IDENTIFICATION OF PROTEIN PARTNERS OF ACIDIC CALPONIN

by

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To my family

ABSTRACT

IDENTIFICATION OF PROTEIN PARTNERS OF ACIDIC CALPONIN

The up-regulation of acidic calponin gene in different brain tumors has been revealed by a bioinformatic tool termed Oncoreveal. Expression pattern has been further confirmed with an independent brain tumor panel by our group members. Previous studies reported the interaction of acidic calponin with Smad1/5 and the negative effect of this interaction on TGFβ/BMP signaling pathway. A link between TGFβ/BMP signaling pathway activation and reduction of brain tumor populations, hints at a role of acidic calponin in tumorogenesis. Moreover, non-tissue specific expression of acidic calponin raised our interest in investigating the function of acidic calponin in liver cells. As a starting point, identification of protein partners of acidic calponin by using yeast two hybrid screening was performed. Screening pretransformed human liver cDNA library resulted in the identification of ring finger protein 10 (RNF10) and RAS p21 protein activator (RASA1) proteins. The interaction between RNF10 and acidic calponin was further verified by co-expression and co-immunoprecipitation experiments. Elucidation of protein partners, which is the main aim of this study, may provide clues about the role of acidic calponin in liver cells. Due to the cross communication of TGFβ/BMP signaling pathway with variety of signaling pathways at different stages further studies may be conducted to investigate the function of acidic calponin in different signaling pathway context, too.

ÖZET

ASİDİK CALPONİN ETKİLEŞİM İÇERİSİNDE BULUNDUĞU PROTEİNLERİN TANIMLANMASI

Asidik calponin proteini kodlayan genin anlatımının farklı beyin tümörlerinde arttığı labımızda geliştirilen Oncoreveal adlı araç tarafından tespit edilmiştir. Bu sonuçlar farklı beyin dokuları kullanılarak teyit edilmiştir. Yayınlanmış olan makalelerde asidik calponin proteinin TGFβ/BMP yolağında sinyal iletimini sağlayan Smad1/5 proteinleri ile etkileşim içerisine girerek bu yolağın çalışmasını engellediği tespit edilmiştir. TGFβ/BMP yolağının çalışmasını artırarak beyin tümörlerinde azalma gözlenmesi bu yolak ile birlikte onu etkileyen proteinlerin de beyin tümörlerinde önemli görevler alabileceği düşünülmekte. Asidik calponin bu yolakla etkileşim içerisinde bulunması ve beyin tümörlerinde anlatımında artış göstermesi bu genin de beyin tümörlerinde bir önem taşıyabileceğini düşündürmekte. TGFβ/BMP yolağının farklı yolaklar ile etkileşim içerisinde bulunması ve asidik calponin proteinin farklı dokularda üretildiği göz önüne alarak bu protein karaciğer dokularındaki görevini tespit etmeyi amaçlamaktayız. Bu genel amacı gerçekleştirme yolunda ilk adım olarak asidik calponin proteinin etkileşim içerisinde bulunduğu proteinleri tespit ederek asidik calponinin karaciğer dokusundaki görevi ile ilgili ipucu elde edilmeye çalışıldı. Bu amacı gerçekleştirmek için insan karaciğer kütüphanesi maya iki hibrid yöntemi kullanılarak tarandı. Asidik calponin proteinin ring finger protein 10 (RNF10) ve RAS p21 protein activator (RASA1) proteinleri ile etkileşim içerisinde olduğu tespit edildi. RNF10 ile olan etkileşim memeli hücreleri kullanılarak birlikte immün çöktürme yöntemiyle teyit edilmiştir. Asidik calponin etkileşim içerisinde bulunduğu diğer proteinlerin tespit edilmesi hem karaciğer hem de başka yolaklar ile bir etkileşimin olup olmadığını tespit etmek için yardımcı olacaktır.

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LIST OF ABBREVIATIONS

AD	Activator Domain
Ade	Adenine
Amp	Ampicillin
APC	Adenomatous Polyposis Coli
APS	Amonium Perdulfate
BD	DNA Binding Domain
BMP	Bone Morphogenetic Protein
bp	Base Pair
BPB	Bromophenol Blue
$CaCl_2$	Calcium Chloride
cDNA	Complementary Deoxyribonucleic Acid
CNN3	Acidic Calponin
CKIα	Casein Kinase Iα
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleosidetriphosphate
EDTA	Ethylenediaminetetraacetate
EtOH	Ethanol
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
Leu	Leucine

LiAc	Lithium Acetate
LRP	Low Density Lipoprotein receptor
mg	Milligram
$MgCl_2$	Magnesium Chloride
µg	Microgram
µl	Microliter
µM	Micromolar
min	Minute
ml	Millilitre
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
OD	Optical Density
pAb	Polyclonal Antibody
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PMSF	Phenylmethylsulphonyl fluoride
QRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNF10	Ring Finger Protein 10
rpm	Revolution per Minute
RT-PCR	Reverse Transcriptase - 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
TEMED	N, N, N, N-tetramethylethylenediamine
Trp	Tryptophan
YPD	Yeast extract peptone dextrose

YPDA	Yeast extract peptone dextrose with adenine

1. INTRODUCTION

1.1. Search for Cancer Related Genes

To identify novel targets of Wnt/β-catenin signaling in liver cancer cells (Huh7), gene expression profiling was conducted in our laboratory (Kavak *et al*., 2010). For this purpose, mutant β-catenin (S33Y) that is phosphorylation and degradation resistant due to C to A missence mutation which change Ser33 to Tyr was transfected to Huh7 cells. Those cells along with Huh7 cells transfected with pCI-Neo empty vector have been injected into imunodeficient nude mice and large tumor formation in nude mice was observed. Those cells were further used for genome-wide microarray and SAGE analysis, where the results were analyzed by bioinformatic tool. The results of these analyses were subjected to in silico promoter analysis where the promoter regions of differentially expressed genes were searched for TCF binding elements (TBS). The selected Wnt/β-catenin signaling putative target genes were further analyzed by real time, RT-PCR, luciferase assay, chromatin immunoprecipitation assay (ChIP), and lithium threatment experiments (Kavak *et al.,* 2010).

1.2.1. SAGE

SAGE (Serial Analysis of Gene Expression) is a method that can be used to quantitatively analyze gene expression profiles of different samples. It can be applied to find candidate cancer-related genes by comparing gene expression profiles of normal and tumor tissues (Yamashita *et al*., 2008 and Porter *et al*. 2006). In comparison to microarray, SAGE is not based on knowledge of the genes to be analyzed. It is based on mRNA of specific tissues or cells. The principle of this technique involves formation of short sequence tags of mRNAs. These tags are combined to form long concatemers that can be cloned and sequenced. Sequencing data of the concatemers provides quick identification of tags and corresponding genes. The number of tag provides quantification of specific gene that was expressed in analyzed sample (Maillard *et al.,* 2005).

1.2.2. Oncoreveal

A bioinformatic tool named Oncoreveal was developed by Dr. Erşen Kavak. The tool utilizes publicly available SAGE data to list genes that are over/under-expressed in different types of tumors. It suggests genes that may be considered as candidate for specific type of tumors. One of these tumor-related candidate genes is *CNN3* gene encoding an actin-binding acidic calponin. In Oncoreveal, it was revealed that *CNN3* gene is over-expressed in several tumors. These are Astrocytoma Grade 2, Astrocytoma Grade 3, Medulloblastoma, Glioblastoma, Ependymoma On the other hand, down-regulation of *CNN3* gene in Breast Carcinoma was also observed (Kavak *et al.,* 2010).

The expression pattern of *CNN3* gene obtained from Oncoreveal predictions was further verified by an independent brain tumor panel. RT-PCR and QRT-PCR methods were used to compare expression pattern of *CNN3* gene in several type of brain tumors and non-tumor brain tissues. The result obtained from brain tumor panel has confirmed the increase of *CNN3* gene expression in brain tumors in comparison to normal tissues (Kavak *et al.*, 2009).

1.3. Calponin Family

Calponin is an actin-binding protein that was first identified in chicken gizzard smooth muscle (Takahashi *et al*. 1986). It has been characterized as an F-actin, tropomyosin and calmodulin-binding protein (Takahashi *et al*., 1988; Winder *et al*., 1990). Its role in muscle contraction, stabilizing the actin cytoskeleton, and signaling has been proposed (Abe *et al.,* 1990; Horiuchi *et al.*, 1991; Gimona *et al.,* 2002).

The structure of calponin is composed of four major domains. The first domain is the Calponin Homology (CH) domain located in amino-terminus. This domain is part of various cytoskeletal and signal transduction proteins including utrophin (Gimona *et al.,* 2002). It facilitates binding to Ca^{2+}/calmodulin, mitogen-activated protein kinases (MAPKs), and tropomyosin (Winder *et al.,* 1993). The second domain is actin-binding site 1 (ABS1) which mediates actin binding together with the third domain. The interaction between actin and calponin is performed predominantly by this domain. The third domain

consists of three Calponin-like repeats (CLIK) motifs in the carboxyl-terminal of calponin (Rozenblum *et al.,* 2009). The fourth domain which determines distinct isoelctrical points of calponin proteins is highly variable region located at the end of carboxyl-terminus. Depending on carboxyl-terminus, three isoforms of calponin encoded by different genes have been identified (Strasser *et al*. 1993).

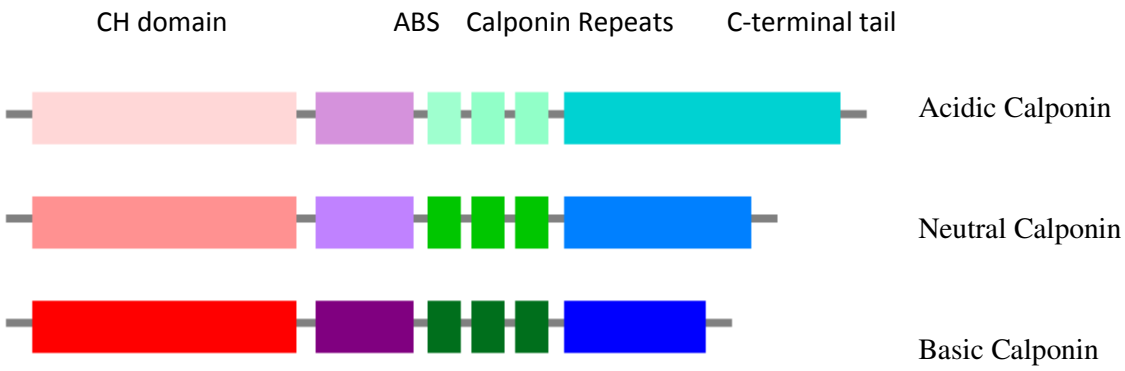


Figure 1.1. Schematic representation of three calponin isoforms. CH, calponin homology domain; ABS, actin binding surface

Table 1.1. Characteristics of different calponin isoforms

Calponin Isoform	Number of amino acids	Molecular Mass (Da)	Isoelectrical point	Chromosome location
Acidic	329	36,413	5.51	1p21
Neutral	309	33,697	7.58	19p13
Basic	297	33,170	8.99	19p13

1.2.1. Basic Calponin

The first calponin identified and characterized was basic isoform of calponin. It is predominantly expressed in differentiated smooth muscles (Takahashi *et al*., 1988). Its role in smooth muscle contraction has been proposed based on its inhibitory property on actin-activated myosin ATPase activity (Winder *et al.,* 1998). A positive correlation between

survivels of patients with osteosarcoma and expression of basic calponin in tumors was observed (Yamamura *et al.,* 1998). Several studies on tumor and metastasis revealed the importance of basic calponin as tumor suppressor (Rozenblum *et al.,* 2008).

1.2.2. Neutral Calponin

The second calponin isoform is neutral calponin. Its expression in cardiomyocytes and other tissues including fibroblasts was denoted (Masuda *et al*., 1996). On the other hand, its function within cell has not been investigated intensively. Depending on localization studies, its role in the organization of actin cytoskeleton in non-muscle cells was proposed (Fukul *et al.,* 1997; Danninger *et al.* 2000). In a zebra fish model, it was shown that over-expression of neutral calponin increases endothelial cell migration (Tang *et al.,* 2006).

1.2.3. Acidic Calponin

The third isoform of calponin with long carboxyl-terminus is acidic calponin (Applegate *et al*., 1994). Like neutral calponin, its expression is not tissue specific. Its expression in variety of rat and ferret tissues, including brain, liver, kidney, lung, heart, aorta has been observed (Applegate *et al.,* 1994; Trabelsi-Terzidis *et al.,* 1995; Appel *et al*., 2010). Its accumulation in glial cells and astrocytes of the cortex, hippocampus and cerebellum in rat brain was detected (Plantier *et al.,* 1999). It was suggested that acidic calponin is an important factor that regulate dendritic spine morphology, density, and synaptic activity by reorganization of the actin cytoskeleton (Rami *et al.,* 2006).

Interaction of acidic calponin with F-actin in a bacterial system and its localization at actin filaments in fibroblast cells was demonstrated (Applegate *et al.,* 1994; Appel *et al.,* 2010). Moreover, change in cell morphology in HEK 293 cells transfected with acidic calponin by actin filament reorganization was reported (Ferhat *et al.,* 2001). In a recent article, the inhibition of fibroblast migration in vitro wound healing assay by acidic calponin knockdown was observed. Based on these finding it has been proposed that acidic calponin enhances cell motility by interacting and activating extracellular signal-related kinase (ERK)1/2 which is a member of the MAPK family (Appel *et al*., 2010).

Acidic calponin was identified as a novel Smad-1 and Smad-5 binding protein in chondrocytes. It was suggested that this interaction function as negative regulatory mechanism for the BMP signaling pathway (Haag *et al.,* 2007).

1.3. BMP Signaling Pathway

Bone morphogenetic proteins (BMP) are members of the Transforming Growth Factor β (TGFβ) superfamily. These cytokines have important functions in brain development, liver, kidney, bone, and cartilage (Miyazono *et al.*, 2010; Shimogori *et al.*, 2004). BMPs transducer their intracellular signal through BMP receptors. Binding of BMP ligand activates transmembrane serine/threonine kinase receptors, which phosphorylate the transcription factors Smad1/5/8 at their carboxyl terminal serines. Phosphorylated Smads bind to Smad 4 and translocate into the nucleus where activation of BMP signaling target genes occurs.

