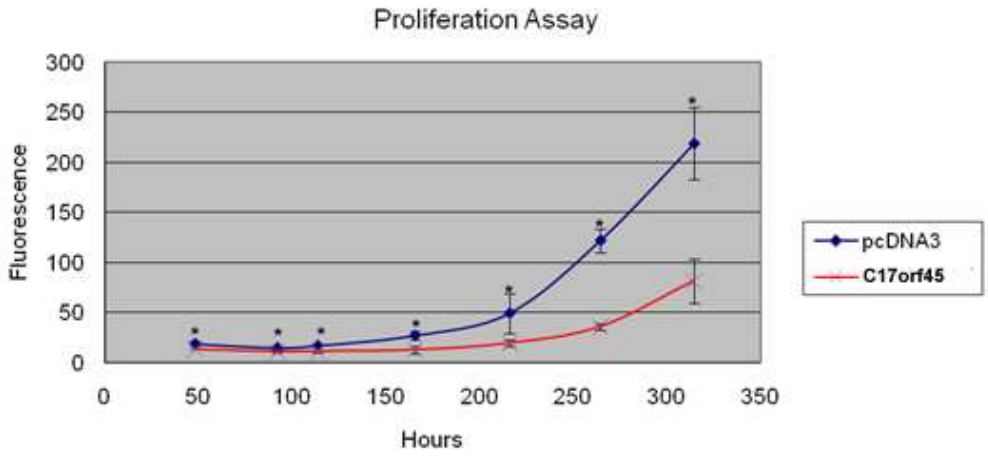

Figure 5.10. Over-expression of *C17orf45* inhibits the proliferation of U373-MG cells. Equal number of cells (500 to 1500 cells) was seeded into 96 well plates 24hrs after transfection. Y axis is the fluorescence intensity which is proportional to number of living cells in the wells. Error bars represent standard deviation. pcDNA3 is the empty control vector. The graph is representative of two experiments.

6. DISCUSSION

In this study, *C17orf45* was identified as a novel transcriptional target of Wnt/β-catenin pathway in Huh7 cells (a hepatocellular carcinoma cell line). *C17orf45* was picked as Wnt target candidate from the combined SAGE and microarray screens. LiCl mediated inhibition of GSK3β, luciferase/GFP reporter assay and chromatin immunoprecipitation assay were performed to verify *C17orf45* as a Wnt target.

Following SAGE-microarray combined screen and verification, lithium treatment assay (Figure 5.2) was performed on Huh7, Mahlavu, SNU449 and SNU182. A human astrocytoma cell line U373-MG was also used to show the finding in another cell type that originated from a different tissue source. Because lithium-treatment assay is a simple and cheap assay and widely used in studies mimicking hyperactivation of Wnt/β-catenin pathway [91], we used this assay to get evidence supporting our hypothesis that *C17orf45* is a target of the Wnt/β-catenin pathway. The result showed statistically insignificant but consistent increases in LiCl treated cells with respect to NaCl treated control samples by a factor of $\sim$1.3 for Huh7, $\sim$1.4 for Mahlavu, $\sim$1.3 for Hep3B, and $\sim$1.7 for U373-MG cells.

Although lithium treatment assay was performed at least twice by using 5 different cell lines and *Axin2* as a positive control, the result by itself cannot be considered as a proof for transcriptional regulation of *C17orf45* by the the Wnt/β-catenin pathway. While lithium ion is widely used it cannot be considered as specific inhibitor for GSK3β [95]. Thus, in order to get more evidence about *C17orf45* as a potential target, the promoter of C17orf45 was cloned into a promoterless luciferase plasmid and its promoter activity was analyzed using luciferase activity upon activation of Wnt/β-catenin pathway by overexpression of β-catenin and TCF4.

Before testing the promoter activity change by Wnt/β-catenin, basal activity of two promoter fragments of *C17orf45* were examined first (Figure 5.3). Two upstream promoter fragments (-2500 -1538) and (-1600 +81) were used for this experiment.

Luciferase activity was only observed from the sample including the fragment (-1600 +81). Luciferase expression under the control of the other fragment was negligible as the luminescence signal was lower than the background signal. So it can be said that for the transcription process the regulatory sequences in the fragment (-1600 +81) is required.

