MENA, BRI3 AND HSF2 AS NOVEL TRANSCRIPTIONAL TARGETS OF THE WNT/β-CATENIN PATHWAY

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To my family

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ABSTRACT

MENA, BRI3 AND HSF2 AS NOVEL TRANSCRIPTIONAL TARGETS OF THE WNT/β-CATENIN PATHWAY

Wnt/β-catenin signaling pathway plays important roles in embryonic development and carcinogenesis, which makes the pathway and its targets important subjects in developmental biology and cancer research fields. In its inactive form, when the Wnt ligand is not bound to the Frizzled receptor, β-catenin is constantly being phosphorylated by glycogen-synthase kinase 3β (GSK3β) and degraded. When the Wnt ligand binds the receptor, activated pathway leads to inhibition of GSK3β, allowing β-catenin to accumulate and translocate into nucleus, where it activates T-cell factor / lymphoid enhancer factor (Tcf/Lef) family of transcription factors, which regulate expression of developmental and cell cycle-related genes.

In this study, which was based on genome-wide differential transcriptome screens, we aimed to identify novel Wnt/β-catenin pathway targets and we focused on three genes, namely *MENA* (Mammalian enabled homologue), *BRI3* (Brain protein I3) and *HSF2* (Heat-shock factor 2), since their expression increases significantly upon β-catenin overexpression and because their promoters contain putative Tcf4-binding motifs. In order to test their candidacies of being novel targets of the pathway we employed lithium-treatment assay to inhibit GSK3β and mimic the pathway activation. We found that the expression of *MENA*, *BRI3* and *HSF2* increases upon lithium-treatment. Also, luciferase reporter assay and chromatin immunoprecipitation showed interaction of β-catenin and promoters of *MENA*, *BRI3* and *HSF2* genes. Finally, comparative RT-PCR analysis of cancer cell lines and human brain tumors was used to test a possible correlation between *MENA* and *CTNNB1* (β-catenin) expression. Our results strongly suggest that *MENA*, *BRI3* and *HSF2* transcription is regulated by the Wnt/β-catenin pathway.

ÖZET

MENA, BRI3 VE HSF2'NİN WNT/β-KATENIN YOLAĞININ YENİ HEDEF GENLERİ OLARAK TESPİTİ

Wnt/β-katenin yolağı embriyonik gelişimde ve kanser oluşumunda önemli roller oynadığından, bu yolağın elemanları ve hedef genleri, gelişim ve kanser biyolojisinde önemli çalışma alanları oluşturmaktadır. β-katenin bir transkripsiyon faktörü olup birçok kanserde düzensizdir. Wnt proteini Frizzled reseptörüne bağlanmaması durumunda, β-katenin sitoplazmada glikojen sintaz kinaz 3β (GSK3β) tarafından fosforile edilip degrede edilmektedir. Wnt proteinin reseptöre bağlanması halinde GSK3β inaktif hale getirilmekte ve β-kateninin sitoplazmada birikerek hücre çekirdeğine transfer edilip, embriyonik gelişim ve hücre devri ile alakalı çeşitli genlerin anlatımını başlatan T-hücre faktörü / lenfoid enhancer faktörü (Tcf/Lef) transkripsiyon faktörleri ailesini aktive etmektedir.

Genom-boyu karşılaştırmalı transkriptomiks çalışmalarına dayanan bu çalışmada, Wnt/β-katenin yolağının yeni hedef genlerin tespiti amaçlanmıştı. Bu bağlamda *MENA* (Mammalian enabled homologue), *BRI3* (Brain protein I3) ve *HSF2* (Heat-shock factor 2) genleri üzerinde yoğunlaştık. Bu genler, degrede-edilemeyen β-kateninin anlatımı sonrasında anlatımları artığından ve promotör bölgelerinde olası Tcf4-bağlanma sekansları bulundurmaları nedeniyle seçildi. *MENA*, *BRI3* ve *HSF2* genlerinin Wnt/β-katenin yolağı tarafından kontrol edilip edilmediğni tespit etmek için GSK3β'yi inhibe eden ve Wnt/β-katenin yolağının suni aktivasyonunu sağlayan lityum klorür tuzuna hücreler maruz bırakılarak *MENA*, *BRI3* ve *HSF2* genlerinin anlatımının arttığı gözlemlendi. Ayrıca, lusiferaz rapörtör analizi ve kromatin immünoçöktürme analizi ile β-katenin ile *MENA*, *BRI3* ve *HSF2* genlerinin promotör bölgeleri arasında bağlantı olduğu tespit edildi. Son olarak, karşılaştırmalı RT-PCR analizi ile altı farklı kanser hücre hattı ve insan beyin tümör örneği *MENA* ve β-katenin (*CTNNB1*) anlatımı için analiz edildi ve bir korelasyon gözlemlendi. Sonuçlarımız, *MENA*, *BRI3* ve *HSF2* genlerinin Wnt/β-katenin yolağının yeni hedef genleri olduğunu kanıtlamaktadır.

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LIST OF ABBREVIATIONS

APC	Adenomatosis polyposis coli
APS	Ammonium peroxodisulphate
bp	Base pair
BPB	Bromophenol blue
BRI3	Brain protein I3
$CaCl_2$	Calcium chloride
Cdk5	Cyclin-dependent kinase 5
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CK1α	Phosphorylated by casein kinase 1α
CMYA1	Cardiomyopathy associated 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNET	Dysembryoplastic neuroepithelial tumor
dNTP	Deoxyribonucleotide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
Fz	Frizzled
GSK3β	Glycogen synthase kinase 3β
HER2	Human epidermal growth factor receptor 2
hrs	Hours
HSF2	Heat-shock factor 2
IP	Immuno precipitation
kb	Kilobase
kDa	Kilodalton

LEF	Lymphoid enhancer factor
LiCl	Lithium chloride
LRP	Low density lipoprotein receptor-related protein
mAb	monoclonal antibody
MAPK	Mitogen-activated protein kinase
MENA	Mammalian enabled homologue
µg	Microgram
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
min	Minute
min	Minute
mL	Milliliter
µl	Microliter
µM	Micromolar
mm	Millimeter
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OD	Optical density
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Triton X-100
PCR	Polymerase Chain Reaction
PMSF	Phenyl methyl sulfonyl fluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse-transcriptase polymerase chain reaction
SAGE	Serial analysis of gene expression
SDS	Sodium Dodecyl Sulphate
TBE	Tris-Borate-EDTA (buffer)

TBE	Tcf4-binding elements
TCF	T-cell factor
TEMED	N,N,N’,N’,-tetramethylethylenediamine
UV	Ultraviolet

1. INTRODUCTION

1.1. Wnt/β-catenin Signaling Pathway

Wnt/β-catenin signaling pathway is critical for early and late embryonic development (Cadigan and Nusse, 1997; Peifer and Polakis, 2000), and it plays important roles in tumorigenesis of various cancers (Polakis, 2000). β-catenin is the central intracellular molecule of the pathway and its function is to transduce the signal into the nucleus, where it acts as a co-activator of Tcf/Lef family of transcription factors (Fig 1.1.) (Behrens *et al.*, 1996; Stambolic *et al.*, 1996).

When the Wnt signaling cascade is not stimulated (the "off" state, Fig 1.1), β-catenin is continuously translated, phosphorylated by casein kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK3β) and subsequently targeted for degradation via the ubiquitin-proteasome pathway, after it is ubiquitinated by βTrcp, an E3 ubiquitin ligase (Rubinfeld *et al.*, 1996; Yost *et al.*, 1996). Adenomatosis polyposis coli (APC) and axin are the scaffold factors that together with CK1α and GSK3β constitute an assembly of proteins called "destruction complex", which is responsible for targeting β-catenin for degradation. Being able to degrade β-catenin in the "off" state of the pathway is vital for the cell to remain in its wild-type phenotype, as loss-of-function mutations in the components of the destruction complex or mutations of β-catenin, which renders it degradation-resistant, lead to various types of cancers (Polakis, 2000).

In the "on" state of the Wnt/β-catenin signaling, when a Wnt glycoprotein ligand binds to a Frizzled receptor (Fz), GSK3β is inhibited as a consequence of a yet unclear mechanism that involves Dvl (dishevelled) protein and LRP5/6 co-receptor (Polakis, 2007). Inhibition of GSK3β leads to disruption of the destruction complex and thus, degradation of β-catenin is prevented, allowing its accumulation in the cytosol and translocation into the nucleus, where β-catenin activates Tcf/Lef family of transcriptional factors that control expression of cell cycle- and differentiation-related target genes (Behrens *et al.*, 1996; Stambolic *et al.*, 1996).

β-catenin can be stabilized by mutations of serine and threonine residues in the N-terminal region of the protein, which are phosphorylated by GSK3β (Behrens *et al.*, 1998; Hart *et al.*, 1998; Itoh *et al.*, 1998; Kishida *et al.*, 1998). Many researchers employ a substitution of Serine[33] to Tyrosine (S33Y), which is present in human colon carcinoma cell line SW48 (Morin *et al.*, 1997), to render the mutant β-catenin non-phoshorylatable by GSK3β and thus protected from being targeted to the 26S proteasome (Kolligs *et al.*, 1999). Another approach to stabilize β-catenin is to inhibit the GSK3β, either via activation of the Wnt pathway with a recombinant Wnt ligand or by treating cells with lithium chloride (Stambolic *et al.*, 1996). These artificial methods of β-catenin stabilization mimic the active status of the Wnt/β-catenin pathway and therefore are commonly used for identification of novel transcriptional targets of the pathway.

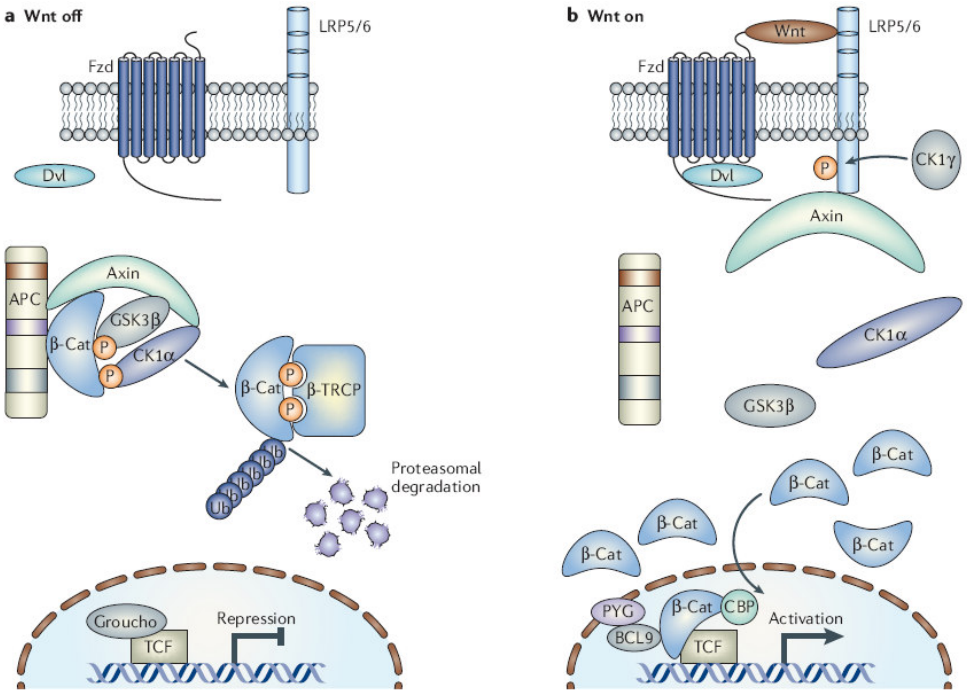

Figure 1.1. Wnt/β-catenin signaling cascade in its "off" (a) and "on" (b) states (adapted from Barker and Clevers, 2006). β-catenin is being ubiquitinated degraded when the pathway is inactive. Upon activation of the pathway, β-catenin accumulates in the cytosol, translocates into the nucleus and activates Tcf/Lef transcription factor family, enhancing expression of the target genes.

1.2. Importance of the Wnt/β-catenin Pathway Target Identification for Cancer Research

The degree of importance of the Wnt/β-catenin pathway in development and carcinogenesis becomes obvious when the plethora of naturally-occurring mutations and the wide range of phenotypes in which they result are considered. Table 1.1 presents a partial list of Wnt signaling component alterations and corresponding associated pathologies (or predispositions to diseases) (Luo *et al.*, 2007). Such a broad spectrum of diseases, in which the Wnt/β-catenin pathway is implicated, makes the pathway both interesting and important to study and offers high-impact fruition in cancer research.

Identification of novel targets of this pathway, serves an important purpose for the cancer research field and many other clinical research fields, since genes regulated by the pathway are potential drug and gene therapy targets (Barker and Clevers, 2006). Also, identification of novel transcriptional targets of Wnt/β-catenin pathway improves our understanding of mechanisms through which the pathway contributes to embryonic development, neoplastic transformation and pathogenesis of diseases, such as osteoarthritis, diabetes and schizophrenia (Table 1.1) (Luo *et al.*, 2007). Knowledge of transcriptional targets of the Wnt/β-catenin pathway, in a certain cancer context, sheds light on the different carcinogenesis mechanisms, better understanding of which increases the chances of successful cancer therapies. In addition, each cancer can be said to have a certain pattern of Wnt/β-catenin pathway targets differentially expressed at any given stage, giving it a "signature". This signature can be recognized and employed for diagnostic purposes, in order to assess the stage of a developing cancer or tumor (Mimeault and Batra, 2006; Pishvaian and Byers, 2007).