The pathway induces its function by interacting with other signaling pathways. As an example, the cross-talk between BMP and Wnt/β-catenin pathway can be given. Deregulation of Wnt/β-catenin signaling pathway due to mutation in the members of the pathway is reason for development of abnormalities and cancer. As examples, LRP5 mutation that was correlated with abnormal bone density and mutations in β-catenin and APC genes, which were observed in hepatocellular carcinomas and human colon cancers can be mentioned (Nusse, 2005, Giles *et al*., 2003). The tumor formation was correlated with mutations in APC, axin and β-catenin that induce stabilization of β-catenin a key player of this pathway (Klaus *et al.,* 2008).

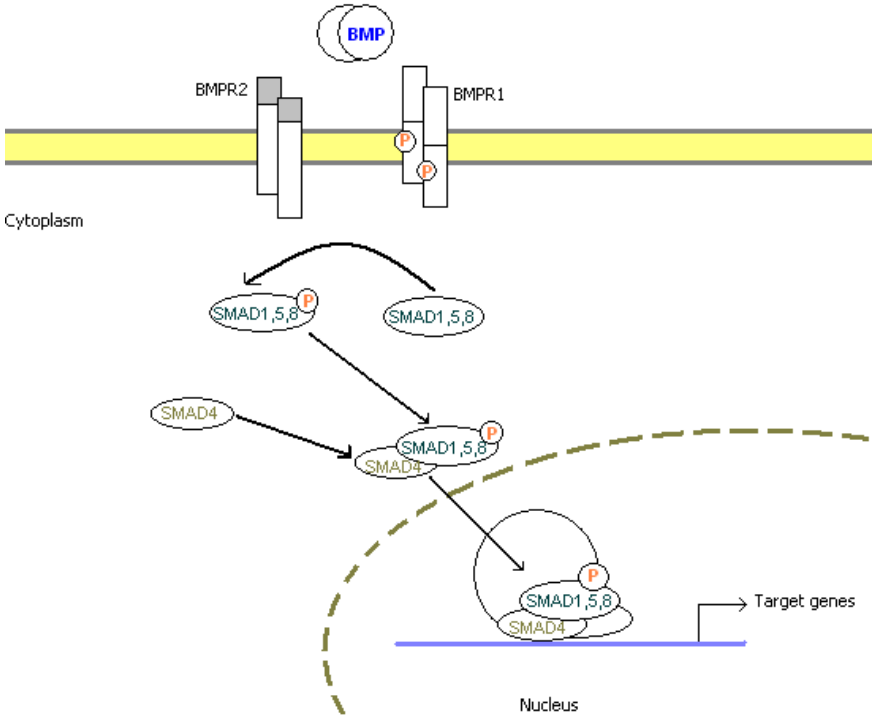


Figure 1.2. Bone morphogenetic protein (BMP) signaling pathway

Wnt/β-catenin pathway and TGFβ/BMP signaling pathways influence each other at different level such as ligand expression. It was shown that a complex of Smads/β-catenin/LEF induces expression of genes which are important in neural development (Hussein *et al.,* 2003). Moreover, it was hypothysed that BMP signal responsive genes may act as Wnt signaling inhibitors by interacting with Dhv (Andersson *et al*., 2008). Identification of molecules that influence or are influenced by these pathway may help to figure out the underlying reasons for diseases and development of novel therapeutic treatments.

1.4. Yeast Two-Hybrid Screening

Yeast two-hybrid screen has been developed in 1989 by Fields and Song as system for detecting in vivo protein-protein interaction (Fields *et al*., 1989). It is used to assess protein-protein interaction between any two proteins that interact. It also has the potential to identify cellular protein partners of a newly identified protein (Gietz *et al.,* 1997). Yeast two hybrid system is very cost effective, convenient to use, and easily adaptable for requirements (Suter *et al*., 2008).

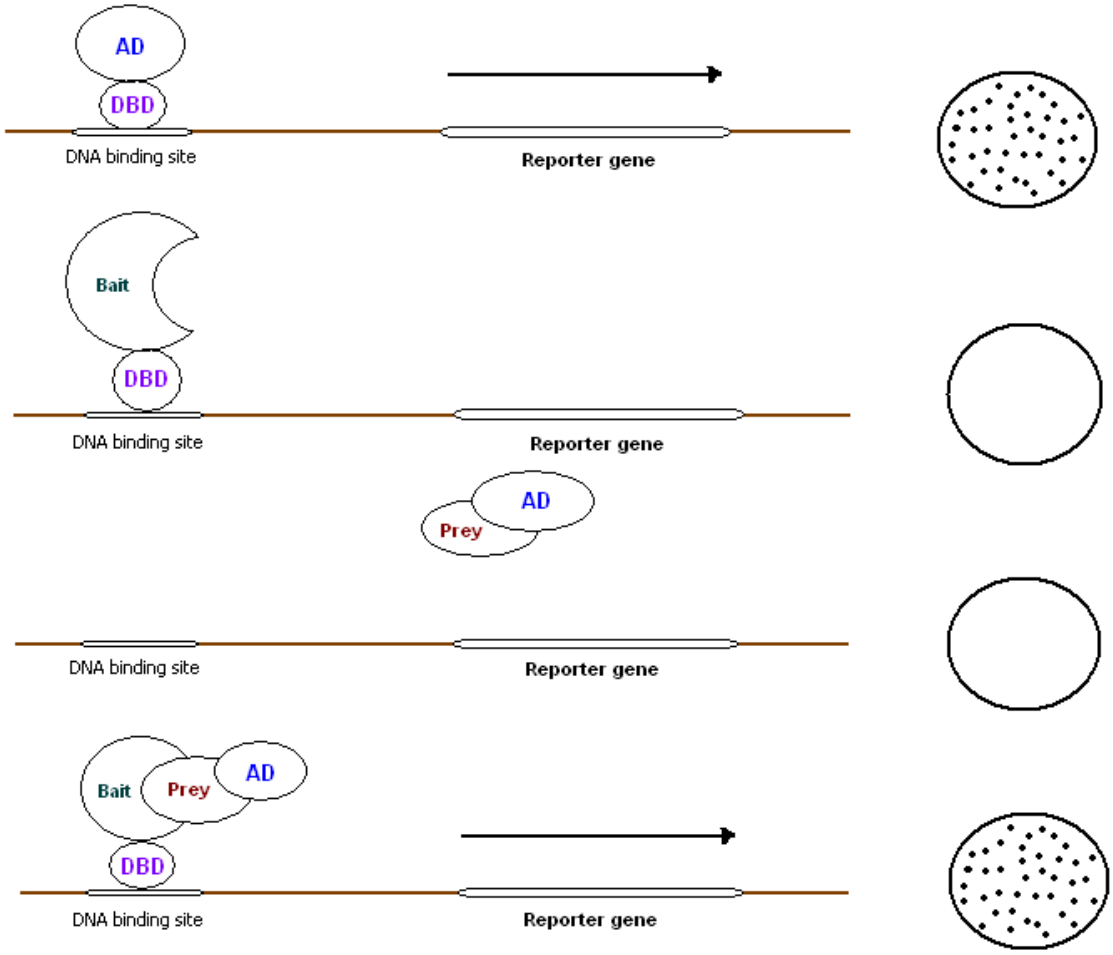


Figure 1.3. Schematic representation of Yeast Two-Hybrid Screening. DBD, DNA binding site; AD, activating domain

The system utilizes the yeast transcriptional factor GAL4, a protein with two functional domains. It is based on the ability of separate domains to form a functional transcription factor when brought into proximity. In this system, the bait protein is expressed as a fusion to the DNA binding domain (BD) of the GAL4 protein which interacts with DNA sequence within the promoter region. The prey protein is expressed as a fusion to transcriptional activating domain (AD) of GAL4 protein which stimulates transcription of reporter genes. When the bait and prey proteins interact, BD and AD of GAL4 protein come close and form a functional transcription factor. Reconstitution of transcription factor induces reporter gene expression and survival of yeast strain in selective medium.

2. PURPOSE

The over-expression of acidic calponin in different brain tumors was detected by Oncoreveal, a bioinformatic tool. The same expression pattern was observed in anindependent brain tumor panel. The correlation of acidic calponin with TGFβ/BMP signaling pathway which has an inhibitory role in brain tumors and cross communication of this pathway with other cancer related signaling pathways catched our interest on the role of acidic calponin in liver cells. To find the interacting partners of acidic calponin in liver we screened a human liver cDNA library by yeast two hybrid assay by using CNN3 as a bait.

3. MATERIALS

3.1. Buffers and Solutions

3.1.1. Agarose Gel Electrophoresis

Table 3.1. Agarose gel electrophoresis buffers and solutions

50X Tris-acetic acid EDTA (TAE)	2 M Tris-acetate
	50 mM EDTA
	pH 8.5
TE Buffer	10 mM Tris–HCl
	1 mM EDTA, pH 8.0
Ethidium bromide (EtBr)	10 mg/ml
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 Fermentas (Burlington, Canada)

3.1.2. Western Blot

Table 3.2. Western blot bufferes and solutions

10 per cent SDS-PAGE gel (running gel)	10 per cent Acrylamide : Bisacrylamide (37.5:1)
	375 mM Tris-HCl (pH 8.8)
	0.1 per cent SDS
	0.1 per cent Ammoniumm persulfate (APS)
	0.1 per cent N, N, N, N-

Table 3.2. Western blot bufferes and solutions (continued)

	Tetramethylethylenediamine (TEMED)
5 per cent SDS- PAGE (stacking gel)	5 per cent Acrylamide : Bisacrylamide (37.5:1)
	125 mM Tris-HCl (pH 6.8)
	0.1 per cent SDS
	0.1 per cent APS
	0.1 per cent TEMED
1X SDS Sample Buffer	2 per cent SDS
	80 mM Tris-HCl (pH 6.8)
	20 per cent Glycerol
	10 per cent β-mercaptoethanol
	0.005 per cent Bromophenol blue
Electrophoresis Buffer	25 mM Tris base
	192 mM Glycine
	0.1 per cent SDS
Transfer Buffer	200 mM Glycine
	25 mM Tris
	10 per cent Methanol
Tris Buffered Saline with Tween 20 (TBST)	150 mM NaCl
	20 mM 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
Coomassie Blue Fixing-Staining Solution	50 per cent Methanol
	0.05 per cent Coomassie R250
	10 per cent Acetic acid

Table 3.2. Western blot bufferes and solutions (continued)

Coomassie Blue Destaining Solution	5 per cent Methanol
	7 per cent Acetic acid
Ponceau S Staining Solution	0.5 per cent Ponceau S
	1 per cent Acetic acid

3.1.3. Bacterial Media

Table 3.3. Bacterial media 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
	10 g NaCl
	15 g Agar
	Distilled water up to 1 L
	Autoclaved
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
	Sterilized by filtration
	Stored at -20°C

3.1.4. Yeast Media and Solutions

Table 3.4. Yeast media and solutions

X-α-Gal Stock	20 mg/ml in dimethyl formamide
	Stored at -20°C in the dark
L-Adenine Hemisulphate	0.2% stock solution
	0.003% working consentration
YPDA Liquid Media	50 g YPD medium
	15 ml 0.2 per cent Adenine Sulfate
	Distilled water up to 1 L
	pH 6.5
	Autoclaved at 121°C for 15 min
Dropout (DO) Liquid Media	26.7 g Minimal SD Base
	DO Supplement
	Distilled water up to 1 L
	pH 5.8
	Autoclaved at 121°C for 15 min
	Stored at 4ºC in dark
Dropout Agar Plate	26.7 g Minimal SD Base
	DO Supplement
	20 g Agar
	Distilled water up to 1 L
	pH 5.8
	Autoclaved at 121°C for 15 min
	Stored at 4ºC in dark

YPD Medium, Minimal SD Base Medium, -Trp DO Supplement, -Leu DO Supplement, -Leu/-Trp DO Supplement, -His/-Leu/-Trp DO Supplement, -Ade/-His/-Leu/-Trp DO Supplement, X-α-Gal, Adenine Sulfate and L-Leucine were purchased from Clontech (CA, USA).

3.1.5. Co-Immunoprecipitation

Table 3.5. Co-immunoprecipitation buffers

Lysis Buffer	50 mM Tris-Cl (pH 8.0)
	300 mM NaCl
	5 mM EDTA
	5 mM EGTA
	2 mM DTT
	0.5 per cent TritonX-100
IP Buffer	20 mM HEPES (pH 7.4)
	150 mM NaCl
	0.1 per cent TritonX-100
	10 per cent Glycerol

3.1.6. Antibiotics

Table 3.6. Antibiotic solutions

Ampicillin stock	100 mg/ml in 50 per cent ethanol
	Sterilized by filtration
	Stored at -20°C
	100 µg/ml (working concentration)
Kanamycin stock	50 mg/ml in distilled water
	Sterilized by filtration
	Stored at -20°C
	50 µg/ml (working concentration)
Chloramphenicol stock	30 mg/ml in absolute ethanol
	30 ng/ml (working concentration)
	Stored at 4°C

3.2. Cell Lines and Strains

3.2.1. Bacterial Strains

TOP10 *E. coli* strain genotype	F- mcrA Δ (mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ (araleu) 7697 galU galK rpsL (StrR) endA1

3.2.2. Yeast Strains

AH109 strain 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
Y187 strain genotype:	MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met^-, gal80Δ, URA3 : : $GAL1_{UAS}$ $GAL1_{TATA}$-lacZ

3.2.3. Mammalian Cell Lines

Huh7 (human hepatocellular carcinoma; kindly provided by Dr. Mehmet Öztürk, Bilkent University) cell lines were used.

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), calcium and magnesium-free phosphate buffered saline (PBS), Penicillin/Streptomycin mixture were commercially obtained from BIOCHROM AG (Berlin, Germany).

3.3. Enzymes

Taq Polymerase together with $MgCl_2$ (25mM) and the 10X reaction buffer was purchased from Fermentas (Burlington, Canada). Expand Long Template PCR System, which contains a high-fidelity DNA polymerase Tgo, was purchased from Roche Applied

Biosciences (Indianapolis, IN, USA). Restriction endonucleases, Calf Intestinal Alkaline Phosphatase and T4 DNA ligase were purchased from Promega (Madison, WI, USA). Trypsin (0.025 per cent, ready to use) was purchased from Gibco (Paisley, UK).

