IDENTIFICATION OF NOVEL PROTEINS THAT INTERACT WITH BRI3, A PUTATIVE TRANSCRIPTIONAL TARGET OF THE WNT/β-CATENIN SIGNALING PATHWAY

by

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To my beloved family...

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ABSTRACT

IDENTIFICATION OF NOVEL PROTEINS THAT INTERACT WITH BRI3, A PUTATIVE TRANSCRIPTIONAL TARGET OF THE WNT/β-CATENIN SIGNALING PATHWAY

The Wnt/β-catenin signaling pathway is an evolutionary conserved pathway which has important functions in vertebrate early development, axis formation, cellular proliferation and morphogenesis. The activation of this pathway leads to translocation of the transcriptional activator β-catenin into the 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. The results of SAGE (Serial Analysis of Gene Expression) performed recently in our lab indicated that *BRI3* (Brain Protein I3) is one of the genes displaying increased expression in the presence of mutant - and thus more stable- form of β-catenin. In previous studies, *BRI3* expression was found to be upregulated in Huh7 cell lines upon lithium treatment. Moreover, with regard to previous literature, BRI3 was found to have a very important role in the TNF-α mediated cell death pathway. Also, its promoter activity was analyzed by luciferase reporter assays and found to have been increased due to overexpression of β-catenin. In this study, we screened a human liver cDNA library by yeast two-hybrid assay using BRI3 as bait, with the aim to find novel binding partners of BRI3 and provide clues for the functional study of BRI3 with respect to the Wnt/β-catenin pathway. Library screening by yeast mating resulted in the identification of 2 candidate positive clones, which have been confirmed by cotransformation to the competent yeast cells, but the results still need to be verified by co-immunoprecipitation using mammalian cells. In conclusion, this study mainly will lead to the discovery of novel BRI3-interacting proteins which is essential for identification of the action mechanism of BRI3 in Wnt/β-catenin signaling. Furthermore, any possible interaction between Wnt/β-catenin pathway and TNF-α mediated cell death pathway can be revealed ultimately.

ÖZET

WNT/β-KATENIN SİNYAL YOLAĞININ VARSAYILAN TRANSKRİPSİYONEL HEDEF GENLERİNDEN BİRİ OLAN BRI3 İLE İLETİŞİM KURAN YENİ PROTEİNLERİN TANIMLANMASI

Evrim süresince korunmuş bir yolak olan Wnt/β-katenin sinyal yolağının omurgalı erken gelişiminde, eksen oluşumunda, hücre çoğalması ve morfojenezde önemli görevleri vardır. Bu yolağın aktivasyonu, transkripsiyonel aktivatör olan β-kateninin çekirdeğe girmesine ve orada gelişimsel ve hücre döngüsü bağlantılı genlerin anlatımını düzenleyen Tcf/Lef transkripsiyon faktörlerini aktive etmesine yol açar. Labımızda önceden gerçekleştirilen SAGE analizi sonuçlarının ortaya koyduğu üzere, *BRI3*, β-kateninin mutant formunun varlığında anlatımı önemli derecede artış gösteren genlerden bir tanesidir. Önceki çalışmalarda, *BRI3* anlatımının lityum ile işlemden geçirilen Huh7 hücre hatlarında arttığı tespit edilmiştir. Öte yandan, yayınlanmış olan makaleleri göz önüne alırsak, BRI3'ün TNF-α aracılı hücre ölüm yolağında çok önemli bir rolü olduğu ortaya konulmuştur. *BRI3*'ün promotör aktivitesi lüsiferaz raportör deneyleri ile analiz edilmiş ve β-kateninin aşırı anlatımından dolayı bu promotör aktivitesinin arttığı tespit edilmiştir. Bu çalışmada, BRI3 proteini yem olarak kullanılarak, insan karaciğeri cDNA kütüphanesi maya iki hibrit yöntemi kullanılarak taranmış ve BRI3'ün Wnt/β-katenin sinyal yolağı dahilindeki işlevini açığa çıkartmaya olanak sağlayacak yeni bağlanma partnerlerinin keşfedilmesi amaçlanmıştır. Maya çiftleştirilmesi yoluyla kütüphane taramasının sonucunda tanımlanan 2 aday klon, kompetan maya hücrelerinde gerçekleştirilen kotransformasyon yöntemiyle doğrulanmıştır, fakat sonuçların memeli hücreleri kullanılarak yapılacak olan birlikte immün çöktürme yöntemi ile de doğrulanması gerekmektedir. Sonuç olarak, bu çalışma en başta, BRI3'ün Wnt/β-katenin sinyal yolağındaki aksiyon mekanizmasının tanımlanması için gerekli olan, BRI3 ile iletişime geçen yeni proteinlerin keşfedilmesine öncülük edecektir. Buna ilaveten, Wnt/β-katenin yolağı ile TNF-α aracılı hücre ölüm yolağı arasındaki olası bir etkileşimin açığa çıkmasına olanak sağlanacaktır.

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LIST OF SYMBOLS / ABBREVIATIONS

µg	Microgram
µl	Microliter
µM	Micromolar
3AT	3-Amino-1-2-4 trizole
AD	Activator domain
Ade	Adenine
Amp	Ampicillin
APC	Adenomatous Polyposis Coli
APS	Ammonium persulfate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BPB	Bromophenol blue
BRI3	Brain protein I3
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CKIα	Casein Kinase Iα
DDO	Double Dropout
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleosidetriphosphate
DO	Dropout medium

Dsh	Dishevelled
EB	Elution Buffer
EDTA	Ethylenediaminetetraacetate
EtOH	Ethanol
FAP	Familial Adenomatous Polyposis
Fz	Frizzled
GSK3β	Glycogen Synthase Kinase 3β
HCl	Hydrochloric Acid
His	Histidine
hrs	Hours
Huh	Human hepatoma
Kan	Kanamycin
kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani
LDL	Low density lipoprotein
LEF	Lymphoid Enhancer Factor
Leu	Leucine
LiAc	Lithium Acetate
LiCl	Lithium Chloride
LRP	Low density lipoprotein receptor-related protein
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
mL	Milliliter
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide

ng	Nanogram
OD	Optical density
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCP	Planar Cell Polarity
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
QDO	Quadruple Dropout
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse-transcriptase mediated polymerase chain reaction
SAGE	Serial Analysis of Gene Expression
SD	Synthetically defined medium
SDS	Sodium Dodecyl Sulphate
SOC	Super optimal catabolite repressed broth
TAE	Tris-acetic acid EDTA (buffer)
TBE	Tris-Borate-EDTA (buffer)
TCF	T-cell factor
TE	Tris-EDTA (buffer)
TEMED	N, N, N, N-tetramethylethylenediamine
Trp	Tryptophan
UV	Ultraviolet
v/v	Volume to volume
w/v	Weight to volume
YPD	Yeast extract-peptone-dextrose medium
YPDA	Yeast extract-peptone-dextrose medium supplemented with adenine

1. INTRODUCTION

The Wnt Signaling pathway is conserved in various organisms from worms to mammals, and plays important roles in development, differentiation, cellular proliferation, morphology, motility and fate. Wnt proteins constitute a family of secreted cysteine-rich glycoproteins that exhibit distinct expression patterns in embryo and adult organisms (Cadigan and Nusse, 1997). Functions of the Wnt proteins have been extensively investigated through genetics studies in *Drosophila melanogaster*, *Caenorhabditis elegans*, mouse, zebrafish, and through biochemical and cell biology studies in *Xenopus laevis*, sea urchin, chicken embryos and mammalian cultured cells (Wodarz and Nusse, 1998). Binding of the Wnt protein to its receptors stimulates 3 different pathways; which are canonical Wnt pathway, planar cell polarity pathway (PCP) and Wnt/calcium pathway.

1.1. Canonical Wnt/β-catenin signaling pathway

The canonical Wnt/β-catenin signaling pathway is the best understood Wnt signaling pathway. β-catenin, which is localized with the membrane bound E-cadherin or is free in the cytoplasm, plays a major role in the transduction of the canonical Wnt/ β-catenin signal (Takeichi *et al*., 1991; Ikeda *et al*., 1998). Cytoplasmic β-catenin levels are normally kept low through continuous proteasome-mediated degradation, which is controlled by a complex containing GSK-3β/APC/Axin. The canonical Wnt/β-catenin signaling pathway is initiated by the binding of a Wnt ligand to the Frizzled receptor and progresses through sequential events leading to the stabilization and translocation of β-catenin into the nucleus where it interacts with the TCF/LEF family of transcription factors in order to activate target gene expression (Yamamoto *et al*., 1999). The canonical Wnt/β-catenin signaling pathway is summarized in Figure 1.1.

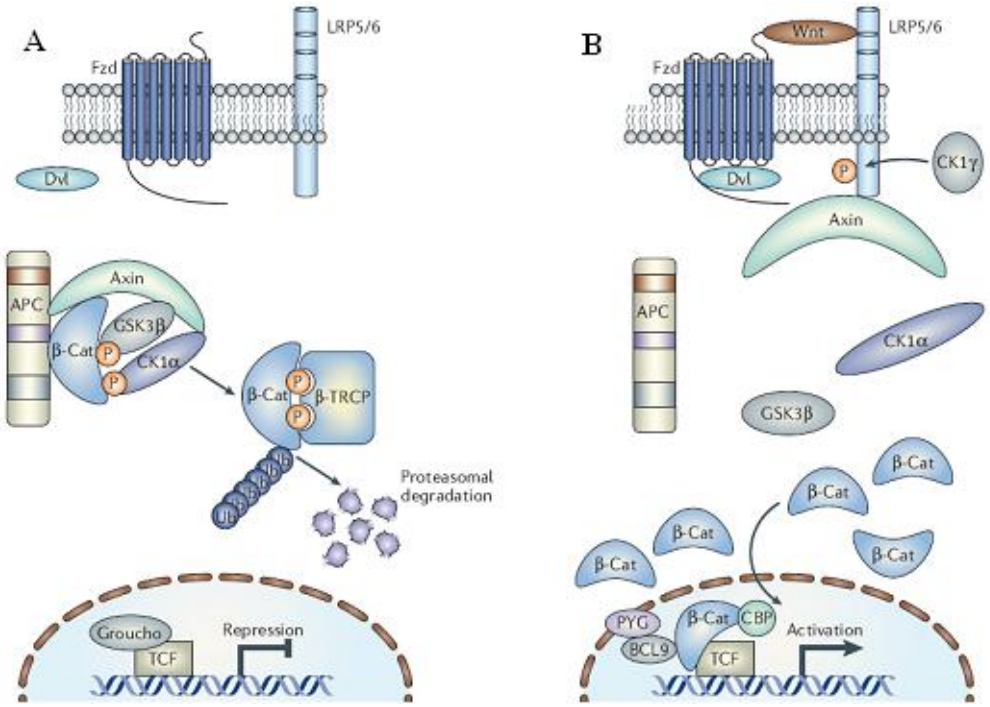


Figure 1.1. Wnt/ β-catenin signaling cascade in its 'inactive' (A) and 'active' (B) states (adapted from Barker and Clevers, 2006). β-catenin is constitutively phosphorylated and degraded when the pathway is inactive. Upon activation of the pathway by binding of a Wnt ligand, β-catenin begins to accumulate in the cytosol and then translocates into the nucleus which results in the activation of TCF/LEF transcription factor family.

1.1.1. Major components of the Canonical Wnt pathway

A total of 19 highly conserved Wnt genes were identified in the human genome. These genes direct the synthesis of secreted signaling molecules that regulate cell differentiation during development and tissue homeostasis, stem cell number in adult organism. Wnt proteins released from or presented on the surface of signaling cells act on target cells by binding to the Frizzled (Fz)/ Low density lipoprotein (LDL) receptor-related protein (LRP) complex at the cell surface. These receptors transduce a signal to several intracellular proteins that include Dishevelled (Dsh), Glycogen Synthase Kinase-3β (GSK-3β), Axin, APC and the transcriptional regulator β-catenin.