In summary, a vast number of diseases in which the pathway is implicated, possibility for drug therapies, prospect for better understanding of carcinogenesis, potential use in cancer diagnostics and other biological and medical reasons, make identification of novel Wnt/β-catenin pathway targets an important research field with-high impact contributions to both basic and clinical research. In order to identify such targets, transcriptome-profile analyses were performed in our laboratory on stable Huh7 (a hepatocellular carcinoma) cell lines

Table 1.1. Alterations in Wnt signaling and associated diseases / phenotypes (adapted from Luo *et al.*, 2007)

Gene	Alteration	Associated diseases / phenotypes
APC	Loss of function	Familial adenomatosis polyposis (FAP), >80 per cent sporadic colorectal cancer (CRC)
Axin1	Loss of function	Hepatocellular carcinoma (HCC), sporadic medulloblastomas, CRC, esophageal squamous
Axin2	Loss of function	Familial tooth agenesis; predisposed to colorectal cancer
β-catenin	Oncogenic mutation	CRC, HCC, melanoma, endometrial cancers, prostate cancer; bone/cartilage phenotypes
β-catenin	Reduced activity	Alzheimer's disease
β-catenin	Increased activity	Dupuytren skin disease
Dkk1	Overexpression	Increased osteolytic metastasis of multiple myeloma
Dvl1	Loss of function	Myocardial infarction
FRP3	Reduced activity	Higher incidence of osteoarthritis in females
Fz2	Increased expression	Cardiac hypertrophy
Fz3	Polymorphic SNPs	Susceptibility to schizophrenia
GSK3b	Altered activity	Schizophrenia
LRP5	Loss of function	OPPG (osteoporosis pseudoglioma syndrome)
LRP5	Gain of function	A high bone mass phenotype
LRP5 or Fz4	Loss of function	Familial exudative vitreoretinopathy (FEVR)
Tcf4	Transcript variants	Risk of type II diabetes (T2D)
Wnt 1	Increased expression	Schizophrenia
Wnt 10B	Loss of function	Associated with early-onset obesity, decreased bone mass
Wnt 3	Homozygous mutation	Tetra-amelia phenotype
Wnt 4	Gene duplication	Mullerian-duct regression and virilization, intersex phenotype

overexpressing degradation-resistant β-catenin. Both SAGE (Serial Analysis of Gene Expression) and genome-wide microarray screens were used to analyze differential expression of potential target candidates. In this work we focused on three of these candidates: *MENA*, *BRI3* and *HSF2*.

1.3. *MENA* (Mammalian homologue of Enabled) and Its Role in Actin Remodelling and Carcinogenesis as a Novel Wnt/β-catenin Pathway Target Gene

Mena is an actin-associated protein that was found to be overexpressed in ~75 per cent of primary breast cancers (Di Modugno *et al.*, 2004). We found *MENA* expression to be upregulated upon β-catenin overexpression in Huh7 cell line, in a SAGE screen performed in our laboratory (Kavak et al, unpublished data). The SAGE screen data, presence of two putative Tcf4-binding sites in the promoters of human, macaque, mouse and rat *MENA* homologues (see Results section), the well-established link between Wnt/β-catenin pathway and breast cancer (reviewed below) and the potential importance of *MENA* to the cancer research field were considered, and the hypothesis that *MENA* is a novel Wnt/β-catenin pathway target was pursued in this study.

It is interesting to note that mouse mammary tumor virus induces breast carcinomas by insertion near the Wnt1 gene, which leads to its overexpression (Nusse and Varmus, 1982). Importantly, the Wnt1 gene is known to induce the Wnt/β-catenin pathway and stabilize β-catenin (Hinck *et al.*, 1994). Moreover, transgenic mice expressing stabilized β-catenin in mammary gland tissue were shown to develop breast tumors (Imbert *et al.*, 2001; Michaelson and Leder, 2001). According to immunohistochemical analysis, nuclear and/or cytoplasmic β-catenin levels have been found to be elevated in about 60 per cent of the breast tumors (Lin *et al.*, 2000; Ryo *et al.*, 2001). Furthermore, reduced levels of extracellular Wnt-inhibitory molecules sFRP1 and WIF1 have been linked to 80 per cent and 60 per cent of breast carcinomas (Ugolini *et al.*, 2001; Wissmann *et al.*, 2003). Additionally, β-catenin has been associated with epidermal growth factor receptor (EGFR) family members (Hoschuetzky *et al.*, 1994; Shibamoto *et al.*, 1994; Kanai *et al.*, 1995; Adam *et al.*, 2001; Schroeder *et al.*, 2002) and the stability of β-catenin and its Tcf/Lef-

activating function has been suggested to be regulated via tyrosine phosphorylation by the EGFR family (Playford *et al.*, 2000; Adam *et al.*, 2001; Danilkovitch-Miagkova *et al.*, 2001; Kim and Lee, 2001), which may be significant for the breast carcinogenesis, since human epidermal growth factor receptor 2 (HER2) is overexpressed in about 30 per cent of human breast tumors (Arteaga, 2002; Normanno *et al.*, 2003). These *in vitro* and *in vivo* evidences implicate Wnt/β-catenin pathway in breast carcinogenesis at different levels/phases of the signaling cascade.

Many actin-associated proteins play important roles in carcinogenesis of various types of cancers (Pawlak and Helfman, 2001). *MENA* is an actin-regulatory protein that belongs to Ena/VASP protein family (*MENA*, VASP and Evl (Kwiatkowski *et al.*, 2003)). This protein family localizes to tips of protruding lamellipodia and filopodia and adhesion foci; and they are involved in control of cell motility and cell-cell adhesion, which are important subjects for development of metastatic potential (Kwiatkowski *et al.*, 2003). *MENA* was shown to interact with the following proteins: 1) Abi-1, which promotes c-Abl-mediated phosphorylation of *MENA* at Tyr-296 (Tani *et al.*, 2003); 2) zyxin, a well-known component of focal adhesions (Drees *et al.*, 2000); 3) TES, a putative tumor-suppressor gene that is also localized to focal adhesions (Coutts *et al.*, 2003); 4) profilin, an actin-binding protein that regulates microfilament polymerization (Lanier *et al.*, 1999); 5) IRSp53, a Rac-binding protein, together with which *MENA* promotes filopodia formation in fibroblasts (Krugmann *et al.*, 2001); and 6) Xin, a Cardiomyopathy associated 1 (CMYA1) gene product, that directly binds to F-actin and plays role in heart morphogenesis, early myofibrillogenesis and maintenance of striated muscle integrity (van der Ven *et al.*, 2006).

Di Modugno *et al*. (2004) showed that human *MENA* (*hMENA*) is overexpressed in ~75 per cent of primary breast cancers. In a recent report by the same group (Di Modugno *et al.*, 2007), *hMENA* was shown to have two splicing isoforms that differ in length by a single exon (exon 11), or 21 amino acids. Overexpression of shorter isoform (hMena) is present in various types of cancer cell lines, whereas the longer isoform ($hMena^{+11a}$) was found to be expressed exclusively in cancer cell lines of epithelial origin. In breast cancer cell lines, epidermal growth factor (EGF) treatment was found to increase of expression of both isoforms. Also, ectopic overexpression of $hMena^{+11a}$ in breast cancer cell lines, led to

increase in p42/44 (p38) mitogen-activated protein kinase (MAPK) activation. On the other hand, *hMENA* knockdown led to reduced p42/44 MAPK activation in response to EGF treatment. In another report, neuregulin-1 treatment was shown to upregulate, while herceptin treatment was found to downregulate *hMENA* expression (Di Modugno *et al.*, 2006).

Since Wnt/β-catenin pathway is obviously implicated in breast carcinogenesis and since *MENA* is overexpressed in ~75 per cent of breast tumors, together with our SAGE data showing that β-catenin overexpression leads to increase in *MENA* transcription, these results suggest a potential link between Wnt/β-catenin pathway, breast cancer and *MENA*, and imply that *MENA* may be a Wnt/β-catenin pathway target candidate.

In addition to its supposed role in carcinogenesis, *MENA* was found to be important in nervous system development. *MENA*-null mice display subtle deficiencies in forebrain commissure formation (Menzies *et al.*, 2004). Also, in a recent gene-wide screen, *MENA* was found to be significantly associated with schizophrenia (Kahler *et al.*, 2008). It is intriguing that Wnt1, Fz3 and GSK3β - major Wnt/β-catenin pathway signaling components - have also been associated with schizophrenia or susceptibility to schizophrenia (reviewed in Luo *et al.*, 2007).

1.4. *BRI3* (Brain protein I3) and *HSF2* (Heat-shock factor 2) as Two Novel Wnt/β-catenin Pathway Target Genes with Putative Roles in Brain Development

Two other potential Wnt/β-catenin pathway targets selected from the SAGE screen described above (and an equivalent microarray screen) were *BRI3* (Brain protein I3) and *HSF2* (Heat-shock factor 2) (Kavak *et al.*, unpublished data). These genes were selected by Erşen Kavak, based on their expression changes observed in the screens, presence of Tcf4-binding elements in their promoters, the degree of characterization of their functions and their potential importance to the field.

BRI3 is a member of a new family of brain integral membrane proteins, which has at least three members (*BRI1*, *BRI2* and *BRI3*) (Vidal *et al.*, 1999; Vidal *et al.*, 2000; Vidal *et*

al., 2001). Mutations in *BRI2* were associated with a neurodegenerative disease similar to Alzheimer's disease (Vidal *et al.*, 2000). Whereas *BRI2* was found to be expressed in majority of tissues, the expression of *BRI3* was detected principally in brain as judged by Northern blotting, with comparably very low expression in other tissues (Vidal *et al.*, 2001). Thus, *BRI3* is referred to as the "brain-specific BRI family member" (Gong *et al.*, 2008). *BRI3* was found to interact physically with BACE1 (β-amyloid protein converting enzyme) (Wickham *et al.*, 2005) and SCG10 (superior cervical ganglia, neural specific 10) and block the latter's microtubule disassembly-inducing activity, stabilizing the microtubules and diminishing NGF-induced neurite outgrowth in PC12 cells (Gong *et al.*, 2008).

HSF2 is a DNA-binding transcription factor that belongs to the same family as the well-known HSF1 – the major heat-response transcription factor (Shamovsky and Nudler, 2008). However, unlike HSF1, HSF2 activity is not induced by heat-shock, but rather abolished by it, even though both HSF1 and HSF2 bind to the same consensus sequence of heat-shock element (HSE) (Loones *et al.*, 1997). HSF2 has been associated with the promoter of hsp70, although the promoter lacks any HSE (Sarge *et al.*, 1994). During mouse development, *HSF2* is expressed at significant levels, but its expression in the brain persists longer (until E15.5) than in other tissues. HSF2 has two alternative splicing isoforms HSF2α and HSF2β, which are differentially expressed in various tissues. (Loones *et al.*, 1997).

Moreover, *HSF2*-null mice showed either embryonic lethal phenotype likely due to central nervous system problems, such as collapse of the lateral ventricles and ventricular haemorrhages, or displayed major brain abnormalities such as dramatic dilation of ventricles, in addition to gametogenesis defects (Wang *et al.*, 2003). In addition, p35, an activator of cyclin-dependent kinase 5 (Cdk5) – a kinase vital for radial migration of neurons – was found to be a transcriptional target of HSF2 (Chang *et al.*, 2006).

Wnt signaling pathway has been associated with brain development and neurological disorders for more than a decade (De Ferrari and Moon, 2006; Malaterre *et al.*, 2007) and given putative roles of *BRI3* and *HSF2* in brain development and our observation that β-catenin overexpression in Huh7 cells leads to increase in mRNA levels of these genes

(Kavak *et al.*, unpublished data), we pursued the hypothesis that *BRI3* and *HSF2* are novel Wnt/β-catenin pathway targets.

2. PURPOSE

The aim of this study was to pursue the hypotheses that *MENA* (Mammalian homologue of Enabled), *BRI3* (Brain protein I3) and *HSF2* (Heat-shock factor 2) are transcriptional targets of the Wnt/β-catenin pathway.

The hypotheses arose from genome-wide microarray and SAGE screens performed in our laboratory. The aforementioned Wnt/β-catenin pathway target candidates were picked from these screens, based on their expression changes upon β-catenin overexpression and the presence of putative Tcf4-binding sites in their promoters, in addition to other factors mentioned in the Introduction part.

In order to obtain *in vitro* and *in vivo* evidence supporting hypothesis that *MENA*, *BRI3* and *HSF2* are transcriptional targets of the Wnt/β-catenin pathway, the following experimental approaches were used. A degradation-resistant β-catenin mutant was overexpressed in various cell lines and changes in the target candidate mRNA levels were assayed using RT-PCR. Also, GSK3β inhibition using LiCl was performed as a standard assay to stimulate Wnt/β-catenin pathway and again RT-PCR was used to assay the target candidate expression changes. Furthermore, the promoter regions of the target candidate genes were cloned and their activity in response to overexpression of β-catenin, dominant-negative Tcf4 and Wnt ligands were analyzed using luciferase reporter assay. Finally, chromatin immunoprecipitation using anti-β-catenin antibody was performed from several cell lines and mouse tissues, in order to establish a physical interaction between β-catenin and the promoters of the target candidates.