3.4. Antibodies

Antibodies used in this thesis are tabulated in Table 3.1.

Table 3.7. Monoclonal (mAb) and polyclonal (pAb) antibodies

Name	Type	Host	Catalog #	Compny
anti-Myc-Tag	pAb	rabbit	2272	Cell Signaling
anti-HA-Tag	mAb	mouse	H3663	Sigma
anti-Acidic Calponin	pAb	rabbit	ab24688	Abcam
pan-actin antibody	pAb	rabbit	4968	Cell Signaling
anti-mouse/HRP	pAb	sheep	NA931	Amersham
anti-rabbit/HRP	pAb	donkey	NA934	Amersham

3.5. Nucleic Acids

3.5.1. Oligonucleotide Primers

Primers were designed by using Invitrogen's OligoPerfect^{TM} Designer online primer design software (http://tools.invitrogen.com/content.cfm?pageid=9716). Primers were purchased from Harvard University MGH Sequencing Core (Boston, USA). Primers used in this study are tabulated in Table 3.2.

Table 3.8. Primers used in this study

Primer Name	Primer Sequence 5'-3'	RE Sites
Cnn3-3F	aaa gaattc atgacccacttcaacaaggg	EcoRI
Cnn3-4R	aaa gtcgac ctaataatcaatgccttggtcg	SalI
Cnn3-5F	aaa gcggccgc atgacccacttcaacaaggg	NotI

Table 3.8. Primers used in this study (continued)

Primer Name	Primer Sequence 5'-3'	RE Sites
Cnn3-6R	aaa tctaga ctaataatcaatgccttggtcg	XbaI
Cnn3-7F	aaa tctaga atgacccacttcaacaaggg	XbaI
Cnn3-8R	aaa gaattc ctaataatcaatgccttggtcg	EcoRI
Cnn3-9R	aaa gtcgac tataatcaatgccttggtcgc	SalI
Cnn3-10F	aga aagctt atgacccacttcaacaaggg	HindIII
Cnn3-11R	aaa ggatcc ataatcaatgccttggtcgc	BamHI
Rnf10-1F	aaa gaattc atgccgctgagctccccc	EcoRI
Rnf10-2R	agg tctaga aggagtgtccagagaaca	XbaI
Rnf10-3F	cct tctaga cctcttgctctccctc	XbaI
Rnf10-4R	aaa gtcgact cttggtgtggacgactgaggt	SalI
Rnf10-5R	aaa ggtacc cttggtgtggacgactgaggtg	KpnI
pACT2F	ctattcgatgatgaagataccccaccaaacc	-
pACT2R	gtgaacttgcggggtttttcagtatctacgatt	-

3.5.2. Plasmids

pEGFP-N2, pGBKT7 and pGADT7 plasmids were commercially obtained from Clontech, CA, USA. The full length open reading frame of *CNN3* in pOTB7 vector (IRAUp969B0777D) was purchased from RZPD German Resource Center for Genome Research (Berlin, Germany). pcDNA3-HA and pcDNA-Myc plasmids were kindly provided by Dr. Nesrin Özören, Bogazici University. pcDNA-dsRED was modified from MTS-dsRED that was kindly provided by Dr.Mikhail F. Alexeyev, University of South Alabama, USA.

3.5.3. DNA Molecular Weight Markers

pBR322 DNA / Bsu RI (Hae III) Marker, 5	Fermentas (Burlington, Canada)
Lambda DNA / Eco 47I (Ava II) Marker, 13	Fermentas (Burlington, Canada)
Lambda DNA / Eco 130I (Sty I) Marker, 16	Fermentas (Burlington, Canada)
1kb Ladder	Roche (Mannheim, Germany)

3.6. Western Reagents

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

3.7. General Chemicals and Kits

All solid and liquid chemicals used in this study were analytical grade 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, Qiagen Plasmid Midi Kit, MinElute PCR Purification Kit, MinElute Gel Extraction Kit, and RNAeasy Mini Kit were purchased from Qiagen (Hilden, Germany). Genopure Plasmid Midi Kit for Plasmid Purification was purchased from Roche (Mannheim, Germany).

3.8. Equipments

Autoclave	Midas 55, Prior Clave, UK
	AMB430T, Astell, 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
	-80°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
Fluoroscence Microscope	Observer.Z1, Zeiss, GERMANY
Heat-blocks	DRI-Block DB-2A, Techne, UK
Heating Magnetic Stirrer	M221 Elektro-mag, TURKEY
	Clifton Hotplate Magnetic Stirrer, HS31, UK
Hemocytometer	Improved Neubauer, Weber Scientific International Ltd, 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
Magnetic Stirrers	M221 Elektro-mag, TURKEY
	Clifton Hotplate Magnetic Stirrer, HS31, UK
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
Spectrophotometers	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 bath	TE-10A, Techne, UK
Water purification	WA-TECH Ultra Pure Water Purification
	System, GERMANY

4. METHODS

4.1. Preparation of Chemically Competent *E.coli*

5 ml of liquid LB medium supplemented with 25 µg/ml of streptomycin was inoculated with a 50 µl aliquot of *E. coli* strain TOP10 glycerol stock. Cells were grown overnight at 37°C while shaking at 220 rpm. After 16 hours, 25 ml LB was inoculated with 250 µl of the overnight culture. Cells were grown at 37°C while shaking at 220 rpm until OD_{595} reached 0.4-0.6. Cells were centrifuged at 4000xg for 10 min at 4°C. Pellet was resuspended in 12.5 ml of ice-cold sterile 50 mM $CaCl_2$. The suspension was chilled on ice for 30 min and centrifuged at 4000xg for 10 min at 4°C. The bacterial pellet was resuspended in 2.5 ml ice-cold sterile 50 mM $CaCl_2$. For immediate transformations 50 µL of the suspension was used. For long term storage at −80°C, glycerol as 10 per cent final concentration was added. The bacterial cells were aliquoted, 100 µl each, and stored until used.

4.2. Transformation of Chemically Competent Cells

A tube containing 50 µl competent cells stored at -80°C was thawed on ice for 15 min. 1 µl of plasmid with 100-150ng/µl concentration was added. Cells were incubated on ice for 10-30 min. The tube was placed in 42°C heat-block for 1 min, and then immediately on ice for 2 min. 700 µl SOC or LB medium was added. Cells were grown for 1 hr at 37°C while shaking at 220 rpm. After 1 hour incubation, 150-200 µl of the cell suspension was spread on antibiotic-containing plates near a bunsen burner. Plates were incubated overnight at 37°C in up-side-down position.

4.3. Molecular Cloning

4.3.1. PCR Amplification

PCR amplifications were carried out with high-fidelity Taq polymerase for cloning procedures. Primers with appropriate restriction endonuclease site at 5' ends were used in these PCR mixes. Colony PCR was performed by using Fermentas Taq DNA polymerase. The PCR reactions were carried out in a total volume of 25 µl, consisting of 1 X PCR buffer, 0.2 mM of each dNTP, 400nM of each primer pair, 20-100 ng of template DNA, 0.2 U of Taq DNA polymerase, and sufficient dH_2O to adjust the volume. The cycling conditions for amplifications were as follows: an initial denaturation step at 94°C for 2-5 min, followed by 23-30 cycles of 30 sec denaturation at 94°C, 30-40 sec annealing at appropriate temperature and 1-2 min elongation at 72°C, and a final extension step for 10 min at 72°C. The primer sequences are given in Table 3.2.

4.3.2. Restriction Enzyme Digestion of DNA

Restriction enzyme digestions were performed in 10-20 µl reaction volumes with the appropriate reaction buffer at 37°C for 2- 4 hours. Enzymes were inhibited at either 65°C or 80ºC for 20 min according to manufacturer's protocol. Restriction was confirmed by agarose gel electrophoresis. Subsequently, purification of restriction fragment was carried out.

4.3.3. Purification

Plasmid isolation was performed according to the manufacturer's protocol by using Qiagen QIAprep Spin Miniprep, Midi, Maxi, and Roche Genopure Plasmid Midi Kit. Plasmids used in cell culture were purified with Midi and endotoxin free Maxi kits. PCR fragments and restriction digested vectors were purified by Qiagen PCR purification and gel extraction kits. The concentrations and purity of purification was checked by spectrophotometric measurements at Nanodrop.

4.3.4. Ligation

Ligation reactions were carried out in 10 µl reaction volume using 100-200 ng plasmid and appropriate amount of insert by considering insert to vector ratio. The reaction was carried out at room temperature for 3 hours. T4 DNA Ligase activity was inhibited at 70°C for 10 min.

4.3.5. Agarose Gel Electrophoresis

DNA fragments were resolved in either 1 or 2 per cent 1X TAE agarose gel. Appropriate amount of agarose was dissolved in 1X TAE Buffer by heating. After cooling for several minutes at room temperature, ethidium bromide was added to final concentration of 0.05 µg/ml. Appropriate amount of the DNA sample was mixed with 6X loading dye to get 1X final concentration and subjected to electrophoresis in 1X TAE Buffer at constant 90 voltage for 15-30 min. The bands were subsequently visualized over a UV light transilluminator.

4.4. Cell Culture

4.4.1. Growth Conditions of Cells and Passaging

Huh7 cell line was used in this study. Cell line was maintained in DMEM supplemented with 10 per cent FBS (fetal calf serum) and 1 per cent penicillin/streptomycin. Cells were incubated at 37ºC in humidified 5 per cent CO_2 incubator. All media were kept at either 4ºC or -20°C and warmed in 37ºC sterile water bath before use. Containers and the hood were cleaned with 70 per cent ethanol prior and after use.

Cells were passaged before reaching 90 per cent confluence. The growth medium was aspirated and the cells were rinsed once with 1X calcium and magnesium-free PBS. To detach 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. The cells were dispersed

by pipetting. 5 volumes of fresh medium were added to inactivate trypsin. The cells were seeded to fresh dishes in a 1:10 ratio for standard passaging.

4.4.2. Freezing and Thawing

The medium of cells was aspirated and cells were rinsed with PBS. Detachment with trypsin and pelleting as in passaging was performed. Cells were resuspended and stored at growth medium supplemented with 10 per cent DMSO in one million cells per milliliter density. The vials were kept at -20°C for two hours and re located to -80°C for long storage.

Before seeding to a new culture dish, a vial of frozen cells was thawed under running tap water. Cells were combined with 4 ml growth medium and pelleted at 1500 rpm for 3 min. The pellet was resuspended and seeded to 100 mm culture dish.

4.4.3. Transfection

Pure DMEM medium was combined with plasmids and Turbofect transfection reagent (Fermentas) according to the manufacturer's protocol. The mixture was incubated for 15 min at room temperature and added drop by drop to 70 per cent confluent Huh7 cells. Medium was changed after 4 hours of transfection. In co-localization experiment, six-well plates were transiently transfected with either 500 ng or 1µg of two plasmids. Huh7 cells in 100mm plate were co-transfected with 10 µg of plasmid DNA and 1µg of GFP plasmid.

4.5. Yeast Two Hybrid Screening

4.5.1. Preparation of Yeast Competent Cells

AH109 cells from a frozen yeast stock were streaked onto an YPDA agar plate. The plate was incubated at 30°C until colonies appeared (3-4 days). 3 ml YPDA medium was inoculated with a single AH109 colony and incubated at 30°C with shaking at 200 rpm for 8-12 hours. 5 µl of the culture was used to inoculate 50 ml of YPDA medium. Then,

incubation at 30°C until the OD_{600} reached 0.15-0.3 was performed. The cells were pelleted at 700xg for 5 min at room temperature. The supernatant was discarded. The pellet was resuspended in 100 ml of YPDA medium and incubated at 30°C until the OD_{600} reached 0.4-0.5. The culture was centrifuged at 700xg for 5 min at room temperature. The pellet was resuspended in 60 ml sterile water and re- pelleted. The pellet was resuspended in 5 ml of 1.1xTE/LiAc and centrifuged at high speed for 15 sec. The pellet was resuspended in 1200 µl of 1.1xTE/LiAc. 50µl of competent cells was used for transformation.

4.5.2. Transformation to Yeast Competent Cells

100 ng plasmid DNA, 5µl denatured herring carrier DNA, 50 µl competent cells, and 0.5 ml PEG/LiAc were combined and mixed in a prechilled microfuge tube. The cells were incubated at 30°C for 30 min by mixing every 10 min. 20 µl DMSO was added. The cells were incubated at 42°C water bath for 15 min by mixing every 5 min. Yeast cells were centrifuged at high speed for 15 sec and cells were resuspended in 1 ml of YPD Plus liquid medium. The yeast cells were centrifuged at high speed for 15 sec and the pellet was resuspended in 1 ml of 0.9% (w/v) NaCl. 100 µl of 1/10 dilution was spread onto plates containing the appropriate SD selection medium. Plates were incubated upside down at 30°C until colonies appear (3-5 days).

4.5.3. Yeast Mating

50 ml of SD/-Trp liquid medium was inoculated with a single colony of AH109 [pGBKT7-CNN3]. The culture was incubated at 30°C with 250-270 rpm shaking until the OD_{600} reaches 0.8. The cells were collected by centrifugation at 1000xg for 5 min. The pellet was resuspended in 5 ml SD/-Trp liquid medium and combined with 1 ml of Library Strain. 45 ml of 2xYPDA liquid medium with 50 µg/ml kanamycin was added. The library vial was rinsed twice with 1 ml 2xYPDA to collect all remaining yeast cells. The culture was incubated at 30°C overnight by slowly shaking at 30-50 rpm. After confirming the zygote formation, the mating mixture was centrifuged at 1000xg for 10 min. The flask where cell culture was incubated was rinsed twice with 0.5xYPDA containing 50µg/ml kanamycin and combined with pelleted cells. Centrifugation at 1000xg for 10 min was

performed. Cells were resuspended in 10 ml of 0.5xYPDA/Kan liquid medium. 200 µl of the resuspended culture was spread on 150 mm SD/-Ade/-His/-Leu/-Trp plates. The plates were incubated at 30°C for 3-8 days.