The pRL-TK vector contains the herpes simplex virus thymidine kinase (HSV-TK) promoter and is commonly used as an internal control. Using the internal control synthetic Renilla gene transcription for normalization minimizes variability in transfection efficiency between different samples of transfected cells. For this reason co-transfection with other plasmids or treatments should not change the activity of the TK promoter. But our data suggest that TK promoter activity is enhanced with the activation of the Wnt/β-catenin pathway (Figure 5.4b). Over-expression of β-catenin and co-overexpression of β-catenin and TCF4 leads to increase in Renilla luciferase activity consistently in 8 independent experiments by an average of $\sim$2-fold ($p < 0.005$, student's t-test) and an average of $\sim$8-fold ($p < 0.005$, student's t-test) respectively. Using ΔN-TCF4 instead of TCF4 in co-overexpression suppresses these increases. ΔN-TCF4 lacks the N-terminus which corresponds to the β-catenin-binding region. Thus, if the examined promoter fragment (HSV-TK) is really regulated by TCF activity in the cell it is expected that promoter activity for the sample (β-catenin + ΔN-TCF4) will be lower than the promoter activity for the sample (β-catenin + TCF4). In the latter sample a functional transcriptional complex cannot be formed other than the endogenous one. Besides, ΔN-TCF4 has still functional DNA binding domain and can bind to TCF regulatory elements in target gene promoter. So, if a promoter is regulated by TCF/β-catenin complex it is expected that ΔN-TCF4 can decrease the effect of β-catenin on promoter activity.

There are studies regarding the HSV-TK promoter that TK promoter activity can be changed upon specific stimulation such as by liganded nuclear receptor RXR [96], GATA transcription factor [97] and overexpression of orphan nuclear receptor Nur77 [98]. Hence, the luciferase reporter assay result of *C17orf45* was presented without normalization to Renilla luciferase activity under the control of the TK promoter.

C17orf45 promoter activity increased by an average of ∼2-fold ($p < 0.005$, student's t-test) when cells were transfected with β-catenin. When TCF4 is added to the transfection mixture activity increases to ∼7-fold ($p < 0.01$, student's t-test). Reaching this value may results from a synergistic effect, because β-catenin and TCF4 cannot form a functional transcription complex independent of each other. For instance, over-expression of β-catenin increases the probability of forming TCF complex but it depends on the amount of endogenous TCF4. But to confirm this, over-expression of TCF4 sample is also required. Using ΔN-TCF4 instead of TCF4 suppresses elevations of promoter activity in all experiments (8/8) of co-overexpression and in 3 experiments (3/7) of β-catenin over-expression. Thus, luciferase assays without normalization suggest that *C17orf45* promoter is regulated by β-catenin and TCF4.

As explained in Section 5.4.3. GFP reporter assays can overcome the variation in transfection efficiency between samples. To support the luciferase assay results, the luciferase reporter gene is substituted with GFP reporter gene in promoter plasmids. The analysis is done by taking relative GFP mean fluorescence intensity as reference for the comparison. In luciferase assays the total luminescence signal is measured. Thus, assay results represent both the transfected and untransfected cell population. But in GFP reporter assays cells that are transfected and express GFP are selected and untransfected cells are not considered. Relative promoter activity in GFP reporter assay is determined by taking the mean values of GFP intensities for each cell population. This value is reliable since depending on the experiment the mean value is calculated from fluorescence reading of 10.000-500.000 cells. The disadvantage of the GFP reporter assay is that it is not very sensitive compared to the luciferase assay.

Promoter activity of C17orf45 is increased in cells overexpressing β-catenin by ∼1.2-fold (Figure 5.5). Co-overexpression of β–catenin and TCF4 increased the promoter activity of C17orf45 by ∼1.6-fold and is consistently higher than the β-catenin sample as in the luciferase assay. The fold increases are lower compared to luciferase folds. The reason might lie in the length of the post-transfection period before the analysis. Cells were incubated for ∼24 hours in GFP reporter assays for ∼24 hours in luciferase assays. Additional time results in more accumulated reporter protein and

if the analysis is allowed to wait for another 24hrs the fold values may increase even further. So GFP reporter and non-normalized luciferase reporter assays were taken together, these findings support that *C17orf45* promoter is regulated by β-catenin and TCF4.

Chromatin Immunoprecipitation assay (ChIP) can be used to detect DNA-protein interactions. β-catenin binding to *C17orf45* promoter fragment was detected after PCR analysis of immuno-precipitated genomic DNA by anti-β-catenin antibody in three HCC cells Huh7, SNU182 and Hep3B (Figure 5.6). Anti-mouse IgG was used to show the specifity of immunoprecipitation. Cells used in ChIP assays were not stimulated by overexpression or chemical treatments. So all kinds of possible artifacts resulting from phenomena such as unspecific inhibition or interaction are eliminated. When compared to other techniques like luciferase assay in which overexpression takes place, ChIP reflects the *in vivo* condition more. Because cell lines were used for ChIP, their cellular context and mechanisms might be different leading to a cell line-specific promoter interaction. So the results can be verified with *in vivo* system using human liver tissue.