3. MATERIALS

3.1. Electrophoresis and Western Blotting Buffers and Solutions

50X Tris-acetic acid EDTA (TAE)	2M Tris-acetate 50mM ethylenediaminetetraacetic acid pH 8.5
TE Buffer	10mM Tris–HCl 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 BromophenolBlue (BPB) 6 ml 100 per cent glycerol 1.2 ml 0.5M EDTA
10 per cent SDS-PAGE gel (running gel)	10 per cent Acrylamide : bisacrylamide (37.5:1) 375mM Tris-HCl (pH 8.8) 0.1 per cent SDS

	0.1 per cent Ammonium persulfate (APS) 0.1 per cent N,N,N',N',-tetramethylethylenediamine (TEMED)
5 per cent SDS- PAGE (stacking gel)	5 per cent Acrylamide : bisacrylamide (37.5:1) 125mM Tris-HCl (pH 6.8) 0.1 per cent SDS 0.1 per cent APS 0.1 per cent TEMED
1X SDS-PAGE dye	2 per cent SDS 80mM Tris-HCl (pH 6.8) 20 per cent Glycerol 10 per cent β-mercaptoethanol 0.005 per cent Bromophenol blue
Running Buffer	25mM Tris 192mM Glycine 0.1 per cent SDS
Transfer Buffer	200mM Glycine 25mM Tris 10 per cent Methanol
Coomassie Blue Fixing-Staining Solution	50 per cent Methanol 0.05 per cent Coomassie R250 10 per cent Acetic acid
Coomassie Blue Destaining Solution	5 per cent Methanol 7 per cent Acetic acid

Ponceau S Solution Staining Solution	0.5 per cent Ponceau S 1 per cent Acetic acid
Tris Buffered Saline with Tween 20 (TBST)	150mM NaCl 20mM Tris-HCl (pH 8.0) 0.1 per cent Tween 20
Blocking Solution TBST	5 per cent non-fat milk powder in
Stripping Solution	62.5mM Tris-HCl, pH 6.8 2 per cent SDS 0.7 per cent β-mercaptoethanol

3.2. Bacterial Culture Buffers and Solutions

Luria-Bertani medium (LB)	10 g tryptophan 5 g yeast extract 10 g NaCl Distilled water up to 1 L, autoclaved
Luria-Bertani Agar	10 g tryptophan 5 g yeast extract 5 g NaCl 15 g Agar Distilled water up to 1 L, autoclaved
Ampicillin stock	100 mg/ml in 50 per cent ethanol Sterilized by filtration and stored at -20°C. 100 μg/ml (working concentration)
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 $MgSO_4$ 20 ml of 1M glucose Distilled water up to 1L Sterilized by filtration and stored at -20°C

3.3. Chromatin Immunoprecipitation Assay Buffer and Solutions

SDS Lysis Buffer	1 per cent SDS 10mM EDTA 50mM Tris-HCl, pH 8.1
ChIP Dilution Buffer	0.01 per cent SDS 1.1 per cent Triton X-100 1.2mM EDTA 16.7mM Tris–HCl, pH 8.1 167mM NaCl
Elution Buffer	1 per cent SDS 0.1M NaHCO3
Low Salt Buffer	0.1 per cent SDS 1 per cent Triton X-100 2mM EDTA 20mM Tris–HCl, pH 8.1 150mM NaCl

Low Salt Buffer	0.1 per cent SDS 1 per cent Triton X-100 2mM EDTA 20mM Tris–HCl, pH 8.1 500 mM NaCl
LiCl Buffer	0.25M LiCl 1 per cent NP-40 1mM EDTA 10mM Tris–HCl, pH 8.1

3.4. Immunofluorescence Buffer and Solutions

10X PBS	81.8 g NaCl 2 g KCl 14.2 g Na_2HPO_4 2.45 g KH_2PO_4 Up to 1L with distilled water.
1X PBST	1X PBS 0.5 per cent Triton X-100
20 per cent Paraformaldehyde stock	10 g paraformaldehyde 50 µl 10N NaOH Up to 50ml with distilled water. Heat to 65°C to dissolve. Store at -20°C
4 per cent Paraformaldehyde in PBS	10 ml 20 per cent paraformaldehyde 5 ml 10X PBS 35 ml distilled water

	Filter and store at 4°C for up to 2 weeks.
Blocking solution: 3 per cent BSA in PBS	1.5 g Bovine Serum Albumin 5ml 10X PBS Up to 50ml with distilled water.
Cell Permeabilization Solution	1X PBS 0.5 per cent Triton X-100

3.5. Enzymes

Taq Polymerase was purchased from Fermentas (Burlington, Canada) together with $MgCl_2$ (25mM) and the 10X reaction buffer. For cloning purposes, Expand Long Template PCR System, which contains a high-fidelity DNA polymerase Tgo, was purchased from Roche Applied Biosciences (Indianapolis, IN, USA). Restriction enzymes, Calf Intestinal Alkaline Phosphatase and T4 DNA ligase were purchased from Promega (Madison, WI, USA).

3.6. Antibodies

Antibodies used during the course of this thesis are tabulated in Table 3.1

Table 3.1. Monoclonal (mAb) and polyclonal (pAb) antibodies employed.

Name	Type	Host	Catalog #	Company
anti-cytosolic b-catenin	mAb	mouse	44206B	Invitrogen
anti-nuclear b-catenin	mAb	mouse	44207B	Invitrogen
pan-actin antibody	pAb	rabbit	4968	Cell Signaling
anti-mouse/HRP	pAb	sheep	NA931	Amersham
anti-rabbit/HRP	pAb	donkey	NA934	Amersham
anti-mouse/AlexaFluor®488	pAb	goat	A-11001	Invitrogen

3.7. Western Reagents

Hybond-P nitrocellulose membrane and ECL Plus Western Blotting Detection Reagents was from Amersham Biosciences (Uppsala, Sweden). Kaleidoscope pre-stained molecular weight marker was purchased from BioRad (Hercules, CA, USA).

3.8. Nucleic Acids

DNA molecular weight markers and deoxyribonucleotides were purchased from Fermentas (Burlington, Canada). CS2+/β-catenin-4m (S33A, S37A, T41A, S45A quadruple-mutant) plasmid and LNCX-Wnt1, LNCX-Wnt3a, LNCX-Wnt4, and LNCX-Wnt5a plasmids were kindly provided by Dr. Xi He, Harvard Medical School. pcDNA3-S33Y-β-catenin mutant was kindly provided by Dr. Mehmet Öztürk, Bilkent University. Primers used in polymerase chain reaction and sequencing were purchased from Harvard University MGH Sequencing Core (Boston, USA).

3.9. Bacterial Strains and Mammalian Cell lines

E. coli bacterial strain TOP10 (genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG) was routinely used for cloning purposes. This strain was propagated in Streptomycin-containing media (25 μg/ml).

The following cell lines were used during the course of this study: Huh7 (human hepatocellular carcinoma; kindly provided by Dr. Mehmet Öztürk), U373MG (human astrocytoma kindly provided by Dr. P.-O. Couraud), 293T (T-antigen transformed human embryonic kidney cells; kindly provided by Dr. Nesrin Özören), Sk-Mel-19, Sk-Mel-103, Sk-Mel-147 and MeWo (human melanoma cell lines; kindly provided by Dr. Nesrin Özören).

3.10. General Chemicals and Kits

Chemicals were either molecular biology or electrophoresis 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 reagent, Exgen 500 was purchased from Fermentas (Burlington, Canada). QIAprep Spin Miniprep Kit and Qiagen Plasmid Midi Kit, for Plasmid Purification, and RNeasy Mini Kit, for RNA Purification, were from Qiagen (Hilden, Germany). Dual-glo Luciferase assay kit were and ImPromII Reverse Transcription System was from Promega (Madison, WI, USA).

3.11. General Equipment

Autoclave	Midas 55, PriorClave, UK
Balances	DTBH 210, Sartorius, GERMANY
Carbon dioxide tank	2091, Habaş, TURKEY
Cell culture incubator	Hepa Class 100, Thermo, USA
Centrifuges	Ultracentrifuge J2MC, BeckmanCoulter, USA
	Mini Centrifuge 17307-05, Cole Parmer, USA
	Centrifuge 5415R, Eppendorf, USA
	Centrifuge, Allegra X-22, Beckman Coulter, USA
Freezers	-20°C, Arçelik, TURKEY
	-86°C ULT Freezer, ThermoForma, USA
Documentation System	Gel Doc XR System, Bio-Doc, ITALY
Electrophoresis Systems	Mini-sub Cell GT, BioRad, USA
	Mini-Protean III Cell, BioRad, USA
Heat-blocks	DRI-Block DB-2A, Techne, 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
Microscope	Inverted Microscope, Observer.Z1, Zeiss, GERMANY
Microwave oven	M1733N, Samsung, MALAYSIA
pH meter	WTW, GERMANY
Pipettor	Pipetus-akku, Hirscmann Labogerate, GERMANY
Power Supply	Biorad, USA
Refrigerators	2082C, Arçelik, TURKEY
Shakers	VIB Orbital Shaker, InterMed, DENMARK
	Lab-Line Universal Oscillating Shaker, USA
Software	Quantity One, Bio-Rad, ITALY
	Scion Image Analysis software (Scion Corporation)
Spectrophotometer	NanoDrop-1000, USA
Thermocyclers	GeneAmp. 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 *E. coli* and Transformation

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. After 16 hrs, 25ml LB was inoculated with 250µl of the overnight culture. Cells were grown until OD595 reached 0.4-0.6. Cells were centrifuged at 4000×g for 10 min at 4°C. Pellet was resuspended in 12.5ml of ice-cold sterile 50mM $CaCl_2$ and incubated on ice for 30 min. Cells were centrifuged again (4000×g for 10 min at 4°C) and 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.

For transformation, a vial (100 µl) of the chemically competent cells was thawed on ice for 15 min and 1µl of 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 and the cells were incubated for 1 hr at 37°C, while shaking at 200rpm. 100µl of the cell suspension was then spread on antibiotic-containing plates and cells were grown overnight at 37°C, in an inverted position.

4.2. Plasmid Purification

All plasmid purifications were done using Qiagen's QIAprep Spin Miniprep Kit and Plasmid Midi Kit, according to the manufacturer's protocol. Concentrations and qualities of plasmids were checked by spectrophotometric measurements using a NanoDrop-1000 spectrophotometer. OD260/280 ratio was 1.8-2.0. Only midi preparations of plasmids were used for transfections.

4.3. Cell Culture Techniques

4.3.1. Cell growth and handling

HCC derived cell line Huh7 was grown in DMEM containing 10 per cent FBS and 1 per cent penicillin/streptomycin (complete DMEM) in an incubator at 37°C, with 5 per cent CO2 and 95 per cent air. Media were kept at 4°C and warmed to 37°C in a water bath before use. Containers were wiped with 70 per cent ethanol prior to use. Cells were routinely passaged before reaching ~90 per cent confluence. After aspiration of growth medium and wash with calcium and magnesium-free PBS, cells were treated with trypsin-EDTA solution (per cent 0.025 trypsin, 0.5mM EDTA) at 37°C for 5min. 5 volumes of fresh medium were added to inactivate trypsin and the suspension was pipetted with a 10ml serological pipette to disperse the cells. The cells were transferred to fresh Petri dishes in a 1:10 ratio for routine passaging.

4.3.2. Freezing and thawing

Cells were frozen in 10 per cent DMSO-containing complete DMEM at about 1 million of cells per 1ml density. Tubes were put into a Styrofoam rack and the rack was enclosed in a Styrofoam box, which was kept in a -80°C freezer for 16hrs for slow-cryopreservation. For long-term storage (up to 1 year), cells were kept in liquid nitrogen tanks. Thawing was done by quickly immersing a tube taken out of a -80°C freezer into 37C water-bath and seeding cells into a 100mm culture dish with 10ml complete DMEM after about 1min.

4.3.3. Transfection

Transfections were done using Exgen500^{TM} (Fermentas) according to ratios DNA:reagent ratios suggested by the manufacturer. Confirmation of successful transfection was done by transient transfection of pEGFP-N2 plasmid.

4.4. RNA Isolation and Reverse Transcription

Qiagen's RNeasy Mini Spin Kit was used according to manufacturer's protocol for all routine RNA isolations during the course of this study. cDNA synthesis was performed with ImProm-II™ Reverse Transcription System kit according to manufacturer's protocol. 1μg total RNA was used as starting material.

4.5. Primers and Polymerase Chain Reaction

Primer design was done using Invitrogen's OligoPerfect™ Designer online primer design software (http://tools.invitrogen.com/content.cfm?pageid=9716). For cloning purposes, appropriate restriction endonuclease sites were designed at the 5' ends of the primers and either "TAA" or "CAT" sequences were added to the 5' ends as "spacers" for restriction enzyme binding. For detection (ChIP or RT-PCR) experiments, the best primer pair suggested by the software was used, considering the match of the Tm values of the primers, the range of Tm values (50-80°C), GC-richness range (50-60 per cent) and primer length range (18-30). Primers used for this study are given in Table 4.1.

For semi-quantitative RT-PCR the following reaction composition and cycling conditions were used. 1X Taq Buffer, 1.5-2mM $MgCl_2$, 0.2-0.25mM dNTP, 5 per cent DMSO, 0.4 μM of each primer, 0.05u/μl Taq DNA polymerase (Fermentas); 94°C 5min, 25 cycles [94°C 30sec, 55°C 30sec, 72°C 30sec], 72°C 5min; Hot-start at 94°C.

For ChIP PCR the following reaction composition and cycling conditions were used. 1X Taq Buffer, 2.5mM $MgCl_2$, 0.2mM dNTP, 0.4 μM of each primer, 0.05u/μl Taq DNA polymerase (Fermentas); 95°C 5min, 40 cycles [94°C 30sec, 52°C 30sec, 72°C 30sec], 72°C 5min; Hot-start at 95°C.

4.6. Plasmids and Molecular Cloning

Standard cloning procedures were used for cloning promoter regions of target candidate genes into promoterless luciferase reporter vector pGL3-basic (Promega). pRL-TK (Promega), a thymidine kinase promoter-driven Renilla luciferase mammalian expression vector, was used an internal control for luciferase assays.

Table 4.1. Sequences, target amplicon sizes, restriction enzyme (RE) sites and applications (applic.) for primers used in this study.