4.5.4. Yeast Colony PCR and Restreaking

After yeast mating step, yeast colonies were formed on SD/-Ade/-His/-Leu/-Trp plates. In order to confirm that the interaction between prey and bait are real interactions, yeast colonies were restreaked on SD/-Ade/-His/-Leu/-Trp/X-α-Gal plates. Colonies that were grown blue were subjected to yeast colony PCR.

Single yeast colonies were picked with sterile pipette tip and resuspended in 3 µl of 20 mM NaOH. The suspensions were boiled at 98°C for 15 min. Master Mix supplemented with pACT2F and pACT2R sequencing primers were added to the suspensions. The cycling condition for amplification was 94°C for 5 min, [94°C for 30 sec, 55°C for 30 sec, 72°C for 1-2 min] 30 cycles, 72°C for 10 min. PCR results were run in 2% agarose gel.

4.5.5. Yeast Plasmid Isolation

Yeast plasmid isolation was performed according to Clontech's YeastMaker Yeast Plasmid Isolation Kit's protocol. Briefly, a single yeast colony was inoculated into the appropriate SD liquid medium. Cell culture was grown at 30°C for 20-24 hours. The cells were pelleted and resuspended in Potassium Phosphate. Addition of Lyticase solution and incubation at 37°C for 1 hour were performed. After SDS addition yeast cells were vortexed vigorously for 1 min. Several freeze/thaw cycles at -20°C were performed. CHROMA SPIN-1000 DEPC-H2O Columns were used to elute the plasmid DNA.

4.5.6. Co-transformation

100 ng from each of bait and prey plasmids were co-transformed into AH109 yeast strain. 100 µl of transformed culture was spread on –Trp/-Leu and –Leu/-Trp/-Ade/-His SD media supplemented with and without X-α-gal plates.

4.6. SDS/PAGE and Western Blotting

10 per cent separating gels with 37.5:1 acrylamide:bisacrylamide ratio were cast in Bio-Rad Mini-Protein III cell. Distilled water was added on top of the gels. After the separating gels had set, 5 per cent stacking gels were prepared and poured on top of the separating gels. The combs were inserted immediately. After 1 hour of polymerization, samples were loaded. Samples were diluted to 1X SDS sample buffer and heated at 95°C for 10 min. 5µl of Kaleidoscope (Bio-Rad) or Fermentas pre-stained markers were used as the molecular weight standards. Initial running was performed at 80 V until the BPB front entered the separating gel. The voltage was increased up to 120 V. Running was continued until all bands of the molecular weight marker separated and BPB front reached the end of the separating gel. Separated proteins were transferred onto nitrocellulose membranes at 100V for 1 hour by using ice-cold transfer buffer and ice-block. Transfer efficiency was verified by staining with Ponceau S staining solution. TBST solution was used for destaining. 5 per cent non-fat milk in TBST was used for blocking the blots for 1 hour at room temperature while gently shaking. Primary and secondary antibodies were diluted in appropriate concentrations according to the manufacturer. The blots were incubated with primary antibody at 4°C overnight. Blots were washed with TBST for 5 min while shaking. ECL Plus Western Blotting Detection Reagents (Amersham) were used to develop the blots. Blots were analyzed by Stella Imaging Station (Raytest) and Xstella image acquisition software (Raytest) according to manufacturer's manual.

4.7. Co-Localization

Transfection of Huh7 cell lines with either 500 ng or 1000 ng of each plasmid was performed. Plasmid combinations were as following: pEGFP-N2/*CNN3*, pcDNA3-dsRED/*RNF10*, and both. Cells were analyzed with fluorescent microscopy under 5X, 10X, and 20X objectives either 24 hours or 48 hours after transfection.

4.8. Co-Immunoprecipitation

Huh7 cell line was cotransfected with combination of 10 µg from each constructs expressing Myc-tag, Myc-tag-RNF10, HA-tag-RNF10, Myc-tag-CNN3, HA-tag-CNN3, HA-tag. 24 hours after transfection, cells were rinsed with ice-cold 1X PBS. Ice-cold Lysis buffer supplemented with Protease Inhibitor Cocktail (Roche) was added. Adherent cells were scraped off the dish by a plastic cell scraper on ice. The cell suspension was collected into microcentrifuge tubes and incubated on ice for 30 min. Centrifugation at 12,000 rpm for 20 min at 4°C was performed. The supernatant was pre-cleared by incubating with 30 µl of 50 per cent slurry of Protein G-agarose beads for 30 min at 4°C while gently agitation. The beads were pelleted by centrifugation. 50 µl of the supernatant was saved as 'input'. The rest of the supernatant was incubated with anti-HA-antibody conjugated Protein G-agarose beads for 4 hours at 4°C by gently agitation. Conjugation of anti-HA-antibody to Protein G-agarose beads was performed as following: 50 µl of 50 per cent slurry of Protein G-agarose beads were washed twice with ice-cold IP buffer and incubated with 4µg of anti-HA antibody for 2 hours at room temperature with gently agitation. After 4 hours of incubation, the beads conjugated with anti-HA antibody and proteins were pelleted by centrifugation at 4°C for 1 min. The beads were washed twice with ice-cold IP buffer supplemented with protease inhibitor and once with 1X PBS. The supernatants were removed and proteins were eluted at 2X Sample buffer at 75°C for 5 min. The eluted samples were subjected to SDS-PAGE separation.

5. RESULTS

5.1. Preparation of the Bait Construct

Yeast two-hybrid system was used to screen a pretransformed human liver cDNA library. The first step of this system is to prepare the 'bait' construct. A bait construct is a plasmid expressing a protein of interest in fusion to the DNA binding domain of the yeast GAL4 transcription factor and a Myc epitope tag. Acidic calponin, which is encoded by the *CNN3* gene was used as a bait protein. The open reading frame of *CNN3* gene was amplified from pOBT7-CNN3 vector in different conditions by using Cnn3-3F and Cnn3-4R primers supplemented with *EcoRI* and *SalI* restriction sites, respectively (Figure 5.2.A). The purified PCR products were then ligated into pGBKT7 vector, which was digested with the same enzymes. After the ligation process was completed, the transformation procedure followed. The bacteria containing pGBKT7 vector were able to form colonies on kanamycin medium, which acts as a selective marker of the vector. Those colonies were subjected to colony PCR screening (Figure 5.2.B). Plasmids from positive colonies were isolated and sequenced to verify that they encode acidic calponin in frame to GAL4-BD and the Myc epitope (Figure 5.1).

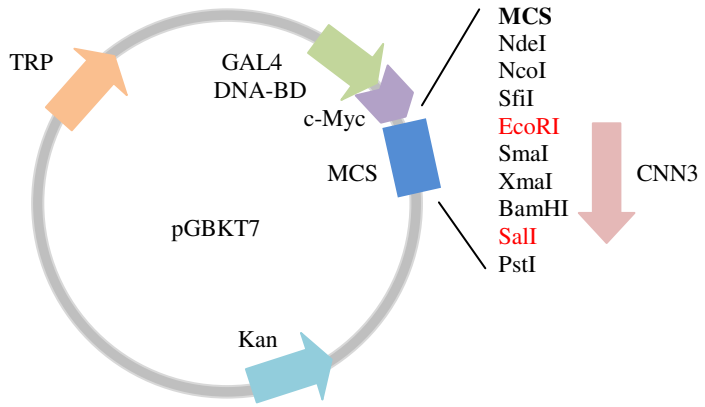


Figure 5.1. Schematic representation of pGBKT7/*CNN3* vector. TRP, tryptophan; Kan, kanamycin; MCS, multiple cloning site; BD, binding domain; CNN3, acidic calponin

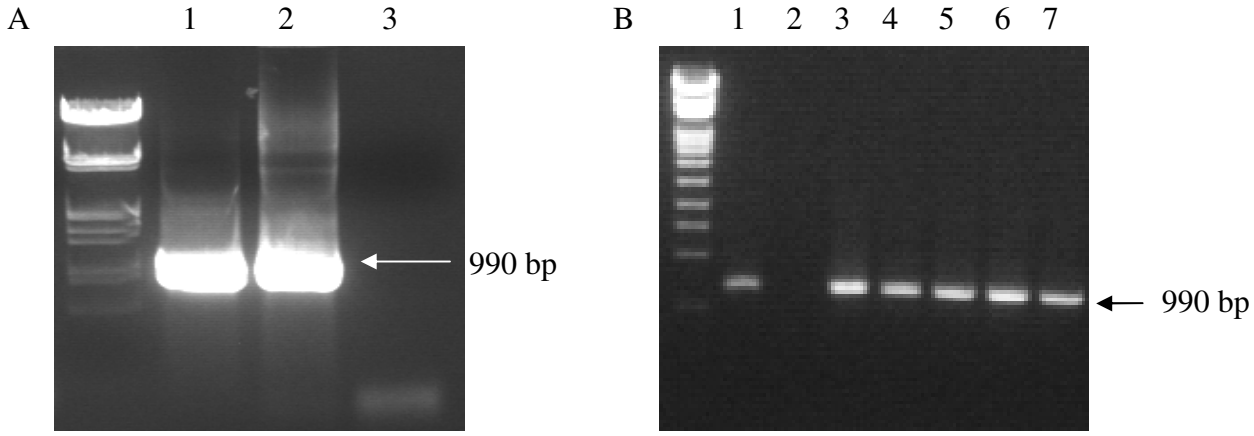


Figure 5.2. Cloning *CNN3* into pGBKT7 vector. (A) PCR amplification of *CNN3* gene by high fidelity Taq polymerase. PCR reactions include 2 mM MgCl2 in lane 1 and 4 mM MgCl2 in lane 2. Lane3 represents negative control. (B) Colony PCR of kanamycin resistant colonies by T7 and Cnn3-4R primers

5.2. Control Experiments

The second step of the yeast two-hybrid system is to test the bait construct for protein expression, transcriptional autoactivation, and toxicity to the yeast cells. This step is important because some proteins like membrane localized proteins may not be suitable for use as bait.

5.2.1. Verification of Bait Expression

The expression of the fusion protein encoded by the bait construct was verified by using the Western blot technique. For this purpose, either the bait construct or empty pGBKT7 pasmid was introduced into the yeast strain AH109 using lithium acetate method according to the manufacturer's guidelines of Matchmaker GAL4 Two-Hybrid System 2 (Clontech). Transformed yeast cultures were spread on SD/-Trp plates. pGBKT7 vector contains Trp selective marker, which allows selection on SD/-Trp media. After three days of incubation at 30°C, two colonies which carry the bait construct and one colony, which carried the empty vector were prepared for Western blotting. Kaleidoscope (BioRad) was used as pre-stained molecular weight marker and polyclonal rabbit anti-myc antibody (Cell Signaling) was used for detection. A band of 54 kDa size corresponding to GAL4-

BD/cMyc-tag/ CNN3 fusion protein and 19 kDa band corresponding to GAL4-BD/cMyc-tag were observed.

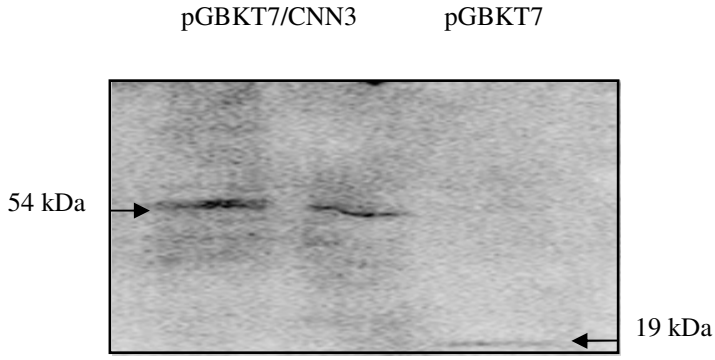


Figure 5.3. Expression of bait construct in yeast cells. GAL4 DNA-binding domain/c-Myc epitope-tag/Acidic calponin and GAL4 DNA-binding domain/c-Myc epitope-tag fusion proteins are detected in AH109 yeast strain by polyclonal rabbit anti-Myc antibody

5.2.2. Test for Toxicity

Toxic proteins may slow down or inhibit yeast growth in liquid and solid selective media. It is important to check whether the bait construct is toxic if it is expressed in yeast cells. For this purpose, the yeast strain AH109 was transformed with either pGBKT7/CNN3-bait construct or empty pGBKT7 vector. Transformed yeast cells were spread on medium lacking Trp. The size and the numbers of the colonies were compared. As the Figure 5.4 shows the size of the colonies carrying pGBKT7/CNN3-bait construct are smaller in comparison to the yeast cells with the empty pGBKT7 vector. On the other hand, no difference in the number of colonies between two sides was observed. The growth rate of the yeast cells having different plasmids was checked in liquid culture. Slow growth in yeast cells with pGBKT7/CNN3-bait construct was not observed. It was concluded that pGBKT7/CNN3-bait construct is not toxic for the yeast strain AH109.

