

**IDENTIFICATION OF KATANIN P60  
INTERACTING PROTEINS**

**MSc. Thesis by  
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**JUNE 2008**

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**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

**KATANİN P60 İLE ETKİLEŞEN PROTEİNLERİN  
AYDINLATILMASI**

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**İstanbul, 2008**

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## ABBREVIATIONS:

<b>AAA</b>	: <u>A</u> TPases <u>A</u> ssociated with Various Cellular <u>A</u> ctivities
<b>AD</b>	: Activation domain
<b>Ade</b>	: Adenine
<b>Amp</b>	: Ampicillin
<b>ATP</b>	: Adenine tri-phosphate
<b>ATPase</b>	: Adenine tri-phosphate
<b>Bax</b>	: Bcl-2 associated x protein
<b>Blast</b>	: The Basic Local Alignment Search Tool
<b>cDNA</b>	: Complementary DNA to mRNA
<b>Cfu</b>	: Colony forming unit
<b>DMSO</b>	: Dimethyl Sulfoxide
<b>DNA-BD</b>	: DNA binding domain
<b>EDTA</b>	: ethylene diamine tetraacetic acid
<b>EtBr</b>	: Ethidium bromide
<b>GAL4</b>	: Galactose promoter binding transcription factor
<b>GC</b>	: Guanine-cytosine
<b>GDP</b>	: Guanosine di-phosphate
<b>GTP</b>	: Guanosine tri-phosphate
<b>His</b>	: Histidine
<b>Kan</b>	: Kanamycin
<b>LB</b>	: Luria bertani
<b>Leu</b>	: Leucine
<b>LiAc</b>	: Lithium Acetate
<b>MCS</b>	: Multiple cloning site
<b>mRNA</b>	: Messenger ribonucleic acid
<b>NADH</b>	: Nicotinamide adenine dinucleotide
<b>NCBI</b>	: National Center for Biotechnology Information
<b>Op18</b>	: Oncoprotein 18
<b>PCR</b>	: Poly chain reaction
<b>PEG</b>	: Polyethylene glycol
<b>PIPES</b>	: Piperazine-1,4-bis(2-ethanesulfonic acid)
<b>SD</b>	: Synthetic Dextrose
<b>SDS</b>	: Sodium dodecyl sulfate
<b>TAE</b>	: Tris-acetate EDTA
<b>Taq</b>	: <i>Thermus aquaticus</i>
<b>TE</b>	: Tris-EDTA
<b>Trp</b>	: Tryptophan
<b>UAS</b>	: Upstream activation sequences
<b>X-gal</b>	: 5-Brom-4-chlor-3-indoxyl- $\beta$ -D-galactopyranosid
<b>YPD</b>	: Yeast Peptone Dextrose
<b><math>\gamma</math>-TuRC</b>	: $\gamma$ -tubulin ring complex

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## KATANİN P60 PROTEİNİ İLE ETKİLEŞEN PROTEİNLERİN BELİRLENMESİ

### ÖZET

Katanin mikrotubul kesme özelliği ile hücre iskeleti dinamiğinde önemli görevlere sahiptir. Uzun mikrotubuller hareketsiz iken katanin tarafından kesilip küçük parçalara ayrılan mikrotubuller hareketlilik kazanırlar ve böylelikle hücre iskeleti organizasyonlarına katılabilirler. Bu kısa ve hareketli mikrotubuller, yeni nörit oluşumunda kullanılmaları bakımından özellikle nöronlar için son derece önemlidirler. Katanin, p60 katalitik ve p80 düzenleyici olmak üzere iki alt birime sahiptir. p60, p80 tarafından yönlendirildiği sentrozomlarda altılı bir yapı oluşturup, ATP hidroliziyle mikrotubulu keser. Ancak katanin p60 alt biriminin p80 alt biriminden farklı bir protein ile etkileşimi henüz gösterilmemiştir. Bu nedenle çalışmanın amacı katanin p60 ile etkileşime giren proteinlerin aydınlatılması olarak belirlenmiştir. Yeni protein etkileşimlerinin aydınlatılması için cDNA havuz çalışmalarında kullanılabilecek en iyi yöntem olan maya ikili hibrid sistemi kullanılmıştır. Etkileşime girecek olan proteinler insan embriyonik beyin cDNA kütüphanesinden seçilmiştir. Katanin p60 geni işaretçi gen transkripsiyon faktörünün DNA dizisine bağlandığı alt birimle birleştirilmiştir. P60'ın etkileşebileceği olası genleri içeren kütüphane de transkripsiyon faktörünün aktivasyon alt birimiyle birleştirilmiştir. Her bir plazmid farklı çiftleşme tipine sahip mayalara (*S. cerevisiae*) yerleştirildikten sonra bu suşların çiftleşmesi sağlanmıştır. Oluşan diploit mayalar, işaretçi genin aktivasyonu ile seçilmiştir. Mayaların çiftleşmesi sonucunda p60 ile etkileşen 22 proteinin etkileşimi doğrulanmıştır.

Katanin mikrotubul kesme özelliği ile mikrotubul dinamiği ve hücre iskeleti oluşumunda önemlidir. Bu sebeple katanin p60 ile etkileşime giren proteinlerin aydınlatılması, kataninin bu proteinlerle etkileşmesi sonucu sinir hücresi içindeki olası diğer rollerinin ortaya çıkartılması açısından sinir bilimi literatürü için çok önemlidir.

## IDENTIFICATION OF KATANIN P60 INTERACTING NEURONAL PROTEINS

### SUMMARY

Katanin has important functions in cytoskeletal dynamics through its microtubule severing activity. Long microtubules are stationary, whereas short microtubule fragments severed via katanin achieve motility and could contribute into cytoskeletal organizations. These short and motile microtubules are especially essential for neuronal branching. Katanin protein consists of two domains; p60 catalytic domain and p80 regulatory domain. P80 directs p60 to the centrosomes where p60 forms hexameric structure and severs microtubule by ATP hydrolysis. Nevertheless, there is no identified interaction of katanin with any protein except p80. Thus, purpose of this study was to identify katanin p60 interacting proteins in neuronal cells. In this study Yeast Two Hybrid System, which is the best method for detection of novel protein interactions from cDNA pool, was chosen for identification of new katanin p60 interacting proteins. “Clontech Matchmaker Yeast Two Hybrid” kit was used for this study and new interacting proteins were selected from fetal human brain cDNA library. Katanin p60 gene was fused with DNA binding domain of transcription factor of reporter gene and cDNA library was fused with activation domain of the same transcription factor. Each fusion construct was inserted into different mating types of *S. cerevisiae* and strains were mated. Following mating, diploid yeast cells were selected through reporter gene activation. 22 new protein interactions were identified and confirmed.

Katanin is a critical protein in microtubule dynamics and has roles in many important cellular activities. Therefore, elucidation of katanin p60 interacting proteins, revealing possible diverse roles of katanin in neurons through discovered interactions would provide significant data for neuroscience literature.

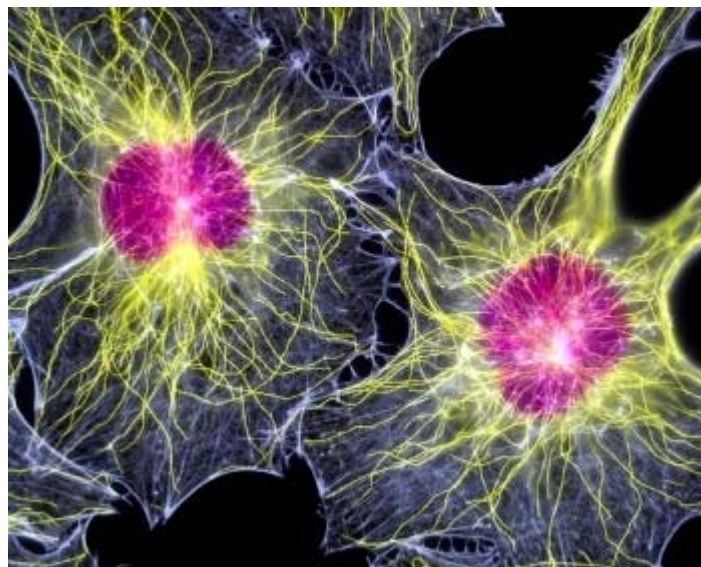
## 1. INTRODUCTION

### 1.1. Theoretical Background

#### 1.1.1. Cytoskeleton

Cytoskeleton is a scaffold of proteins present in cytoplasm (immunostained cytoskeletal proteins can be seen in Fig. 1.1). Cytoskeleton is a dynamic structure; thus, none of the construction is stable. In fact they are continuously assembling and disassembling. Cytoskeletal fibers assemble via polymerization of protein subunits, and disassemble via depolymerization into protein subunits.

Cytoskeleton has many important functions in the cell. Foremost, preservation of cell shape and maintenance of mechanical strength of the cell are attained by cytoskeleton. Furthermore, cell movement is achieved through cytoskeletal fiber organizations, e.g. flagella, cilia and pseudopodia. Moreover, intracellular transportation of molecules, mRNA, filaments, vesicles, and even organelles, is accomplished via motor proteins “walking” over cytoskeletal fibers. Last, but not the least, cytoskeletal elements function in cell division.



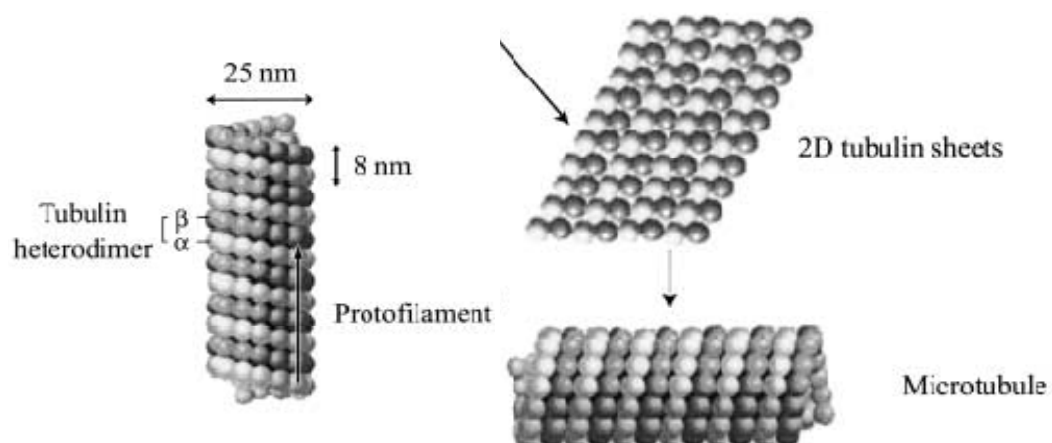
**Figure 1.1** Cytoskeleton (tubulins are yellow, actins are blue). (Wittmann, 2006)

Cytoskeletal fibers are separated into 3 groups, each formed from different subunits; intermediate filaments, microfilaments and microtubules. Intermediate elements are responsible of the shape and strength. These filaments are more durable than other cytoskeletal elements. Microfilaments are composed of bead-like dimeric actin molecules and also function in the attainment of cell shape and mechanical support and also in movement. Actin fibers take role in muscle contraction in vertebrates and form pseudopodia in amoeba and erythrocytes. Microtubules are hollow cylinders composed of  $\alpha$ -tubulin and  $\beta$ -tubulin. Microtubules are involved in cell division in the course of drawing chromosomes to the poles. They also supply the intracellular transport and cellular movement (Alberts et al., 2003).