Primer Name	Primer Sequence	Size (bp)	Applic.	RE Sites
MENA.F.1	gctggcacccacttcttatt	108	RT-PCR	-
MENA.R.1	ctggtggggaagcctctg			-
MENA.clngF.9	atgagtgaacagagtatctgtcag	1776	Cloning	-
MENA.clngR.10	ctatgcagtatttgacttgctcag			-
MENA.F.prmtr	aagctagcgggatgaagtcatccctatacctagtgtgc	1256	Cloning	NheI
MENA.R.prmtr	aaaaagcttcatggtgccggcggc			HindIII
BRI3.pr.1F	taagctagcaacccgtgaagccttgtatg	660	Cloning	NheI
BRI3.pr.2R	cataagcttgccgctgctctctagacct			HindIII
HSF2.pr.3FNhe	taagctagcctgagaatttcaagaggatgag	1714	Cloning	NheI
HSF2.pr.2RXho	taactcgagggagaccgtgtttggatgtt			XhoI
MENA.mus.ChIP.F	gctgacagggctgttttctc	447	ChIP	-
MENA.mus.ChIP.R	ccaaaatggaggacctagca			-
*HSF2*FmusChIP	atctgctcacaagcagctca	299	ChIP	-
*HSF2*RmusChIP	cggatgcactacagagcaaa			-
*BRI3*FmusChIP	gctgcagaatctgtcaacca	284	ChIP	-
*BRI3*RmusChIP	gggtgttgtcctcgttcagt			-

4.7. Agarose Gel Electrophoresis

DNA fragments were resolved on standard 1X TAE-based agarose gel slabs (1.0 per cent to 2 per cent). Agarose was boiled in 1X TAE Buffer in a microwave oven and ethidium bromide was added to final concentration of 0.05 μg/ml, after cooling the solution for 10 min at room temperature. Gels were run in 1X TAE Buffer at constant voltage, until desired resolution of the bands to be analyzed.

4.8. SDS-PAGE Electrophoresis and Western Blotting

SDS-PAGE gels were cast, run and transferred using Mini-Protean III cell and Mini Trans-blot cell (BioRad). Routinely, 10 per cent running gels (with 37.5:1, acrylamide:bis-acrylamide ratio) were cast, water was added on top of the gels, and after 30min of polymerization, a 5 per cent stacker gel (with the same crosslinker ratio) was cast. Gels were run after 1hr of stacker gel polymerization. Samples were prepared in 1X SDS-PAGE dye. 5 µl of Kaleidoscope pre-stained marker (BioRad) was used as a molecular weight estimate. Samples were heated at 95°C for 10min, vortexed, flash-centrifuged and loaded into the wells formed by the stacker gel. Two gels in a single Mini-Protean III cell were ran at 60-70V until the BPB front entered the running gel and then the voltage was increased up to 100V. Runs were stopped when the BPB front left the running gel. Proteins were transferred onto nitrocellulose membranes for 1hr at 100V. Ice-block supplied with the Trans-blot cell was used routinely. Transfer efficiency was verified by staining the blots with Ponceau S staining solution and TBST was used for destaining. Blots were then blocked in 5 per cent non-fat milk, for 1hr at room temperature, while shaking at about 100rpm. Incubations with primary and secondary antibodies were done by diluting them to manufacturer-suggested concentrations and incubating for 1hr at room temperature, while shaking at about 100rpm. Washes after antibody incubations were done with TBST, thrice, 5min each wash, while shaking at about 120rpm. ECL Plus Western Blotting Detection Reagents (Amersham) were used to develop the blots. Blots were either exposed to X-ray film (Amersham) or analyzed using Stella Imaging Station (Raytest) and Xstella image acquisition software (Raytest), according to manufacturer's manual. Images were quantified using Scion Image Analysis software (Scion Corporation).

4.9. Chromatin Immunoprecipitation Assay

The procedure was adapted from a previously published protocol (Nelson *et al.*, 2006). Huh7 cells in 150mm plates were crosslinked by adding 0.8mL of 37 per cent formaldehyde into 20mL medium and incubating at 25°C for 15min. To quench the crosslinking, 1.1ml of 2.5M glycine were added and incubated at 25°C for 5 min. Cells were harvested by scraping and centrifuged at 1000×g for 5min at 4°C. The cell pellet was

washed 2 times with 10mL ice-cold PBS + 1mM PMSF. The cell pellet was resuspended in 2.5 ml of ice-cold IP buffer (150mM NaCl, 5mM EDTA, 1 per cent Triton X-100, 0.5 per cent NP-40, 50mM Tris-HCl (pH 7.5) and 0.5mM DTT) containing protease inhibitor cocktail (Sigma) and an extra 1mM of PMSF. The cells were sonicated three times for 30 seconds at power setting 5 with 50 per cent power efficiency. The debris was removed by centrifugation at 14,000×g for 10min at 4°C. 100µl of the supernatant was saved as "input". The rest of the supernatant was pre-cleared by incubating with 80µL of 50 per cent slurry of Protein A-agarose with salmon sperm DNA (10µg/mL) for 30min at 4°C with gentle agitation. The supernatant (~2mL) was incubated with anti-β-catenin monoclonal antibody or a control antibody (5-10 µg), overnight at 4°C. Then, 80µL of Protein A-agarose beads with salmon sperm DNA (10µg/mL) were added and incubated at 4°C for 2 hrs with gentle agitation. The beads were then washed sequentially with 1ml of the following buffers for 5 min at room temperature with mild rocking: (1) Low Salt Buffer; (2) LiCl Buffer; and (3) TE Buffer. DNA was eluted twice by adding 250µL of Elution Buffer and incubating at room temperature for 15min with vortexing every 5min. The two 250µL elutions were then combined, 20µL of 5M NaCl were added and incubated at 65°C overnight to reverse the crosslinking. Next, 10µL of 0.5M EDTA, 20µL of 1M Tris-HCl, pH 6.5 and 2µL of 10mg/ml proteinase K were added and the mixture was incubated for 1hr at 45°C. DNA was extracted using Qiagen's PCR purification kit. PCR was done using a primer pair amplifying promoter regions of the target genes.

For ChIP from mouse tissues, the tissue samples were first chopped into small pieces and then crosslinked (10 µL of 1 per cent formaldehyde to 1 mg tissue) at 37°C for 10min, with shaking at 200rpm. Next, SDS Lysis Buffer was added (10 µL per 1 mg tissue) and samples were incubated on ice for 10 min. The tissues were sonicated 10 times for 10-15 seconds at power setting 3 with 50 per cent power efficiency. The rest of the ChIP procedure was that of the ChIP done for cells.

4.10. Luciferase Assay

Luciferase assays were performed according to Levy *et al.* (2002) with some modifications. Briefly, 0.5 µg (per well of a 12-well plate) of a pGL3-promoter plasmid were co-transfected with 25ng of pRL-TK (internal control, Renilla luciferase) and 0.9 µg

of either an empty CS2+ vector or CS2+/β-catenin-4m plasmid into Huh7, HeLa or HEK293T cells by using Exgen500 reagent (Fermentas), following the manufacturer's instructions. Cells were harvested 48hrs post-transfection using 200 µl (per well of a 12-well plate) 1X PLB (Passive Lysis Buffer) provided by the Dual-Glo™ Luciferase Assay System (Promega). The lysates were vortexed and spun at 16,000×g for 5min at 4°C, to pellet the cell debris. 100 µl 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 Fluoroskan Ascent FL (Thermo Electron). Next, 100 µl of Renilla luciferase substrate reagent (Stop&Glo™) 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 1-5 seconds. Firefly luciferase reads were normalized to Renilla luciferase reads and graphs were plotted in Microsoft Excel.

4.11. Immunofluorescence Methods

For immunostaining procedures, cells were grown on glass coverslips, washed twice with PBS, fixed in 4 per cent paraformaldehyde in PBS (freshly diluted from frozen 20 per cent stock) or 3.7 per cent formaldehyde in PBS for 15min at 37°C (no shaking), rinsed twice with PBS and permeabilized with 0.5 per cent Triton X-100 for 15min (mild shaking). Cells were then incubated in 3 per cent bovine serum albumin in PBS for 30min at 37°C (no shaking), incubated with the primary antibody (dilutions recommended by the manufacturer) for 1hr and washed with PBST (PBS + 0.5 per cent Triton X-100) twice for 10min (with shaking at 100rpm). After incubating the cells with secondary antibody (dilutions recommended by the manufacturer) for 1hr, cells were washed twice for 10 min with PBST (with shaking at 100rpm). Antibody incubations and washes were done at room temperature. The coverslips were then mounted with Vectashield mounting medium onto glass slides and observed under Observer.Z1 (Zeiss) inverted fluorescent microscope.

4.12. Lithium-treatment Assay

Lithium treatment assay was done as previously described by Levy *et al.* (2002). Briefly, cells were seeded into 12-well plates and at indicated time-points (usually 0, 1, 2, 6, 12, 24, 48, 72hrs), but in reverse order, lithium chloride was added to a final concentration of 25mM. Cells in all wells were then harvested at t=0 time-point by adding 350 µL of RLT lysis buffer provided by RNeasy® Mini Kit (Qiagen). For Western Blotting analysis, cells were harvested in 1X SDS-PAGE sample dye. Genomic DNA was sheared with a 23G syringe needle before proceeding with RNA isolation or Western Blotting.

4.13. *In silico* analysis and statistics

Promoter sequences were analyzed using Transcriptional Regulatory Element Database located at Cold Spring Harbor Laboratory website (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home) and TFexplorer (http://www.tfexplorer.org) (Kim *et al.*, 2005). First 2000bp upstream of predicted transcriptional start site were analyzed.

Each experiment was performed at least twice. All data are shown as means. Error bars represent standard deviations. Statistical significances were tested by Student's t-test. P-values less than 0.05 were considered significant (one stars) and p-values less than 0.005 were considered very significant (two stars).

5. RESULTS

5.1. SAGE and Microarray Analysis Reveals that *MENA*, *BRI3* and *HSF2* Expressions Increase Upon Degradation-Resistant β-catenin Overexpression

From a Serial Analysis of Gene Expression (SAGE) screen previously performed in our laboratory (Kavak *et al.*, unpublished data), mRNA levels of *MENA* and *BRI3* were found to be upregulated by 5.5-fold and 2.4-fold, respectively ($p < 0.01$ for both), when degradation-resistant S33Y mutant β-catenin (S33Y-β-catenin) was overexpressed in a hepatocellular carcinoma cell line, Huh7 (Table 5.1). No data was obtained for *HSF2* expression from the SAGE screen. However, genome-wide microarray screen of the same samples revealed a 1.55- and 2.12-fold increase (as detected by two different Affymetrix tags) in *HSF2* expression upon β-catenin overexpression, even though average expression changes for *MENA* or *BRI3* were not as high as observed from the SAGE screen (Table 5.2). These screen data were verified by RT-PCR, using the same RNA used for the screens (Figure 5.1a and Kavak *et al.*, unpublished data). Also, RT-PCR analysis showed that the mRNA levels of *MENA*, *BRI3* and *HSF2* genes increase upon transient overexpression of S33Y-β-catenin in Huh7 cells and U373 cells, a neuroblastoma cell line (Figure 5.1b and 5.1c).

Table 5.1. Increase of *MENA* and *BRI3* mRNA levels upon stable overexpression of S33Y-β-catenin in Huh7 cells as detected by SAGE screen performed by Kavak *et al.*, (unpublished data). Fold increase in expression is shown as "β-catenin / vector" ratio - normalization of signal obtained from Huh7-S33Y (S33Y-β-catenin-overexpressing stable cell line) cDNA sample to signal obtained from Huh-vector (stable cell line which has been generated by transfecting with the empty vector, pCI-Neo) cDNA sample.

Gene	Fold increase (β-catenin/vector)
MENA	5.5
BRI3	2.4

Table 5.2. Increase of *MENA*, *BRI3* and *HSF2* mRNA levels upon stable overexpression of S33Y-β-catenin in Huh7 cells as detected by genome-wide Affymetrix microarray screen performed by Kavak *et al.*, (unpublished data). "Fold" denotes the fold increase described in Table 5.1.

MENA	
Affymetrix Tag	**Fold**
1553672_at	1.18
217820_s_at	1.23
222433_at	1.1
222434_at	0.82
228310_at	0.78
228553_at	1.13

BRI3	
Affymetrix Tag	**Fold**
223376_s_at	1.01

HSF2	
Affymetrix Tag	**Fold**
209657_s_at	2.12
211220_s_at	1.55

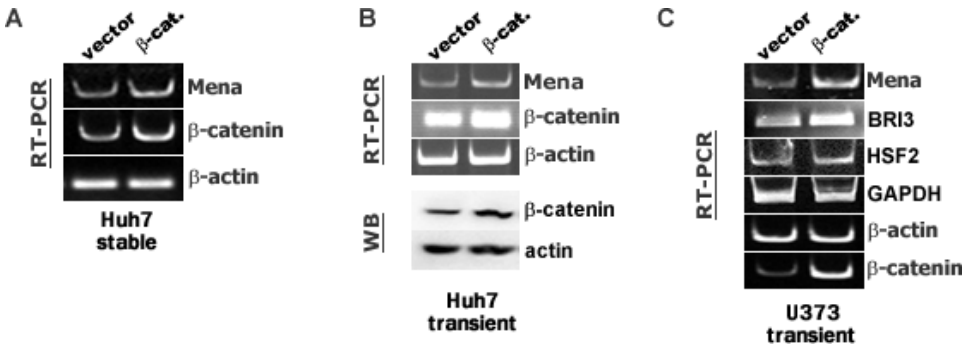

Figure 5.1. Confirmation of SAGE and microarray data by transient overexpression of S33Y-β-catenin in Huh7 and U373 cells. Stable overexpression of S33Y-β-catenin (denoted by "β-cat",) in Huh7 cells (A) and its transient overexpression in both Huh7 (B) and U373 (C) cells leads to increase in *MENA*, *BRI3* and *HSF2* transcription, as judged by RT-PCR. β-actin or GAPDH were used as internal controls, where indicated. For transient overexpression of S33Y-β-catenin in Huh7 cells (B), whole cell lysates were analyzed for β-catenin overexpression by Western Blotting (WB) using monoclonal anti-β-catenin antibody and anti-pan-actin antibody as internal control.