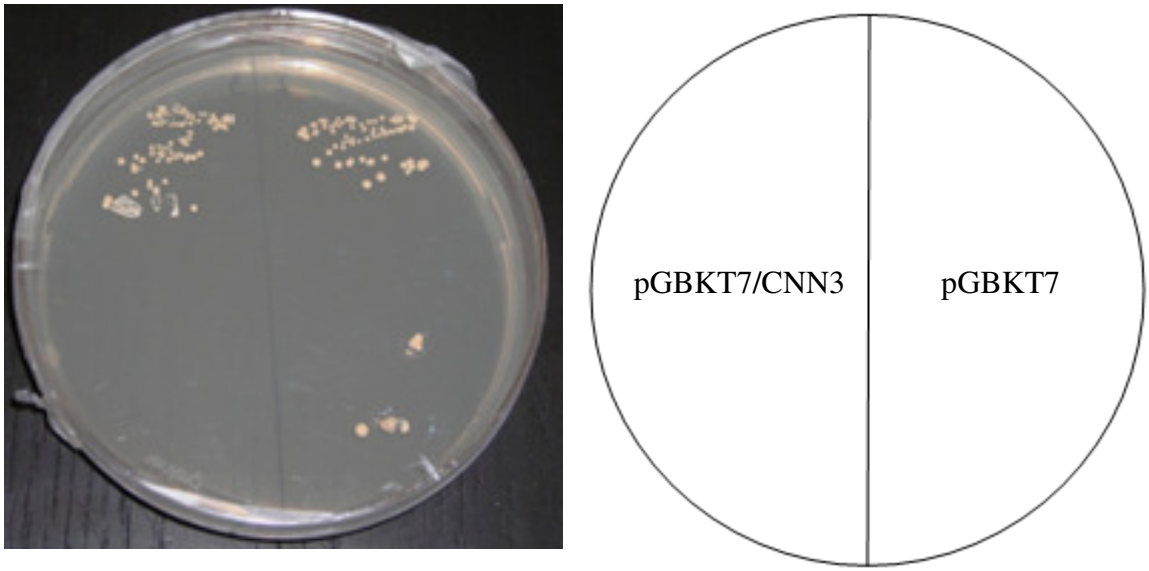


Figure 5.4. Toxicity test of pGBKT7/CNN3-bait construct. The yeast strain AH109 was transformed with the indicated plasmids and grown on SD medium lacking Trp

5.2.3. Test for Autoactivation

Using bait proteins that induce expression of the reporter genes in the absence of the prey protein in the yeast two-hybrid system may lead to identification of false positive clones (Moller *et al*., 2007). It is important to perform an autoactivation test before the library screening. To confirm that the pGBKT7/CNN3-bait protein does not induce expression of the reporter genes, the bait construct along with the pGADT7 vector was transformed into the AH109 yeast strain. Transformed yeast cells were spread on different selective SD medium as indicated in table 5.1. After three days of incubation at 30°C, the yeast colonies which are co-transformed with pGBKT7-53 and pGADT7-T antigen showed growth in all type of selective medium. This is because pGBKT7-53 encodes a GAL4-BD/murine p53 fusion protein and pGADT7-T antigen plasmid encodes GAL4-AD/SV40 large T antigen fusion protein. Interaction between T antigen and p53 is known one that brings GAL4 domains together and enables yeast cells to grow in all selective media. The combination of pGBKT7-53 and pGADT7-T antigen was used as positive control. pGBKT7-Lam plasmid express a fusion protein of GAL4 BD/Lamin C that does not interact with T antigen. The combination of these two plasmids was used as negative control. As it can be seen in the representative plate in Figure 5.5, yeast cells sustaining positive control plasmids were able to grow onto media lacking Trp, Leu, Ade, and His

whereas yeast cells carrying negative control plasmids did not show any growth. Yeast cells that have either CNN3-bait construct or CNN3-bait construct with pGADT7 vector encoding the GAL4-AD were not able to form colonies onto –Trp/-Leu/-Ade/-His SD selective media. These results verify that CNN3-bait construct does not induce expression of reporter genes in absence of the prey-protein.

Table 5.1. Colony formation of different constructs in different selective media

Selective Plate	Plasmids	pGBKT7-CNN3		pGBKT7-CNN3 pGADT7		pGBKT7-53 pGADT7-T ag		pGBKT7-Lam pGADT7- T ag	
		Colony	Color	Colony	Color	Colony	Color	Colony	Color
-Trp		Yes	White	Yes	White	Yes	Blue	Yes	White
-Leu		No	-	Yes	White	Yes	Blue	Yes	White
-Trp/-Leu		No	-	Yes	White	Yes	Blue	Yes	White
-Trp/-Leu/-Ade/-His		No	-	No	-	Yes	Blue	No	-

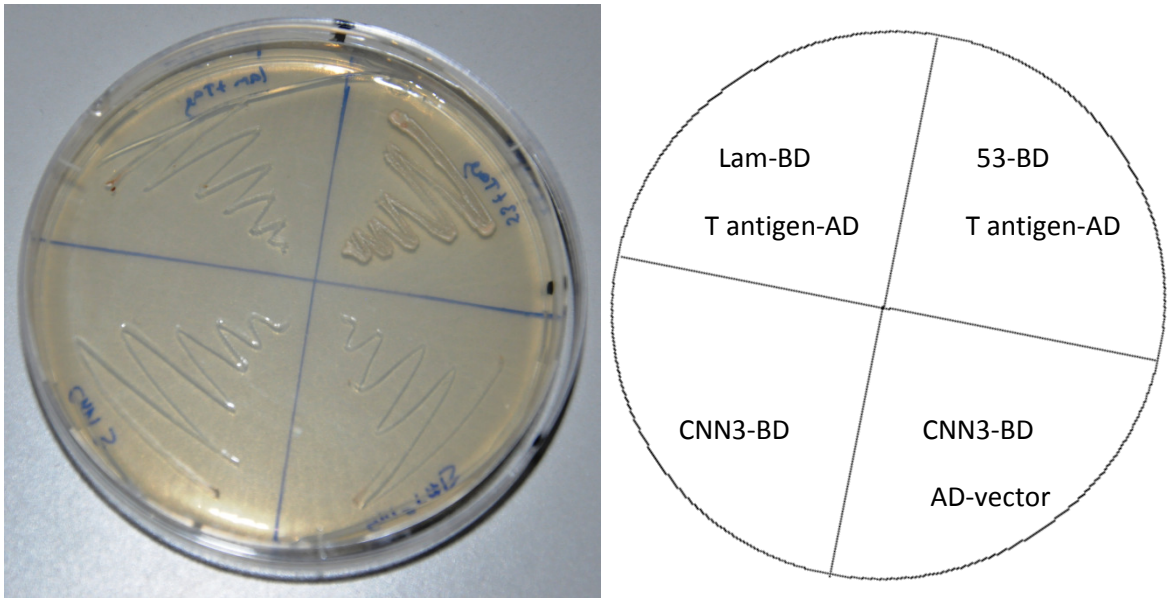


Figure 5.5. Autoactivation test of CNN3-bait construct on –Trp/-Leu/-Ade/-His SD selective media. The yeast strain AH109 was transformed with the indicated plasmids

5.3. Yeast Mating and Screening

5.3.1. Yeast Mating

Screening of the cDNA library was performed by mating MATa type AH109 yeast strain with MATα type Y187 yeast strain. For this purpose, pGBKT7/CNN3 was transformed into yeast strain AH109 and mated with a pretransformed human liver cDNA library cloned in pACT2 and carried in yeast strain Y187. The diploid colonies were plated on -Leu/-Trp/-Ade/-His SD agar plates to screen for interacting partners.

5.3.2. Screening

According to the colonies obtained after mating, 2.1 x 10^6 independent clones were screened. Among these transformants, 224 colonies grew on SD medium lacking Trp, Leu, His, and Ade. To distinguish true positives from false positives, colonies that grew successfully in the selective media were further screened for the expression of reporter gene, α-galactosidase. Colonies surviving on medium and showing strong galactosidase activity on medium containing X-α-gal were subjected to yeast colony PCR to identify protein partners of acidic calponin.

5.3.2.1. Phenotype Confirmation by Restreaking

224 colonies were obtained after 8 days of the yeast mating. These diploid colonies were further analyzed by re-streaking three times on –Trp/-Leu/-Ade/-His SD selective media with and without X-α-gal. 175 colonies out of 224 turned blue in presence of X-α-gal. This indicates that there is an interaction with proteins from the liver cDNA library and this interaction induces transcription of the MEL1 reporter gene. Colonies that were not able to grow or turn blue were considered as false positive and were eliminated.

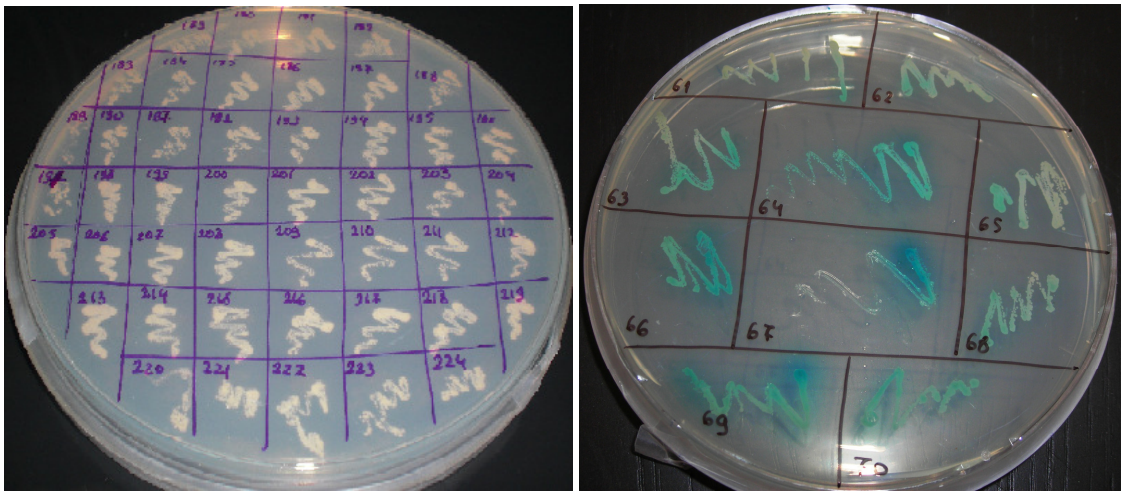


Figure 5.6. Phenotype conformation of colonies on –Trp/-Leu/-Ade/-His selective media with and without X-α-gal

5.3.2.2. Yeast Colony PCR

Colonies that passed X-α-gal selection were subjected to yeast colony PCR amplification. The reaction was carried out with pACT2F and pACT2R primers that can bind to pACT2 vector and amplify the liver cDNA clones. The PCR products were analyzed and in some cases double band patterns were observed. It was assumed that these colonies may contain double plasmids or they may be two different yeast colonies close to each other. To eliminate these possibilities, colonies with two or more PCR band patterns were re-streaked and re-amplified. PCR products with single band patterns were sequenced. Sequence results were analyzed by blast software of NCBI. The results of DNA sequencing and homology search revealed identity of 132 colonies. 74 per cent of these colonies were eliminated because they were out of frame with respect to the GAL4-activating domain. 24 per cent of the remaining colonies included partial proteins that were identified as false positive as they were fished out in other yeast two hybrid systems. Some examples of false positives are zinc finger proteins, mitochondrial proteins such as NADH and cytochrome subunits. The results of the screen are summarized in Table 5.2.

The yeast cells coding for a part of Ring Finger Protein 10 (RNF10) and RAS p21 protein activator (RASA1) were able to grow on –Trp/-Leu/-Ade/-His selective medium.

They also showed positive signal to α-galactosidase activity. They were selected for further analysis.

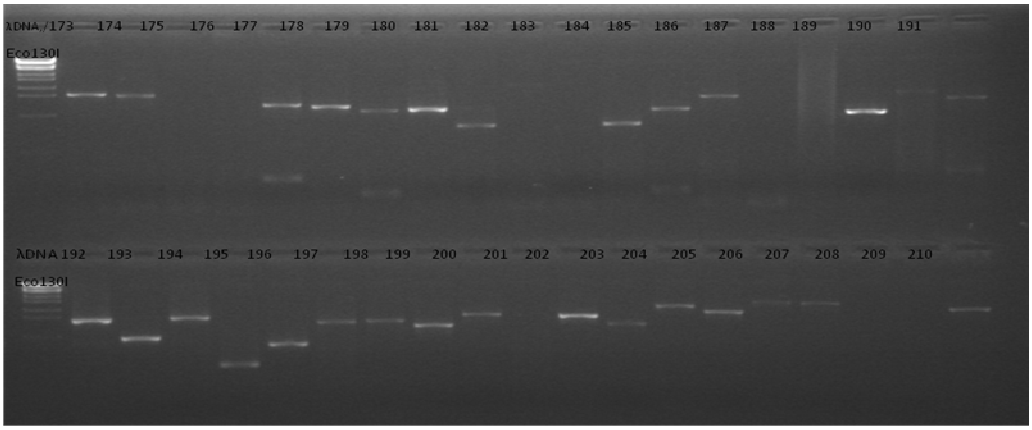


Figure 5.7. Yeast Colony PCR of the colonies formed on selective medium. pACT2F and pACT2R praimers were used for amplification cDNA clones

Table 5.2. The results of liver cDNA screening by acidic calponin

	In Frame		Out of Frame
Numbers of colonies	34		98
	Candidate	False Positives	False Positives
Numbers of colonies	2	32	98
Percent	1,50%	24%	74%

5.4. Co-Transformation of bait and prey vectors

Partial RNF10 and RASA1 cDNA clones encoded by the library plasmids were able to pass the selections. To further confirm that the interaction between these proteins and acidic calponin were not false positives, co-transformation of two plasmids into the AH109 yeast strain was performed. The plasmids encoding partial *RNF10* and *RASA1* genes were isolated with the YeastMaker Yeast Plasmid Isolation Kit. They were co-transformed with either pGBKT7/*CNN3* or empty pGBKT7 vector along with control plasmids. As it can be seen in a representative plate in Figure 5.8 for RNF10, yeast cells carrying *RNF10* and *CNN3* were able to grow whereas cells sustained RNF10 and activating domain of GAL4 were not able to form colonies on selective media. Blue colony formation in yeast carrying

pGBKT7/*CNN3* and pACT2/*RNF10* was observed on Leu/-Trp/-Ade/-His SD media supplemented with X-α-gal (data not shown). The same results were obtained for yeast cells transformed with pGBKT7/*CNN3* and pACT2/*RNF10*. These results indicated that the activation of the reporter genes in the yeast two-hybrid system occurred as a result of interactions between CNN3 and two candidate prey proteins, RNF10 and RASA1.