The main receptors of Wnt proteins, Frizzleds, are seven-transmembrane receptors with a highly conserved amino-terminal cysteine rich domain (CRD). On the other hand, LRP5/6 are Wnt co-receptors which consist of long single pass transmembrane molecules. LRPs are phosphorylated in a Wnt-induced manner on the intracellular domain by GSK-3β and CKIγ (Mao *et al*., 2001). The binding of Wnts to Frizzled and LRP5/6 results in the formation of a trimeric complex.

Axin is the core scaffold protein of the β-catenin destruction complex. It possesses binding sites for APC, GSK-3β, β-catenin, Dishevelled, and PP2A. GSK-3β and β-catenin can bind simultaneously to different sites of Axin, forming a ternary complex. In the Axin complex, GSK-3β efficiently phosphorylates β-catenin, APC and Axin itself, while Dishevelled and PP2A prevent the phosphorylation.

Adenomatous Polyposis Coli Gene Product (APC) is an important component of the β-catenin destruction complex. Within the destruction complex, APC interacts with both Axin and β-catenin, leading to the proteasomal degradation of β-catenin. The central part of APC contains three ‘15 amino acid repeat’ regions and seven ‘20 amino acid repeat’ regions, such that both of them can interact with β-catenin.

The serine/threonine kinase Glycogen Synthase Kinase 3 beta (GSK-3β) is another crucial component of the β-catenin destruction complex. Phosphorylation of β-catenin by GSK-3β is essential for down regulation of Wnt signaling. GSK-3β dependent phosphorylation of β-catenin takes place at the serine 33, serine 37 and threonine 41 residues (Behrens *et al*., 1998). Thus, β-catenin can be stabilized by point mutations of these serine and threonine residues in the N-terminal region of the protein, which renders protection from phosphorylation and makes it degradation resistant. This, in turn, results in an overall increase in Wnt signaling (Aberle *et al*., 1997). Furthermore, inhibition of GSK-3β, either via the activation of Wnt pathway by using a recombinant Wnt ligand or lithium chloride (LiCl) treatment of the cells in order to inactivate GSK-3β, can be considered as alternative approaches to stabilize β-catenin.

β-catenin was originally identified as a protein which interacts with the cytoplasmic domain of cadherin and links cadherin to α-catenin, which in turn mediates the anchorage

of the cadherin complex to the cortical actin cytoskeleton. Additionally, it is the key component of the canonical Wnt/β-catenin signaling pathway through its role as a transcriptional activator. In the absence of Wnt signals, free β-catenin in the cytosol is constitutively degraded in proteasome, whereas in their presence, β-catenin is stabilized and translocated into the nucleus where it can associate with members of the T cell factor/Lymphoid enhancer factor (TCF/LEF) family of transcription factors and act as transcriptional activators of the Wnt target genes (van de Wetering *et al*. 1997). β-catenin contains a large central region composed of 12 repeats of three helices each (called the armadillo repeats), forming a superhelix of helices (Huber *et al*., 1997). This structure forms a rigid scaffold for the binding of many factors, including the TCF transcription factor, the cell adhesion protein cadherin, APC, Axin and GSK-3β.

1.1.2. The On/Off States of the Canonical Wnt pathway

β-catenin is present in the Axin complex in the absence of a Wnt signal. In this complex, cytosolic β-catenin, but not the cadherin-bound β-catenin, is continuously phosphorylated, ubiquitinated and degraded by proteasome. The multiprotein complex that is responsible for β-catenin degradation is assembled around the scaffold protein axin. Within this "destruction complex" complex, β-catenin is firstly phosphorylated by casein kinase Iα (CKIα) at serine 45 and then by GSK-3β on its serine/threonine residues 33, 37 and 41. Subsequently, β-catenin is targeted for degradation via the ubiquitin-proteasome pathway, after it is ubiquitinated by β-transduction repeat containing protein (β-TrCP), which is an E3 ubiquitin ligase (Rubinfeld *et al*., 1996).

The "on" state of the canonical Wnt/β-catenin signaling pathway is initiated by the binding of a Wnt ligand to Frizzled (Fz) and LRP 5/6 co-receptor. In this case, it is hypothesized that Dishevelled binds to Axin and antagonizes Axin activity in response to Wnt. Thus, Dishevelled is thought to inhibit GSK-3β dependent phosphorylation of β-catenin and APC through a still unclear mechanism. As a consequence, β-catenin is dissociated from the destruction complex and starts to accumulate in the cytosol. The accumulated β-catenin is then translocated into the nucleus, and binds to and activates TCF/LEF family of transcription factors that results in the expression of cell cycle and

differentiation-related target genes (Behrens *et al*., 1996). Overall, in the absence of Wnt signaling, TCF acts as a repressor of Wnt / Wingless (Wg) target genes, by forming a complex with Groucho. Once in the nucleus, β-catenin is thought to convert the TCF repressor complex into a transcriptional activation complex which possibly occurs via displacement of Groucho from TCF/LEF and recruitment of the histone acetylase CBP/p300 (CREB-binding protein).

1.1.3. Role of canonical Wnt/β-catenin signaling pathway in Cancer

Aberrant activation of the Wnt/β-catenin signaling pathway can result in a wide range of pathological phenotypes or predisposition to diseases. Mutations that cause constitutive activation of the Wnt/β-catenin signaling pathway lead to different types of cancer (Polakis, 2007). The best known example of a disease involving a Wnt pathway mutation that produces tumors is Familial Adenomatous Polyposis (FAP), an autosomal dominant disease in which patients display hundreds or thousands of polyps in the colon and rectum. This disease is most frequently caused by truncations in APC. Mutations in β-catenin itself have also been detected in sporadic colon cancers (Giles *et al*., 2003) and a large variety of other tumor types including melanoma (Rubinfeld *et al*., 1997), hepatocellular tumor (Kim *et al*., 2000) and gastrointestinal tumor (Kolligs *et al*., 2002). Loss-of-function mutations in Axin have been detected in hepatocellular carcinomas (Satoh *et al*., 2000). Furthermore, in vertebrate development, loss of a single Wnt gene can produce dramatic phenotypes that range from embryonic lethality and CNS abnormalities to kidney and limb defects (Bienz, 2002).

To conclude, the normal Wnt signaling control of β-catenin regulation and the proper functioning of the β-catenin destruction complex are essential for normal embryogenesis and stem cell growth, with alterations resulting in the genesis of many cancer types. Therefore, identification of novel transcriptional targets of Wnt/β-catenin signaling pathway is crucial with respect to cancer research and several clinical research fields, since genes regulated by the pathway are potential drug and gene therapy targets.

1.2. BRI3 (Brain Protein I3) as a novel Wnt/β-catenin pathway target gene

In order to identify novel transcriptional targets of Wnt/β-catenin signaling pathway, transcriptome-profile analyses were recently performed in our laboratory using stable Huh7 (hepatocellular carcinoma) cell lines overexpressing a mutant form of β-catenin, which is degradation-resistant. Subsequently, both SAGE (Serial Analysis of Gene Expression) and genome-wide microarray screens were utilized to analyze differential expression of potential target candidates. BRI3 (Brain protein I3) has been selected among the potential Wnt/β-catenin signaling pathway targets based on the SAGE screen and an equivalent microarray screen (Kavak *et al*., unpublished data). Moreover, lithium treatment of Huh7 cell lines and overexpression of the Wnt ligands in the same cell lines resulted in the upregulation of *BRI3* gene expression, as determined by quantitative RT-PCR. The results obtained from luciferase reporter gene assay, in which *BRI3* promoter activity was found to be increased due to overexpression of β-catenin, also supported the former data (Kavak *et al*., unpublished data).

BRI3 gene product belongs to a family of integral membrane proteins containing at least three members (*BRI1*, *BRI2* and *BRI3*) (Vidal *et al*., 1999; Vidal *et al*., 2000; Vidal *et al*., 2001). Northern blot analysis has indicated that *BRI3* mRNA has high levels of expression in the brain, especially in the cerebral cortex, medulla, hippocampus, thalamus and spinal cord, but relatively low level of expression in other organs. Therefore, it was concluded that BRI3 is a brain-specific type II integral membrane protein (Vidal *et al*., 2001). Contrary to the specific *BRI3* expression, *BRI2* gene has been expressed in all tissues tested so far, with high levels of expression in the heart, placenta, kidney, and pancreas. Nevertheless, BRI3 protein contains similar structural features to the other two isoforms, BRI1 and BRI2, namely a single transmembrane domain, a single potential N-glycosylation site and no amino terminal signal peptide (Vidal *et al*., 2001). On the other hand, the results of SOSUI (a web-based protein analysis tool for prediction of transmembrane domains) analysis suggest that BRI3 is an integral membrane protein with 2 transmembrane helices, with both N- and C-termini being intracellular (Figure 1.2). Submission of the protein sequence for SOSUI analysis was done online at http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html.

BRI3 was originally identified as a 125 amino acid transmembrane protein that is overexpressed in TNF-α treated L929 cells, which are murine fibrosarcoma cells (Wu *et al*., 2003). The blocking of new BRI3 protein synthesis by using BRI3-antisense RNA, resulted in increased resistance of these cells to TNF-induced cell death at greater than 1000-fold. Although the exact action mechanism of BRI3 within the TNF-induced cell death pathway still remains unknown, it is hypothesized that, BRI3 synthesis acts as a negative checkpoint of this pathway (Wu *et al*., 2003). With respect to previous studies, BRI3 has been determined as the interaction partner of BACE1 (β-amyloid protein converting enzyme) (Wickham *et al*., 2005) and SCG10 (superior cervical ganglion, neural specific 10) (Gong *et al*., 2007) by using yeast two-hybrid assay.

Considering the fact that, Wnt/β-catenin 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), greater knowledge on the function of BRI3, as a nerve-specific member of the BRI protein family, can essentially lead to the discovery of its roles in neuronal development and in certain neural diseases.

A

N terminal	Transmembrane region	C terminal	Type	Length
67	YPANSIVVVGGCPVCRVGVLEDC	89	SECONDARY	23
91	TFLGIFLAIILFPFGFICCFAL	112	PRIMARY	22

B

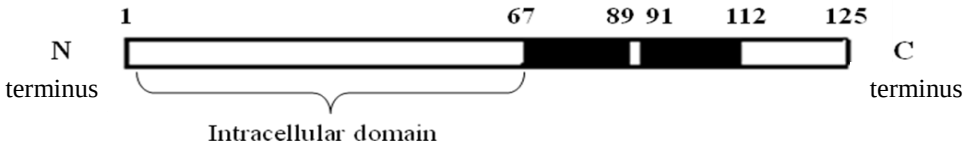


Figure 1.2. SOSUI analysis of BRI3 for prediction of transmembrane domains, suggested two transmembrane helices, one of which is a primary helix, whereas the other one is a secondary helix (A). BRI3 protein is schematized in (B) according to the SOSUI prediction analysis. The black regions correspond to transmembrane domains of BRI3.

1.3. Principle and Experimental Outline of the Yeast Two-Hybrid System

Yeast Two-Hybrid system originally described by Fields and Song (1989) has been developed as a high-throughput technique for detection of in vivo protein-protein interactions. In general, it is used primarily for initial identification of interacting proteins, but not for detailed characterization of the interaction. Nevertheless, it is still a very useful and efficient technique for this purpose due to its relative simpleness and low cost.

This method takes advantage of the modular properties of GAL4 and other transcription factors. Many eukaryotic transcription factors have at least two distinct functional domains, one binding to a promoter DNA sequence (DNA-binding domain) and another one activating transcription (activator domain). Thus, as in the case of our experiment, the DNA-binding domain of GAL4 is not activating transcription unless interacting with the activator domain. Our protein of interest (the bait protein) is produced as fusion to the GAL4 DNA-Binding Domain and the proteins from the prey library are expressed as fusion to the GAL4 Activator Domain. So that, in the case of an interaction, a functional GAL4 transcription factor will be reconstituted. This will lead to the regular transcription of the reporter genes, which in turn, enables the auxotrophic yeast two-hybrid strains to grow on nutritionally selective media.