5.2. *MENA*, *BRI3* and *HSF2* Genes Have Putative Tcf4-Binding Elements in Their Promoter Regions

Since Tcf4 is the transcription factor through which the Wnt/β-catenin pathway operates, we analyzed the promoter regions of *MENA*, *BRI3* and *HSF2* genes for the presence of putative Tcf4-binding elements (TBE). The consensus sequence for TBE is WWCAAWG, where W stands for adenine or thymine (Roose and Clevers, 1999).

We used Transcriptional Regulatory Element Database located at Cold Spring Harbor Laboratory website (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home) and TFexplorer (http://www.tfexplorer.org) to analyze the promoter sequences for putative TBEs. Indeed, we have identified two putative TBEs in *MENA* and *HSF2* promoters and one putative TBE in *BRI3* promoter (Figure 5.2). For *MENA*, promoters of different species were also analyzed for the presence of TBEs. We have found that the sequences of the putative TBEs in *MENA* ortholog promoters and their locations in the promoters (with respect to transcriptional start site) are strikingly similar (Figure 5.3). Putative binding sites for other transcription factors, namely p300, NF-κB, MZF1, Ubx, Oct-1 and HNF3β, were also identified (Figure 5.2 and 5.3). The presence of TBEs in the *MENA*, *BRI3* and *HSF2* promoters and in the four *MENA* ortholog promoters suggests that these genes may be regulated by the β-catenin/Tcf4 complex.

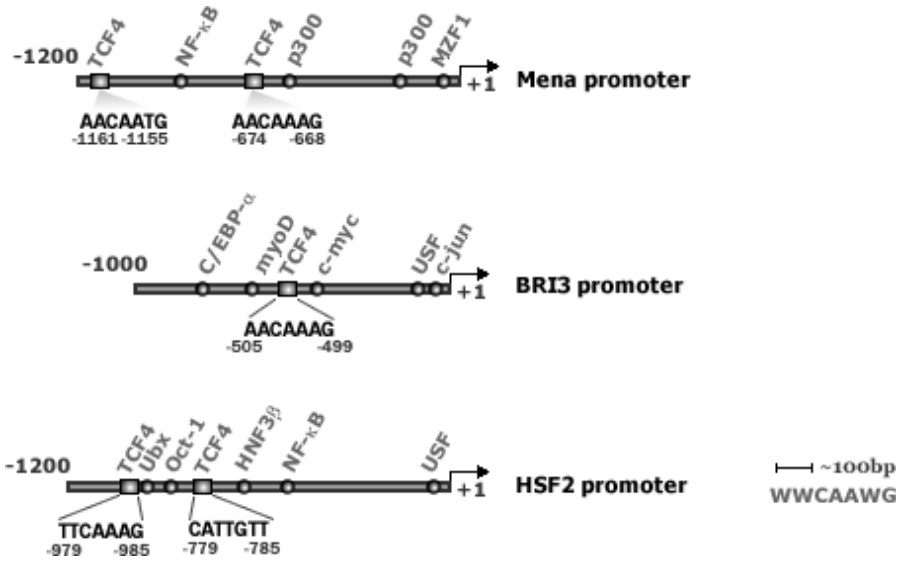

Figure 5.2. *MENA*, *BRI3* and *HSF2* promoters have putative Tcf4-binding elements (TBEs). Transcriptional Regulatory Element Database located at Cold Spring Harbor Laboratory website (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home) and TFexplorer (http://www.tfexplorer.org) were used to analyze the promoter sequences. The location of the TBEs is indicated by boxes and the sequences and locations with respect to transcriptional start sites (denoted by arrows) are shown under the boxes. Predicted binding motifs for other transcription factors are also indicated. The length of the bar is 100bp. The WWCAAWG sequence is the consensus sequence for TBEs.

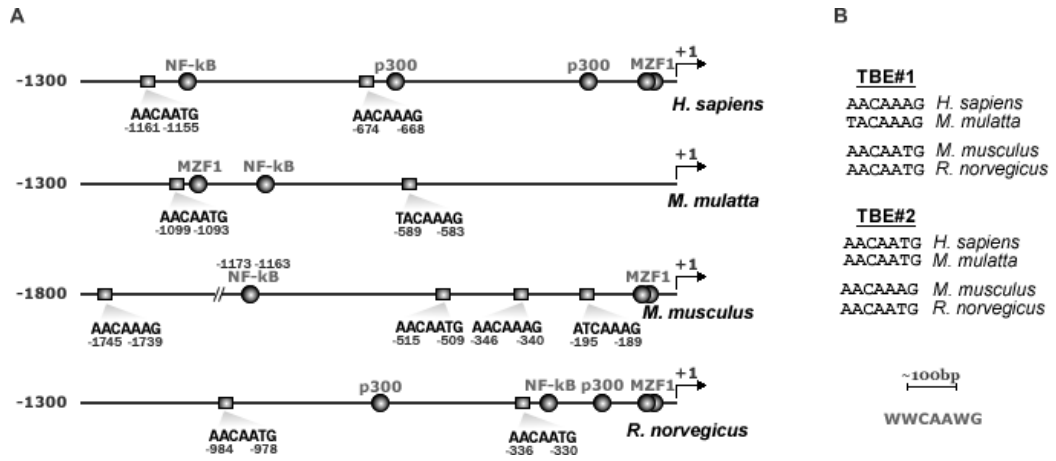

Figure 5.3. *MENA* ortholog promoters have putative Tcf4-binding elements. (A) Schematic representation of human, macaque, mouse and rat *MENA* ortholog promoters and the identified putative TBEs. (B) Alignment of proximal and distal TBEs. Transcriptional Regulatory Element Database located at Cold Spring Harbor Laboratory website (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home) and TFexplorer (http://www.tfexplorer.org) were used to analyze the promoter sequences. The location of the TBEs is indicated by boxes and the sequences and locations with respect to transcriptional start sites (denoted by arrows) are shown under the boxes. Predicted binding motifs for other transcription factors are also indicated. The length of the bar is 100bp. The WWCAAWG sequence is the consensus sequence for TBEs.

5.3. Lithium-Mediated Inhibition of GSK3β Leads to Accumulation of β-catenin, Its Nuclear Translocation and Induction of *MENA*, *BRI3* and *HSF2* Gene Transcription

GSK3β is a central inhibitory kinase of the Wnt/β-catenin pathway, artificial inhibition of which leads to increase in target gene expression. Lithium salts have been widely used as potent inhibitors of this kinase (Rubinfeld *et al.*, 1996; Stambolic *et al.*, 1996; Levy *et al.*, 2002). In order to obtain *in vitro* evidence supporting the hypothesis that β-catenin, and hence Wnt/β-catenin pathway, controls the expression of *MENA*, *BRI3* and *HSF2* genes, we treated Huh7 and U373 cell lines with either 25mM LiCl or 25mM lithium acetate (or 25mM NaCl as a control) and (1) confirmed that lithium-treatment of these cell lines leads to the expected β-catenin accumulation (Figure 5.4); (2) confirmed that β-catenin

translocates into the nuclei of these cells upon LiCl-treatment (Figure 5.5); and (3) showed that this nuclear localization of β-catenin induces an increase in *MENA*, *BRI3* and *HSF2* mRNA levels (Figure 5.6 and 5.7). The confirmation of β-catenin accumulation and its nuclear translocation was done in order to demonstrate that lithium-treatment works as predicted in Huh7 and U373 cell lines.

Significant β-catenin accumulation was observed after 24hrs LiCl-treatment of Huh7 and U373 cells (Figure 5.4). However, no change in β-catenin protein levels was observed in NaCl-treated cells. Subcellular distribution of β-catenin was assessed by immunostaining of Huh7 and U373 cells treated with LiCl for 24hrs (Figure 5.5). β-catenin was localized to cell membrane periphery in NaCl-treated Huh7 cells, whereas in LiCl-treated Huh7 cells β-catenin was strongly localized to the nuclei. In U373 cells, β-catenin was localized mainly to cytosol and cell membrane periphery, when the cells were treated with NaCl (control), whereas LiCl-treated cells showed strong nuclear localization of the β-catenin signal in addition to cytosolic and membranal localization.

MENA mRNA levels increased significantly ($p<0.05$) after 12hrs of lithium-treatment of Huh7 cells (~1.4-fold, Figure 5.6). β-catenin protein accumulation also peaked at 12hrs time-point. On the other hand, the same extent of increase (~1.4-fold) was observed only after 72hrs of lithium-treatment of Huh7 cells for c-myc, a known Wnt/β-catenin pathway target, whereas *MENA* mRNA levels increased up to 2-fold after 72hrs lithium-treatment. *BRI3* mRNA levels increased significantly only after 48hrs of lithium-treatment of Huh7 cells (Figure 5.7a), however this expression change reached ~2-fold after 72hrs. *HSF2* mRNA did not change significantly when Huh7 cells were treated with lithium (data not shown). These *in vitro* data strongly suggest that *MENA*, *BRI3* and *HSF2* transcription is induced by higher β-catenin levels and thus supports the hypothesis that these genes are targets of the Wnt/β-catenin pathway.

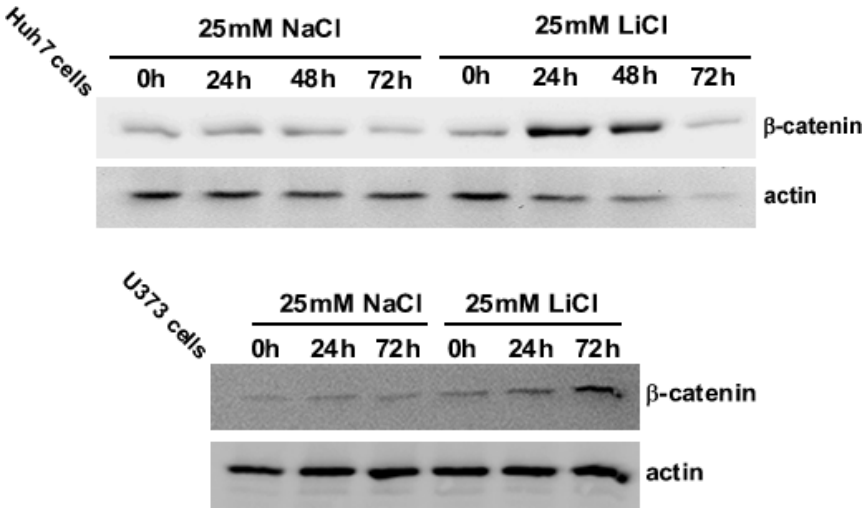

Figure 5.4. Lithium-treatment leads to β-catenin accumulation in Huh7 and U373 cells. Western Blotting using monoclonal anti-β-catenin antibody and anti-pan-actin antibody as internal control. Huh7 and U373 cells were treated with either 25mM LiCl or 25mM NaCl, over the indicated time-points and whole cell lysates were analyzed for β-catenin accumulation. Each image is a representative of at least two independent experiments.

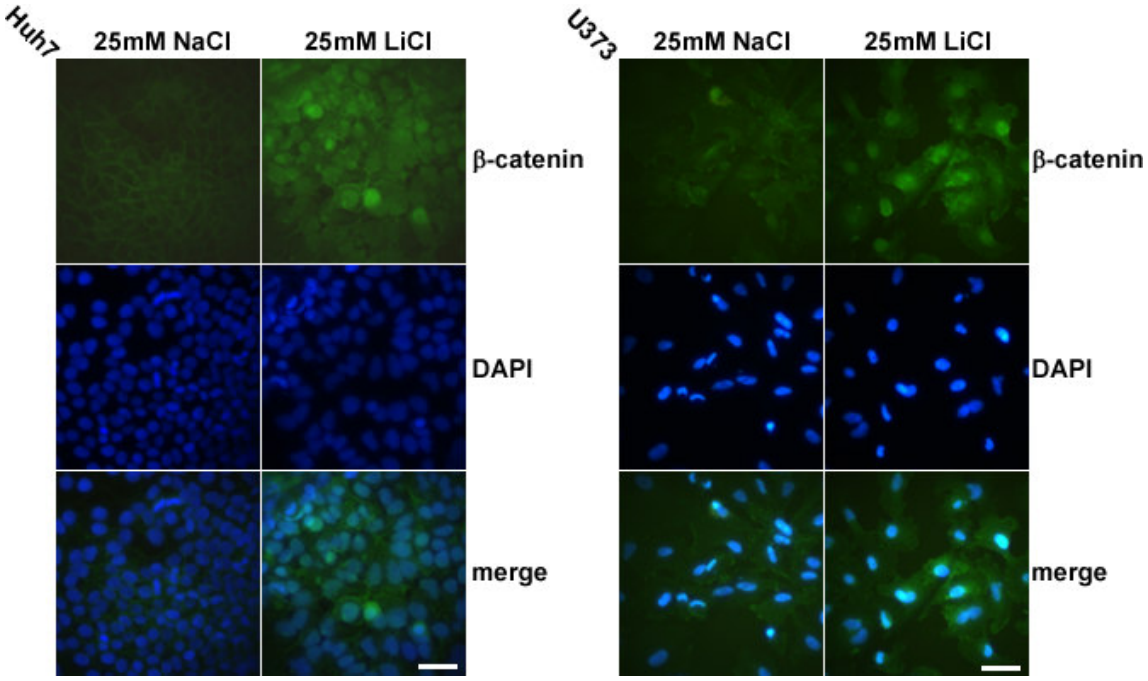

Figure 5.5. Lithium-treatment leads to accumulation and nuclear translocation of β-catenin in Huh7 and U373 cells. Immunofluorescence analysis of Huh7 and U373 cells treated with either 25mM LiCl or 25mM NaCl for 24hrs; cells were fixed with 4 per cent paraformaldehyde and stained with a cocktail of two mouse monoclonal anti-β-catenin antibodies which recognize nuclear and cytosolic β-catenin respectively (see Materials section). Anti-mouse/AlexaFluor®488 conjugated IgG was used as secondary antibody. Nuclei were stained with DAPI. The bar indicates 50μm. Each image is a representative of at least two independent experiments.