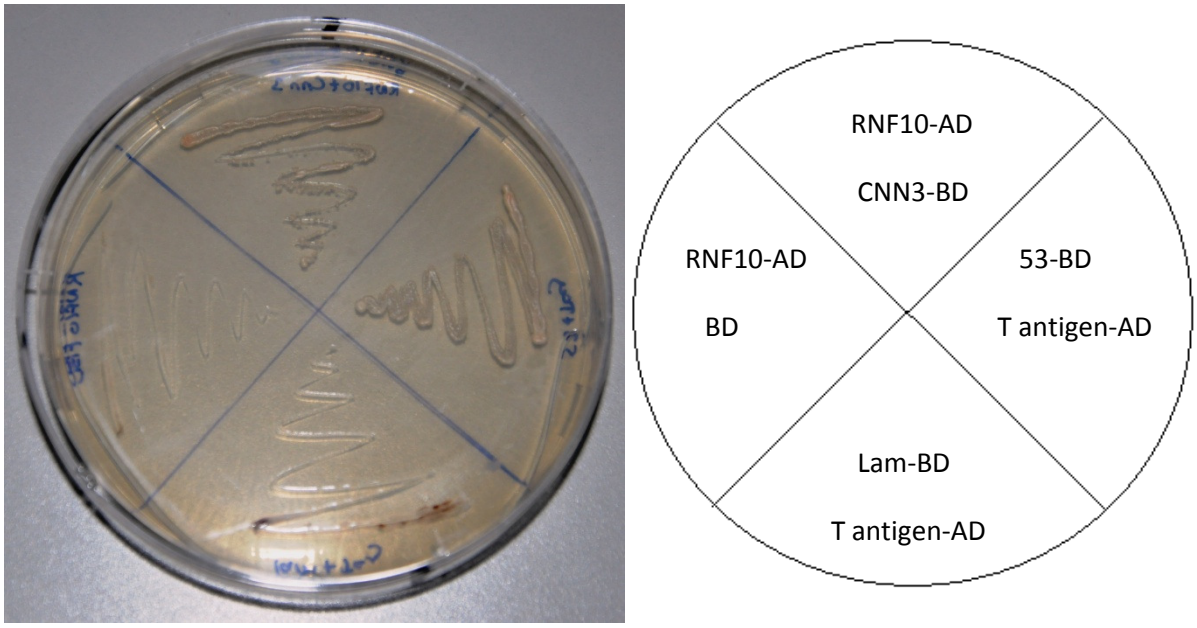


Figure 5.8. Co-transformation of *CNN3* and partial-*RNF10*. The yeast strain AH109 was transformed with the indicated plasmids and grown on SD medium lacking Trp, Leu, His, and Ade. Each colony was restreaked on same medium

5.5. Co-expression in Mammalian Cell Lines

In the yeast two-hybrid system, Ring finger protein 10 was detected as protein partner of acidic calponin. Further investigation of this interaction was performed with transient co-transformation of pcDNA3-dsRED/*RNF10* and pEGFP/*CNN3* in the Huh7 mammalian cell line. A PCR product was not observed after PCR amplification of the second candidate gene *RASA1*. For this reason cloning was not performed. The full length of *RASA1* was purchased.

To perform co-localization experiments, the open reading frame of RNF10 gene was amplified from a human brain cDNA by using primers with flanking restriction sites. The coding sequence of *RNF10* gene was cloned into double digested pcDNA-dsRED vector at *HindIII* and *KpnI* restriction sites. On the other hand, the open reading frame of *CNN3* gene was sub-cloned into pEGFP-N2 plasmid at *EcoRI* and *KpnI* restriction sites. As a result, fusion proteins of GFP/*CNN3* and dsRED/*RNF10* were obtained. These constructs were transiently transfected to Huh7 cell lines and analyzed with fluorescent microscopy.

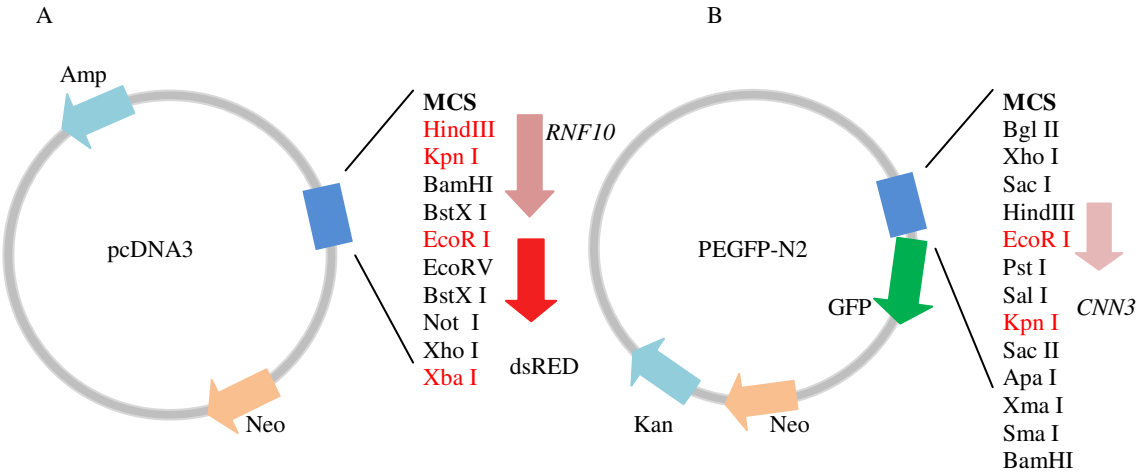


Figure 5.9. Schematic representation of pcDNA3-dsRED/*RNF10* and pEGFP-N2/*CNN3*. Amp, ampicilin, Kan, kanamycin; Neo, neomycin; RNF10, ring finger protein 10; CNN3, acidic calponin, GFP, green fluorescent protein

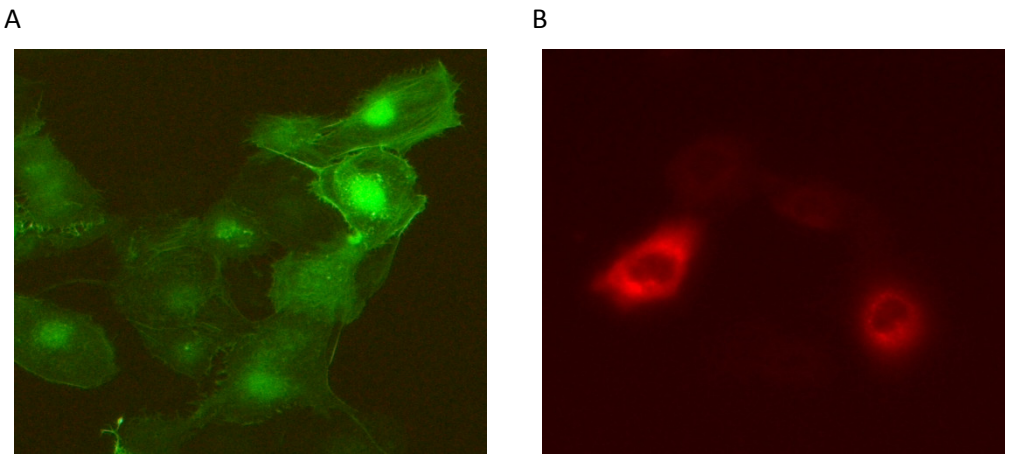


Figure 5.10. Expression of pEGFP/*CNN3* and pcDNA-dsRED/*RNF10* in Huh7 cells. Cells were transiently transfected with (A) pEGFP/*CNN3* and (B) pcDNA-dsRED/*RNF10*. All transfections were repeated at least twice

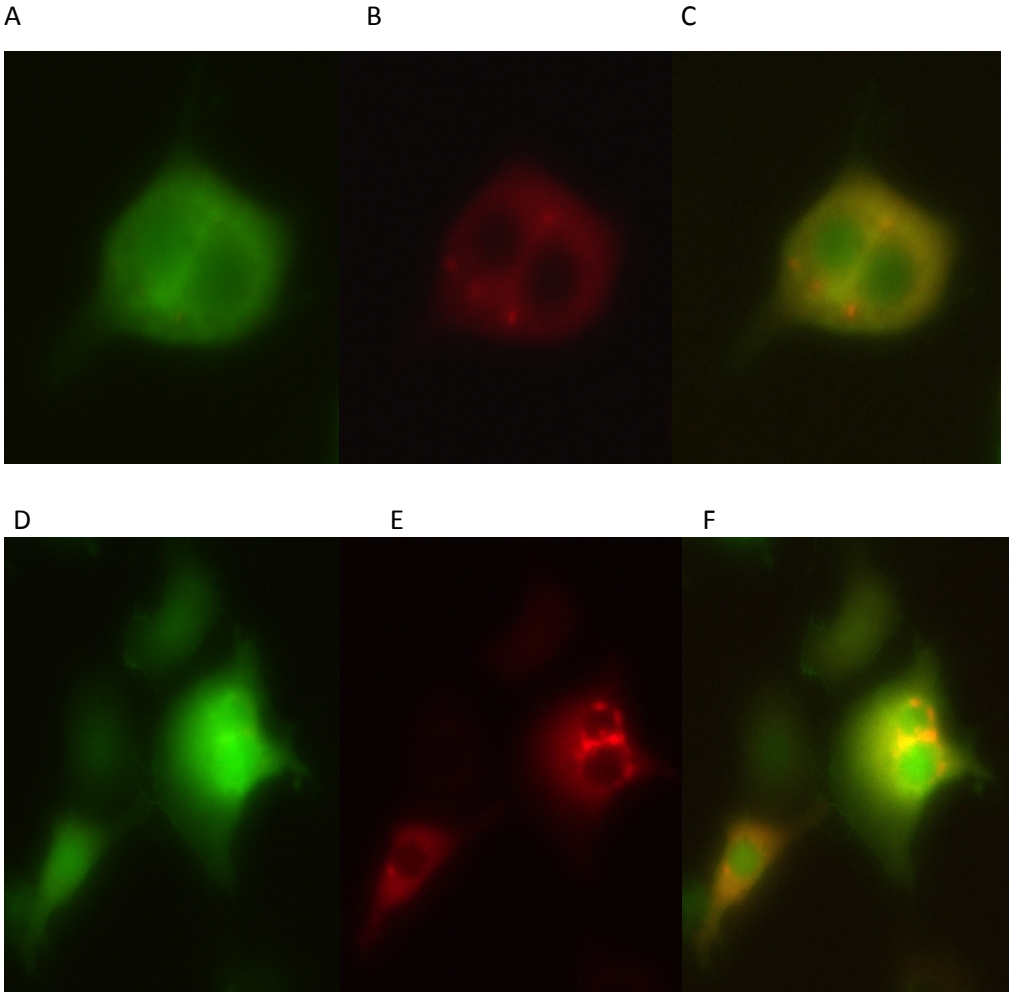


Figure 5.11. Co-localization of pEGFP/*CNN3* and pcDNA-dsRED/*RNF10* in Huh7 cells. Huh7 cells were transiently transfected with (A-F) pEGFP/*CNN3* and pcDNA3-dsRED/*RNF10.* C and F are merged images. All transfections were repeated at least twice

Cytoplasmic and nuclear localization of the fusion protein GFP/CNN3 was observed (Figure 5.10.A). dsRED/RNF10 fusion protein expression was largely detected in the cytoplasm (Figure 5.10.B). When images of mammalian cells, which were co-transfected with GFP/CNN3 and dsRED/RNF10 fusion proteins were merged, cytoplasmic co-localization of proteins was observed as it can be seen in Figure 5.11.C and F.

5.6. Conformation of Interaction by Co-Immunoprecipitation

In yeast two-hybrid system, the polypeptide that interacts with acidic calponin encodes the amino acid 524-766 region of 811 amino acid long ring finger protein 10. To confirm that the in vivo interaction between CNN3 and RNF10 can occur between full length sequences of same proteins, direct interaction of these proteins was analyzed with co-immunoprecipitation experiment.

For co-immunoprecipitation experiments, the open reading frames of *CNN3* and *RNF10* genes were sub-cloned in frame to HA and Myc epitope tag containing vectors, respectively. Huh7 cell lines were transiently transfected with different plasmid combinations including pcDNA3-HA/*CNN3* and pcDNA3-Myc/*RNF10*, pcDNA3-HA and pcDNA3-Myc/*RNF10*, pcDNA3-HA and pcDNA3-Myc/*RNF10* where the last two combinations were used as negative control. Cells were harvested and subjected to immunoprecipitation with monoclonal anti-HA antibody. Co-precipitated proteins were separated by SDS/PAGE and detected with polyclonal anti-Myc antibody. Anti-HA antibody was used to detect the efficiency of the experiment.

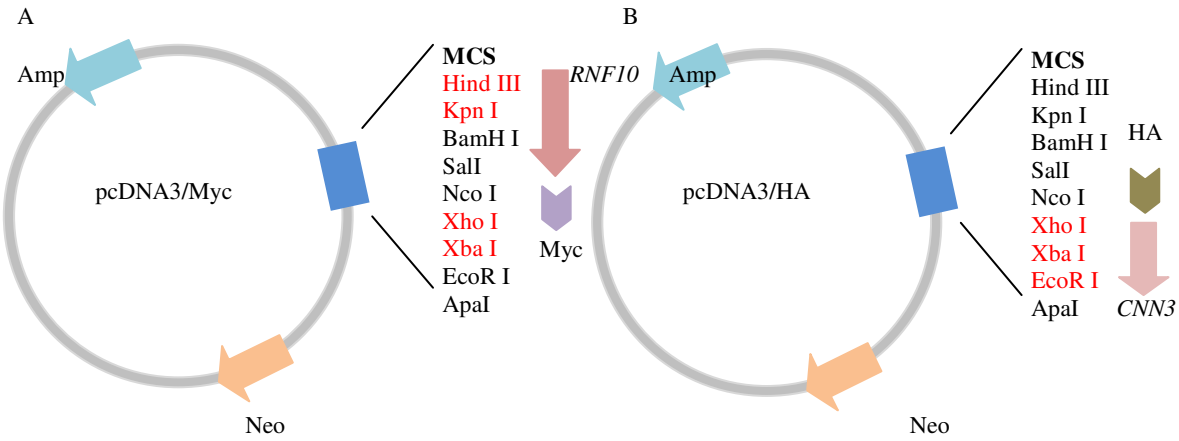
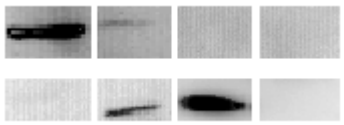


Figure 5.12. Schematic representation of (A) pcDNA3-Myc/*RNF10* and (B) pcDNA3-HA/*CNN3* constructs. Amp, ampicilin; Neo, neomycin; RNF10, ring finger protein; CNN3, acidic calponin; Myc, Myc epitope tag; HA, hemagglutinin epitope tag

Western blot results obtained after immunoprecipitation with monoclonal anti-HA antibody revealed bands corresponding to ring finger protein 10 as it can be seen in Figure

5.13. The first lane corresponds to 2 per cent of total cell lysate obtained from mammalian cells co-transfected with pcDNA3-HA/*CNN3* and pcDNA3-Myc/*RNF10*. A band of Myc/RNF10 fusion protein was detected. A faint version of the same band was observed in the second lane which is a co-immunoprecipitation result of the same cell lysate. Immunoprecipitated HA-CNN3 fusion protein was also detected when the blot was analyzed with anti-HA antibody. The third line and fourth lanes represent co-immunoprecipitation results of Huh7 cells, which were co-transfected with pcDNA3-HA/*CNN3* : pcDNA3-Myc and pcDNA3-HA : pcDNA3-Myc/*RNF10*, respectively. In the third lane, a band of HA/CNN3 fusion protein was observed whereas no band was detected in the blot incubated with anti-Myc antibody. In the fourth lane, bands were not seen in membranes which were blotted with either anti-HA or anti-Myc antibodies. This experiment revealed that ring finger protein 10 could be co-immunoprecipitated with Myc tagged acidic calponin.