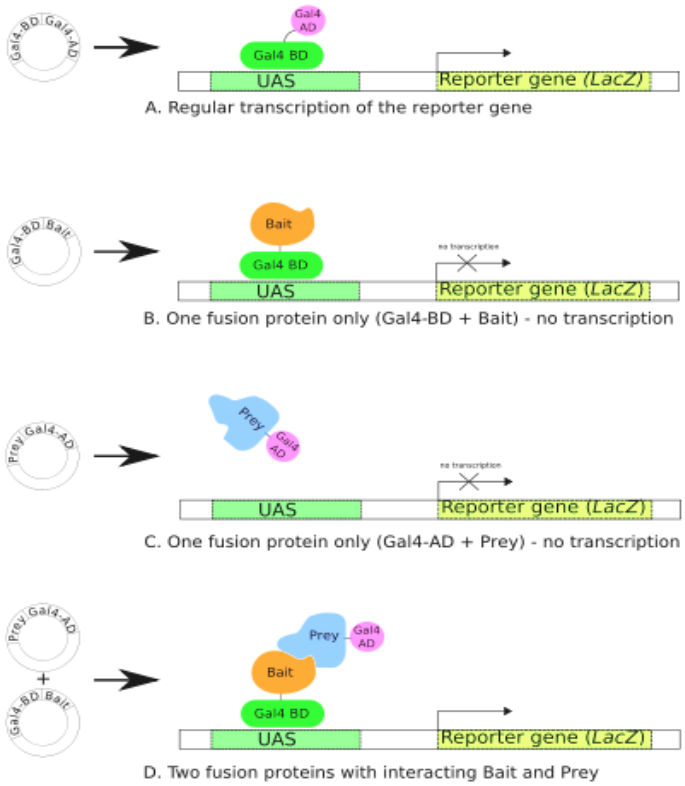


Figure 1.3. Overview of Yeast Two-Hybrid screening (adapted from Joung *et al.*, 2000). DNA Binding and Activating domains of transcription factor GAL4 are split and fused to two proteins being tested for interactions.

2. PURPOSE

The aim of this study was to identify novel interaction partners of BRI3 (Brain protein I3), which is recently determined to be one of the putative transcriptional targets of the Wnt/β-catenin signaling pathway.

The hypothesis that *BRI3* is a transcriptional target of the Wnt/β-catenin pathway, has been first revealed from genome-wide microarray and SAGE screens performed in our laboratory. *BRI3* was picked as one of the Wnt/β-catenin pathway target candidates, based on evidence its expression changes upon β-catenin overexpression, the presence of a putative TCF4-binding site in its promoter and additional factors mentioned in the Introduction part.

In this study, we screened a human liver cDNA library by yeast two-hybrid assay using BRI3 as bait, with the aim to find novel binding partners of BRI3 and characterize the molecular pathways regulating BRI3 function, which will provide clues for the action mechanism of BRI3 in Wnt/β-catenin pathway. Furthermore, any possible interaction between Wnt/β-catenin pathway and TNF-α mediated cell death pathway can be revealed ultimately.

3. MATERIALS

3.1. Electrophoresis and Western Blotting Buffers and Solutions

50X Tris-acetic acid EDTA (TAE)	2M Tris-acetate 50mM EDTA pH 8.5
TE Buffer	10mM Tris–HCl 1mM EDTA, pH 8.0
Ethidium bromide (EtBr)	10 mg/ml (stock solution) 30 ng/ml (working solution)
10X Tris Borate EDTA (TBE)	108 g Tris base 55 g Boric acid 9.3 g EDTA Double distilled water up to 1 L
Loading buffer	6X Loading Buffer purchased from Fermentas (Burlington, Canada)
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 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
Tris Buffered Saline with Tween 20 (TBST)	150mM NaCl 20mM Tris-HCl (pH 8.0) 0.1 per cent Tween 20
Blocking Solution	5 per cent non-fat milk powder in TBST
Stripping Solution	62.5mM Tris-HCl, pH 6.8 2 per cent SDS 0.7 per cent β-mercaptoethanol

3.2. Bacterial Culture Solutions and Antibiotics

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 Filter-sterilized and stored at -20°C 100 μg/ml (working concentration)
Kanamycin stock	50 mg/ml in distilled water Filter-sterilized and stored at -20°C 50 μg/ml (working concentration)
Chloramphenicol stock	30 mg Chloramphenicol in 1 ml Absolute ethanol 30 ng/ml (working concentration)
SOC medium	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
Filter-sterilized and stored
at -20°C

3.3. Bacterial Strains

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).

3.4. Yeast Growth Media

The basic yeast media and supplements required for a two-hybrid screen were purchased from Clontech (CA, USA). These include YPD Medium (500 g), Minimal SD Base Medium (267 g), -Trp DO Supplement (10 g), -Leu DO Supplement (10 g), -Leu/-Trp DO Supplement (10 g), -His/-Leu/-Trp DO Supplement (10 g) and -Ade/-His/-Leu/-Trp DO Supplement (10 g). Additional media supplements include X-α-Gal (25 mg) as a stock solution of 20 mg/ml in dimethyl formamide, Adenine Sulfate (0.2 per cent stock solution) and L-Leucine.

For the preparation of rich YPDA media, 50 g YPD medium and 15 ml 0.2 per cent Adenine Sulfate were mixed with distilled water up to 1 L. pH of the solution was adjusted to 6.5 and then autoclaved.

For the preparation of Dropout (DO) media, 26.7 g Minimal SD Base was mixed with the appropriate DO Supplement (the amount as indicated in the manufacturer's recipe) and distilled water was added up to 1 L. pH of all Dropout Media was adjusted to 5.8 and then autoclaved. The solutions were stored at 4ºC in subdued light.

If agar plates were to be prepared, 20 g agar was included in the mixture prior to autoclaving. The plates were allowed to harden at room temperature and then stored in a plastic sleeve at 4ºC.

In order to make X-α-Gal supplemented agar plates, X-α-Gal was diluted to 4 mg/ml in dimethyl formamide and spread 100 µl onto 10 cm plates or 200 µl onto 15 cm plates using a sterile glass rod.

3.5. Yeast Strains

The yeast host strains used in this study were obtained from Clontech (CA, USA). These are AH109 (genotype: MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3 : : $MEL1_{UAS}$-$MEL1_{TATA}$-lacZ) and Y187 (genotype: MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met^{-}, gal80Δ, URA3 : : $GAL1_{UAS}$-$GAL1_{TATA}$-lacZ).

3.6. Mammalian Cell Lines

Human Hepatocellular Carcinoma (HCC) derived Huh7 cell line was kindly provided by Dr. Mehmet Öztürk, Bilkent University.

3.7. Nucleic Acids

DNA molecular weight markers and deoxyribonucleotides were purchased from Fermentas (Burlington, Canada). pcDNA3-S33Y-β-catenin mutant was kindly provided by Dr. Mehmet Öztürk, Bilkent University. CS2+/β-catenin-4m (S33A, S37A, T41A, S45A quadruple-mutant) plasmid was kindly provided by Dr. Xi He, Harvard Medical School. Control plasmids for the Matchmaker GAL-4 Yeast Two-Hybrid System, pGBKT7/p53 and pGADT7/T-antigen, were purchased from Clontech (CA, USA). Primers used in polymerase chain reaction were purchased from Harvard University MGH DNA

Sequencing Core (Boston, USA). Sequencing service for confirmation of plasmids with cloned inserts and for the identification of DNA sequence in yeast colony PCR samples, was commercially provided by Iontek (Istanbul, Turkey), Harvard University MGH DNA Sequencing Core (Boston, USA) and Macrogen Inc. (Seoul, South Korea).

3.8. Enzymes

Taq DNA Polymerase together with $MgCl_2$ (25mM), 10X reaction buffer and all conventional restriction enzymes used in this study were purchased from Fermentas (Burlington, Canada). High-fidelity Taq DNA Polymerase PCR System, used for cloning, was purchased from Roche Applied Biosciences (Indianapolis, IN, USA). T4 DNA Ligase was purchased from Promega (Madison, WI, USA).

3.9. General Chemicals and Kits

All chemicals used in this study were analytical grade and purchased from Sigma (St. Louis, MO, USA) and Merck (Schucdarf, Germany), unless stated otherwise in the text. Matchmaker GAL-4 Yeast Two-Hybrid System, YeastMaker Yeast Transformation System, YeastMaker Yeast Plasmid Isolation Kit and Pretransformed Human Liver cDNA Library were purchased from Clontech (CA, USA). 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, Turbofect was purchased from Fermentas (Burlington, Canada). QIAprep Spin Miniprep Kit (250) and Qiagen Plasmid Midi Kit (50), for Plasmid Purification; QIAquick PCR Purification Kit (250), MinElute PCR Purification Kit (250), QIAquick Gel Extraction Kit (250) and MinElute Gel Extraction Kit (250), for DNA purification; RNAeasy Mini Kit, for RNA Purification, were purchased from Qiagen (Hilden, Germany). Genopure Plasmid Midi Kit for Plasmid Purification was purchased from Roche (Mannheim, Germany).

3.10. 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
Cold room	Birikim Elektrik Soğutma, Turkey
Deep freezers	-20°C, Arçelik, TURKEY
	-70°C, Harris, UK
	-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, Bio-Rad, ITALY
Heat-blocks	DRI-Block DB-2A, Techne, UK
Heating Magnetic Stirrer	M221 Elektro-mag, TURKEY
	Clifton Hotplate Magnetic Stirrer, HS31, UK
Ice Machine	Scotsman Inc., AF20, ITALY
Incubators	Blue M, USA
	Weiss Gallenkamp, Plus Series, UK
Inverted Microscope	Zeiss, Axio Observer Z1, USA
Laboratory Bottles	Isolab, GERMANY
Laminar flow cabinet	Labcaire BH18, UK
Liquid Nitrogen Tank	Air Liquide, TR21, FRANCE
Micropipettes	Finnpipette, Thermo, USA
Microwave ovens	Philips Whirlpool, USA
	M1733N, Samsung, MALAYSIA
pH meter	WTW pH330i, GERMANY
Pipettor	Pipetus-akku, Hirscmann Labogerate, GERMANY

Power Supply	Bio-Rad, USA
Refrigerators	2082C, Arçelik, TURKEY
	4030T, Arçelik, TURKEY
Shakers	VIB Orbital Shaker, InterMed, DENMARK
	Lab-Line Universal Oscillating Shaker, USA
	Thermo EC, Forma Orbital Shaker 420, USA
Spectrophotometer	NanoDrop 1000, USA
	Agilent 8453, USA
Speed Vacuum	Thermo EC, SPD111V, USA
Thermocyclers	Applied Biosystems, GeneAmp PCR System 2700, USA
Vacuum pump	KNF Neuberger, USA
Vortex	Vortexmixer VM20, Chiltern Scientific, UK
Water baths	TE-10A, Techne, UK
Water purification	WA-TECH Ultra Pure Water Purification System, GERMANY

4. METHODS

4.1. Preparation of Chemically Competent Cells by Calcium Chloride Method

5 ml LB medium containing streptomycin (25 µg/ml) was inoculated with E.coli strain TOP10 and grown overnight at 37ºC with shaking at 200 rpm. After 16 hours, 25 ml LB was inoculated with 250 µl of the grown culture to make 1:100 dilution. Cells were grown in the shaker till OD 595 reached 0.4. Cells were harvested by centrifuging at 3000 rpm for 10 minutes at 4ºC. The pellet was resuspended in 12.5 ml of ice-cold sterile 50 mM $CaCl_2$ and incubated on ice for 30 min. Cells were centrifuged again (3000 rpm for 10 min at 4ºC) and the pellet was resuspended in 2.5 ml of ice-cold sterile 50 mM $CaCl_2$. 100-150 µl of this preparation was used for transformations. For long term storage at -80 ºC, glycerol was added to make 10 per cent final concentration and cells were rapid-frozen in liquid nitrogen.

4.2. Transformation of the Chemically Competent Cells

A vial (100-150 µl) of competent cells was thawed on ice for 10-15 min. 1 µl plasmid or 10 µl of the ligation product was added onto the competent cells and incubated in ice for 15-20 min. Next, the vial was placed in 42ºC heat block for 1 min and then immediately on ice for 2 min. 500 µl LB or SOC medium was added and the cells were incubated at 37ºC for 1 hour with shaking at 200 rpm. The cells were centrifuged for 2-3 min and resuspended in 100-150 µl LB or SOC medium and the suspension was spread onto appropriate antibiotic resistance plates. The plates were incubated overnight at 37ºC in inverted position.