### 1.1.2. Microtubules

#### 1.1.2.1. Structure and Organization of Microtubules

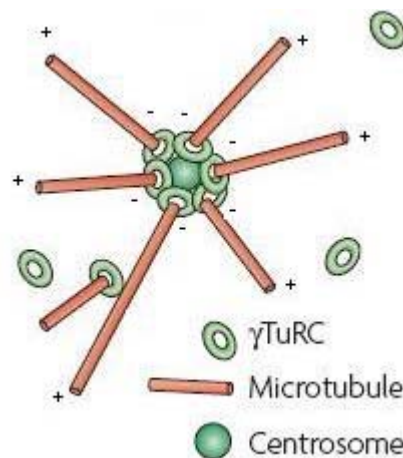
Microtubules, hollow cylinders with 25 nm diameter, are composed of  $\alpha$ -tubulin and  $\beta$ -tubulin. Initially,  $\alpha$ -tubulin and  $\beta$ -tubulin form heterodimer, subsequently these heterodimers build the structure of microtubules. Microtubule structure is demonstrated in Fig. 1.2.  $\alpha$ -tubulin and  $\beta$ -tubulin consist of 40 amino acids and amino acid sequences are 50 % identical (Burns, 1991). Each monomer of heterodimer binds to a GTP molecule;  $\alpha$ -tubulin binds to GTP non-exchangeable, whereas  $\beta$ -tubulin binds exchangeable fashion. GTP bound to  $\beta$ -tubulin is utilized during polymerization of tubulin.



**Figure 1.2** Microtubule structure (Valiron et al., 2001)

The centrosomes, consisting of 2 centrioles surrounded by an amorphous cloud of pericentriolar material, are the primary source of microtubule nucleation in eukaryotes. Pericentriolar material contains the  $\gamma$ -tubulin ring complexes ( $\gamma$ -TuRC) which are in charge of nucleating microtubules (Ahmad et al. 1994).  $\gamma$ -tubulins are arranged into circular ring structures in centrosomal pericentriole regions and nucleation from these structures apparently restricts the lattice structure of microtubule to 13 protofilaments (Baas, 1997).  $\gamma$ -TuRC has proteins additional to  $\gamma$ -tubulin, for instance ninein (Mogensen et al. 2000), functions in attaching  $\gamma$ -tubulin rings to pericentriole.

The organization of  $\alpha$ - and  $\beta$ -tubulin heterodimers in the microtubule lattice is polarized, and structural and kinetic differences are resulted from this characteristic at the microtubule ends. The faster growing end (plus end) has the  $\beta$ -tubulin subunit of each heterodimer exposed, whereas the slower growing end (minus end) has the  $\alpha$ -tubulin subunit exposed. *In vivo*, the minus end of the microtubule is associated with the  $\gamma$ -TuRC complex. Microtubule nucleation and polarity are illustrated in Fig 1.3.

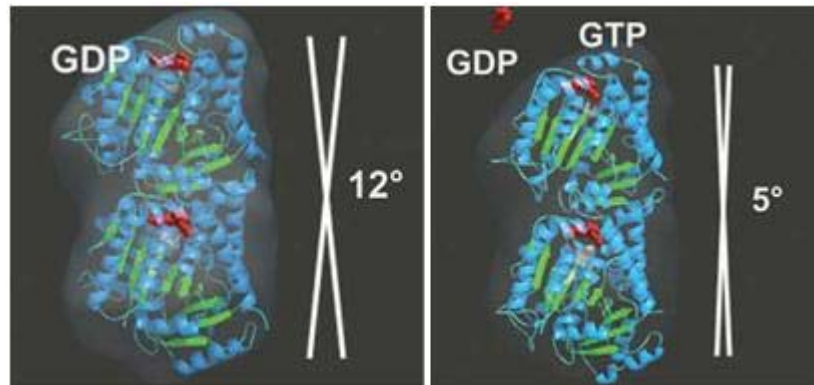


**Figure 1.3** Microtubule nucleation and polarity (Lüders and Stearns, 2007)

#### 1.1.2.2. Microtubule Dynamics

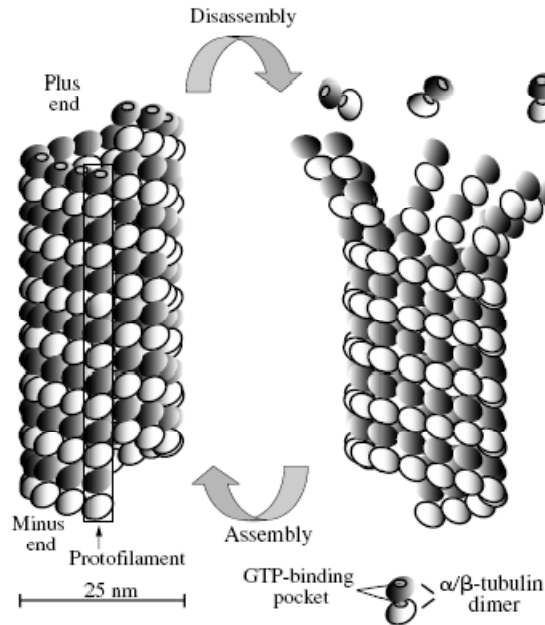
Elongation of the microtubule is arisen from the polymerization of tubulins. As mentioned before,  $\alpha$ - and  $\beta$ -tubulins initially assemble into dimmers; afterward, they construct protofilaments. Each of the protofilaments consists of a head-to-tail arrangement of  $\alpha$ -/ $\beta$ -tubulin heterodimers. When bound to GDP, the tubulin dimer is in a bent conformation, which assembles poorly into microtubule lattice. These conformation differences are demonstrated in Fig. 1.4. Exchange of GTP into its

active site straightens the dimer, facilitating its incorporation into a sheet at the growing end of the microtubule (Walczak, 2000). When sheet of 13 protofilament is sealed as a hollow cylinder, consequently microtubule lattice is build.



**Figure 1.4** Illustrations of conformation change due to bound nucleotide in tubulin heterodimer (Nogales and Wang, 2006)

Microtubules comprising GTP-bound tubulin at the plus end are stable since these GTP caps strengthen the lattice of microtubule. Therefore, microtubules with tubulin-GTP continue to elongate (Caplow and Shanks, 1996). This phase is emphasized as microtubule growth phase. As soon as the GTP cap of microtubule is detached, the lattice of microtubule becomes unstable and protofilaments are peeled from the lattice (Desai and Mitchison, 1997). This phase is known as shrinkage. Microtubules are switching between growth and shrinkage phases. This feature is entitled as dynamic instability (Fig. 1.5).



**Figure 1.5** Demonstration of dynamic instability (Wiese and Zheng, 2006)

Many microtubule interacting proteins are found. These proteins regulate the dynamics of microtubule organization. Microtubule interacting proteins are classified into two main groups: proteins that stabilize microtubules and proteins that destabilize microtubules. Microtubule associated proteins (MAPs) are known to stabilize microtubules by enhancing the rate of microtubule growth and suppressing the transitions from a growing phase to a shrinking state catastrophe (Drechsel et al., 1992; Kowalski and Williams, 1993). Microtubule destabilizing proteins are discovered since microtubule turnover *in vivo* is faster than *in vitro* (Cassimeris et al., 1988; Simon et al., 1992). Op18 (Belmont and Mitchison, 1996), XKCM1 (Walczak et al., 1996), katanin (Vale, 1991) are best known microtubule destabilizing proteins.

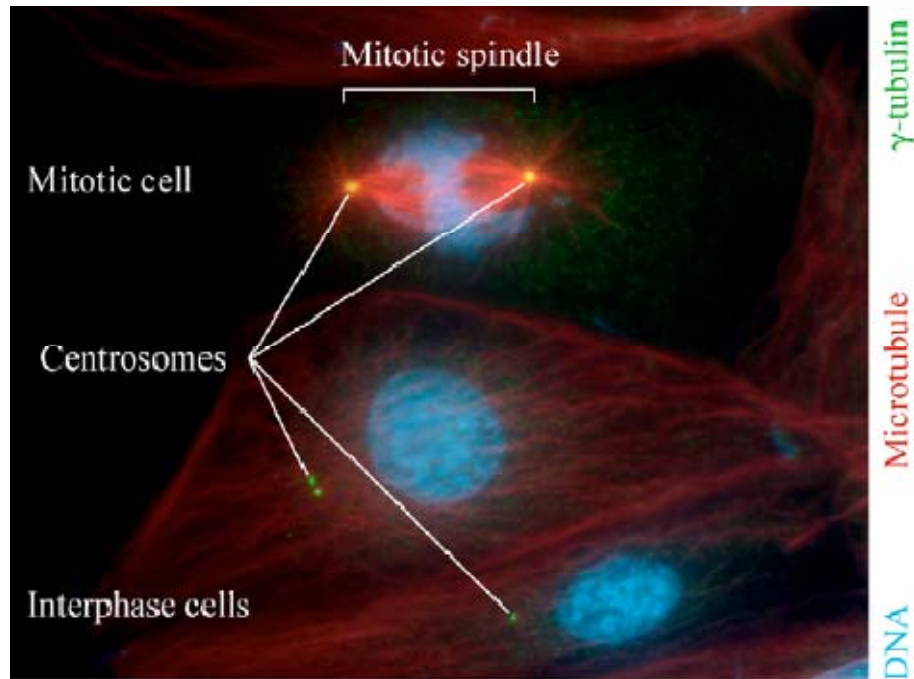
Both the structure of the lattice and the polarity of the microtubule are central to the function of microtubule motor proteins which are able to move on microtubules. There are two basic types of microtubule motors: plus-end motors and minus-end motors, depending on the direction in which they "walk" along the microtubules. Kinesins and dyneins are examples of microtubule motor proteins.