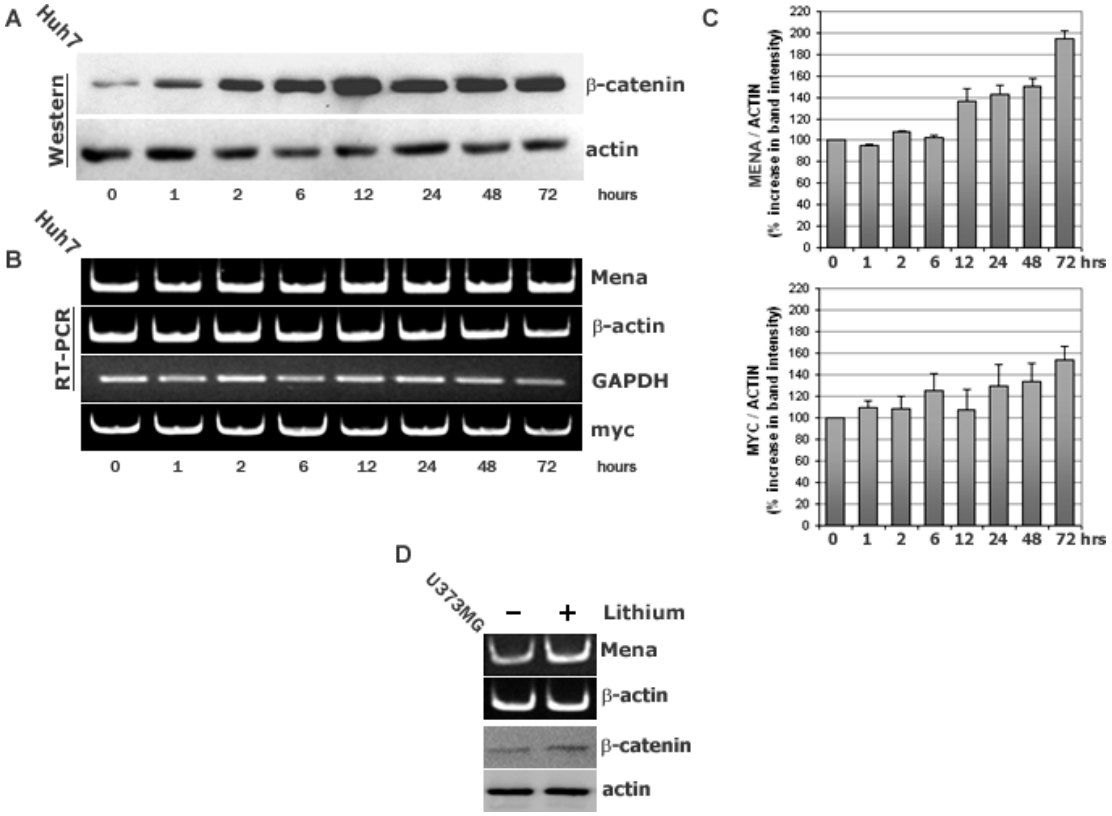

Figure 5.6. Lithium -treatment of Huh7 and U373 cells leads to β-catenin accumulation and induction of *MENA* transcription. Huh7 cells (A) and U373 cells (D) were treated with 25mM lithium acetate over indicated time-points (24hrs for U373 cells) and whole cell lysates were analyzed for β-catenin accumulation by Western Blotting using monoclonal anti-β-catenin antibody and anti-pan-actin antibody as internal control. Control cells in (D) did not receive any treatment. (B) *MENA* and *MYC* mRNA levels were assessed using RT-PCR and *ACTB* (β-actin) and *GAPDH* were used as internal controls. (C) *MENA* and *MYC* RT-PCR band densities from (B) were digitized using Scion Image Analysis software. The bars are given as averages (n=3) normalized to β-actin and then shown with respect to 0hrs treatment. Error bars represent standard deviation. Each image is a representative of at least two independent experiments.

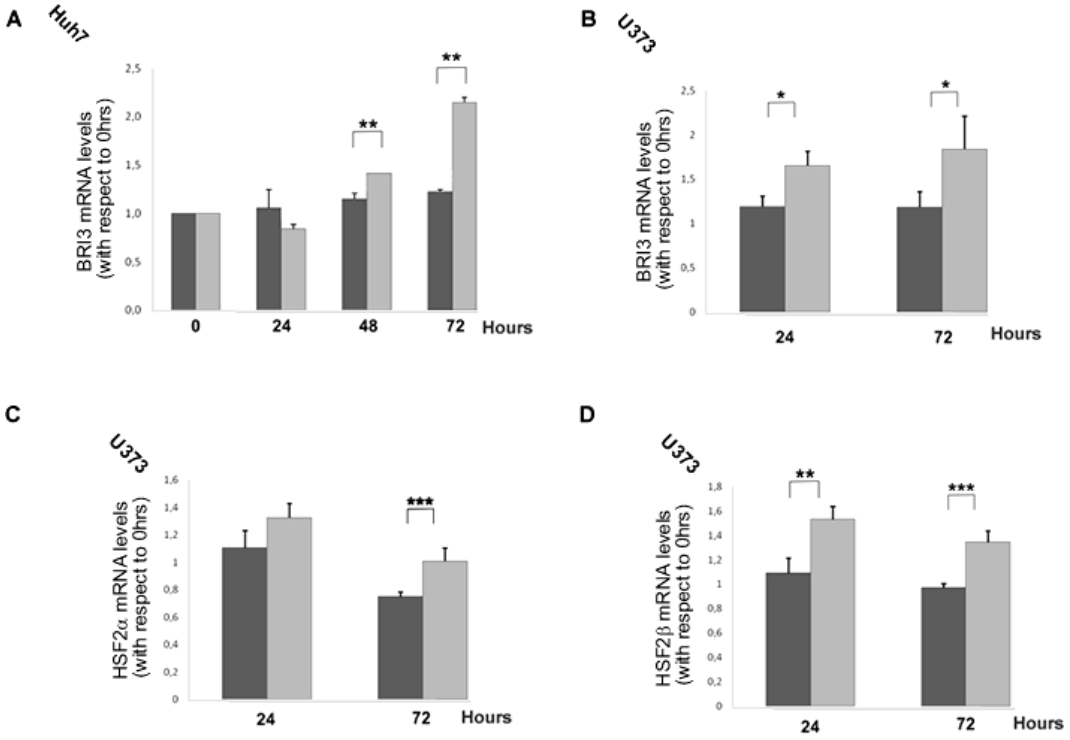

Figure 5.7. *BRI3* and *HSF2* transcription is induced by lithium-treatment. Huh7 (A) and U373 (B, C, D) cells were treated with 25mM LiCl over indicated time-points and *BRI3* and *HSF2* transcription was assessed using Real-Time RT-PCR. Dark-gray bars and light-gray bars represent NaCl- and LiCl-treatments, respectively. The bars are given as averages (n=3) normalized to β-actin and then shown with respect to 0hrs treatment. Error bars represent standard deviation. (* = $p<0.05$, ** = $p<0.01$ and *** = $p<0.005$, Student's t-test). Each graph is a representative of at least two independent experiments.

5.4. Promoters of *MENA*, *BRI3* and *HSF2* are Regulated by Wnt/β-catenin Pathway Components

Luciferase reporter assay is a widely employed method for studying promoter function. We cloned the 5' regions of the *MENA* (1250bp), *BRI3* (660bp) and *HSF2* (1700bp) genes into the promoterless luciferase reporter plasmid pGL3-basic, with an aim of inspecting the functionality of the putative Tcf4-binding elements. The experiments were performed using Dual-Glo^{TM} Luciferase Assay system (Promega) (Figure 5.8). Overexpression of degradation-resistant β-catenin with four point mutations (S33A, S37A, T41A, S45A) in its N-terminal region (β-catenin-4m) in Huh7 cells, lead to ~5-fold increase in *MENA* promoter activity (Figure 5.8a) , ~2-fold increase in *BRI3* promoter activity (Figure 5.8c) and about ~4.5-fold increase in *HSF2* promoter activity (Figure 5.8d). In addition, co-overexpression of a dominant-negative Tcf4 (ΔNTcf4) and β-catenin-4m, in Huh7 cells, diminished the promoter activity-stimulating effect of β-catenin-4m overexpression, since ΔNTcf4, which competes with the wild-type Tcf4 for the TBEs, lacks β-catenin-binding region and cannot be activated. Also, for *MENA* promoter we observed a synergic effect of β-catenin-4m and Tcf4 overexpression in HeLa cells (Figure 5.8b). However, such synergy was not observed in Huh7 cells (data not shown). Overexpression of β-catenin and Tcf4 was not performed for pGL3-*BRI3* and pGL3-*HSF2* reporter plasmids.

Taken together, these luciferase reporter assay results strongly suggest that *MENA*, *BRI3* and *HSF2* promoters are regulated by β-catenin and Tcf4.

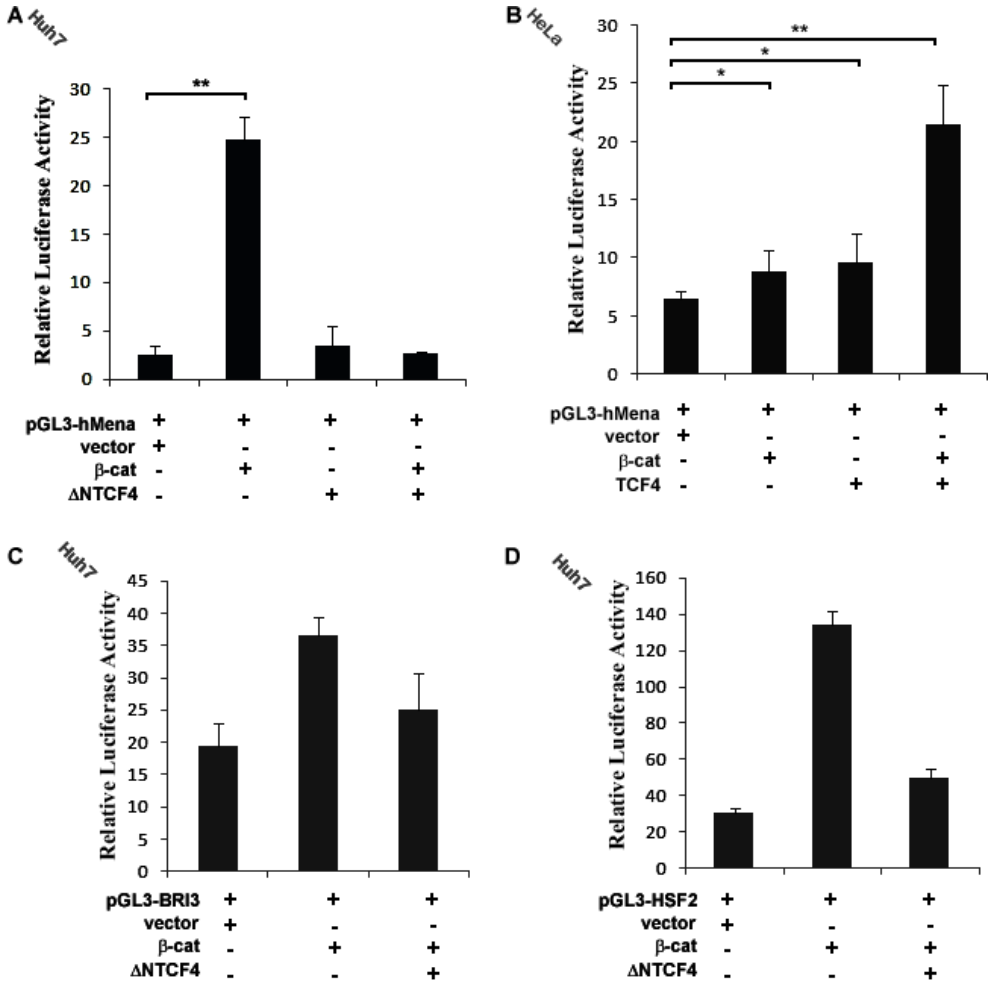

Figure 5.8. β-catenin and Tcf4 regulate activities of *MENA*, *BRI3* and *HSF2* promoters. Huh7 cells were transfected with pGL3-MENA (A), pGL3-BRI3 (C) or pGL3-HSF2 (D) reporter plasmids carrying corresponding gene promoters. The promoter activities were stimulated by overexpression of β-catenin-4m and inhibited by overexpression of ΔNTcf (A, C, D). (B) HeLa cells were transfected with pGL3-MENA and promoter activities were stimulated by overexpression of β-catenin-4m and Tcf4. The bars are given as averages (n=3) normalized to Renilla luciferase activity, which was used an internal control. Error bars represent standard deviation (* = $p<0.05$, ** = $p<0.01$ and *** = $p<0.005$, Student's t-test). Each graph is a representative of at least two independent experiments. "β-cat" denotes the quadruple-mutant β-catenin-4m.