4.3. Plasmid Purification

Plasmid purifications were performed using Qiagen QIAprep Spin Miniprep Kit, Plasmid Midi Kit, Plasmid Maxi Kit and Roche Genopure Plasmid Midi Kit, according to

the manufacturer's protocol. Only midi and maxipreps of plasmids were used for the transfection of cell lines. Quality and concentration of the isolated plasmids was checked by spectrophotometric measurements using a NanoDrop-1000 spectrophotometer. OD260/280 ratio was 1.8-2.0.

4.4. Agarose Gel Electrophoresis

DNA fragments were fractionated by horizontal electrophoresis using standard 1X TAE-based agarose gels (1 per cent to 2 per cent). Agarose is mixed with 1X TAE Buffer and allowed to boil in a microwave oven. After cooling for a couple of minutes, ethidium bromide was added to final concentration of 30 ng/ml and the solution was poured into the gel casting tray. Appropriate amounts of the DNA samples were mixed with 6X loading buffer to get 1X final concentration. The solidified gels were run in 1X TAE buffer at varying voltage and time depending on the size the fragments.

4.5. Primers and Polymerase Chain Reaction

Primer design was done using CLC Bio Workbench software (http://clcbioworkbench.com). For cloning purposes, appropriate restriction endonuclease sites were attached at the 5' ends of the primers and "AAA" sequence is also added to the 5' ends as "spacers" for restriction enzyme binding. All Primers used in this study are given in Table 4.1.

For semi-quantitative RT-PCR, the following reaction composition was used. 1X Taq Buffer, 2 mM $MgCl_2$, 0.25 mM dNTP, 5 per cent DMSO, 0.4 µM of each primer, 0.05u/µl Taq DNA polymerase (Fermentas). The PCR reaction was started with an initial denaturation step at 94ºC for 5 min. Cycling conditions of PCR were as following : denaturation step at 94ºC for 30 sec, annealing step at 55ºC for 30 sec and elongation step at 72ºC for 30 sec or 1 min. After 28-32 cycles, the PCR reaction was ended with a final elongation step at 72ºC for 7 min.

Table 4.1. Primers used throughout this study for cloning and diagnostic purposes

Primer Name	Primer Sequence (5'- 3')	Restriction Enzyme Sites
BRI3_7F_cl	AAAGAATTCATGGACCACAAGCCGCT	EcoRI
BRI3_8R_cl	AAAGTCGACTTAAGCGAAGGTGGCTCCA	SalI
BRI3_9F_cl	AAAGCGGCCGCATGGACCACAAGCCGCT	NotI
BRI3_10R_cl	AAATCTAGATTAAGCGAAGGTGGCTCCA	XbaI
pACT2F	CTATTCGATGATGAAGATACCCCACCAAACC	-
pACT2R	GTGAACTTGCGGGGTTTTTCAGTATCTACGATT	-
pGADT7_seq_R	AGATGGTGCACGATGCACAG	-
briFmyc	AAATCTAGAATGGACCACAAGCCGCT	XbaI
briRmyc	AAAGAATTCTTAAGCGAAGGTGGCTCCA	EcoRI
Bri3/1-59-rev	AAAGTCGACTCAGATGTTGTAGACCCTGGG	SalI
CTNNB1_7Fcl	GACTAGATCTATGGGTAAAGGCAATCCTGAGGAA	BglII
CTNNB1_6Rcl	CCTCATCTAATGTCTCAGGG	-
CTNNB1_10Fcl	AAAGGGCCCAGCGAAGGTGGCTCCACAG	ApaI
CTNNB1_9Rcl	AAAGGATCCCAGGTCAGTATCAAACCAGGC	BamHI
bcr_gfp_R	AAAGGGCCCAGCGAAGGTGGCTCCACAG	ApaI
IFITM_3F	AAAAAGCTTATGAATCACACTGTCCAAACC	HindIII
IFITM_3R	AAAGGATCCTCCATAGGCCTGGAAGATCA	BamHI
IFITM_4F	AAATCTAGAAATCACACTGTCCAAACCTTC	XbaI
CTNNB1_11Fcl	AAAGGATCCATGGCTACTCAAGCTGATTTGAT	BamHI
CTNNB1_12F	AAAGGATCCATGGAACCAGACAGAAAAGCG	BamHI

4.6. Molecular Cloning

Standard cloning procedures were used for cloning the PCR amplified insert with flanking restriction sites into the desired linearized vector. In some cases where the insert of interest was present in another vector, sub-cloning was performed by using the appropriate restriction enzyme pair.

4.6.1. Restriction Enzyme Digestion of DNA

Restriction reactions were carried out at 37 ºC for 3-4 hours in suitable buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 100 mM NaCl. One unit of enzyme was used for the digestion of 1µg of DNA. Subsequently, inhibition of the restriction enzyme was performed at 80ºC for 20 min.

4.6.2. Extraction by Phenol – Chloroform

For phenol-chloroform extraction, equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) were mixed with the DNA samples. After high speed centrifugation for 5 min, the aqueous layer was transferred to a new tube and equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed. After another centrifugation, the aqueous layer was collected into a new tube. 1/10 volume of 3M sodium acetate (pH 5.0) was added and mixed by inverting the tube. 2 volumes of absolute ethanol were added and then the mixture was incubated at -80ºC for 20 min. After a high-speed centrifugation for 15 min, the pellet was washed with 70 per cent ethanol, air dried and dissolved in sterile water.

4.6.3. Ligation of DNA molecules

The concentrations of vector and insert DNAs were determined by absorbance measurements at 260 nm and their purity was confirmed by agarose gel analysis. Ligations were performed in 10 µl of total reaction volume containing 100-150 ng of plasmid DNA and molar excess of insert DNA in the presence of 1 unit of T4 DNA Ligase and 1X T4 Ligation Buffer at room temperature for 3 hours. Inhibition of T4 DNA Ligation enzyme was performed by heating the reaction tube at 70 ºC for 10 min.

4.7. SDS/PAGE 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 30 min of polymerization, a 5 per cent stacker gel (with the same cross linker 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 the molecular weight standard. Samples were heated at 95ºC for 10 min, vortexed, flash-centrifuged and loaded into the wells formed by the stacker gel. Two gels in a single Mini-Protean III cell were run 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 1 hr at room temperature, while shaking at about 100 rpm. Incubations with primary and secondary antibodies were done by diluting them to appropriate concentrations suggested by the manufacturer and incubating for 1 hr at room temperature, while shaking at about 100 rpm. After antibody incubations, washes were performed using TBST, twice, 5 min each wash, while shaking at about 120 rpm. ECL Plus Western Blotting Detection Reagents (Amersham) were used to develop the blots. Blots were 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).

4.8. Library Screening by Yeast Mating

A fresh and large colony of the bait strain (AH109 [pGBKT7/Bri3]) was inoculated into 50 ml of SD/-Trp liquid medium and incubated at 30ºC with shaking (230-250 rpm) until the OD_{600} reaches 0.8. The cells were centrifuged at 1000 g for 5 min, and then resuspended in 5 ml of SD/-Trp. In a sterile 2 L flask, 1 ml aliquot of the library strain was combined with 5 ml bait strain. 45 ml of 2x YPDA liquid medium was added to the flask and incubated at 30ºC for 22 hr with slow shaking (50 rpm). After 22 hr, the cells were centrifuged. The 2L flask was rinsed twice with 50 ml 0.5x YPDA. The rinses were then combined and used to resuspend the pelleted cells. The cells were again centrifuged for 10 min and all pelleted cells were resuspended in 10 ml of 0.5x YPDA liquid medium. The

mated culture was spread on SD/-Ade/-His/-Leu/-Trp plates (200 µl per 150 mm plate). The plates were incubated at 30ºC for 5-6 days.

4.9. Yeast Colony PCR

The identification of cDNA clone in prey vector was done by yeast colony PCR and sequencing of the PCR samples. For this purpose, single and fresh colonies were picked with a sterile pipette tip and resuspended in 3µl of NaOH (25mM) in separate PCR tubes. Liquid nitrogen was used to quick-freeze the samples. Then the samples were placed into the PCR machine and boiled at 95ºC for 10 min. Master mix was prepared (using the pACT2F - pACT2R primer pair) and distributed to the tubes. For the PCR reaction, the following cycling conditions were used. 94ºC for 5min, 30 cycles [94ºC for 30sec, 55ºC for 30sec, 72ºC for 1min], 72ºC for 10min; Hot-start at 94ºC.

4.10. Co-transformation of Competent Yeast Cells

Clontech's YeastMaker Yeast Transformation System was used according to manufacturer's protocol for all transformations of yeast cells. PEG/LiAc (polyethylene glycol 3350/lithium acetate) based method was provided for the preparation and transformation of competent yeast cells. For the co-transformation of the bait and prey vectors, 0.2µg of each vector was used together with 5µl of Herring Testes Carrier DNA (10mg/ml).

4.11. Yeast Plasmid Isolation

Clontech's YeastMaker Yeast Plasmid Isolation Kit was used according to manufacturer's protocol. A single yeast colony containing the plasmid of interest was inoculated into the appropriate SD minimal medium and grown overnight at 30ºC. The cells were centrifuged at high speed for 5 min and then resuspended in 50 µl of Potassium Phosphate (67 mM KH_2PO_4; pH 7.5) provided with the kit. 10 µl Lyticase solution was

added, mixed thoroughly and incubated at 37ºC for 1 hr. 10µl of 20 per cent SDS was added at the end of 1 hr and vortexed vigorously for 1 min. Finally, the samples were treated with one freeze/thaw cycle (-20ºC) to ensure complete lysis. CHROMA SPIN-1000 DEPC-H_2O Columns, provided with the kit, were used to purify the plasmid DNA. After elution of the DNA solution from the column, 5µl of this eluate was used for transformation of chemically competent *E. coli*.

4.12. Cell Culture Techniques

4.12.1. Growth conditions of cells 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 37ºC, with 5 per cent CO_2 and 95 per cent air. Media were kept at 4ºC and warmed to 37ºC 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. For this purpose, the growth medium was aspirated and the cells were washed once with 1X calcium and magnesium-free PBS. In order to remove the monolayer cells from the surface, the cells were treated with trypsin-EDTA solution (0.025 per cent trypsin, 0.5mM EDTA) and incubated at 37ºC for 5 min. 5 volumes of fresh medium were 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.

4.12.2. Thawing

One vial of frozen cell line was taken from -80ºC freezer and thawed under 40ºC (at most) running tap water or immersing the vial into 37ºC water bath. Immediately after cells are thawed, they were transferred to 100 mm culture dishes with 10 ml complete DMEM.

4.12.3. Transfection

Transfections were carried out using the Turbofect transfection reagent (Fermentas) according to DNA: reagent ratios suggested by the manufacturer. For the co-localization assay 250 ng, 500 ng and 1500 ng of each of the two vectors were used for the transfection of the cells in six-well plates.

5. RESULTS

5.1. Preparation of the Bait Vector Constructs

5.1.1. Cloning of Full Length BRI3

The Open Reading Frame (ORF) of BRI3 was PCR amplified from the vector pOBT7/Bri3 using the primers Bri3_7F and Bri3_8R with flanking EcoRI and SalI restriction sites, respectively, for cloning into the yeast two-hybrid vector pGBKT7 (bait vector). Selection of pGBKT7 as the bait vector has enabled us to create out bait construct in which BRI3 is cloned in frame with the GAL4 DNA-BD, by using the primers with the indicated restriction enzyme sites. PCR analysis of BRI3 in three different reactions is indicated in Figure 5.1a. Both the BRI3 PCR product and pGBKT7 vector were double-digested with EcoRI and SalI. After ligation and transformation reactions, the resulting colonies were analyzed by colony PCR. The isolated plasmids corresponding to 3 of the positive colonies were confirmed by sequencing.