Another improvement on the presented experiments for both luciferase/GFP reporter and ChIP assays might be using a universal negative control. A universal negative control is a gene whose transcription is not regulated by alteration in Wnt pathway activation. A gene or genes whose transcription is/are not implicated in Wnt pathway may be found by searching the literature or databases including high-throughput screening.

The next step is investigating potential roles of C17orf45 in the Wnt/β-catenin pathway. Because there is no study on *C17orf45* in the literature, addditional characterization of C17orf45 is required for further studies before this step. For this purpose, initial motivation was to identify the subcellular localization and interaction partners of C17orf45 using yeast two hybrid screening. For the yeast two hybrid screening *C17orf45* open reading frame was cloned and identification of potential interactants is going on. Another point about C17orf45 is that there is no evidence of its protein prod-

uct other than prediction. So in parallel, the preparation of an antibody for C17orf45 is in progress.

C17orf45 open reading frame without stop codon was cloned into a GFP plasmid. Co-transfection of GFP fused C17orf45 with dsRED plasmids revealed that fused protein co-localizes with mitochondrial marker MTS-dsRED but not with DAPI (Figure 5.7). When the predicted mitochondria targeting sequence was deleted, GFP fused protein didn't localize to mitochondria (Figure 5.7c). Thus, GFP fused C17orf45 was found to be localized to mitochondria and has a mitochondria localization signal in its amino acid sequence. Subcellular localization will also be determined if immunofluorescence grade antibody against C17orf45 is produced that this will be direct evidence for protein coding and localization of C17orf45.

C17orf45 is in the candidate list that sagereveal bioinformatic tool [90] suggests as potential tumorigenesis related gene and its expression is altered in human tumors (Table 1.4.). So in order to support this hypothesis mRNA levels of C17orf45 was examined in various human brain tumors (Figure 5.8). mRNA level of *C17orf45* is up-regulated in all tumors except one DNET sample. Besides, two more meningioma samples were added to the study and three meningioma samples were compared with four normal samples according to their C17orf45 mRNA level. C17orf45 mRNA levels were increased significantly ($p < 0.05$, student's t-test) compared to the four normal samples. As a next step to investigate a potential link of *C17orf45* with tumorigenesis, the effect of *C17orf45* on cell growth of U373-MG cells were examined. Overexpression of C17orf45 inhibits the proliferation of U373-MG cells when growth curves of empty vector control sample and cells over-expressing C17orf45 were compared (Figure 5.10). Most of the Wnt/β-catenin targets can be considered as oncogene. But there are also tumor suppressor Wnt targets as well. *Axin2* [93] and Dickkopf-1 (*DKK1*) [99] are known to be tumor suppressors and negative regulators of Wnt signaling. Also *C17orf45* might be a negative regulator of Wnt pathway that it may have a role in a negative feedback mechanism upon the Wnt/β-catenin pathway activation. According to the proliferation assay and expression analysis in human brain tumor findings it is worth to expand the studies with techniques such as xenograft experiments on nude mice in

order to get *in vivo* evidence about a potential link of C17orf45 with tumorigenesis. Furthermore, over-expression studies bring many questions and doubts. Overexpression of a protein may allow unspecific events and protein level may reach toxic level. Hence, before the in *vivo* experiments it will be better to examine effect of siRNA or shRNA mediated C17orf45 knock-down on cell growth.

The *C17orf45* open reading frame is not conserved in vertebrates according to the UCSC Genome Browser multiple alignment (Figure 1.3). High percentage conservation is observed only in primates. Between human and non-primate vertebrates interestingly snoRNA coding regions are conserved but the exons are not. So we can argue that C17orf45 gene encodes both snoRNAs whose functions and related mechanisms probably are conserved in vertebrates and a protein whose function is probably primate specific. Because of being potential primate specific protein, we can speculate that *C17orf45* might be important for brain development. C17orf45 localized in mitochondria and overexpression inhibits the proliferation of U373-MG cells (Figure 5.10). Thus, it might be related with the intrinsic pathway in apoptosis that many important interaction and mechanisms exist in mitochondria. In mammals, the intrinsic pathway plays an important role in the formation of the central nervous system and brain development that during mammalian brain development both neurons and glia production is under the control of apoptosis [100–102].

To sum up, results of this study strongly suggest a gene that has not been studied before as a novel target of Wnt/β-catenin pathway that is potentially related with tumorigenesis. Therefore, we will expand our studies in order to investigate eventual roles of *C17orf45* in Wnt pathway and tumorigenesis.

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