### 1.1.2.3. Microtubule Functions

Microtubules are crucial for many intracellular functions. During interphase, microtubules are required for organizing large intracellular membrane compartments,



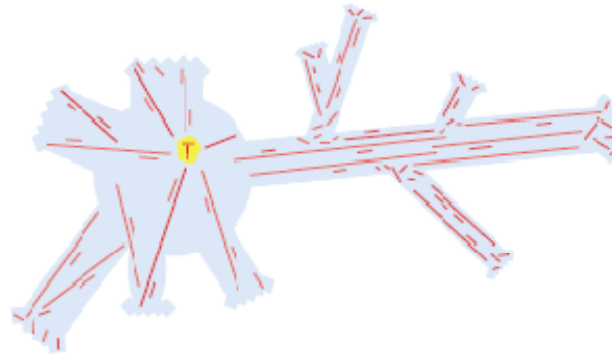
such as the Golgi apparatus (Ho et al., 1990) and the endoplasmic reticulum (Terasaki et al., 1986), as well as for transporting small membrane carrier vesicles in the endocytotic and secretory pathways (Vale, 1987). During mitosis, microtubules are the primary constituents of the mitotic spindle and are needed for proper segregation of chromosomes (Rappaport, 1985).



**Figure 1.6** Organisation of microtubule in mitotic and interphase cells (Weise and Zheng, 2006)

Microtubules serve extensively important functions in some specialized cells like neurons. Microtubules in neurons are not employed for spindle formation; but rather, they function in elongation of axons (Karabay et al., 2004). Crucial events for axonal differentiation such as elongation, branching, navigation, retraction, are accomplished by changes in the configuration and behavior of microtubules (Baas and Buster, 2004). Microtubules also serve as roadway for transport of organelles in both directions. Although microtubules are nucleated from centrosomes, they are transported to different intracellular domains in neurons (Fig. 1.7). One important target for microtubule transport is the axon. Neurons comprise a unique microtubule organization wherein microtubule bundles are located in axons and dendrites, and these microtubules are originally constructed at centrosomes (Baas, 1999). Microtubule is released from minus end or severed by a severing enzyme and

transport of these non-centromal microtubules are conveyed by motor proteins (Keating et al, 1997).



**Figure1.7** Microtubule organization in neurons (Baas et al, 2005).

### 1.1.3. Katanin

Microtubule severing is originally discovered in sea urchin lysate through observed shortening of microtubules (Vale, 1991). Microtubule severing protein is later identified as katanin for the namesake of the Japanese samurai sword, katana. Characterization of katanin reveals that katanin disrupts the lattice of microtubule in an ATP hydrolysis driven fashion (McNally and Vale, 1993).

Katanin comprise an AAA ATPase domain (ATPases Associated with various cellular Activities). Members of this domain are involved in various protein-protein interactions and function in a myriad of important cellular activities. Each protein conferred with one or two of 230-residue ATPase module which is conserved extensively with ~30% identity (Confalonieri and Duguet, 1995; Patel and Latterich, 1998). AAA proteins are prevalently act as hexameric rings since AAA core contains subunit-subunit interactions (Vale, 2000).

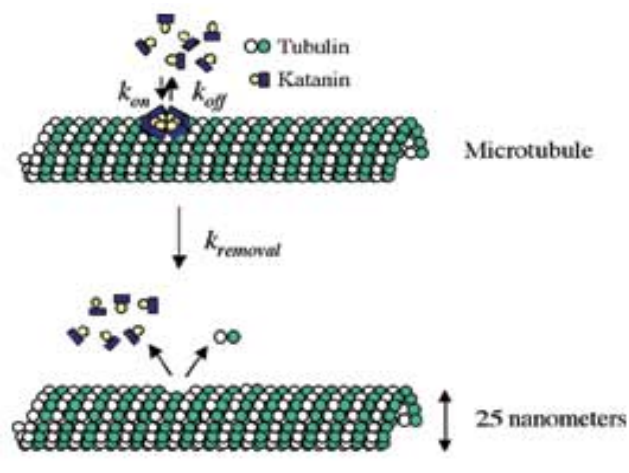
Katanin is a heterodimer consisting of 60-kDa and 80-kDa subunits. 60 kDa subunit is known as katanin p60; it is the catalytic subunit and comprises AAA ATPase domain. 80 kDa subunit is known as katanin p80; it is the regulatory domain and it has WD repeats in its protein structure for protein-protein interactions. p80 subunit is

not responsible for microtubule severing; however, it directs p60 to centrosomes since it has centrosome interaction domains. Catalytic activity of katanin p60 hexameric ring does not require the presence of p80. However, in the presence of p80, severing activity of p60 increases two fold (Hartman et al. 1998). Katanin is a severing protein conferred with microtubule driven ATP hydrolysis (Hartman and Vale, 1999).

Severing of microtubule is substoichiometrical; thus, one katanin protein is able to release more than one tubulin subunit. Moreover, released tubulins are able to be used in polymerization again; thus katanin do not proteolyze or modify structure of tubulins (McNally and Vale, 1993).

Since the first experiments were performed on sea urchin and *Xenopus*, vertebrate homologous proteins are investigated in later studies. McNally and Thomas isolated human katanin homologous protein and also showed the presence of human katanin homologous protein in different tissue types (McNally and Thomas, 1998).

Katanin severs microtubules by generating an internal break on microtubule lattice (Shiina et al., 1992; McNally and Vale, 1993). However, the exact mechanism of microtubule severing still remains to be unsolved. There is a model postulation for katanin severing which states that the microtubules are providing a scaffold for katanin 60 subunits to interact and form hexameric catalytic structure (Fig. 1.8). When the hexameric katanin p60 forms, it cuts the scaffold microtubules (Hartman and Vale, 1999).

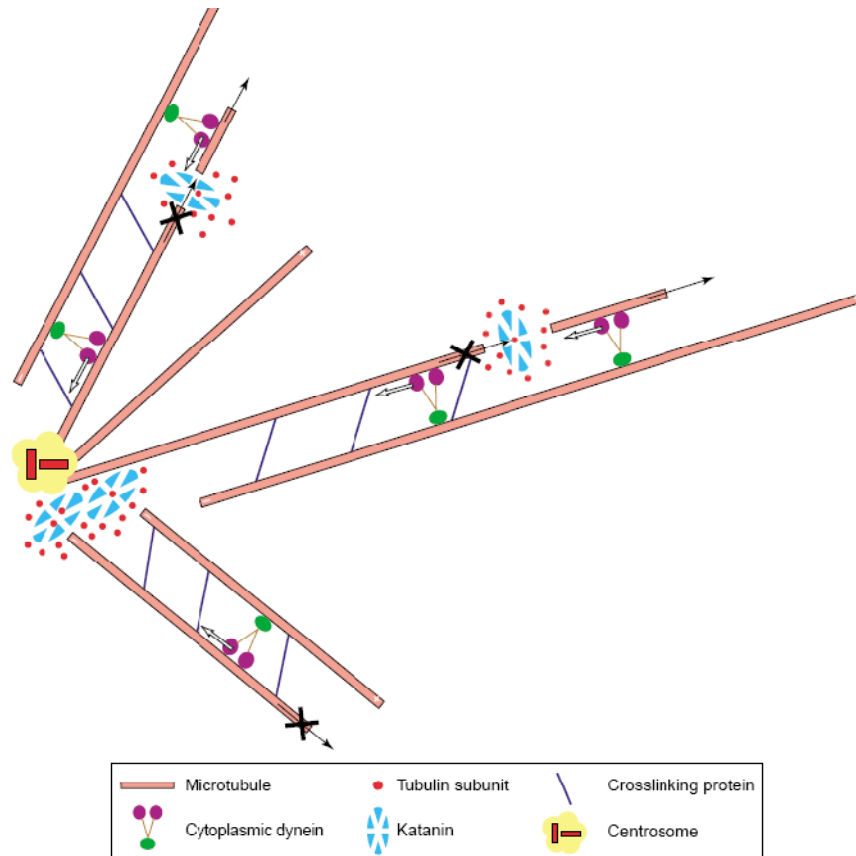


**Figure 1.8** Severing of microtubules with katanin (Hartman and Vale, 1999)

Severing of microtubules with katanin has potential functions in several changes in the microtubule cytoskeleton observed *in vivo*. Katanin is found to be localized near centrosomes (McNally et al., 1996). Poleward flux of tubulin to centrosome in mitotic spindle require microtubule disassembly (Mitchison, 1989), katanin may disassemble microtubules in order to generate poleward flux or it just cuts from  $\gamma$ -tubulin (Moritz et al., 1995; Zheng et al., 1995). Since it is found that microtubules have their minus ends unattached from centrosome (Kitanishi-Yumura and Fukui, 1987) katanin may take role in the release of microtubule from centrosome. Lastly, fast depolymerization of microtubules in G2/M transition (Zhai et al., 1996) may be generated by katanin.

Neurons comprise highly abundant and specialized microtubule organization thus katanin serves extensively important functions in neurons. Structurally, axon is composed of bundles of microtubule where plus ends of axonal microtubules are not GTP capped but it is highly stable. This stabilization may present due to transportation of new microtubules to the axon from centrosomal sites (Karabay et al., 2004). Microtubules are cut in the centrosomal sites and they are conveyed to dendritic or axonal site (Baas et al., 2005) since these locations require microtubules for structural support and transport of materials (Quarmby, 2000).

In order to provide microtubule fragments for axonal and dendritic processes, “microtubule cut and run model” is recruited (Fig 1.9). Rationale of model is as followed; long stationary microtubules are severed into small fragments of microtubules and these newly generated small microtubules are transported with dyneins to axonal and dendritic processes (Baas et al., 2005).



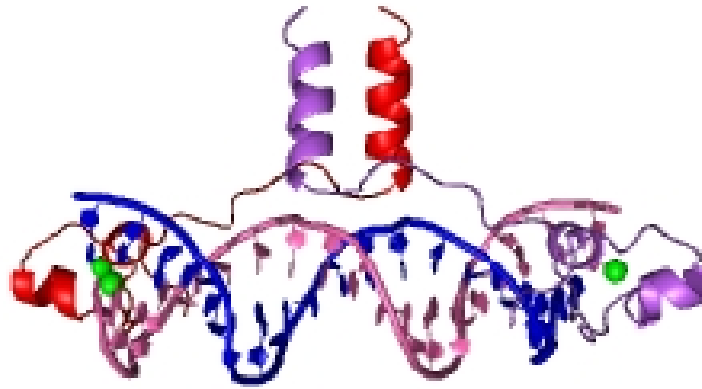
**Figure 1.9** Microtubules cut and run mode (Baas et al., 2005).

Severing activity of katanin is regulated by the presence of MAP4 in mitotic fibroblast cells (McNally et al., 2002) and tau in neurons (Qiang et al., 2006). Both MAP4 and tau are strong microtubule binding proteins; therefore they reduce the access of katanin to microtubule. Recent studies reveal that katanin is present throughout the axonal microtubules; however, katanin is able to reach only tau unoccupied microtubule sites (Yu et al., 2008).