5.5. β-catenin Interacts with *MENA*, *BRI3* and *HSF2* Promoters

In order to obtained evidence for the proposed "β-catenin - target candidate promoter" interaction, we performed chromatin immunoprecipitation assay (ChIP) on adult mouse brain and liver tissues, and Huh7 and HEK293T cell lines (Figure 5.9).

A monoclonal anti-human-β-catenin antibody was confirmed to crossreact with the mouse β-catenin by Western Blotting analysis of mouse liver and brain tissue lysates (Figure 5.9a). *MENA*, *BRI3* and *HSF2* promoter fragments were detected in the chromatin pulled-down by the anti-β-catenin antibody from both mouse brain and mouse liver lysates (Figure 5.9b). *MENA* and *HSF2* promoters were also found to interact with β-catenin in Huh7 and HEK293T cells (Figure 5.9c). Also, β-catenin interacted with the *BRI3* promoter in Huh7 cells (Figure 5.9d).

A monoclonal antibody of the same isotype as the β-catenin antibody was used as a control IgG, with the caution that the target of the control antibody is not a nuclear protein. Interaction between β-catenin and *BRI3* and *HSF2* promoters in Huh7 cells was also detected with another anti-β-catenin monoclonal antibody (data not shown; Kavak *et al.*, unpublished data).

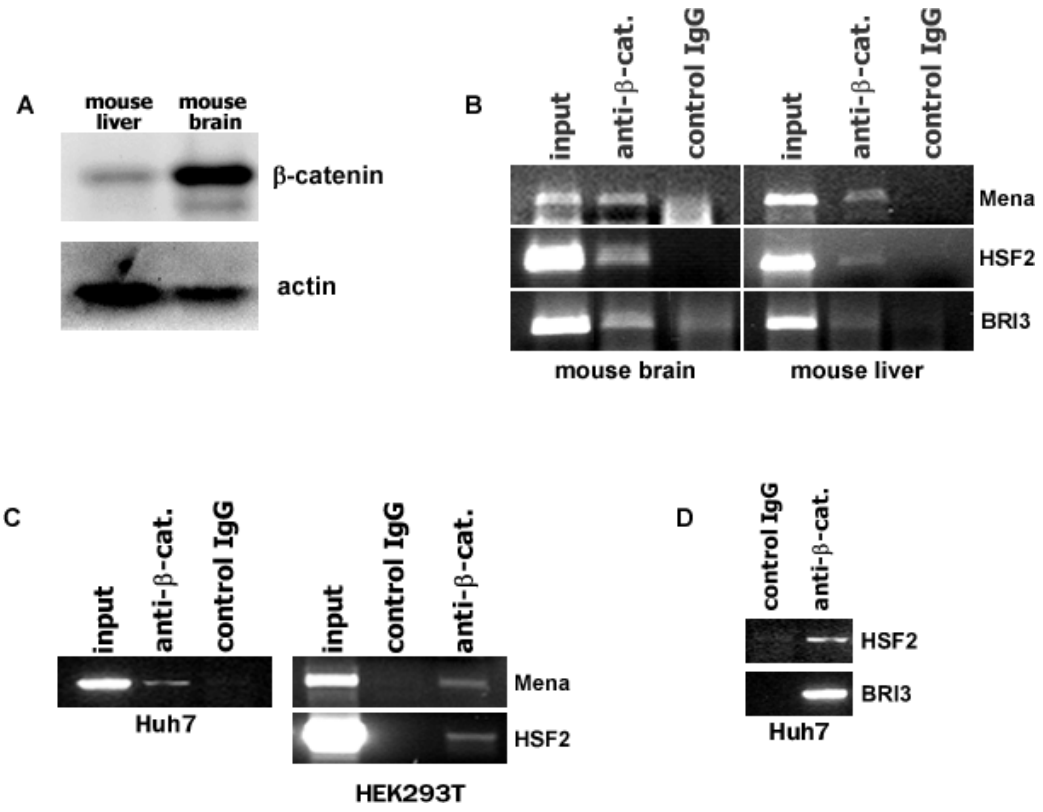

Figure 5.9. β-catenin interacts with *MENA*, *BRI3* and *HSF2* promoters. Chromatin immunoprecipitation assay was done with monoclonal anti-human-β-catenin antibody that was proven to detect mouse β-catenin in mouse liver and brain by analysis of whole tissue lysates using Western Blotting (A). PCR (35-40 cycles) was used to detect the promoter fragments in the immunoprecipitated chromatin and the whole reaction volumes were analyzed by agarose gel electrophoresis (B, C, D). β-catenin was found to interact with *MENA*, *BRI3* and *HSF2* promoters in adult mouse brain tissue and adult mouse liver tissue (B), Huh7 cells and HEK293T cells (C and D). DNA used as "input" was isolated from post-sonication, pre-immunoprecipitation lysates and 33-50 per cent of the "input" reaction volumes were loaded on the gels. All experiments were repeated at least twice. "Control IgG" denotes an unrelated monoclonal antibody.

5.6. Expressions of *CTNNB1* (β-catenin) and *MENA* Correlate in Several Cancer Cell Lines and Human Brain Tumors

We performed comparative RT-PCR analysis, in order to determine if there is a correlation between extent of *CTNNB1* (β-catenin) and *MENA* expression. Six cancer cell lines and six brain tumors were analyzed and such correlation has been observed in all six cell lines (Figure 5.10a and 5. 10b) and in three out of six brain tumor samples (Figure 5. 10c and 5. 10d).

Cells with low *CTNNB1* expression levels (Sk-Mel-103 and Sk-Mel-147) also showed low *MENA* expression levels, whereas cells with high *CTNNB1* expression levels (U373, Sk-Mel-19 and MeWo) also showed high *MENA* expression levels. Huh7 is an obvious exception (it has high *CTNNB1* expression levels, compared to U373, Sk-Mel-103 and Sk-Mel-147, but low *MENA* expression levels), since the β-catenin in this cell line is mostly localized to the periphery of the cell membrane and does not localize to the nucleus (Cha *et al.*, 2004 and see Figure 5.5).

Normal human brain tissue samples showed relatively low (band intensity ratio (BIR) = 0.4) *CTNNB1* and *MENA* mRNA levels when compared to central neurocytoma and pilocytic astrocytoma samples (BIRs varied between 0.8-1.0) (Figure 5.10d). However, DNET (Dysembryoplastic neuroepithelial tumor), gliolastoma multiforme and oligodenrioglioma samples showed low *CTNNB1* mRNA levels (BIR = 0.4), but high *MENA* mRNA levels (BIRs varied between 0.6-1.0).

The correlation of *CTNNB1* and *MENA* mRNA levels in the analyzed cancer cell lines and brain tumor samples supports the idea that *MENA* expression is regulated by the β-catenin.

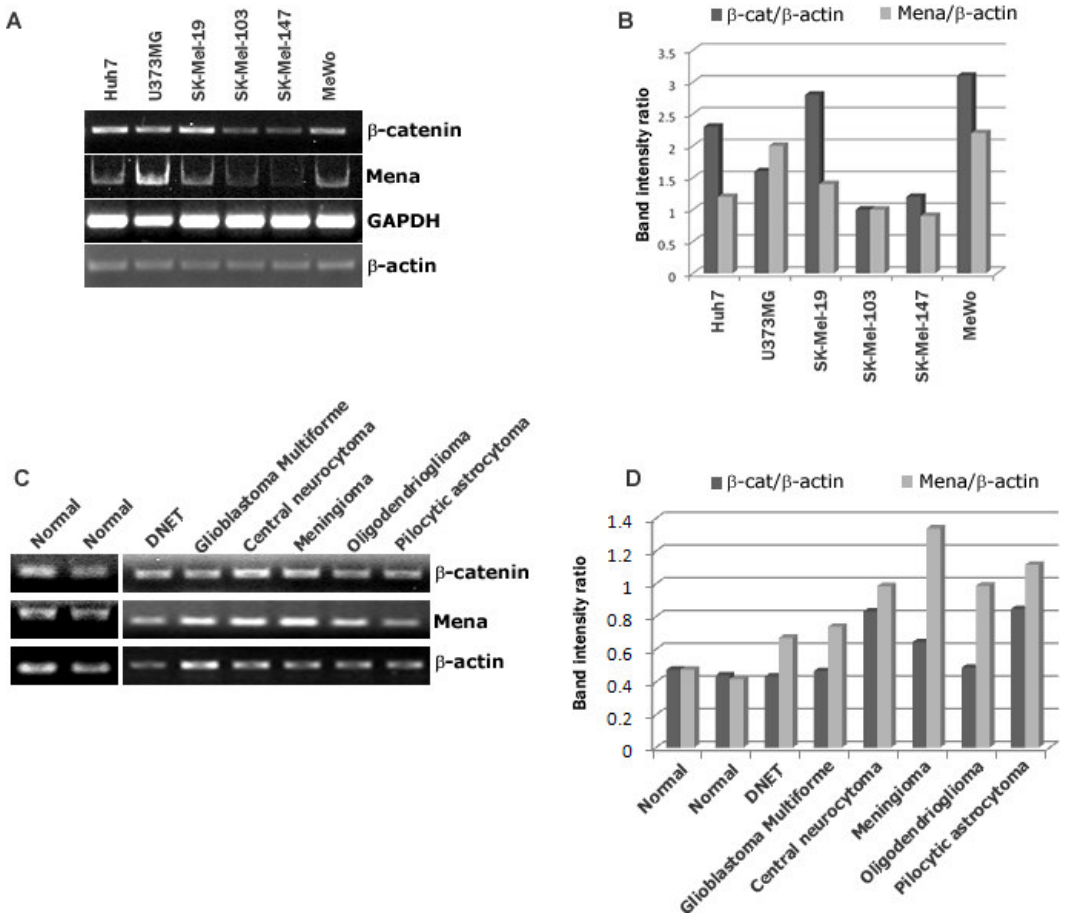

Figure 5.10. Expressions of *CTNNB1* (β-catenin) and *MENA* correlate in several cancer cell lines and human brain tumors. Comparative RT-PCR analysis of Huh7, U373, Sk-Mel-19, Sk-Mel-103, Sk-Mel-147 cell lines (A and B) and human brain samples of DNET (Dysembryoplastic neuroepithelial tumor), gliolastoma multiforme, central neurocytoma, meningioma, oligodenrioglioma and pilocytic astrocytoma origin (C and D) was done to analyze expression of *CTNNB1* and *MENA*. GAPDH and β-actin were used as internal controls. Band densities from (A) and (C) were digitized using Scion Image Analysis software. The bars are given as averages (n=3) normalized to β-actin. Each image is a representative of at least two independent experiments.

6. DISCUSSION

In this study, we identified *MENA*, *BRI3* and *HSF2* genes as novel transcriptional targets of the Wnt/β-catenin pathway. We used overexpression of degradation-resistant β-catenin, lithium-mediated inhibition of GSK3β, luciferase reporter assay and chromatin immunoprecipitation assay as major methods of assessment of the transcriptional regulation of *Mena*, *BRI3* and *HSF2* genes.

MENA and *BRI3* were picked as Wnt/β-catenin pathway target candidates from the SAGE screen and *HSF2* was picked from the genome-wide microarray screen. The fact that no data was obtained for *HSF2* in the SAGE screen can be explained by the sensitivity and specificity of the SAGE system (the tags employed to detect *HSF2* could be not efficient enough) and the abundance/scarceness of the *HSF2* mRNA (and the relative presence of its isoforms, *HSF2*α and *HSF2*β) in the Huh7 context.

MENA and *BRI3* mRNA levels were not observed to be upregulated by β-catenin overexpression in the microarray screen, as significantly as in the SAGE screen (compare Table 5.1 and 5.2). This inconsistency in fold changes between the screens is most likely due to the use of different tags (by SAGE and by Affymetrix microchips) to assess the expression levels and the nature of the screen systems. In the microarray screen, *MENA* expression is characterized by six different tags, four of which indicate an increase in *MENA* expression ranging from 1.1 to 1.23 folds and two of which indicate a decrease in *MENA* expression ranging from 0.78 to 0.82 folds. This inconsistency already indicates that screen results should always be confirmed (Figure 5.1). *BRI3* expression is assessed by only one Affymetrix tag and the expression change fold is only 1.01, which points out the need for many tags to assess a gene's expression in screens.

After analysis of screen data, yet before final decision on candidate genes list, we performed *in silico* analysis of the promoter regions of the genes whose expression was upregulated by β-catenin overexpression. An online promoter database / analysis tool called "Transcriptional Regulatory Element Database" designed by Michael Zhang Lab

(Cold Spring Harbor Laboratory) was used to analyze the promoter regions. We have found that *BRI3* has one, whereas *MENA* and *HSF2* promoters have two putative Tcf4-binding elements (TBEs) (Figure 5.2). The presence of TBEs was a major parameter for picking target candidates, since Wnt/β-catenin pathway operates through this transcription factor. However, since the consensus sequence is moderately-degenerate (WWCAAWG), extreme care was necessary before jumping to conclusions about the strength of candidacy of a given gene whose expression of upregulated by β-catenin overexpression. Therefore, promoters of different species were also analyzed for the presence of TBEs (Figure 5.3). Importantly, similarity of relative locations of the TBEs across the orthologs' promoters and their sequence similarity were strikingly suggestive of the functionality of these sites. In theory, each particular real TBE that fits the consensus sequence will have its own affinity for Tcf4 transcription factor, which will determine the kinetics of recruitment of Tcf4 to the particular promoter, which in its turn will determine the strength of activation of the gene expression upon β-catenin binding. Therefore, the similarity of the TBEs (and their relative locations in the promoter) across the species underlines their functionality, since it is expected that the Tcf4-dependent activation of a given gene's expression is going to be similarly regulated in human and macaque, and in mouse and rat.