	Input	**IP: Anti-HA**		
				WB: Anti-Myc
				WB: Anti-HA
pcDNA-HA/CNN3	+	+	+	-
pcDNA-Myc/RNF10	+	+	-	+
pcDNA-HA	-	-	-	+
pcDNA-Myc	-	-	+	-

Figure 5.13. Co-immunoprecipitation of RNF10 and CNN3 in Huh7 cells. Proteins were immunoprecipitated with monoclonal anti-HA antibody and blotted with either polyclonal anti-Myc antibody or monoclonal anti-HA antibody. WB, Western blot; IP, immunoprecipitation

6. DISCUSSION

To identify protein partners of acidic calponin in liver tissue, a human liver cDNA library was screened by using yeast two-hybrid screening. Ring finger protein 10 (RNF10) and RAS p21 protein activator (GTPase activating protein) 1 (RASA1) were fished out as putative protein partners. The interaction between acidic calponin and RNF10 was further confirmed by co-immunoprecipitation and co-expression in mammalian cell lines.

Yeast two hybrid screening of human liver cDNA library was performed by using acidic calponin as bait. For this purpose, the full-length open reading frame of *CNN3* was cloned as a translational fusion of the GAL4 DNA-binding domain.

Before initiating library screening, control experiments were performed. The expression of CNN3-bait construct was checked by Western blot analysis. Western blot analysis of yeast cells transformed with the bait vector was detected by polyclonal rabbit anti-Myc antibody. A band corresponding to the fusion protein demonstrated that CNN3-bait protein was expressed in the AH109 yeast cells.

Toxicity experiments were performed in order to verify that the bait did not attenuate the growth and colony formation of yeast cells. Yeast cells were transformed with either bait or the empty vector and plated on Trp lacking medium. Although yeast cells transformed with CNN3-bait vector formed smaller colonies than yeast cells transformed with the empty vector, difference of growth rate in liquid medium was not observed.

The proteins that induce expression of reporter genes in absence of prey protein give large number of false positives. To eliminate this possibility autoactivation tests were performed. Yeast transformed with different plasmid combinations were plated on different selective media as indicated in Table 5.1. Blue colony formation on selective media was observed only in the positive control. This indicated that CNN3-bait protein did not activate the expression of reporter genes in the absence of the prey protein.

After it was confirmed that CNN3-bait was suitable for yeast two hybrid assay, mating between AH109 yeast cells transformed with bait and pretransformed human liver cDNA library cells was performed. Diploid cells that were able to grow on media lacking Trp, Leu, Ade, and His were restreaked on the same media supplemented with X-α-gal. Yeast cells were able to form colonies and turn blue only if CNN3-bait protein and cDNA-prey protein interact with each other. The CNN3-bait vector encodes a fusion protein of GAL4-DNA binding domain and CNN3 whereas cDNA-prey vector encodes a fusion protein of GAL4-activating domain and cDNA of the liver library. If CNN3 and a peptide encoded by the library cDNA interact, the GAL4 activation domain and DNA binding domain can come in close proximity and activate the reporter genes that encode essential amino acids of adenine and histidine. Mel1 is the third reporter gene that encodes α-galactosidase as a result of interaction between the prey and the bait. After activation, yeast cells secrete α-galactosidase and turn blue in presence of the chromogenic substrate X-α-gal.

The yeast cells that form blue colonies were subjected to yeast colony PCR. Yeast colonies that showed double band pattern were restreaked and reamplified. The double pattern may be due to presence of two plasmids in single yeast cells. These colonies were restreaked in order to obtain single colonies that carry single prey plasmid. If yeast cells form colonies this may be due to interactions with the bait protein. In this way, false positive plasmids can be eliminated.

Yeast PCR products were sequenced and analyzed by blast software of NCBI. The identity of 135 cDNAs was revealed the 2.1×10^6 independent clones that were screened. 74 per cent of these colonies were not in frame to the GAL4 activation domain. This means that the translated protein is not the same protein encoded by the cDNA clone. The rest of the clones were in frame to the GAL4 activating domain. However, 24 per cent of them were considered as false positives due to the fact that they were proteins that are commonly fished out in yeast two hybrid screening. NADH dehydrogenase and cytochrome C subunits can be given as examples. The reason of high number of false positives may be due to the library that was used in this study. Preferring a normalized liver library may give less false positives. A normalized library has equal representation of

highly abundant transcripts and low copy number transcripts. These libraries may increase the chance of fishing out proteins that are expressed at low amount.

Among the identified clones, ring finger protein 10 (RNF10) and RAS p21 protein activator (GTPase activation protein) 1 (RASA1) were identified as putative interacting proteins of acidic calponin. The cDNA clones identified in the yeast two-hybrid system encoded the carboxyl regions of RASA1 (927-1032 aa out of 1047 amino acid) and RNF10 (524-766 out of 811 amino acid). These clones were isolated and co-transformed to AH109 yeast cells with CNN3- bait construct in order to verify their interaction within yeast cells. As a result of this experiment, colony formation on selective media that indicates interaction between bait and prey proteins was observed.

To check the localization of CNN3 and RNF10 in mammalian cells, cloning to pEGFP-N2 vector and pcDNA3-dsRED vector respectively was performed. The constructs were co-transfected to Huh7 cells. Cytoplasmic and nuclear localization of the fusion protein GFP/*CNN3* was observed. dsRED/*RNF10* fusion protein expression was largely detected in the cytoplasm as previously observed in fibroblast cell lines (Lin *et al.,* 2005). On the other hand, nuclear localization of RNF10 in rat Schwann cells had been detected (Hoshikawa *et al*., 2008). Although co-localization of the two proteins in the cytoplasm was observed, better results would be obtained if indirect immunofluorescence experiments by using antibodies against the endogenous proteins.

The cDNA clone of RNF10 that was isolated from yeast cells as a result of yeast two-hybrid screening, encoded a partial region of identified protein. To verify that the interaction observed in the yeast two hybrid systems occurs between full lengths of proteins in mammalian cells, a co-immunoprecipitation assay was performed. As a result of this experiment, a faint band corresponding to co-immunoprecipitated Myc tagged RNF10 was observed. The same band was not observed in cells co-transfected with empty pcDNA3-HA or pcDNA-Myc vectors. These results were obtained from a single co-immunoprecipitation experiment. Thus the experiment should be repeated to verify the results.

RNF10 contains a RING domain. It has been shown that RING finger proteins have a role in protein turnover by acting as ubiquitin ligases (Freemont, 2000). They have diverse roles in cellular processes including the cell cycle, signaling, apoptosis, and proliferation. Mutations in the RING finger domain were associated with diseases. As an example, mutations in the RING finger domain of the tumor suppressor BRCA1 were associated with breast and ovarian cancer (Joazeiro *et al.,* 2000). In the previous studies, RNF10 has been identified as a protein partner of the Mesenchyme Homeobox 2 (Meox2) transcription factor which is known to regulate the proliferation and differentiation of vascular smooth muscle cells and cardiomyocytes. It was suggested that RNF10 acts as co-activator by enhancing the expression of Meox2 target genes (Lin *et al.,* 2005). In another study, RNF10 was fished out as Myelin-associated glycoprotein (MAG) promoter binding protein as a result of screening a rat Schwann cell derived cDNA library with the yeast one-hybrid system. Based on the observation that RNF10 induces expression of the MAG gene and myelin formation, a role of RNF10 in Schwann cell differentiation and myelination was suggested (Hoshikawa *et al.,* 2008).

RASA1 is the second gene that was fished out as a result of yeast two hybrid screening. The verification of interactions between RASA1 and CNN3 has to be performed in mammalian cells. The open reading frame of RASA1 gene was purchased. After subcloning to the appropriate cloning vectors, co-immunoprecipitation and co-expression in Huh7 cells would be performed. RASA1 gene encodes a Ras GTPase-activating protein, which interacts with Ras and mediates hydrolysis of Ras-bound GTP to GDP, resulting in Ras protein inactivation (Yunoue *et al.,* 2003). It has been noted that it has a role in directional cell movement (Kulkarni *et al.,* 2000).

In conclusion, the results suggest the interaction of RNF10 and RASA1 proteins with acidic calponin in yeast cells. The interaction between RASA1 and CNN3 must be further investigated by co-immunoprecipitation assays in mammalian cells. Although repeating co-immunoprecipitation experiments should be carried out, the results indicate an interaction between RNF10 and CNN3 both in yeast cells and in mammalian cells.

It was noted that acidic calponin expression increases in different brain tumors by using Oncoreveal and independent brain tumor panel results (Kavak *et al.,* 2009).

Moreover, interaction with Smad1/5 proteins and negative regulation of TGFβ/BMP signaling pathway by acidic calponin was reported (Haag *et al*., 2007). An observation shows that BMP treatment reduces the population of stem-like tumor-initiating precursors of human glioblastomas (Piccirillo *et al.,* 2006). This may hint to importance of this pathway and proteins that regulate it in brain tumors. It is tempting to speculate that maybe acidic calponin may have a role in the formation of brain tumors. To investigate this possibility acidic calponin can be used in *in vivo* xenografting experiments on nude mice to observe whether it has a tumor formation capacity.

RNF10, which has a function in myelination was found as protein partner of acidic calponin. Checking whether acidic calponin has any stimulatory or inhibitory effect on RNF10 function and myelination may be performed. As for RNF10, its epression pattern in independent brain tumor panel used for acidic calponin can be analyzed. If its expression pattern shows any correlation with the acidic calponin expression pattern, RNF10 alone or in combination with acidic calponin may be used in *in vivo* xenograft experiments on nude mice.

Investigating the role of acidic calponin in Wnt/β-catenin signaling pathway may be aimed in future experiments. This purpose may be based on observations mentioned below. Five putative Tcf4-binding elements (TBEs) were found within the CNN3 promoter (data not shown). TBEs are located in promoters of Wnt/β-catenin target genes where TCF4 transcription factor and β-catenin bind. The first step then may be testing whether acidic calponin is a target of the Wnt/β-catenin cascade. This may be performed by checking expression levels and protein levels of cells with and without an active pathway. Although differences of expression levels of acidic calponin in cells with active and inactive Wnt/β-catenin pathway was not observed, the protein stability of protein can be checked (data not shown).

Miss-regulation of Wnt/β-catenin signaling pathway was also linked to brain tumors including medulloblastoma (Huse *et al.,* 2010). Mutations in members of the pathway including β-catenin, Axin, APC and accumulation of β-catenin in the nucleus were correlated with the development of brain tumors (Caricasole *et al.,* 2005).

Inactivation of TGFβ/BMP signaling pathway due to BMPR1 and Smad4 gene mutations was linked to prognosis of colon cancer (Hardwick *et al.,* 2008). Wnt/β-catenin signaling pathway is another deregulated pathway that was correlated to colon cancer due to mutations. The cross-talk at different levels between these two pathways has been studied. The TGFβ/BMP pathway inhibits Wnt/β-catenin induced β-catenin translocation due to interaction between Smad1 and Dvl-1 proteins in bone marrow stromal cells (Liu *et al.,* 2006). On the other hand, GSK3β which is a member of the destruction complex of the Wnt/β-catenin pathway phosphorylates Smad1 together with ERK1/2. This phosphorylation leads to Smad1 ubiquitination and degradation (Guo *et al*., 2009).

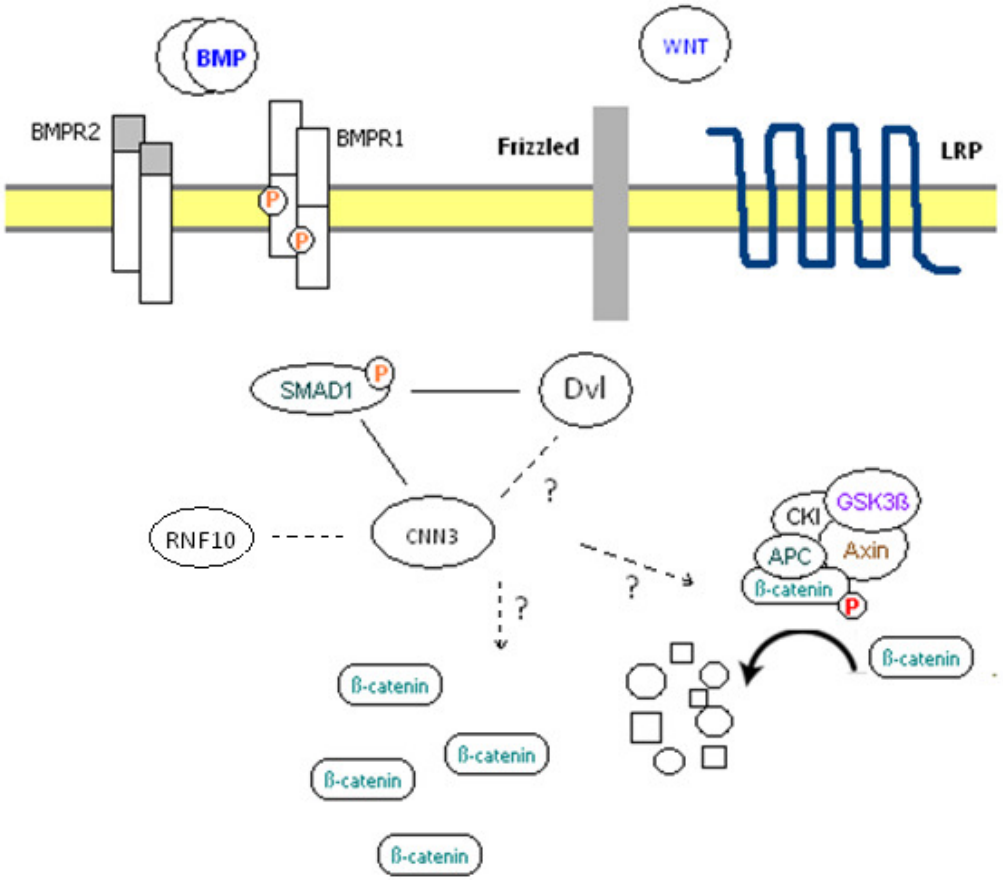


Figure 6.1. Schematic representation of cross-talk between Wnt/ β-catenin and TGFβ/BMP pathways via Smad1. The role of acidic calponin ?