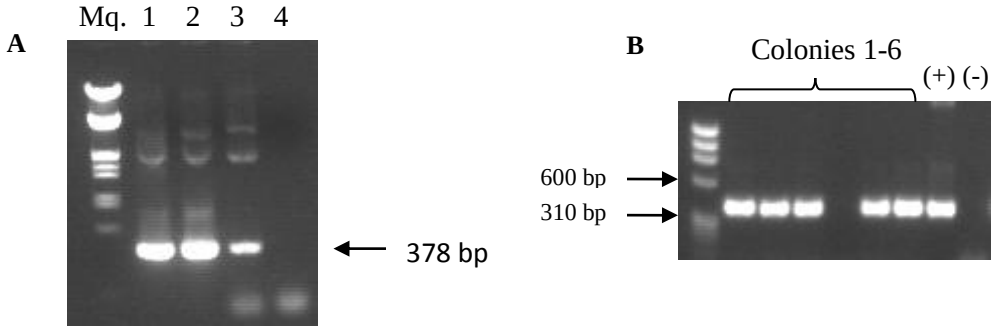


Figure 5.1. (A) PCR amplification of BRI3 using pOBT7/Bri3 vector as the template. Lanes 1-3 represent PCR reactions with 2 mM $MgCl_2$, 4 mM $MgCl_2$ and in the absence of DMSO, respectively. Lane 4 is the negative control. (B) Colony PCR analysis of the selected colonies with BRI3 specific primers. pOBT7/Bri3 was used as the template for positive control of PCR amplification.

5.1.2. Cloning of BRI3 cytoplasmic domain fragment

The cytoplasmic domain fragment of BRI3, which corresponds to amino acid residues 1-59, was PCR amplified from the vector pGBKT7/Bri3, using the Bri3_7F and Bri3/1-59-rev primer pair, with flanking EcoRI and SalI restriction sites, respectively. pGBKT7/Bri3(1-59aa) vector was generated, in which the N-terminal cytoplasmic domain fragment of BRI3 was cloned in frame with the GAL4 DNA-BD, and used as the bait vector in yeast two-hybrid screening.

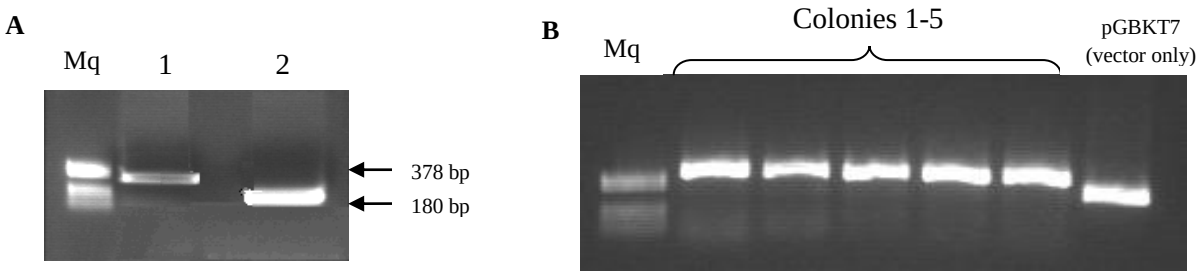


Figure 5.2. (A) Lanes 1 and 2 represent PCR amplification of full-length BRI3 and cytoplasmic fragment of BRI3, respectively. (B) Colony PCR analysis was performed by using T7-M13 primer pair (both on the pGBKT7 vector) in order to detect the presence of the insert.

5.2. Detection of the Bait Protein Expression in Yeast by Western Blotting

Competent yeast cells were transformed with the pGBKT7/Bri3 bait vector and grown on SD/-Trp agar plates since pGBKT7 vector contains the Trp nutritional marker (Figure 5.3b). Wild-type yeasts (AH109 strain) were also plated for control (Figure 5.3a).

In order to determine whether or not out bait protein is expressed well in yeast, Western Blot analysis was performed using the yeast cells transformed with the bait construct. For this purpose, two colonies of pGBKT7/Bri3 transformed yeast from the SD/-Trp plate are inoculated into SD/-Trp liquid medium and allowed to grow overnight. Yeast lysate for Western Blot analysis was prepared. Kaleido marker, which has the indicated

molecular weight standards, was used. Expression of a fusion protein consisting of GAL4 BD (Binding Domain), c-myc tag and full-length Bri3 is detected by Western Blotting (Figure 5.4) using polyclonal rabbit anti-myc antibody (Cell Signaling, Catalog # 2272). As a result, an expected protein band of approximately 31 kDa was observed, corresponding to the fusion protein of GAL4 BD, c-myc and BRI3, together with unspecific bands.

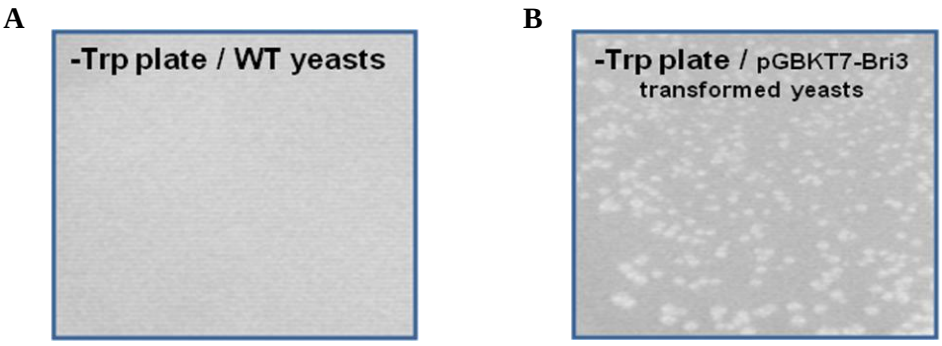


Figure 5.3. Untransformed (wild-type) yeast (A) and pGBKT7-Bri3 transformed yeast cells (B) on SD/-Trp plates.

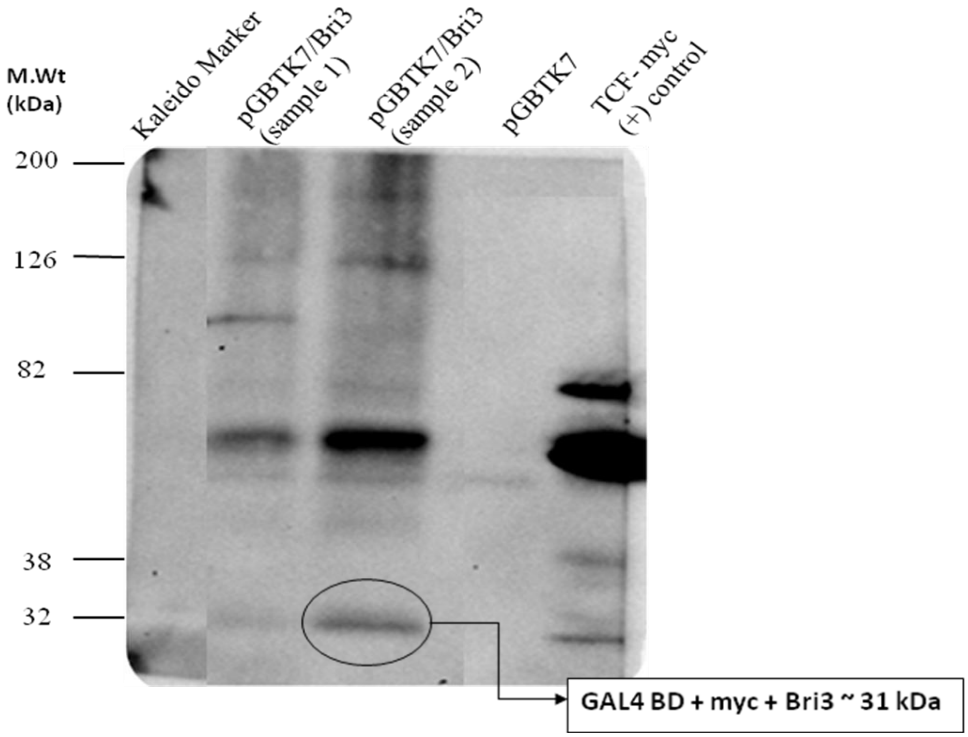


Figure 5.4. Western Blot analysis for the detection of bait protein expression in yeast.

5.3. Control experiments -Autoactivation and Toxicity- prior to Yeast Two Hybrid Assay

Before initiating the Yeast Two-Hybrid assay, in order to confirm that our bait protein does not autonomously activate the reporter genes in the absence of a prey protein in AH109 yeast strain, autoactivation test was performed. The transformed yeast colonies from 3 different DO plates were streaked onto a single SD/-Ade/-His/-Leu/-Trp plate for simplicity (Figure 5.5). The control vectors pGBKT7-53, pGADT7-T and pGBKT7-Lam were provided with the kit. pGBKT7-53 encodes a DNA-BD/murine p53 fusion protein, whereas pGADT7-T encodes an AD/SV40 large T antigen fusion protein. Since the p53 and T antigen proteins are known to be interacting partners, they were used as the positive control. On the other hand, the Lamin C protein encoded by the pGBKT7-Lam vector, does not possess any interaction partner. Therefore, only yeast cells transformed with the positive control vectors (pGBKT7-53 and pGADT7-T) were able to grow on SD/-Ade/-His/-Leu/-Trp agar plates.

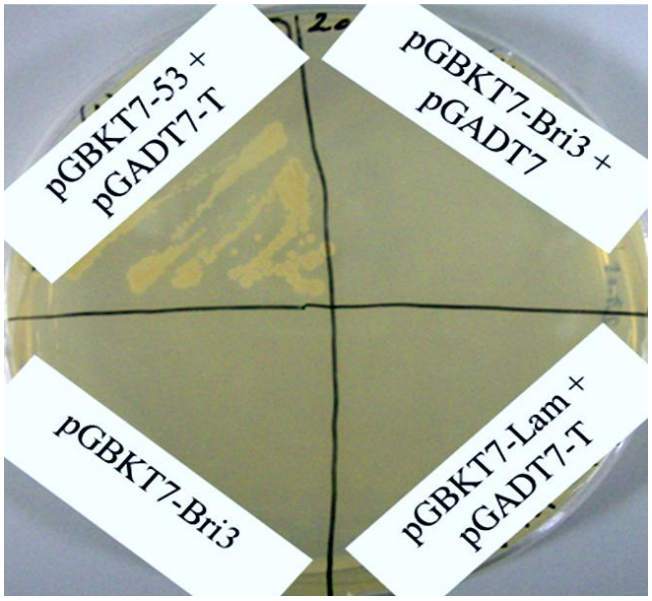


Figure 5.5. Autoactivation test for bait protein. Transformants were streaked on a SD/-Ade/-His/-Leu/-Trp plate without X-α-Gal.

The bait protein BRI3 has also been proved to be non-toxic when expressed in yeast. For this purpose, pGBKT7 (empty) and pGBKT7-Bri3 vectors were separately transformed to competent yeast cells and the transformants were allowed to grow on SD/-Trp agar plates. As a result, yeast colonies of similar size and growth rate were observed in both plates. Furthermore, no noticeable difference was observed when the transformed yeast colonies were inoculated into SD/-Trp medium and grown in liquid cultures.

Table 5.1. Approximate colony number of yeast transformants on the indicated SD agar plates.

Yeasts / Plates	Wild -type + pGBKT7-Bri3	pGBKT7- p53 + pGADT7 - T ag.	pGBKT7- Lam + pGADT7 - T ag.	pGBKT7-Bri3 + pGADT7
-Trp	250	200	180	160
-Leu	0	200	160	175
-Trp, -Leu	0	120	100	80
-Trp, -Leu -Ade, -His	0	75	0	0

5.4. Yeast Mating and Library Screening Leads to the Identification of Candidate Positive cDNA Clones

5.4.1. Library Screening

First mating was performed between the MATa strain (AH109) transformed with the pGBKT7/Bri3 bait vector and the MATα strain (Y187) pretransformed with human liver cDNA library. The estimated number of independent clones screened by this mating was calculated to be $2.6x10^6$. The mated culture was allowed to grow on SD/-Ade/-His/-Leu/-Trp agar plates, so that high stringency was used in order to detect the activation of the reporter genes ADE2 and HIS3. Another mating was also performed with using the

cytosolic N-terminal fragment of BRI3, which corresponds to 1-59 aminoacid residues of BRI3. Library screening was done by the mating of the AH109 strain transformed with pGBKT7/Bri3(1-59aa.) and the Y187 strain pretransformed with human liver cDNA library. A schematic overview of library screening by yeast mating is represented in Figure 5.6.