## 1.2. Experimental Background

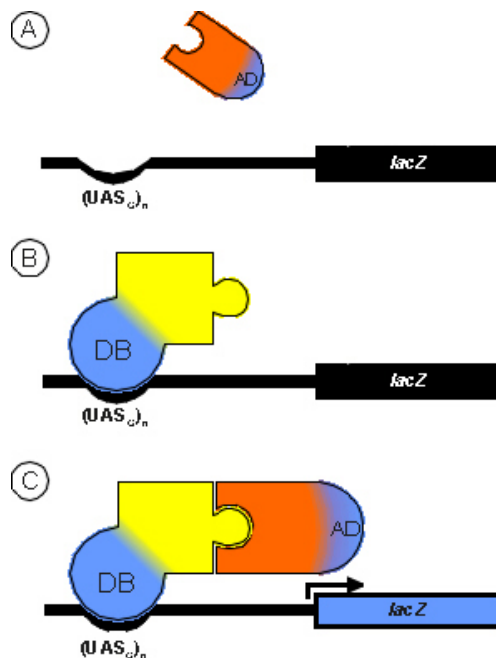
### 1.2.1. Yeast Two hybrid

The method is based on the modular properties of GAL4 transcription factor which consists of two separable domains of DNA-binding and transcriptional activation (Fig 1.10) (Keegan et al., 1986). In fact, many eukaryotic transcription activators comprise at least two distinct functional domains, one that directs binding to a promoter DNA sequence and one that activates transcription (Hope and Struhl, 1986).



**Figure 1.10** Native GAL4 protein dimer in association with DNA (Protein Database, P04386)

Plasmids encoding two hybrid proteins, one consisting of the GAL4 DNA-binding domain fused to protein of interest (bait) and the other consisting of the GAL4 activation domain fused to cDNA library (prey), are constructed and introduced into yeast (Fig. 1.11). Reporter gene with GAL4 promoter region is activated with interaction between bait and prey domains (Fields and Song, 1989; Chien et al., 1991).

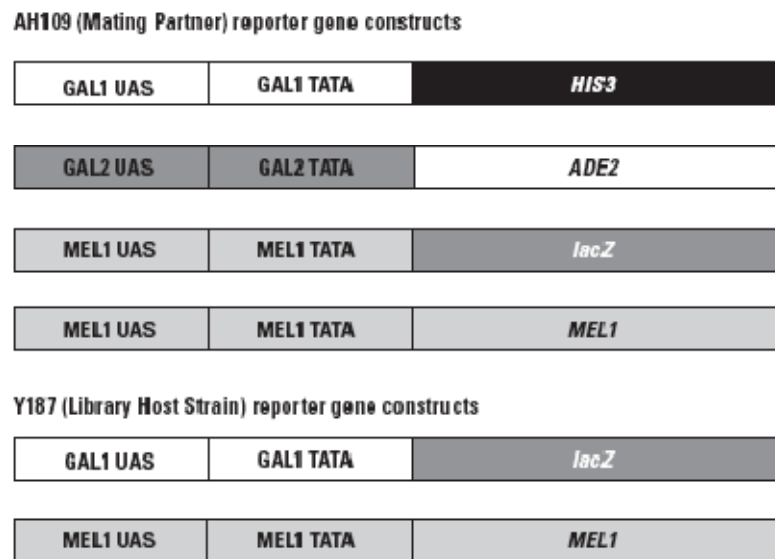


**Figure 1.11** Principle of Yeast Two Hybrid Technique, (A), (B) Two fusion proteins, one containing the DNA-binding domain (DB: blue circle) and one that contains an activation domain (AD: half blue circle), are co-transfected into an appropriate host strain. (C) If the fusion partners (yellow and red) interact, the DB and AD are brought into proximity and can activate transcription of reporter genes (Van Criekinge and Beyaert, 1999).

This technology can be used for identifying novel protein interactions, confirming suspected interactions, and defining interacting domains.

### 1.2.2. Reporter Genes

In this study, Clontech Yeast Two Hybrid product family is employed; since Clontech's Matchmaker Yeast Strain AH109 expresses four integrated reporter genes in response to two-hybrid interactions under the control of distinct GAL4-responsive upstream activating sequences (UASs) and TATA boxes (Fig. 1.12). Therefore, each gene is transcribed from a distinct promoter sequence. Consequently library proteins that bind to flanking regions of promoter, are eliminated.



**Figure 1.12** Reporter genes: Promotor regions for GAL4 protein in AH109 and Y187 strain. (Clontech, 2005)

Here is the list of reporter genes:

***HIS3***. AH109 is unable to synthesize histidine and is therefore unable to grow on media that lack this essential amino acid. When bait and prey proteins interact, Gal4-responsive His3 expression permits the cell to biosynthesize histidine and grow on a his- minimal medium.

***ADE2***. AH109 is also unable to grow on minimal media that does not contain adenine. However, when two proteins interact, Ade2 expression is activated, allowing these cells to grow on ade- minimal medium.

**MEL1.** MEL-1 encodes  $\alpha$ -galactosidase, an enzyme occurring naturally in many yeast strains. As a result of two-hybrid interactions,  $\alpha$ -galactosidase (MEL1) is expressed and secreted by the yeast cells. Yeast colonies that express Mel1 turn blue in the presence of the chromagenic substrate X- $\alpha$ -Gal.

**LacZ.** Lac Z encodes  $\beta$ -galactosidase ( $\beta$ -Gal), an E.coli enzyme which is integrated into the AH109 chromosome. As a result of two-hybrid interactions,  $\beta$ -galactosidase is expressed, but not secreted. Thus, only if the cells are lysed, as in a colony lift assay, can a blue color be detected in the presence of X-Gal. In contrast, X- $\alpha$ -Gal detection allows blue colonies to be visualized directly on the growth medium.

### 1.2.3. Advantages and Limitations for Yeast Two Hybrid

Yeast Two Hybrid technique comprises advantageous and disadvantageous properties, as with any technique. On one hand it is cheap, easy to perform and allows screening a large number of proteins. On the other hand, interaction medium is restrained to yeast nucleus, false positives and false negatives can arise, membrane bound proteins are not assayed properly (Hollingsworth and White, 2004).

**Table 1.1** Advantages and Limitations for Yeast Two Hybrid

Advantages	Limitation
<ul style="list-style-type: none"> <li>• Cheap, simple and versatile</li> <li>• <i>In vivo</i> protein interaction analysis</li> <li>• Capable of detecting weak or even transient interactions</li> <li>• Can be used to identify novel interactors by cDNA library screening</li> <li>• Several variations allow multiple applications</li> <li>• Can be scaled up through automation for genomic-scale protein interaction mapping</li> </ul>	<ul style="list-style-type: none"> <li>• Yeast cell environment may not fully mimic mammalian cells (e.g. post-translational modifications may not be replicated in yeast)</li> <li>• Interactions are assayed in the yeast nucleus rather than the correct cellular compartment</li> <li>• Membrane-bound proteins and transcription are often not suitable (since Y2H forces proteins into the nucleoplasm and relies on transcriptional activation as a read-out).</li> <li>• False positives and false negatives can occur</li> </ul>



### **1.3. Aim of the study**

Katanin is an extremely important protein for all cell types because of cytoskeletal arrangements which are essential for each cell.

Since microtubules are employed for axonal elongation instead of spindle formation in neurons, microtubule organizations are extensively involved in axonal differentiation. Axonal differentiation occurs at distant places from the cell body and rearrangements of cytoskeletal elements are required for axon processes; therefore, microtubules should be transported to dendritic and axonal process sites. For this purpose, microtubules are severed with katanin and transported to differentiation site with motor proteins. Consequently, katanin is involved in neuronal differentiation via microtubule severing property.

Katanin may also comprise a function in regeneration of neuronal injuries. The damaged axons may be reconstituted with microtubules that are generated via microtubule severing. Moreover, katanin may also function in regeneration of new branching sites. Importance of katanin with neurodegenerative diseases may be derived from this property.

In molecular learning, the hypothesis is that neurons form new connection with their target sites. This neuron targeting requires new cytoskeletal rearrangements. Katanin may involve in molecular learning models with its recruitment in formation of new axonal processes to prospective target neurons via its microtubule severing property.

Although the structure of katanin is studied extensively, protein interaction pattern of katanin still remains to be discovered. Known katanin p60 interactions are restricted with microtubule, p80 subunit and p60 itself. Since katanin has exceptionally essential functions in neurons; it possibly interacts with many more proteins and accordingly involves in many neuronal pathways.

In this study, protein interactions of katanin in whole human fetal brain are examined. Results of the study can enlighten the protein interactions hence other possible functions of katanin.

## 2. MATERIALS AND METHOD

### 2.1. MATERIALS

#### 2.1.1. Equipments

The equipments employed in this study are illustrated in the table below:

**Table 2.1** Equipment

<b>Equipment</b>	<b>Supplier Company</b>
DNA sequencer	Applied Biosciences 3100 Avant
Electrophoresis Gel System	E-C Apparatus Corporation, EC250-90 Minicel Primo
Microcentrifuge	Beckman Coulter
pH Meter	Mettler Toledo MP220
Precision Weigher	Precisa 620C SCS
Shaker	Forma
UVIPhoto MW Version 99.05 for Windows 95 & 98	UVItec Ltd.
UV Transilluminator	Biorad UV Transilluminator 2000
Vortex	Heidolph, Reaxtop
Water Bath	Memmert

## 2.2. Chemicals

The chemicals used in this study are listed in the table below:

**Table 2.2** Chemicals

<b>Chemical</b>	<b>Supplier Company</b>
Low Melting Agarose X-gal	Applichem
PIPES	BDH Laboratory
YPD liquid Medium YPD AgarMedium SD Medium SD Agar Medium -Trp/-Leu Drop-out Amino acid Mixture -His/-Leu/-Trp Drop-out Amino acid Mixture -Ade/-His/-Leu/-Trp Drop-out Amino Acid Mixture	Clontech
MgCl <sub>2</sub> dNTP Mix DNA ladder DNA loading Dye	Fermentas
Isopropanol CaCl <sub>2</sub> Glycerol NaCl Glacial Acid	Fluka
p60 specific primers	Integrated DNA Technologies
pACT2-pGBKT7 specific primers	Alpha DNA
Tryptone	Lab M TM
Glucose EDTA EtBr Tris Base Yeast Extract Agar KCl MgCl <sub>2</sub>	Merck
Na <sub>2</sub> HPO <sub>4</sub> NaH <sub>2</sub> PO <sub>4</sub>	J.T. Baker

### 2.1.3. Enzymes and Buffers

Enzymes and buffers used in this study are given in the table below:

**Table 2.3** Enzymes and buffers

<b>Product</b>	<b>Supplier Company</b>
Long Pcr Enzyme Mix Taq polymerase Cfr9I (XmaI) restriction enzyme Sal I restriction enzyme HindIII Restriction Enzyme 10X Taq Polymerase Buffer 10X Long PCR Enzyme Mix Buffer	Fermentas
T4 ligase T4 ligase buffer	Roche
Lyticase from <i>Anthrobacter luteus</i>	Sigma