Following the identification of putative TBEs in the promoters of our Wnt/β-catenin pathway target candidates, we employed lithium-treatment assay - a simple and cheap assay that is widely used for identification of novel Wnt/β-catenin pathway targets (Rubinfeld *et al.*, 1996; Stambolic *et al.*, 1996; Levy *et al.*, 2002). We have confirmed that lithium-treatment of Huh7 and U373 cells leads to β-catenin accumulation and its translocation into the nucleus (Figure 5.4 and 5.5). This proof-of-principle was important because lithium-treatment is not a very specific assay and also because Huh7 has most of its β-catenin localized to the membrane periphery and lithium-treatment could have unexpected results in this cell line.

MENA mRNA levels increased significantly after 12hrs of lithium-treatment of Huh7 cells (~1.4-fold, Figure 5.6). It is noteworthy that β-catenin protein accumulation also peaked at 12hrs time-point, which further supports the specificity of the *MENA* mRNA accumulation upon lithium-treatment. *MYC* mRNA levels were analyzed since MYC is a

known target of the Wnt/β-catenin pathway (reviewed in Barker and Clevers, 2006). However, the extent of *MYC* mRNA accumulation upon lithium-treatment of Huh7 cells was not as strong as that of *MENA* mRNA (Figure 5.6). This can be due to the fact that GSK3β regulates *MYC* expression both at transcriptional and translational levels (Jin *et al.*, 2008) and therefore stronger transcriptional activation is not required to achieve stronger *MYC* expression upon activation of the Wnt/β-catenin pathway.

HSF2 mRNA did not change significantly when Huh7 cells were treated with lithium (data not shown).This can be due to the fact that GSK3β regulates the expression of certain genes on two different levels – transcriptional (via β-catenin) and translational (via mTOR pathway) (Jin *et al.*, 2008). In that case, direct inactivation of GSK3β (by lithium) and Wnt ligand-mediated stimulation of the Wnt/β-catenin pathway (or even overexpression of β-catenin) could have a distinct outcome on expression of certain genes. Since we have not analyzed the protein levels of *HSF2* upon lithium-treatment of Huh7 cells, a final conclusion about the nature of the phenomenon (lithium-treatment of Huh7 cells does not lead to *HSF2* transcription stimulation) cannot be reached yet. Nevertheless, lithium-treatment of U373 showed an increase in *HSF2* mRNA levels (Figure 5.7c and 5.7d). We analyzed both alternative splicing isoforms of *HSF2* and found that the *HSF2*α isoform is upregulated by lithium only after 72hrs treatment, whereas the *HSF2*β isoform is significantly upregulated already after 24hrs of treatment. *BRI3* mRNA levels were also upregulated after 24hrs of lithium-treatment of U373 cells (Figure 5.7b) as did *MENA* mRNA levels (Figure 5.6d).

Intriguingly, *MENA* levels have been previously shown to be upregulated by administering of lithium to mice, as a mood-stabilizing drug (Blair *et al.*, 2006) and this increase of *MENA* expression was in parallel with β-catenin accumulation. In other words, this study (*in vivo* lithium-treatment) further supports the link between GSK3β inhibition, β-catenin accumulation and increase of *MENA* mRNA levels, similar with results of our *in vitro* lithium-treatment assay.

As expected, β-catenin-4m overexpression lead to increase in the promoter activities of the *MENA*, *BRI3* and *HSF2* genes. The fold increase in the activity, relative to the basal

activity, ranged from 1.75 to 6.25, which we speculate is due to the differential expression of the *MENA*, *BRI3* and *HSF2* genes in the Huh7 context and/or varying strength of the Tcf4-mediated gene expression activation in the Huh7 context. This can be attributed to different TBE sequences and different positions in the promoters, as well as different transcription factor partners in the transcriptosome.

Dominant-negative Tcf4 (ΔNTcf4) diminished the β-catenin-4m overexpression effect (Figure 5.8a, c, d), as it competed with the endogenous Tcf4 for the TBEs in the pGL3-promoter constructs. Observation of this effect of ΔNTcf4 further supports the functionality of the TBEs identified in *MENA*, *BRI3* and *HSF2* promoters and thus serves as additional evidence for their candidacy of being the Wnt/β-catenin pathway targets (Figure 5.2).

The promoter activity-activating effect of β-catenin-4m also varied depending on the cell context. In HeLa cells, *MENA* promoter activity was activated only by 1.2-1.3 fold upo β-catenin-4m overexpression (Figure 5.8b). However, when extra Tcf4 was supplied, β-catenin-4m induced *MENA* promoter activity to ~3.7-fold, again further supporting that the at least one TBE in the *MENA* promoter is functional.

It has been reported that presence of higher TBE copy numbers in luciferase reporter plasmids correlates with stronger luciferase expression levels, when cultured cells transfected with the reporter plasmids were stimulated with Wnt3a-conditioned medium (Liao *et al.*, 2006). Similarly, presence of only one TBE in the *BRI3* promoter in contrast to two TBEs in *MENA* and *HSF2* promoters is in accord with the luciferase data, which shows that *BRI3* promoter activity is induced by β-catenin-4m overexpression by only 1.75 fold, whereas *MENA* and *HSF2* promoter activities are induced by 6.25 and 5.2 folds, respectively.

A monoclonal anti-β-catenin antibody that was proven to detect mouse β-catenin (Figure 5.9a) was used to pull-down *MENA*, *BRI3* and *HSF2* gene promoter fragments from adult mouse brain and liver tissues (Figure 5.9b) or Huh7 and HEK293T cells (Figure 5.9c). The ChIP data suggets that β-catenin directly interacts with the *MENA*, *BRI3* and

HSF2 promoters in both mouse brain and liver context. *MENA*, *BRI3* and *HSF2* promoters were also pulled-down by the β-catenin antibody from Huh7 cells (Figure 5.9c, 5.9d and Kavak *et al.*, unpublished data) and HEK293T cells (Figure 5.9c and Kavak *et al.*, unpublished data). Since intact fresh tissues and cells grown in the medium were flash-crosslinked with formaldehyde, the ChIP data reflects the *in vivo* interaction of β-catenin and the *MENA*, *BRI3* and *HSF2* promoters. Anti-β-catenin antibody was used to show that the *MENA*, *BRI3* and *HSF2* promoters interact with β-catenin and are thus are being regulated by the Wnt/β-catenin pathway. Anti-Tcf4 antibody could also be used for our ChIPs, however, since Tcf4 may sit on any given promoter with a TBE (without being bound by β-catenin), ChIP with anti-Tcf4 antibody would be a less reliable option.

Even though *BRI3* is identified as a primarily brain-related protein with very weak expressions in other tissues (Vidal *et al.*, 2001), the fact that β-catenin interacts with its promoter in the liver tissue context (in addition to the brain tissue context) supports the idea that it is not unexpected for this gene to be identified as a Wnt/β-catenin pathway target from a screen performed in a hepatocellular carcinoma model (Huh7).

The bands seen in the *MENA* panel (Figure 5.9b) just below the main bands are primer dimes. The smears in the *BRI3* panel (Figure 5.9b) are most likely due to low primer quality. In general, PCR band qualities were lower in the ChIP assays since 35-40 cycles were used to detect the promoter fragments. High cycle numbers were necessary since the washes during the ChIP were stringent and the immunoprecipitated DNA quantities were therefore low.

Cell lines were used for the ChIP assays as initial experimental systems for the pull-downs. However, such results obtained from cell lines must always be verified with more *in vivo* systems before reaching definite conclusions, considering alterations in the cellular properties due to their tumoral characteristics and/or variations during *in vitro* cultivation.

If *MENA* was a real target of the Wnt/β-catenin pathway, one could consider whether *MENA* expression would be elevated in cells and tumors which have an endogenously-high β-catenin levels. In fact, *MENA* was shown to be overexpressed in ~75 per cent of breast

tumors (Di Modugno *et al.*, 2004) and Wnt/β-catenin pathway is strongly implicated in breast carcinogenesis at different levels/phases of the signaling cascade. However, such association may not be true for all cancer types with high β-catenin levels, (even crosstalk of pathways, different cellular contexts and other factors may lead to different downstream effects). Nevertheless, we have found that cancer cells of different origin which have high β-catenin expression mostly have high *MENA* expression and cells which have low β-catenin expression mostly have low *MENA* expression (Figure 5.10a, b). A clear exception was the Huh7 cell line, in which most of the β-catenin is intensly localized to the peri-membranal region.

A similar finding was observed in the six brain tumors analyzed for *MENA* and β-catenin expressions (Figure 5.10c, d). Three out of six tumors which had relative β-catenin expression higher than that of normal samples, also had elevated *MENA* expression levels. Also, all six tumors had elevated *MENA* mRNA levels, especially meningioma sample (more than 3-fold, with respect to normal tissues), which suggest the importance of *MENA* for carcinogenesis of the analyzed brain tumors.

If *MENA*, *BRI3* and *HSF2* are indeed targets of the Wnt/β-catenin pathway, what could be their roles in the pathway perspective? The answer to this question is, of course, going to take not a single researcher and not a single dozen of papers, but there are clues that may shed light on this puzzle.

PP2Ac is a catalytic subunit of large protein phosphatase 2A complex, which is known to interact with axin and APC and modulate the Wnt/β-catenin pathway activity (Figure 6.1, reviewed in Polakis, 2006). It is appealing that *HSF2* is a potential Wnt/β-catenin pathway target, since HSF2 is known to inhibit PP2Ac (Hong and Sarge, 1999). Such a link between HSF2 and PP2A suggest an existence of a positive feedback for *HSF2* regulation (Figure 6.2). This potential link can be tested by determining the effect of HSF2 overexpression on β-catenin/Tcf4 activity using a TBE-containing luciferase reporter plasmid which would reflect the changes in the Wnt/β-catenin pathway activity upon the HSF2 overexpression.

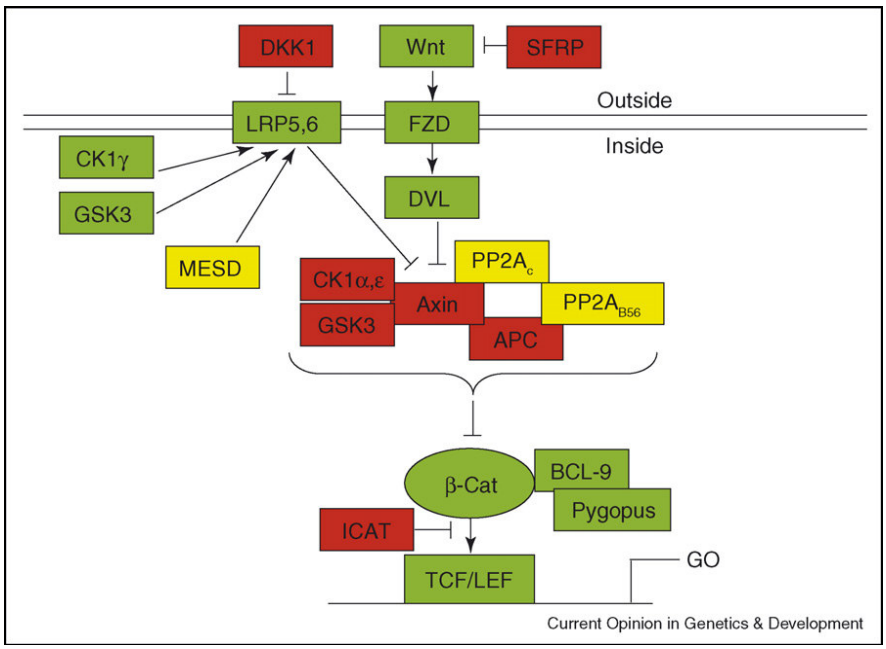

Figure 6.1. The PP2Ac and Wnt/β-catenin pathway (adapted from Polakis, 2006).

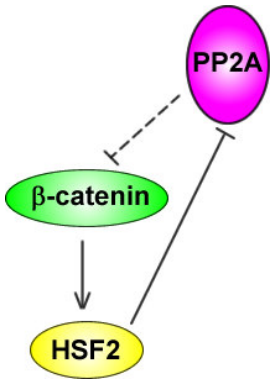

Figure 6.2. PP2A inhibits β-catenin activity through axin and APC (dashed line). β-catenin enhances *HSF2* expression. Is there a positive feedback for HSF2, over the PP2A phosphatase?

Mena is a known actin-associated protein. It is interesting that Dvl (dishevelled), a regulator of β-catenin destruction box downstream of Fz receptor (Figure 6.1), has been associated with F-actin (Torres and Nelson, 2000) and that Dvl has both SH3 and WW

domains (personal observation), to which Mena's proline-rich domain may bind (reviewed in Kwiatkowski, *et al.*, 2003). In this context, Mena might also have a similar feedback self-regulation.

Three manuscripts based on this study and the previous SAGE and microarray screens are presently in preparation for submission. We will continue investigating the potential roles of *MENA*, *BRI3* and *HSF2* in the Wnt/β-catenin pathway. The potential protein-protein interaction between Mena and Dvl will be investigated in the context of the relation of the Wnt/β-catenin pathway and actin cytoskeleton remodelling. Further functional analyses investigating interactions of *MENA*, *BRI3* and *HSF2* with carcinogenesis-related cellular processes, including cell proliferation and apoptosis will be pursued. We will expand our studies with *in vivo* xenograft experiments on *nude* mice in order to study their eventual roles in tumorigenesis.

7. REFERENCES

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