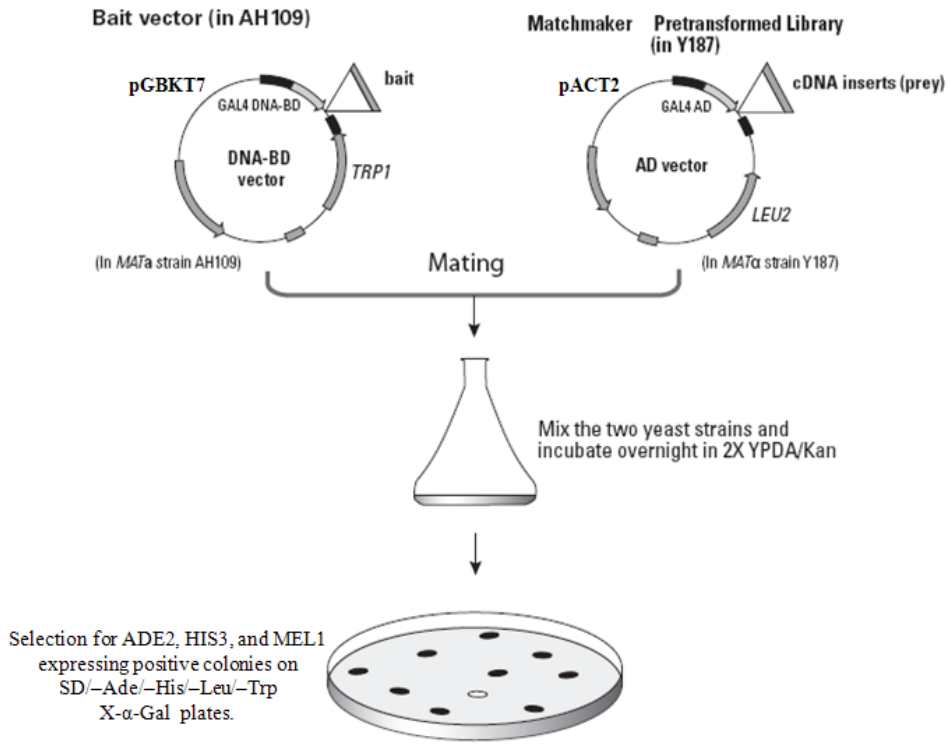


Figure 5.6. Overview of Two-Hybrid Library Screening using Yeast Mating (adapted and modified from Clontech Two-Hybrid systems manual).

First yeast mating (using the full-length BRI3) and two-hybrid library screening initially yielded 90 colonies. On the other hand, the second mating with the cytosolic fragment of BRI3 as bait, resulted in more than 200 colonies. The positive clones obtained from the first mating were re-streaked to single colonies on SD/-Ade/-His/-Leu/-Trp agar plates with X-α-Gal, in order to confirm the phenotype (Figure 5.7). As a result of two-hybrid interactions, in addition to ADE2 and HIS3 reporter genes, MEL1 which encodes the enzyme α-galactosidase will also be expressed. Yeast colonies that express Mel1 turn

blue in the presence of the chromagenic substrate X-α-Gal. Therefore, those single blue colonies were analyzed primarily for being candidate positive colonies.

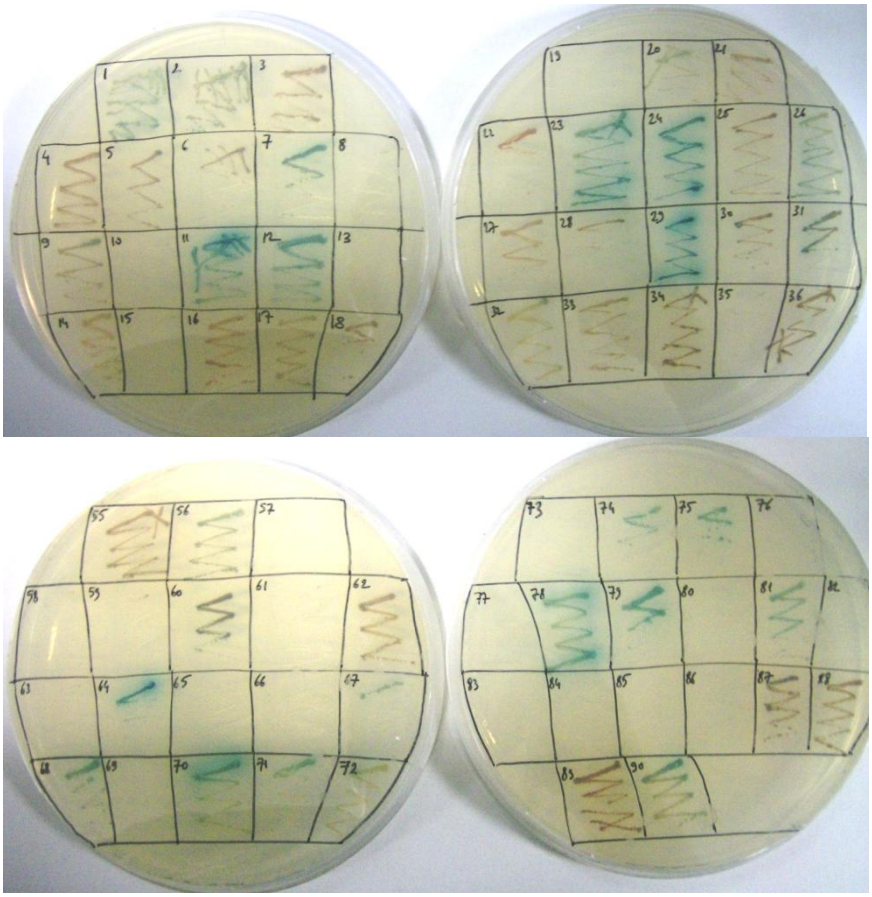


Figure 5.7. Confirmation of phenotype by re-streaking. 26 out of 90 colonies are eliminated after re-streaking since they were unable to grow on SD/-Ade/-His/-Leu/-Trp/ X-α-Gal medium.

5.4.2. Characterization of Candidate Clones

The cDNA inserts corresponding to candidate clones were analyzed by yeast colony PCR and subsequent sequencing of the PCR product. Their identities were revealed by performing a BLAST search of the raw outputs over various databanks in order to find significant matches. Figure 5.8 represents samples of yeast colony PCR from positive colonies of both first and second mating.

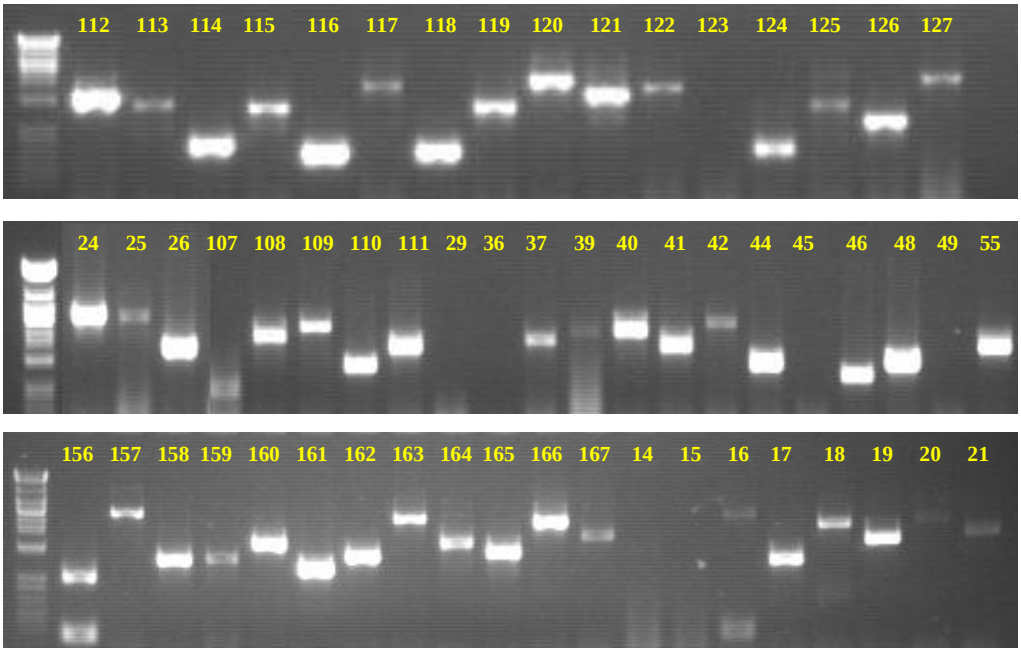


Figure 5.8. Yeast Colony PCR analysis of the indicated colonies. pACT2F and pACT2R primer pair that is present on the prey library vector pACT2 was used for the amplification of the cDNA inserts.

After analysis of the sequencing results by means of homology search, the clones that are “in frame” with the GAL4-AD in the prey vector were determined. A high number of cDNA clones were eliminated at these steps either for being out-of-frame with the GAL4-AD or for being false positives and abundant “sticky” proteins have relatively high number of copies in the library such as albumin, ferritin or dynamin binding protein (further explained in the ‘Discussion’ part).

As a result, 3 candidate positive cDNA clones were determined as the following:

- IFITM3 - Interferon induced transmembrane protein 3
- IL-7R - Interleukin-7 receptor
- MGAT - Mannosyl (α-1,3-) glycoprotein β-1,4-N-acetylglucosaminyl transferase

IFITM3 and IL-7R were determined as candidate interactors with respect to the first mating using the full-length BRI3 as the bait, whereas MGAT emerged as a candidate

interactor from the second mating using the cytosolic N-terminal fragment of BRI3 as bait. The regions of these candidate positive interactants that were fished out in library screening are indicated in Figure 5.9.

Table 5.2. Aminoacid regions of the candidate interacting proteins that were identified by sequencing of the corresponding library prey vector.

	Full-length protein	**Region identified in the prey vector**
IFITM3	133 aa.	Between 31 - 133 aa.
IL-7R	459 aa.	Between 103 – 310 aa.
MGAT	445 aa.	Between 176 – 365 aa.

5.5. Confirmation of the Positive Interaction by Co-Transformation of Bait and Prey Vectors into the Yeast Cells

Co-transformation was performed using selective media in order to verify the positive interactions and distinguish between genuine positives and false positives. The candidate prey vectors responsible for the positive interactions were isolated from yeast cells by using the YeastMaker Yeast Plasmid Isolation Kit. The following pairs of bait and prey vectors together with their controls were co-transformed into the competent AH109 cells by using the YeastMaker Yeast Transformation System.

- pGBKT7/BRI3 + pACT2/IFITM3
- pGBKT7 (empty) + pACT2/IFITM3
- pGBKT7/BRI3 + pACT2/IL-7R
- pGBKT7 (empty) + pACT2/IL-7R
- pGBKT7/BRI3 + pACT2/MGAT

- pGBKT7 (empty) + pACT2/MGAT
- pGBKT7/53 + pGADT7/T (positive control)
- pGBKT7/Lam + pGADT7/T (negative control)

The transformation mixes were spread on SD/-Leu/-Trp (data not shown) and SD/-Ade/-His/-Leu/-Trp agar plates (Figure 5.9). Colony formation can be seen on the plates corresponding to the co-transformants of pACT2/IFITM3 and pACT2/MGAT prey vectors together with the pGBKT7/BRI3 bait vector, whereas no colony was observed on the plate corresponding to the co-transformants of pGBKT7/BRI3 and pACT2/IL-7R vectors.