### 2.1.4. Commercial Kits

Commercial kits used in this study are illustrated in the table below:

**Table 2.4** Commercial Kits

<b>Kit</b>	<b>Supplier Company</b>
High Pure Plasmid Purificatin Kit	Roche
QiaPrep Spin Miniprep Kit	Qiagen
Qiagen Plasmid Maxi Prep	Qiagen
Bid Dye Terminator v 3.1 Cycle Sequencing Kit	Applied Biosystems
The Yeastmaker Yeast Transformation System 2	Clontech
Pretransformed fetal human brain cDNA library	Clontech

### 2.1.5. Buffer and Solutions:

Buffers and solutions used in this study are illustrated in the table below:

**Table 2.5** Buffers and Solutions

<b>Buffer/Solution</b>	<b>Content</b>
TAE Buffer (50X)	40 mM Tris Base 20mM Glacial acetic acid 1 mM EDTA (pH 8.) dH <sub>2</sub> O
CaCl <sub>2</sub> Solution	60 mM CaCl <sub>2</sub> 10mM PIPES 15% glycerol dH <sub>2</sub> O
TE/LiAc Solution	110 mM LiAc 1.1 X TE
PEG/LiAc Solution	%40 (w/v) PEG 4000 100 mM 1X TE
Buffer Z	60 mM Na <sub>2</sub> HPO <sub>4</sub> 40 mM NaH <sub>2</sub> PO <sub>4</sub> 10 mM KCl 10 mM MgSO <sub>4</sub> dH <sub>2</sub> O (pH 7.0 with HCl)

### 2.1.6. Microorganism Strains

#### 2.1.6.1. Bacterial Strains

*Escherichia coli* (*E.coli*) DH5 $\alpha$  strain [F<sup>-</sup>,  $\phi$ 80*dlacZ* $\Delta$ M15,  $\Delta$ (*lacZYA-argF*)U169, *deoR*, *recA1*, *endA1*, *hsdR17*(*rk*<sup>-</sup>, *mk*<sup>+</sup>), *phoA*, *supE44*,  $\lambda$ <sup>-</sup>, *thi-1*, *gyrA96*, *relA1*]

#### 2.1.6.2. Yeast strains

*Saccharomyces cerevisiae* (*S. cerevisiae*) AH 109 strain [MAT $\alpha$ , *trp1-901*, *leu2-3*, *112*, *ura3-52*, *his3-200*, *gal4* $\Delta$ , *gal80* $\Delta$ , *LYS2* : : GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2 URA3 : : MEL1UAS-MEL1TATA-LacZ], Clontech

*Saccharomyces cerevisiae* (*S. cerevisiae*) Y187 strain [MAT $\alpha$ , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3*, *112*, *gal4* $\Delta$ , *gal80* $\Delta$ , *met*<sup>-</sup>, URA3 : : GAL1UAS-GAL1TATA-LacZ MEL1], Clontech

#### 2.1.7. Culture Media

Luria Bertani (LB) Medium: 10 gram (g) tryptone, 5 g yeast extract, and 10 g NaCl were dissolved in distilled water and the volume was adjusted to 1 L. The media was sterilized by autoclaving for 15 minutes. In order to make selection media, antibiotics

were added to the LB medium after sterilization was completed and the media was cooled down to approximately 55 °C. Stock and working concentrations of used antibiotics are given below:

**Table 2.6** Stock and Working Solutions of Antibiotics

<b>Antibiotic</b>	<b>Stock Solution Concentration</b>	<b>Working Solution Concentration</b>
Kanamycin	10 mg/L in distilled water	50 µg/L
Ampicillin	10 mg/L in distilled water	50 µg/L

Agar plates were prepared by adding 20 g/L to LB Medium solution and sterilized at 121 °C with autoclaving for 15 minutes.

SOC medium was used to cultivate *E. coli* cells after heat shock transformation. 2 g tryptone, 5 g yeast extract, 0.058 g NaCl, 0.0186 g KCl, 0.095 g MgCl<sub>2</sub>, and 0.23 MgSO<sub>4</sub> were dissolved in 80 ml distilled water and sterilized at 121 °C with autoclaving for 15 minutes. 0.36 g glucose was dissolved in 20 ml distilled water and solution was sterilized with filter-sterilization. These two sterile solutions were mixed and used.

YPD (Yeast Peptone Dextrose) medium was prepared by dissolving 20 g of Difco peptone, 10 g of yeast extract, and 2 g of dextrose in 1 L distilled water. The media was sterilized by autoclaving for 15 minutes.

In order to make agar plates, 20 g/L agar was added to ingredients then autoclaving for 15 minutes was performed.

In order to make Adenine supplemented YPD, medium was prepared as standard YPD preparation, after autoclaving 1.5 ml of filter-sterilized 2% Adenine hemisulfate (w/v) was added to medium.

SD (Synthetic Dextrose) medium was prepared by dissolving 20 g dextrose, 6.7 g yeast nitrogen base without amino acids, in 1 L distilled water. 20 g agar was added to SD ingredients in order to prepare SD Agar plates. Appropriate drop-out amino acid mixtures were added to SD before sterilization with autoclaving for 15 minutes. Final concentrations of additive amino acids are given in the table below:

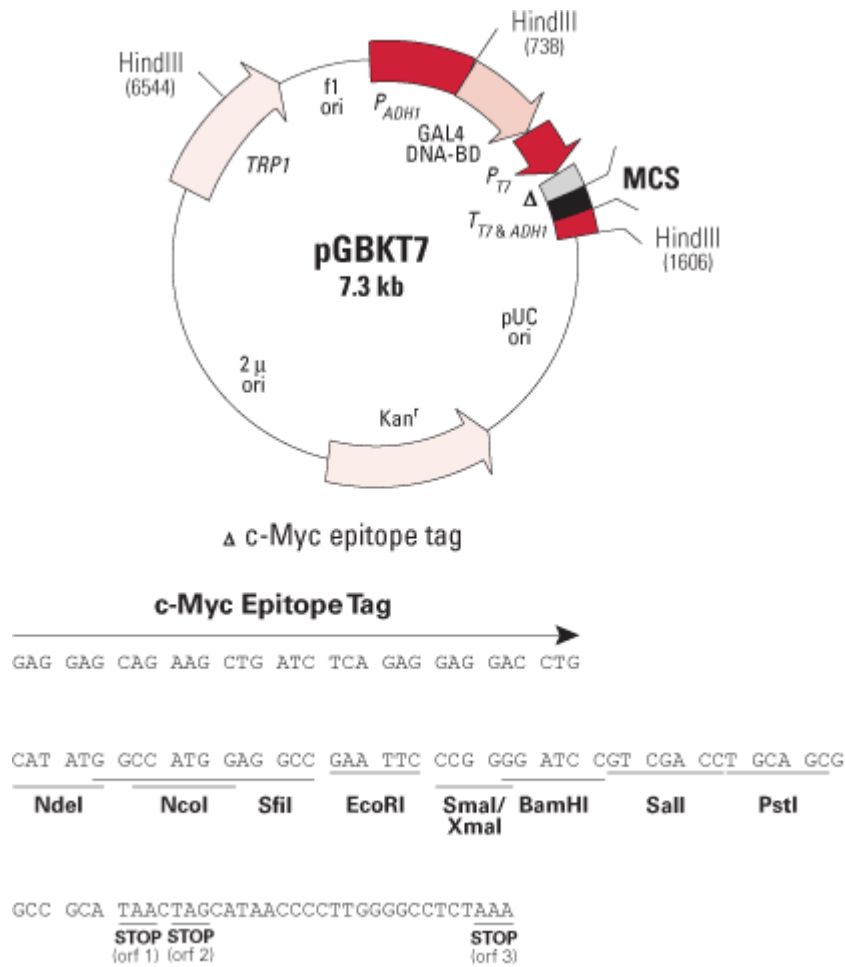
**Table 2.7** Amino acid supplement final concentrations

<b>Nutrient</b>	<b>Final concentration</b>
L-Adenine hemisulfate salt	20 mg/L
L-Arginine HCl	20 mg/L
L-Histidine HCl monohydrate	20 mg/L
L-Isoleucine	30 mg/L
L-Leucine	100 mg/L
L-Lysine HCl	30 mg/L
L-Methionine	20 mg/L
L-Phenylalanine	50 mg/L
L-Threonine	200 mg/L
L-Tryptophan	20 mg/L
L-Tyrosine	30 mg/L
L-Uracil	20 mg/L
L-Valine	150 mg/L

### 2.1.8. Vectors

#### 2.1.8.1. DNA Binding Domain Vector, pGBKT7

The pGBKT7 vector expresses proteins fused to amino acids 1–147 of the GAL4 DNA binding domain (DNA-BD). In yeast, fusion proteins are expressed at high levels from the constitutive *ADHI* promoter (*PADHI*); transcription is terminated by the T7 and *ADHI* transcription termination signals (*TT7* & *ADHI*). pGBKT7 also contains the T7 promoter, a c-Myc epitope tag, and a MCS. pGBKT7 replicates autonomously in both *E. coli* and *S. cerevisiae* from the pUC and 2  $\mu$  ori, respectively. The vector carries the Kan<sup>r</sup> for selection in *E. coli* and the *TRP1* nutritional marker for selection in yeast. Yeast strains containing pGBKT7 exhibit higher transformation efficiency than strains carrying other DNA-BD domain vectors.