The cross-talk between Wnt/ β-catenin and TGFβ/BMP pathways via Smad1 and interaction of acidic calponin with Smad1 may be further investigated in the Wnt/ β-catenin pathway context. Analyzing whether acidic calponin may have any direct or indirect role on DVL function may be performed by overexpression and knockdown of acidic calponin in the presence or absence of Wnt and BMP ligands. Checking β-catenin levels or its targets may give information whether acidic calponin has any affect on the Wnt/β-catenin signaling pathway, too.

APPENDIX A: SEQUENCES

A.1. Partial sequence of pGBKT7/*CNN3*

Gal4 DNA-Binding Domain

TCA TCG GAA GAG AGT AGT AAC AAA GGT CAA AGA CAG TTG ACT GTA **TCG** CCG GAA TTT

c-Myc epitope tag

GTA ATA CGA CTC ACT ATA GGG CGA GCC GCC ATC **ATG** GAG GAG CAG AAG CTG ATC TCA

EcoRI **Start** ***CNN3***

GAG GAG GAC **CTG** CAT ATG GCC ATG GAG GCC GAA TTC **ATG** ACC CAC TTC AAC AAG

GGC CCT TCC TAT GGG CTC TCG GCC GAA GTC AAG AAC AAG ATT GCT TCC AAG TAT GAT

CAT CAG GCA GAA GAA GAT CTT CGC AAT TGG ATA GAA GAG GTG ACA GGC ATG AGC

ATT GGC CCC AAC TTC CAG CTG GGC TTA AAG GAT GGC ATC ATC CTC TGC GAA CTT ATA

AAC AAG CTA CAG CCA GGC TCA GTG AAG AAG GTC AAC GAG TCC TCA CTG AAC TGG CCT

CAG TTG GAG AAT ATT GGC AAC TTT ATT AAA GCT ATT CAG GCT TAT GGT ATG AAG CCA

CAT GAC ATA TTC GAA GCA AAT GAT CTT TTT GAG AAT GGA AAC ATG ACC CAG GTT CAG

ACT ACT CTG GTG GCT CTA GCA GGT CTG GCT AAA ACA AAA GGA TTC CAT ACA ACC ATT

GAC ATT GGA GTT AAG TAT GCA GAA AAA CAA ACA AGA CGT TTT GAT GAA GGA AAA

TTA AAA GCT GGC CAA AGT GTA ATT GGT CTG CAG ATG GGA ACC AAC AAA TGT GCC AGC

CAG GCA GGT ATG ACA GCT TAC GGG ACT AGG AGG CAT CTT TAT GAT CCC AAA ATG CAA

ACT GAC AAA CCT TTT GAC CAG ACC ACA ATT AGT CTG CAG ATG GGC ACT AAT AAA GGA

GCC AGC CAG GCA GGG ATG TTA GCA CCA GGT ACC AGA AGA GAC ATC TAT GAT CAG

AAG CTA ACA TTA CAG CCG GTG GAC AAC TCG ACA ATT TCC CTA CAG ATG GGT ACC AAC

AAA GTT GCT TCC CAG AAA GGA ATG AGT GTG TAT GGG CTT GGG CGG CAA GTA TAT GAT

CCC AAA TAC TGT GCT GCT CCT ACA GAA CCT GTC ATT CAC AAC GGA AGC CAA GGA ACA

GGA ACA AAT GGT TCG GAA ATC AGT GAT AGT GAT TAT CAG GCA GAA TAC CCT GAT GAG

TAT CAT GGC GAG TAC CAG GAT GAC TAC CCC AGA GAT TAC CAA TAT AGC GAC CAA GGC

ATT GAT TAT **TAG** GTC GAC CCT GCA GCG GCC GCA TAA CTA GCA **TAA** CCC CTT GGG GCC

Stop **SalI** **Stop**

TCTAAACGGGTCTT

A.2. Partial sequence of pcDNA3-HA/*CNN3*

AAG CTT GGT ACC GAG CTC CCG GGA TCC AAG AGA GAT ACC ACC ATG GCC CTC GAG TAC

HA- epitope tag **XbaI** **Start** ***CNN3***

CCA TAC GAC GTC CCA GAC TAC GCT TCT AGA **ATG** ACC CAC TTC AAC AAG GGC CCT TCC
TAT GGG CTC TCG GCC GAA GTC AAG AAC AAG ATT GCT TCC AAG TAT GAT CAT CAG GCA
GAA GAA GAT CTT CGC AAT TGG ATA GAA GAG GTG ACA GGC ATG AGC ATT GGC CCC
AAC TTC CAG CTG GGC TTA AAG GAT GGC ATC ATC CTC TGC GAA CTT ATA AAC AAG CTA
CAG CCA GGC TCA GTG AAG AAG GTC AAC GAG TCC TCA CTG AAC TGG CCT CAG TTG GAG
AAT ATT GGC AAC TTT ATT AAA GCT ATT CAG GCT TAT GGT ATG AAG CCA CAT GAC ATA
TTC GAA GCA AAT GAT CTT TTT GAG AAT GGA AAC ATG ACC CAG GTT CAG ACT ACT CTG
GTG GCT CTA GCA GGT CTG GCT AAA ACA AAA GGA TTC CAT ACA ACC ATT GAC ATT GGA
GTT AAG TAT GCA GAA AAA CAA ACA AGA CGT TTT GAT GAA GGA AAA TTA AAA GCT
GGC CAA AGT GTA ATT GGT CTG CAG ATG GGA ACC AAC AAA TGT GCC AGC CAG GCA GGT
ATG ACA GCT TAC GGG ACT AGG AGG CAT CTT TAT GAT CCC AAA ATG CAA ACT GAC AAA
CCT TTT GAC CAG ACC ACA ATT AGT CTG CAG ATG GGC ACT AAT AAA GGA GCC AGC CAG
GCA GGG ATG TTA GCA CCA GGT ACC AGA AGA GAC ATC TAT GAT CAG AAG CTA ACA TTA
CAG CCG GTG GAC AAC TCG ACA ATT TCC CTA CAG ATG GGT ACC AAC AAA GTT GCT TCC
CAG AAA GGA ATG AGT GTG TAT GGG CTT GGG CGG CAA GTA TAT GAT CCC AAA TAC TGT
GCT GCT CCT ACA GAA CCT GTC ATT CAC AAC GGA AGC CAA GGA ACA GGA ACA AAT GGT
TCG GAA ATC AGT GAT AGT GAT TAT CAG GCA GAA TAC CCT GAT GAG TAT CAT GGC GAG
TAC CAG GAT GAC TAC CCC AGA GAT TAC CAA TAT AGC GAC CAA GGC ATT GAT TAT **TAG**

Stop

GAA TTC GCT AGT GGG CCC

EcoRI

A.3. Partial sequence of pcDNA3-Myc/*RNF10*

HindIII **Start** ***RNF10***

AAGCTT CGAATTC **ATG** CCG CTG AGC TCC CCC AAC GCC GCC GCC ACC GCC TCC GAC ATG
GAC AAG AAC AGC GGC TCC AAC AGC TCC TCC GCC TCT TCG GGC AGC AGC AAA GGG CAA
CAG CCG CCC CGC TCC GCC TCG GCG GGG CCA GCC GGC GAG TCT AAA CCC AAG AGC GAT
GGA AAG AAC TCC AGT GGA TCC AAG CGT TAT AAT CGC AAA CGT GAA CTT TCC TAC CCC
AAA AAT GAA AGT TTT AAC AAC CAG TCC CGT CGC TCC AGT TCA CAG AAA AGC AAG ACT
TTT AAC AAG ATG CCT CCT CAA AGG GGC GGC GGC AGC AGC AAA CTC TTT AGC TCT TCT
TTT AAT GGT GGA AGA CGA GAT GAG GTA GCA GAG GCT CAA CGG GCA GAG TTT AGC CCT
GCC CAG TTC TCT GGT CCT AAG AAG ATC AAC CTG AAC CAC TTG TTG AAT TTC ACT TTT
GAA CCC CGT GGC CAG ACG GGT CAC TTT GAA GGC AGT GGA CAT GGT AGC TGG GGA
AAG AGG AAC AAG TGG GGA CAT AAG CCT TTT AAC AAG GAA CTC TTT TTA CAG GCC AAC
TGC CAA TTT GTG GTG TCT GAA GAC CAA GAC TAC ACA GCT CAT TTT GCT GAT CCT GAT

ACA TTA GTT AAC TGG GAC TTT GTG GAA CAA GTG CGC ATT TGT AGC CAT GAA GTG CCA
TCT TGC CCA ATA TGC CTC TAT CCA CCT ACT GCA GCC AAG ATA ACC CGT TGT GGA CAC
ATC TTC TGC TGG GCA TGC ATC CTG CAC TAT CTT TCA CTG AGT GAG AAG ACG TGG AGT
AAA TGT CCC ATC TGT TAC AGT TCT GTG CAT AAG AAG GAT CTC AAG AGT GTT GTT GCC
ACA GAG TCA CAT CAG TAT GTT GTT GGT GAT ACC ATT ACG ATG CAG CTG ATG AAG AGG
GAG AAA GGG GTG TTG GTG GCT TTG CCC AAA TCC AAA TGG ATG AAT GTA GAC CAT CCC
ATT CAT CTA GGA GAT GAA CAG CAC AGC CAG TAC TCC AAG TTG CTG CTG GCC TCT AAG
GAG CAG GTG CTG CAC CGG GTA GTT CTG GAG GAG AAA GTA GCA CTA GAG CAG CAG
CTG GCA GAG GAG AAG CAC ACT CCC GAG TCC TGC TTT ATT GAG GCA GCT ATC CAG GAG
CTC AAG ACT CGG GAA GAG GCT CTG TCG GGA TTG GCC GGA AGC AGA AGG GAG GTC
ACT GGT GTT GTG GCT GCT CTG GAA CAA CTG GTG CTG ATG GCT CCC TTG GCG AAG GAG
TCT GTT TTT CAA CCC AGG AAG GGT GTG CTG GAG TAT CTG TCT GCC TTC GAT GAA GAA
ACC ACG GAA GTT TGT TCT CTG GAC ACT CCT TCT AGA CCT CTT GCT CTC CCT CTG GTA
GAA GAG GAG GAA GCA GTG TCT GAA CCA GAG CCT GAG GGG TTG CCA GAG GCC TGT
GAT GAC TTG GAG TTA GCA GAT GAC AAT CTT AAA GAG GGG ACC ATT TGC ACT GAG TCC
AGC CAG CAG GAA CCC ATC ACC AAG TCA GGC TTC ACA CGC CTC AGC AGC TCT CCT TGT
TAC TAC TTT TAC CAA GCG GAA GAT GGA CAG CAT ATG TTC CTG CAC CCT GTG AAT GTG
CGC TGC CTC GTG CGG GAG TAC GGC AGC CTG GAG AGG AGC CCC GAG AAG ATC TCA
GCA ACT GTG GTG GAG ATT GCT GGC TAC TCC ATG TCT GAG GAT GTT CGA CAG CGT CAC
AGA TAT CTC TCT CAC TTG CCA CTC ACC TGT GAG TTC AGC ATC TGT GAA CTG GCT TTG
CAA CCT CCT GTG GTC TCT AAG GAA ACC CTA GAG ATG TTC TCA GAT GAC ATT GAG AAG
AGG AAA CGT CAG CGC CAA AAG AAG GCT CGG GAG GAA CGC CGC CGA GAG CGC AGG
ATT GAG ATA GAG GAG AAC AAG AAA CAG GGC AAG TAC CCA GAA GTC CAC ATT CCC
CTC GAG AAT CTA CAG CAG TTT CCT GCC TTC AAT TCT TAT ACC TGC TCC TCT GAT TCT
GCT TTG GGT CCC ACC AGC ACC GAG GGC CAT GGG GCC CTC TCC ATT TCT CCT CTC AGC
AGA AGT CCA GGT TCC CAT GCA GAC TTT CTG CTG ACC CCT CTG TCA CCC ACT GCC AGT
CAG GGC AGT CCC TCA TTC TGC GTT GGG AGT CTG GAA GAA GAC TCT CCC TTC CCT TCC
TTT GCC CAG ATG CTG AGG GTT GGA AAA GCA AAA GCA GAT GTG TGG CCC AAA ACT GCT
CCA AAG AAA GAT GAG AAC AGC TTA GTT CCT CCT GCC CCT GTG GAC AGC GAC GGG GAG
AGT GAT AAT TCA GAC CGT GTT CCT GTG CCC AGT TTT CAA AAT TCC TTC AGC CAA GCT
ATT GAA GCA GCC TTC ATG AAA CTG GAC ACA CCA GCT ACT TCA GAT CCC CTC TCT GAA
GAG AAA GGA GGA AAG AAA AGA AAA AAA CAG AAA CAG AAG CTC CTG TTC AGC ACC
TCA GTC GTC CAC ACC AAG GGT ACC GAG CTC CCG GGA TCC AAG AGA GAT ACC ACC ATG

KpnI **Myc-epitope tag**

GCC CTC GAG CAA AAG CTC ATT TCT GAA GAG GAC TTG TCT AGA

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