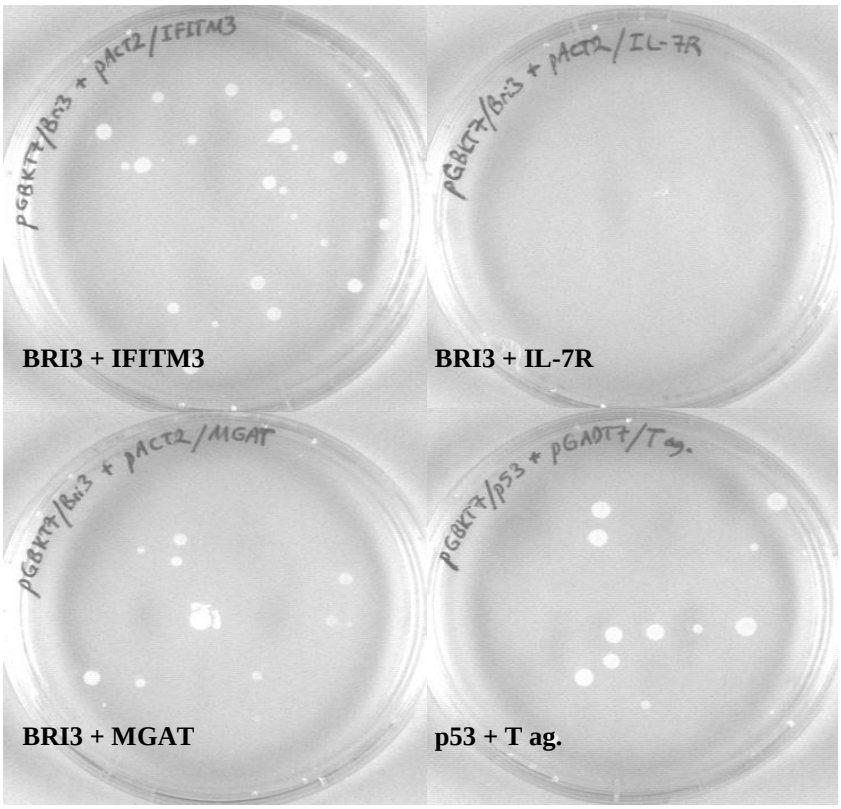


Figure 5.9. Confirmation of positive interactions by Co-Transformation of the indicated bait and prey vectors to competent yeast cells (Only QDO agar plates are shown).

Table 5.3. Co-transformation results for the indicated vectors on DDO and QDO agar plates. (+) denotes presence of colonies, whereas (-) denotes absence of any colony.

	DDO medium (SD/-Leu/-Trp)	QDO medium (SD/-Ade/-His/-Leu/-Trp)
pGBKT7/BRI3 + pACT2/IFITM3	+	+
pGBKT7 (empty) + pACT2/IFITM3	+	-
pGBKT7/BRI3 + pACT2/IL-7R	+	-
pGBKT7 (empty) + pACT2/IL-7R	+	-
pGBKT7/BRI3 + pACT2/MGAT	+	+
pGBKT7 (empty) + pACT2/MGAT	+	-
pGBKT7/53 + pGADT7/T	+	+
pGBKT7/Lam + pGADT7/T	+	-

5.6. Co-expression of BRI3 and IFITM3 in Mammalian Cell Lines

In order to assay for colocalization, BRI3 coding sequence was cloned into the pEGFP-N2 vector in order to express a fusion protein of BRI3-GFP. PCR Amplification of BRI3 from the vector pGBKT7/Bri3 was performed by using the primers BRI3_7F and bcr_gfp_R with flanking EcoRI and ApaI restriction enzyme sites respectively. On the other hand, IFITM3 coding sequence was cloned into the pcDNA3-dsRED vector in order to express a fusion protein of IFITM3-dsRED. IFITM3 was amplified from cDNA by using the primers IFITM_3F and IFITM_3R with flanking HindIII and BamHI restriction enzyme sites respectively. Transfection-gradient midipreps of the prepared constructs and Turbofect transfection reagent (Fermentas) were used for the transfection of Huh7 cell lines. Three transfections for co-expression in Huh7 cells were carried out, in which 250 ng, 500 ng and 1500 ng of each vector were used respectively. Figure 5.10 demonstrates the fluorescent microscopy images of the colocalization assay.

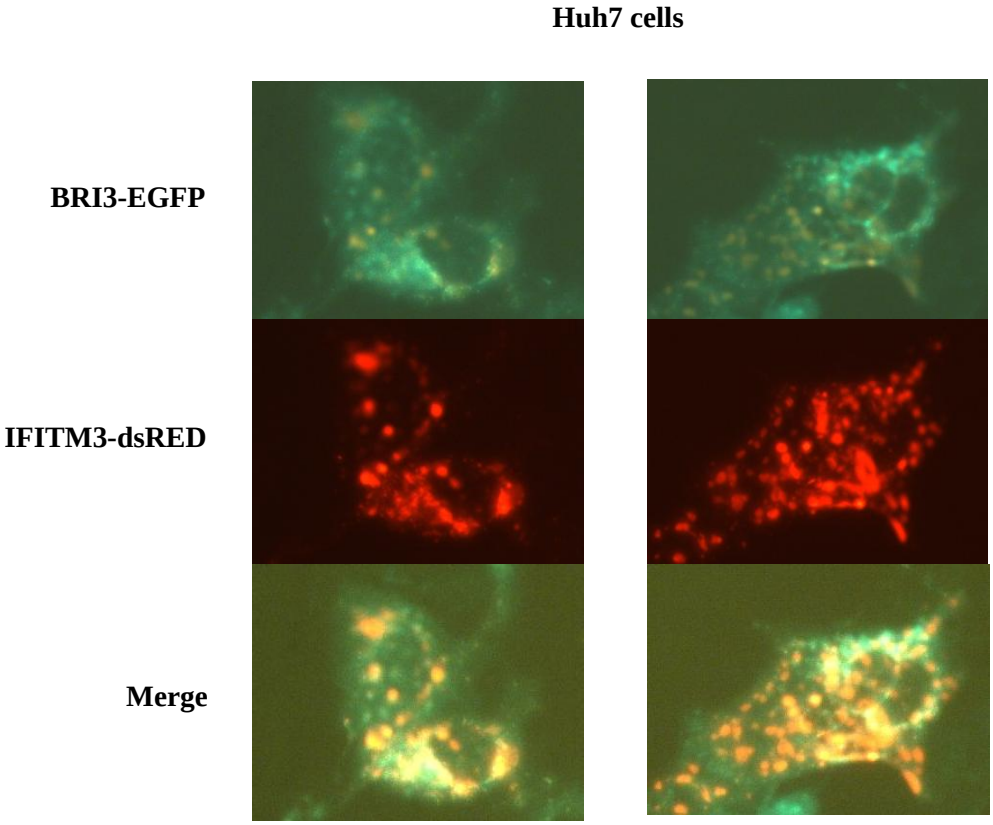


Figure 5.10. Colocalization assay in Huh7 cell lines transfected with pEGFP-N2/BRI3 and pcDNA3-dsRED/IFITM3.

The transfection of Huh7 cells with pEGFP-N2/BRI3 and pcDNA3-dsRED/IFITM3 vectors exhibits that neither BRI3 nor IFITM3 were merely localized in the plasma membrane. The two proteins seemed to be distributed throughout the cytoplasm, with being accumulated more around the nucleus. In fact, a previous research suggested that, BRI3 may be localized in lysosomes and its function may be associated with the lysosomes (Wu *et al*., 2003). In this study, L929 cells were transfected with pEGFP-N2/BRI3, generating the BRI3-EGFP fusion protein, and then the cells were stained with LysoTracker in order to show the subcellular localization of the BRI3-GFP fusion protein in lysosomes.

6. DISCUSSION

The canonical Wnt/β-catenin signaling pathway, named for its most upstream ligands, Wnts and its cytoplasmic component that is a transcriptional activator β-catenin, is a highly conserved pathway and it is involved in various differentiation events during embryonic development, such as axis formation, cellular proliferation, differentiation and morphogenesis. β-catenin is considered to the key molecule in this pathway. Apart from its role in cadherin-based cell adhesion, β-catenin is also essential for the Wnt/Wingless growth factor signaling.

In addition to its functions in vertebrate early development, Wnt/β-catenin pathway has the potential to initiate tumor formation when aberrantly activated. Molecular studies have made it evident that activating mutations of the Wnt/β-catenin signaling pathway are accountable for the cause of approximately 90 per cent of colorectal cancer (CRC), and somewhat less frequently in cancers at other sites, such as hepatocellular carcinoma (HCC). Those characteristics of the Wnt/β-catenin signaling pathway makes the pathway itself and its targets important subjects in cancer research fields.

Mutations mimicking Wnt stimulation which are, in general, inactivating APC mutations or activating β-catenin mutations, result in nuclear accumulation of β-catenin which subsequently complexes with T-cell factor/Lymphoid Enhancing Factor (TCF/LEF) family of transcription factors to activate gene transcription. Expression of the genes like c-myc, cyclinD1, fra1, c-jun, components AP-1 complex and extracellular matrix protease matrilysin, is under the control of TCF/LEF and β-catenin transcriptional complex. Therefore, these genes together with several others are considered as the target genes of the canonical Wnt/β-catenin signaling pathway.

In order to identify novel transcriptional targets of the Wnt/β-catenin pathway, SAGE screen was recently carried out in our laboratory. As the result of analyses, a number of genes were determined to be either upregulated or downregulated significantly by means of mimicking the active status of the Wnt/β-catenin pathway.

BRI3 was determined to be one of the putative transcriptional targets of the Wnt/β-catenin signaling pathway. It has been selected from the SAGE screen owing to the fact that overexpression of the mutant, and thus more stable form of β-catenin resulted in increased BRI3 gene expression. Supplementary data obtained from lithium treatment assays using Huh7 cell lines, luciferase reporter assays, overexpression of Wnt ligands and chromatin immunoprecipitation (ChIP) assay by using the anti-β-catenin antibody (Kavak *et al.*, unpublished data). These recent findings combined with the very low characterization level of BRI3 with respect to previous literature, have prompted us to provide clues for the functional study of BRI3 within the Wnt/β-catenin pathway.

The aim of this study was to discover novel interaction partners of the BRI3 protein in order to shed light on the exact action mechanism of BRI3. Furthermore, as being a nerve-specific member of the BRI protein family, detailed knowledge on the function of BRI3 can be of critical importance for the identification of its possible roles in neuronal development and in certain neural diseases, considering the fact that Wnt/β-catenin pathway has also been established to have essential role in brain development and neurodegeneration (De Ferrari and Moon, 2006).

Additionally, previous studies that primarily lead to the identification of BRI3 as a novel gene of the BRI gene family, have suggested the participation of BRI3 in TNF-α induced cell death pathway. Therefore, a further aim of this study can be stated as the discovery of a possible interaction between the Wnt/β-catenin signaling pathway and TNF-α induced cell death pathway.

As a first step towards the elucidation of the action mechanism of BRI3, we screened a human liver cDNA library by yeast two-hybrid assay using BRI3 as bait, with an expectation to find novel binding partners of BRI3. For this purpose, two constructs were prepared, one encoding the full-length BRI3 and the other one encoding the cytoplasmic N-terminal fragment of BRI3, both of which are fused to the GAL4-BD in the bait vector pGBKT7.

The two constructs were used as bait in two separate two-hybrid library screenings. The screening of very high numbers of coding sequences (more than $1x10^6$ independent

clones) with a relatively simple protocol like yeast mating is very convenient, considering the fact that the assay takes place *in vivo* and there is no need for protein purification. On the other hand, it only gives a first hint about a possible interaction so additional methods must be utilized for confirmation of the positive interaction. Furthermore, membrane proteins have been known to possess an inherent disadvantage in using the yeast two-hybrid assay.

After determining that our bait protein is expressed well in yeast by means of Western Blotting from yeast protein extract, control experiments were initiated and the bait proteins were tested for autoactivation and toxicity. Both the full-length BRI3 and the cytosolic part of BRI3 did not elucidate any self activation or toxic effects in yeast.