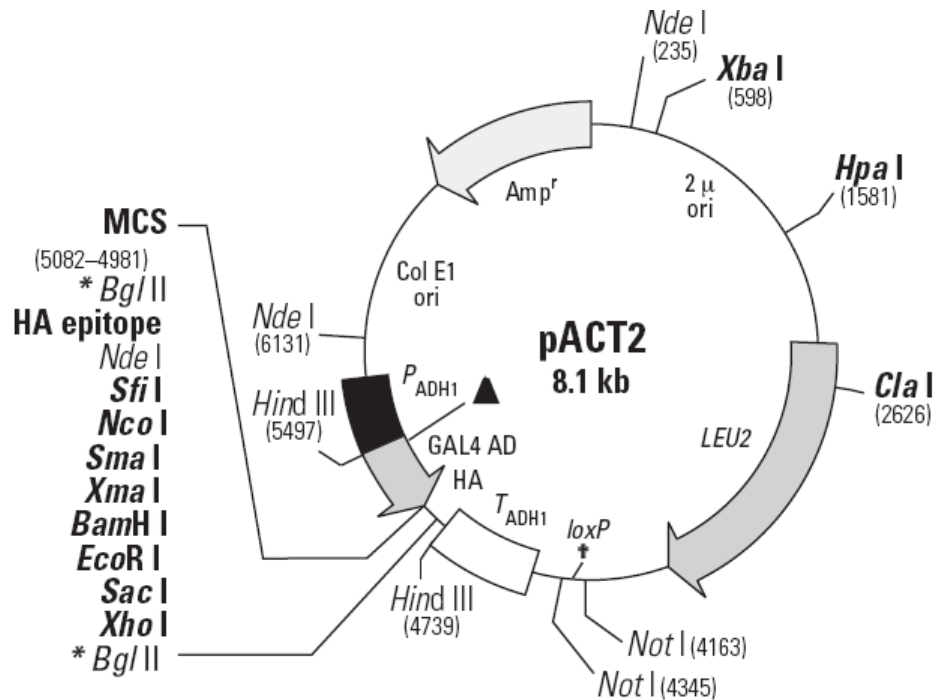
**Figure 2.1** Restriction map and multiple cloning site of pGBKT7 vector

### 2.1.8.2. Activation Domain Vector, pACT2

pACT2 generates a fusion of the GAL4 AD (amino acids 768–881), an HA epitope tag, and a protein of interest (or protein encoded by a cDNA in a fusion library) cloned into the MCS in the correct orientation and reading frame. pACT2, which is derived from pACT, contains a unique *EcoR* I site in the MCS. The hybrid protein is expressed at high levels in yeast host cells from the constitutive ADH1 promoter (*P*); transcription is terminated at the ADH1 transcription termination signal (*T*). The protein is targeted to the yeast nucleus by the nuclear localization sequence from SV40 T-antigen which has been cloned into the 5' end of the GAL4 AD sequence. pACT2 is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae* and carries the *bla* gene, which confers ampicillin resistance in *E. coli*. pACT2 also contains the *LEU2* nutritional gene that allows yeast auxotrophs to grow



on limiting synthetic media. Transformants with AD/library plasmids can be selected by complementation by the *LEU2* gene by using an *E. coli* strain that carries a *leuB* mutation (e.g., HB101).



**Figure 2.2** Restriction map and multiple cloning site of pACT2 plasmid.

## 2.2. METHODS

### 2.2.1. Bait Plasmid Construction

#### 2.2.1.1. Primer Design

Homo sapiens katanin p60 is present as inserted in pEGFP. Since restriction sites in this plasmid are not suitable, primers with appropriate flanking restriction site were designed for the gene by considering the general rules of primer design. For instance, primers should not form a secondary structure which prevents annealing to the template and GC content of primers should not exceed 40%. The sequence information of p60 was taken from <http://www.ncbi.nih.gov/> NM\_007044

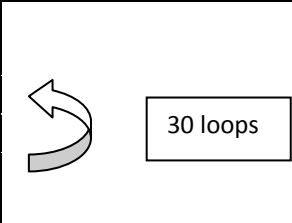
### 2.2.1.2. PCR of p60 cDNA

Polymerase Chain Reaction is used to amplify a fragment from template DNA sequence. Borders of amplified fragments are defined with small oligonucleotides called primers. These primers provide a binding site on template DNA for DNA polymerases. Polymerases are able to work *in vitro* and preserve their natural conformation at high temperature as 95 °C which is used to denature template DNA thoroughly. For this purpose, thermophilic bacteria DNA polymerases are used. The buffers are added to preserve DNA polymerase in its natural confirmation. dNTP molecules are added to be integrated into recently forming DNA molecules. The theory of PCR is as followed; DNA denatures at high temperature, primers bind to single stranded template DNA polymerase creates the new DNA fragment. Since each newly produced fragment is able to serve as template, the number of DNA molecules increases logarithmically. Applied PCR reaction and PCR program is given below:

**Table 2.8** PCR Reaction for cloning p60 cDNA

Ingredients Stock Solutions	Amount for 1X rxn mix
Long enzyme Buffer (10X)	2.5 µl (1X)
Forward primer (25 mM)	0.5 µl (0.5 mM)
Reverse primer (25 mM)	0.5 µl (0.5 mM)
dNTP (10 mM)	0.5 µl (0.2 mM)
Template pEGFP-Hsp60 (2ug/ul)	0.1 µl (0.2 ug DNA)
Long enzyme mix (5u/ul)	0.2 µl (1 unit)
Sterile mq water	20.7 µl
Total	25 µl

**Table 2.9** PCR program for cloning p60 cDNA

Initial denaturation (94 °C)	: 3 min	
Denaturation (94 °C)	: 15 sec	
Annealing (58 °C)	: 30 sec	
Extension (68 °C)	: 30 sec	
Final extension (68 °C)	: 5 min	

PCR products were run on 1% (w/v) low melting agarose-TAE, with 75 mV for 45 minutes in electrophoresis to determine length of DNA product.

### **2.2.1.3. Purification of p60 cDNA PCR product**

After PCR, the fragments were purified with Roche High Pure PCR product purification kit. This kit removes the protein contamination derived from polymerase enzymes and nucleic acid contamination derived from primers. The principle of the kit is given as followed; DNA is bound to glass fleece with aid of high concentrated chaotropic salt; remains of protein are removed with washes. Finally, the DNA molecules are released with low salt concentration elution buffer. The purification protocol is given below:

- Total volume of PCR product was brought to 100  $\mu$ l and 500  $\mu$ l Binding Buffer [3 M guanidine-thiocyanate, 10 mM Tris-HCl, 5% ethanol (v/v), 2 mg RNAase, pH 6.6 (25° C)] was added to each 100  $\mu$ l PCR tube.
- After mixing the sample well, sample was transferred into collection filter tubes and centrifuged for 1 minute at 14000 rpm at table top centrifuge.
- Flow through was discarded and 500  $\mu$ l Wash buffer [20 mM NaCl, 2 mM Tris-HCl, 80% ethanol pH 7.5 (25°C)] was added. Then again the filter tube was centrifuged at 14000 rpm for 1 minute.
- Flow through was discarded and 200  $\mu$ l of Wash Buffer was added. The mixture was centrifuged at 14000 rpm for 1 minute.
- Flow-through was discarded and filter was connected to a clean 1.5 ml eppendorf tube. 50  $\mu$ l of Elution buffer [10 mM Tris-HCl, pH 8.5 (25°C)] was added; then, it was centrifuged at 14000 rpm for 1 minute.
- DNA concentration was determined by subsequent OD<sub>260</sub> measurement (An OD<sub>260</sub> of 1 corresponds to 50  $\mu$ g/ml).

### **2.2.1.4. Restriction of p60 cDNA**

DNA fragments are cut with sticky end restriction enzymes forming nicks in the structure of DNA, this phenomenon serves for generating sticky overhangs which has ability to complement with a fragment containing same overhang. Some restriction enzymes cut bluntly, generating no overhangs. Using this type of restriction enzyme can be excruciating when the gene has to be inserted in a significant direction. Purified DNA fragments and target vector pGBKT7 were cut with Cfr9I and Sall. The reaction mixture is given in the table below:

**Table 2.10** Restriction reaction mixture of pGBKT7 and p60 cDNA

	<b>pGBKT7</b>	<b>Hsp60</b>
DNA template	10 $\mu$ l	10 $\mu$ l
Fast Digest Buffer	2 $\mu$ l	2 $\mu$ l
Cfr9I	0.5 $\mu$ l	0.5 $\mu$ l
Sall	0.5 $\mu$ l	0.5 $\mu$ l
Sterile mq H2O	7 $\mu$ l	7 $\mu$ l
Total	20 $\mu$ l	20 $\mu$ l

The restriction reactions were incubated at 37 °C for 3 hours. The resulting mixture was run on gel to understand if the fragments are cut properly. Later, restricted fragments were again purified with Roche High Pure PCR Product Purification Kit.

#### 2.2.1.5. Ligation of p60 cDNA into pGBKT7 vector

Purified vector and insert gene were ligated with Roche T4 ligase according to 2:15 molecular ratio. The ligation reaction mixture is given below:

**Table 2.11** Ligation mixture of pGBKT7 and p60 cDNA

<b>Ingredients</b>	<b>Amount</b>
Hsp60 (1 $\mu$ g/ $\mu$ l)	15 $\mu$ l
pGBKT7 (0.5 $\mu$ g/ $\mu$ l)	2 $\mu$ l
10X Roche T4 ligation buffer	2.5 $\mu$ l
Roche T4 Ligase	1 $\mu$ l
ATP (5 mM)	4 $\mu$ l (0,8 mM)
Sterile mq H2O	0.5 $\mu$ l
Total	25 $\mu$ l

Ligation mixture was prepared according to concentration in the table and ligation was performed at 4 °C overnight. After reaction was terminated by freezing at -20 °C, 2  $\mu$ l of reaction mixture was loaded onto 1% TAE agarose gel and run at 70 mV for 45 minutes to identify if the fragments were ligated to each other. Then, ligation mixture was transformed into competent DH5 $\alpha$  E.coli.

#### 2.2.1.6. Transformation of Constructed pGBKT7-p60 plasmid

Subsequent to ligation, plasmid constructs were transformed into a bacterial host in order to increase the number of plasmids. Since, plasmids contain a constitutive replication feature by origin of replication of pUC in high copy number plasmids. DNA transfer to bacterial cells is not a spontaneous process since DNA is a hydrophilic molecule and membrane has a hydrophobic interface. In nature, cells

having ability to import DNA molecule are present. However, this ability requires additional specialized channel and proteins for intake of DNA molecule. Cells could be modified in order to intake DNA. For this approach; first, cell wall is disrupted with  $\text{CaCl}_2$  and later, DNA binds to disrupted cell wall fractions and finally, DNA is introduced into cell with membrane scaring by heat shock. DNA introduction can be mediated without chemical treatment. DNA is introduced into cell with an electric voltage which creates holes in the structure of membrane. This method is called electroporation. The latter method is more efficient but viability of cells decreased since voltage application is exceedingly harsh.

In our lab,  $\text{CaCl}_2$  treatment based chemical competent cells are used. *Escherichia coli* (*E. coli*) DH5 $\alpha$  cells were modified into competent cells. The chemical competent cell preparation protocol (Sambrook et al., 2001) is given below:

- LB plate was streaked with DH5 $\alpha$  frozen stock.
- One good separated colony was chosen for inoculation of 3 ml liquid LB media. Media was incubated at 37 °C with 250 rpm shaking overnight (~16 hours).
- 100 ml LB medium was inoculated with overnight culture, and incubated at 37 °C with 250 rpm shaking for 2.5-3 hours until OD<sub>590</sub> reaches 0.612
- Culture was taken into 2 pre-chilled sterile 50 ml falcons and placed on ice for 10 minutes.
- Vials were centrifuged at 2500 rpm for 5 minutes at 4 °C. Supernatant was discarded.
- Each pellet was washed with 10 ml ice cold  $\text{CaCl}_2$  solution, and then, centrifuged at 2500 rpm for 5 minutes at 4 °C.
- Pellets were resuspended with 2 ml ice cold  $\text{CaCl}_2$  solution and 20  $\mu\text{l}$  of resuspension aliquoted into pre-chilled 1.5 ml eppendorf tubes. Aliquots were frozen with liquid nitrogen and stored at -80 °C.