Yeast mating was preferred instead of transformation due to being more practical and relatively high efficiency procedure (Soellick and Uhrig, 2001). Furthermore, we had both the two yeast strains -MATa and MATα- in which the MATα strain was present as frozen aliquots of yeast cells carrying the cDNA library. As a result of two-hybrid library screening by yeast mating, colonies were selected on high stringency growth media and the candidate interaction partner corresponding to the positive colonies were characterized by means of yeast colony PCR and sequencing. The mating with the full length BRI3 as bait, resulted in 64 colonies after restreaking, whereas the mating with the cytosolic domain of BRI3 as bait, resulted in the appearance of a very high number of colonies, which were approximately 200. More than 140 of those colonies were analyzed by colony PCR and subsequent sequencing has identified a relatively high rate of false positives compared to that in the first mating. This might be due to the unspecific binding of the cytosolic N-terminal domain of BRI3 to a number of abundant and ‘sticky’ proteins expressed in the liver cDNA library, since certain proteins have a relatively high rate of expression level depending on the tissue. In fact, among the fished-out proteins in library screening, the frequently appearing ones were found to have a much higher expression level in liver as suggested by their EST profiles with respect to body organs/tissues. Nevertheless, homology search followed by the elimination of cDNA clones that were out-of-frame with the GAL4-AD and those that have an unusually high level of expression in liver, resulted in the identification of a few possible interacting partners. With respect to the second yeast mating with the N-terminal domain of BRI3 as bait, we were able to identify MGAT -

Mannosyl (α-1,3-) glycoprotein β-1,4-N-acetylglucosaminyl transferase- as a candidate positive interactor, which was also confirmed by cotransformation into yeast cells (Figure 5.9). A further reason for MGAT to attract our attention was that it has been enumerated as one of the genes that demonstrated differential expression level with respect to the SAGE analysis performed previously. Thus, MGAT can also be considered as a candidate for being a transcriptional target of the Wnt/β-catenin pathway, but additional data is still required for verification. Apart from MGAT, another positive candidate, namely IFITM3 (Interferon induced transmembrane protein 3), has been fished out by using the full length BRI3 as bait. As shown in Figure 5.9, the positive phenotype was confirmed in yeast cells by cotransformation with the bait vector/BRI3 and prey vector/IFITM3. On the other hand, cotransformation of the competent yeast cells with the bait vector/BRI3 and prey vector/IL-7R, failed to activate the reporter genes and no colonies were observed (Figure 5.9). Therefore, IL-7R was eliminated as a candidate interactant.

According to previous literature, IFITM was identified as a new molecular marker in human colorectal tumors and it has been stated that IFITM gene expression is controlled by the Wnt/β-catenin signaling in mouse and human intestinal epithelium (Andreu *et al*., 2006). Furthermore, the results of a recent study indicate elevated IFITM3 expression in colon cancer cells compared to normal colon cells. The data obtained from this study suggest that IFITM3 plays an important role in early colon cancer development (Fan *et al*., 2008).

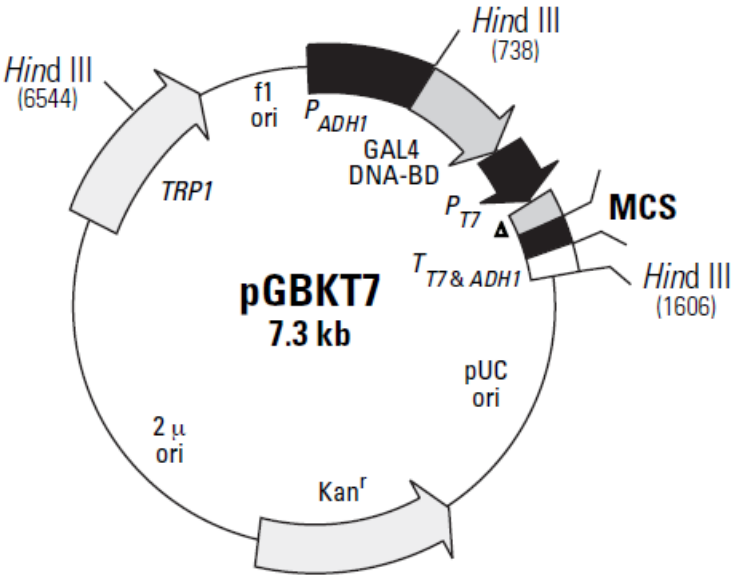
As mentioned earlier, the result of cotransformation to the yeast cells has confirmed IFITM3 and MGAT as a candidate positive interactor but it has eliminated IL-7R due to lack of growth on selective media. At this point, IFITM3 was chosen to further continue with, in order to obtain supplementary data for verification. For this purpose, colocalization assay was performed using mammalian cell lines. After co-expression of BRI3-GFP and IFITM3-dsRED fusion proteins in Huh7 cells, their fluorescent markers were observed to overlap at the subcellular level, which is mostly perinuclear (Figure 5.10). However, with regard to the co-expression data, the exact localization of the two proteins still remains unclear. In fact, a previous study has been carried out in order to determine the subcellular localization of BRI3 (Wu *et al*., 2003). The results obtained from this study suggested that BRI3-GFP fusion protein localizes in the lysosomes within the

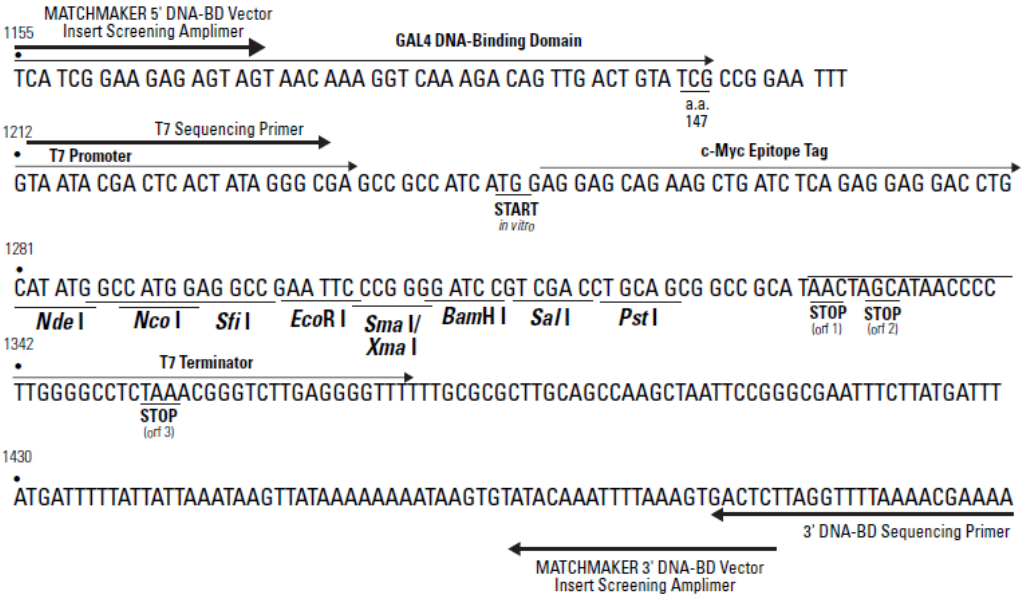
cell and the function of BRI3 may be related to lysosomes. Furthermore, a more recent study was carried out in order to examine the subcellular localization patterns of BRI3 and one of its interaction partners, which is human SCG10 (superior cervical ganglia, neural specific 10). Co-expression of BRI3-EGFP and SCG10-dsRED in neuroblastoma SH-SY5Y cells resulted in a mainly perinuclear colocalization (Gong *et al*., 2007).

To sum up, results obtained in this study suggest two candidate interaction partners for BRI3, which are IFITM3 and MGAT. For the future prospects, those potential interactions need to be further investigated and verification should be done accordingly, by means of coimmunoprecipitation using mammalian cell lines. Moreover, BRI1 and BRI2 can be used as bait proteins in yeast two-hybrid screenings to test whether these proteins also interact with the fished-out candidate interacting proteins, IFITM3 and MGAT. With regard to the previous literature, BRI3 can be considered as a poorly characterized protein. The identification of its interaction partners will be a first step for the determination of the exact action mechanism of BRI3, both as being a novel target of the Wnt/β-catenin pathway and playing an important role in the TNF-α mediated cell death pathway. Furthermore, a possible cross-talk between the Wnt/β-catenin signaling pathway and TNF-α mediated cell death pathway can be elucidated eventually. For the further characterization of BRI3 and its confirmed interaction partner, it is worth to expand the studies with *in vivo* xenograft experiments on nude mice in order to determine their roles in tumorigenesis. Also, in order to investigate the function of BRI3, especially in carcinogenesis-related cellular processes, further experiments can be carried out including knock-down of BRI3 by RNA interference (RNAi) method, knock-out and also overexpression analyses.

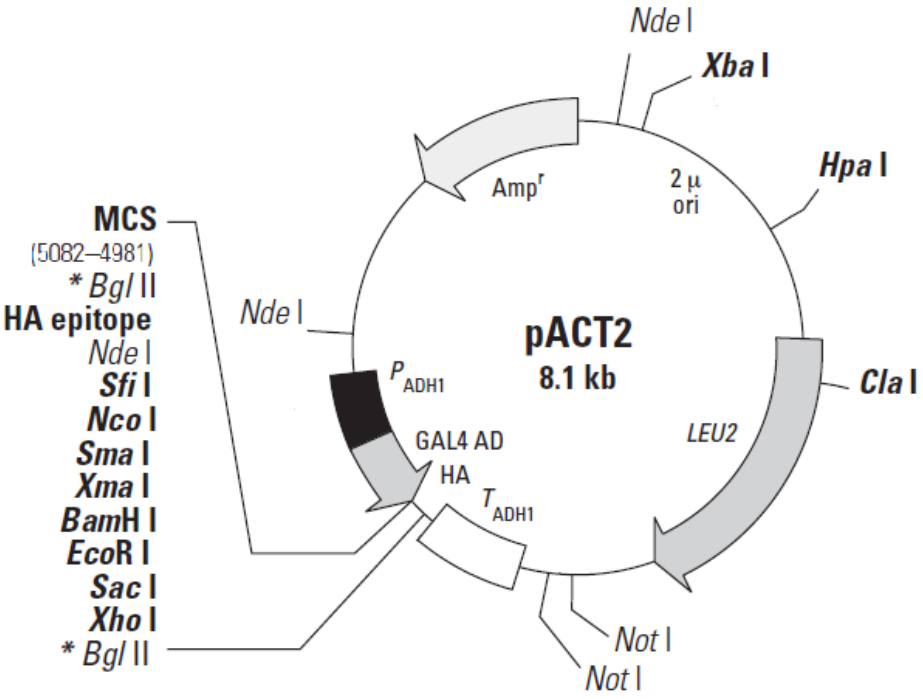
APPENDIX A: VECTOR MAPS

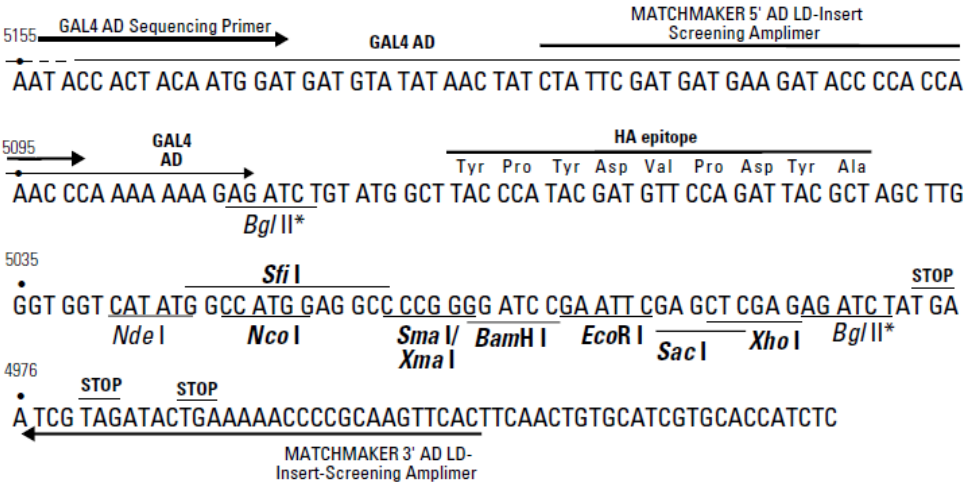
pGBKT7 (Restriction Map and Multiple Cloning Site)





pACT2 (Restriction Map and Multiple Cloning Site)





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