And heat shock transformation was applied accordingly:

- Aliquots were taken from -80 °C, and thawed on ice.
- 1  $\mu\text{g}$  DNA was added to each vial and left on ice for 30 minutes.
- The vial was placed in 42 °C water bath for 45 seconds and put on ice for 2-3 minutes.

- 100 µl of SOC medium was added, and then, culture was incubated at 37 °C with 250 rpm shaking for 1 hour.
- Cultures were plated on selective medium with appropriate antibiotic (LB-Kan)
- Plates were incubated at 37 °C overnight.

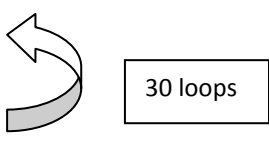
After incubation, recently emerged positive results were screened via colony PCR:

- 30 µl of sterile double distilled H<sub>2</sub>O was added to adequate number of PCR tubes, and a tip of each colony was resuspended.
- Mixture was boiled for 5 minutes.
- The consequential mixture was vortexed and centrifuged.
- Supernatant of the mixture was employed as sample to PCR reaction given in the table below:

**Table 2.12** Colony PCR reaction mix for pGBKT7-p60 transformed cells

Stock Ingredients	Amount for 1X rxn mix (25 µl)
Taq Buffer (10X)	2,5 µl (1X)
MgCl <sub>2</sub> (25 mM)	2 µl (2 mM)
Forward primer (25 mM)	0.5 µl (0.5 mM)
Reverse primer (25 mM)	0.5 µl (0.5 mM)
dNTP (10 mM)	0.5 µl (0.2 mM)
Taq enzyme (5u/ul)	0.2 µl (1 unit)
Colony mixture	1 µl
Sterile double distilled water	17.8 µl
Total	25 µl

**Table 2.13** PCR program for colony PCR of pGBKT7-p60

Initial denaturation (94 °C)	: 3 min	
Denaturation (94 °C)	: 15 sec	
Annealing (61 °C)	: 30 sec	
Extension (72 °C)	: 30 sec	
Final extension (72 °C)	: 5 min	

The resultant PCR products were employed on 1% TAE Agarose gel and insert containing colonies were selected.

### 2.2.1.7. DNA Isolation

DNA was isolated from positive colonies with Roche Miniprep DNA isolation Kit. The principle of the kit is as followed; alkaline lysis of bacteria, clearing RNA traces with RNase, with the aid of chaotropic salt Guanidine phosphate DNA molecules selectively bind to glass fleece; remains of bacterial lysate are cleared with washes and finally eluted with low salt buffer. The protocol of kit is given below:

- 5 ml of LB was inoculated with positive colony and the culture was incubated at 37 °C with 250 rpm shaking overnight.
- Overnight cultures were centrifuged at 5000 rpm for 5 minutes. Supernatant was discarded and pellet was resuspended in 250 µl Suspension Buffer [50 mM Tris-HCl, 10 mM EDTA and 0.1 g/L]. The suspension was taken into 1.5 eppendorf tubes.
- 250 µl Lysis Buffer [0.2 M NaOH and 1% SDS] was added to suspension and mixed gently with inverting 3 to 6 times. Then mixture was incubated at RT for 5 minutes.
- Following 350 µl chilled Binding Buffer was added and mixed gently inverting the tube 3 to 6 times. Afterward, it was chilled on ice for 5 minutes. Cloudy mixture was resulted after chilling.
- Mixture was centrifuged at 14000 rpm for 10 minutes in a standard table top centrifuge. Next, the supernatant was obtained without disturbing the pellet and loaded on the filter column tube.
- The filter tube was centrifuged at 14000 rpm for 1 minute, flow-through was discarded.
- 700 µl of Wash Buffer II was added to filter column and column was centrifuged at 14000 rpm for 1 minute. The flow-through was discarded.
- Filter column was centrifuged an additional 1 minute and the filter was placed on a clean 1.5 ml eppendorf tube.
- 50 µl Elution Buffer was added to upper reservoir of the filter tube and the set-up was left 1 minute on bench.
- The tube was centrifuged at top speed for 1 minute and the concentration was identified with OD 260 nm (An OD<sub>260</sub> of 1 corresponds to 50 µg/ml for double-stranded DNA).

### **2.2.2. Transformation of Yeast**

Constructed bait plasmid was supposed to be inserted into an appropriate mating type strain of *S. cerevisiae* on account of mating. Since commercial cDNA library is transformed into *S. cerevisiae* Mat  $\alpha$  type Y187 strain, bait plasmid was designed to be introduced into *S. cerevisiae* Mat  $a$  type AH109 strain which is supplied with Clontech Pre-transformed cDNA library. Yeast do not comprise competency, consequently competent yeast cells are prepared prior to transformation. In competency of yeast cells, chemical method is also applied. AH109 yeast cells were altered to competent cells with LiAc/TE method which disrupts the cell wall. Transformation of DNA was mediated with heat shock in which holes in membrane structure formed.

#### **2.2.2.1. Competent Cell Preparation**

Competent cell preparation of yeast cells was performed with Yeastmaker transformation kit. The procedure of kit is given below:

- YPDA plate was streaked with AH109 strain stock taken from  $-80\text{ }^{\circ}\text{C}$ ; incubated at  $30\text{ }^{\circ}\text{C}$  for 3-4 days. (This resultant working stock plate could be preserved for 1 month at  $4\text{ }^{\circ}\text{C}$ ).
- 1 colony was inoculated into 3 ml of YPDA medium and incubated at  $30\text{ }^{\circ}\text{C}$  for 8-12 hours.
- 5  $\mu\text{l}$  of culture was inoculated to 50 ml of YPDA medium and incubated until  $\text{OD}_{600}$  was between 0.15 - 0.30 (approximately 18-20 hours).
- Cells were centrifuged at 2000 rpm for 5 minute, and supernatant was discarded. Pellet was suspended in 100 ml YPDA, then mixture was incubated at  $30\text{ }^{\circ}\text{C}$  until  $\text{OD}_{600}$  reaches 0.4 - 0.5 (approximately 3-5 hours).
- Culture was centrifuged at 2000 rpm for 5 minute, and supernatant was removed.
- Pellet was dissolved in 60 ml of sterile double distilled water.
- Solution was centrifuged at 2000 rpm for 5 minutes, supernatant was removed.
- Cells were resuspended in 3 ml 1.1X TE/LiAc solution (1.1X TE, 110 mM LiAc) and cells were divided into two 1.5 ml sterile eppendorf.
- Eppendorf tubes were centrifuged at 14000 rpm for 15 seconds.
- Supernatant was removed and each pellet was dissolved in 600  $\mu\text{l}$  1.1 X TE/LiAc solutions. These mixtures may be left at room temperature for several hours.



#### **2.2.2.2. Yeast Transformation**

The next step to competent cell preparation was transformation of prepared competent yeast cells with constructed bait plasmid. After transformation, *S. cerevisiae* AH109 Mat a strain contained DNA binding vector construct including human katanin p60. Transformation of yeast cells was achieved with LiAc/PEG Transformation protocol in YeastMaker Transformation Kit. Protocol is given below:

- A sterile 1.5 ml microfuge tube was prepared to contain 1 µg construct plasmid and 5 µl denaturated Herring Testes Carrier DNA (10mg/ml).
- 50 µl of previously generated competent cells was added to DNA mixture and mixed gently.
- 0.5 ml PEG/LiAc (%40 PEG, 1X TE, 100mM LiAc; all components were sterile) was added to suspension and gently mixed with inverting tube up and down.
- The competent yeast cells and DNA suspension were incubated at 30 °C for 30 minutes, and mixed with 10 minute intervals.
- 20 µl DMSO was added and cells were suspended. Subsequently, the tubes were placed in a 42 °C water bath for 15 minutes, and mixed with 5 minute intervals.
- Cells were centrifuged and the supernatant was discarded. Pellet was dissolved in 1 ml sterile 0.9% NaCl solution.
- 100 µl mixture was employed on appropriate selection medium (for pGBKT7 plasmid, it was SD/-Trp) and plates were incubated at 30 °C for 3-6 days.
- After colonies emerge, plates were sealed and stored at 4 °C (no longer than 1 month) as working stock.

#### **2.2.3. Generation of Diploid Cells**

*S. cerevisiae* mating strains AH109 and Y187, containing respectively pGBKT7 and pACT2 plasmids were mated in order to place two yeast expression vectors in same yeast diploid organism.

##### **2.2.3.1. Yeast Mating**

- One colony from yeast transformation plate of our construct plasmid was seeded to 50 ml SD/-Trp.
- Culture was incubated at 30 °C, with 250 rpm shaking until the OD<sub>600</sub> reaches 0.8.

- Cells were centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded and the cells were dissolved in the residual liquid (~5ml).
- One aliquot of cDNA library (human fetal brain cDNA library in pACT2 at Y187) was thawed (10 µl of pretransformed library was separated in a separate vial for library control).
- AH109 bait culture and Y187 cDNA library were mixed entirely in a 2 L sterile flask, with 50 mL 2X YPDA/Kan.
- Culture was incubated at 30 °C overnight (20-24 hours) with 30-50 rpm shaking.
- Cells were centrifuged at 1500 rpm for 10 minutes; and supernatant was removed.
- 2L flask was washed 2 times with 50 ml 2X YPDA/Kan and pellet was washed with these flask wash liquids.
- Cells were centrifuged at 1500 rpm for 10 minutes and the pellet was resuspended with 10 ml 0.5X YPDA/Kan.
- Mating suspension was plated on ~180 SD/-his/-leu/-trp triple drop-out plates (less stringent), and a series of dilution (1/10, 1/100, 1/1000, 1/10000) were plated on SD/-Leu, SD/-Trp and SD/-Leu/-Trp for calculating mating efficiency.
- Plates were incubated at 30 °C for 4-5 days until colonies emerge.

### **2.2.3.2. Increasing Stringency for Diploid Selection**

Less stringent media was selected for the first inoculation of diploid cells. Since the mating media is an extensively nutrient-full media, high stringency may lead the death of some proportion of diploid cells. However, after surviving in less stringent media, stringency should be increased in order to obtain less false positive results at the end. For this purpose, mating plates were transferred to SD/-ade/-his/-leu/-trp, quadruple drop-out plate (SD QDO) plates with replica plate method.

The protocol for replica plate is given below:

- A sterile velvet cloth was placed and fixed on the block of replica plate apparatus.
- SD TDO mating plate was placed and pressed on the sterile velvet cloth with an even pressure and dissembled from replica apparatus.
- A SD QDO was placed on velvet having cell pattern of previous plate and evenly pressed.

## **2.2.4. Diploid Cell Phenotype Confirmation**

### **2.2.4.1. Multiple Streaks**

Cells growing on SD QDO media plate were further streaked again onto SD QDO plates in order to decrease the number of multiple activation domain bearing plasmids. Each colony was streaked separately and streaking procedure is continued until fifteenth streaks.

### **2.2.4.1. Colony lift assay**

In order to obtain genuine positive results, all selectivity experiments were performed on diploid cells. GAL4 system, in our experimental design, has three genes downstream, two of these three are essential amino acid genes and used in nutrient selection medium. Third one is MEL1 which is expressing  $\alpha$  and  $\beta$  galactosidase. In colony lift assay, diploid cell were investigated for their ability to utilize  $\beta$  galactose.

- Fresh colonies (grown at 30°C for 2–4 days), 1–3 mm in diameter were used for this experiment.
- Z buffer/X-gal solution was prepared just before the assay.
- For each plate of transformants to be assayed, a sterile Whatman No. 5 filter was presoaked by placing it in 2.5–5 ml of Z buffer/X-gal solution in a clean 100- or 150-mm plate.
- Using forceps, a clean, dry filter was placed over the surface of the plate of colonies to be assayed. The filter was gently rubbed with the side of the forceps to help colonies cling to the filter.
- Holes were poked through the filter into the agar in three or more asymmetric locations to orient the filter to the agar.
- When the filter had been evenly wetted, it was carefully lifted off the agar plate with forceps and transferred (colonies facing up) to a pool of liquid nitrogen. Using the forceps, the filters were completely submerged for 10 seconds.

- After the filter has frozen completely (~10 seconds), it was moved from the liquid nitrogen and allowed to thaw at room temperature. (This freeze/thaw treatment was to permeabilizes the cells.)
- The filter was carefully placed, colony side up, on the presoaked filter. Trapping air bubbles under or between the filters was avoided.
- The filters were incubated at 30°C (or room temperature) and checked periodically for the appearance of blue colonies.

### 2.2.5. Plasmid Selection

There is a high number of interacting diploids in yeast two hybrid studies; isolating plasmids from each clone is laborious and expensive. Therefore, the decision on which plasmid is going to be isolated was made by yeast colony PCR and subsequent restriction enzyme digestion for fingerprinting.

#### 2.2.5.1. Yeast Colony PCR

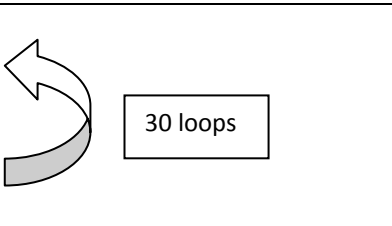
Yeast colony PCR was executed with pACT2 amplification primers. Procedure of yeast colony PCR is given below:

- 30 µl of sterile double distilled H<sub>2</sub>O was added to adequate number of PCR tubes, and a tip of each colony was resuspended.
- Mixture was boiled for 15 minutes.
- The consequential mixture was vortexed and centrifuged.
- Supernatant of the mixture was employed as sample to PCR reaction given below:

**Table 2.14** Yeast Colony PCR constituents

<b>Stock Ingredients</b>	<b>Amount for 1X rxn mix (25 ul)</b>
Taq Buffer (10X)	2,5 ul (1X)
MgCl <sub>2</sub> (25 mM)	2 ul (2 mM)
Forward primer (25 mM)	0.5 ul (0.5 mM)
Reverse primer (25 mM)	0.5 ul (0.5 mM)
dNTP (10 mM)	0.5 ul (0.2 mM)
Taq enzyme (5u/ul)	0.2 ul (1 unit)
Colony +water mix	18.8 µl
Total	25 µl

**Table 2.15** PCR Program for Yeast Colony PCR

Initial denaturation (94 °C) : 3	
Denaturation (94 °C) : 15 sec	
Annealing (61 °C) : 30 sec	
Extension (72 °C) : 30 sec	
Final extension (72 °C) : 5 min	

PCR products were applied on 1% TAE gel in order to identify if there are more than one product for plasmid primers. One product containing samples were selected and digested with frequent cutter *AluI* enzyme for fingerprinting, and fragments were applied on 2% TAE agarose gel. Same restriction pattern containing diploids are determined as same proteins, so diploids with different pattern are chosen in order to isolate plasmid DNA.

#### 2.2.5.2. DNA Isolation

After plasmids were selected through yeast colony PCR and *AluI* digestion pattern, DNA isolation was performed. Yeast cells comprise a thick cell wall; hence general use bacterial plasmid isolation kits can not be applied. Thus, cells were pre-treated with lyticase which disrupts the chemical bond between cell wall components. These pre-treated cells were employed to Qiaprep Spin Miniprep. Pre-treatment protocol of yeast cells is given below:

- Each colony was seeded to 5 ml SD/-ade/his/-leu/-trp medium in a glass tube, and incubated at 30 °C for 24 hours.
- Cells were centrifuged at 4000 rpm for 10 minutes.
- The supernatant was discarded and the pellet was dissolved in residual SD media, the culture was transferred into 1.5 ml microcentrifuge tubes.
- 10 µl lyticase was added and mixture was incubated at 30 °C for 1 hour (alternatively 100 µl of 450 µm diameter glass beads were used and vortexed thoroughly for 10 minutes.)
- After incubation, the mixture was applied in Qiaprep Spin Miniprep Plasmid Isolation Kit.

Qiaprep Spin Miniprep Plasmid Isolation Kit is also working with same principal as Roche Plasmid Kit. Procedure is given below:

- Pre-treated yeast cells were mixed with 250 µl Buffer P1 and transfer to a microcentrifuge tube.

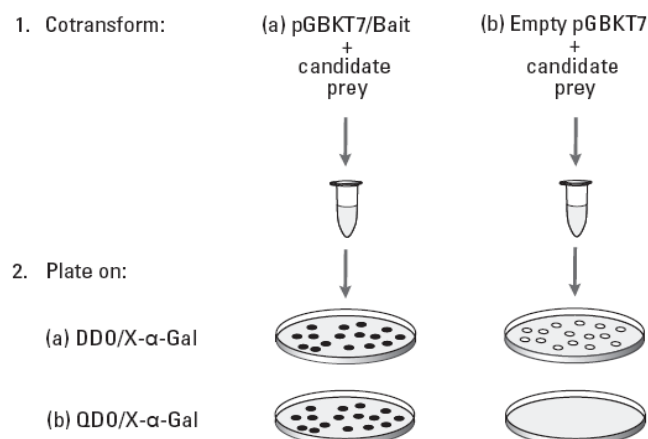
- 250  $\mu$ l Buffer P2 was added and mixed thoroughly by inverting the tube 4–6 times.
- 350  $\mu$ l Buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4–6 times.
- Suspension was centrifuged for 10 minutes at 14,000 rpm in a table-top microcentrifuge.
- The supernatant from previous step was applied to the QIAprep spin column by decanting or pipetting.
- Filter-tube assembly was centrifuged for 30–60 seconds. The flow-through was discarded.
- The QIAprep spin column was washed by adding 0.5 ml Buffer PB and centrifuging for 30–60 seconds. Flow-through was discarded.
- The QIAprep spin column was washed by adding 0.75 ml Buffer PE and centrifuging for 30–60 seconds.
- The flow-through was discarded, and tubes were centrifuged for an additional 1 minute to remove residual wash buffer.
- QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50  $\mu$ l Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the center of each QIAprep spin column. Column was let standing for 1 minute, and centrifuged for 1 minute.

Plasmids isolated from diploid cells are a mixture of plasmids, it contains both bait and prey plasmids as well. The concentration of yeast isolated DNA is very low. Therefore, 10  $\mu$ l of plasmid was transformed into competent *E. coli* DH5 $\alpha$  strain in order to increase the number of prey plasmids to be isolated. Transformations were selected with ampicillin resistance which is conferred to pACT2. DNA isolations were performed with Roche High Pure Plasmid Kit as emphasized in section 2.2.1.6.

#### **2.2.6. Co-transformation**

Since yeast-two-hybrid technique results in many false positive interactions, there are control experiments as co-transformation in order to decrease the number of false positives. In this experiment design, each prey plasmid was co-transformed for 2 times; one with bait plasmid containing p60, one with empty bait plasmid (Fig. 2.3). 100 ng of each plasmid was transformed with LiAc Transformation - enlightened in

2.2.2.2. -, and then each transformation was plated on SD double drop-out and SD quadruple drop-out plates.



**Figure 2.3** Illustration of cotransformation experiment set-up

## 2.2.7. Sequencing

After cotransformation, a list of possible interaction plasmids was in our hands. The identification of the cDNA inserted into prey plasmids was performed by sequencing.

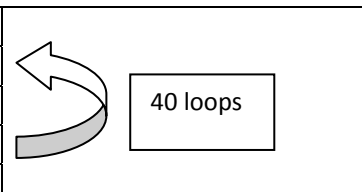
### 2.2.7.1. Sequencing PCR

Sequencing PCR is special kind of PCR in which single strand of DNA is amplified. Each dNTP is labeled with different fluorescent tag; therefore, resultant DNA fragment is fluorescently labeled. Single strand of DNA is desired to be exploited since 2 strands apparently interfere the fluorescence of each other. Thus, only one primer is used in Sequencing PCR. All ingredients are placed on ice and reaction is performed in dark in order to preserve fluorescence. The reaction mixture preparation is given in the table below:

**Table 2.16** Sequencing PCR ingredients

Stock Ingredients	Amounts for 1X rxn mix (10 µl)
Big dye sequencing buffer	2 µl (1X)
Big dye	2 µl
Reverse primer / Forward primer	0.5 µl
Template DNA	1 µg
Sterile mq water	3.5 µl
Total	10 µl

**Table 2.17** Sequence PCR program

Initial denaturation (94 °C) : 3 min	
Denaturation (94 °C) : 15 sec	
Annealing (55 °C) : 15 sec	
Extension (68 °C) : 1 min	
Final extension (68 °C) : 5 min	

### 2.2.7.2. PCR Product Purification for Sequencing

Since PCR product was contaminated with polymerase, a subsequent purification was performed. The protocol is given below:

- 10 µl PCR product was taken in to microfuge tube; then, 2µl of 3M NaAc and 50 µl ice-cold 95% ethanol were added to tube.
- Tubes were incubated on ice for 30 minutes.
- Mixture was centrifuged at 14000 rpm for 15 minutes. Supernatant was discarded and pellet was resuspended in ice-cold %70 ethanol.
- Tubes were centrifuged at 14000 rpm for 15 minutes. Supernatant was discarded.
- Tubes were incubated at 95°C for 5 minutes with caps open (in order to evaporate ethanol)
- 20 µl of formamide was added to DNA pellet and the mixture was vortexed vigorously
- Tubes were again incubated at 95 °C for 5 minutes with caps closed
- Tubes were immediately put on ice and kept at 4 °C until analysis.

### 2.2.7.3. Analysis of Sequence Results

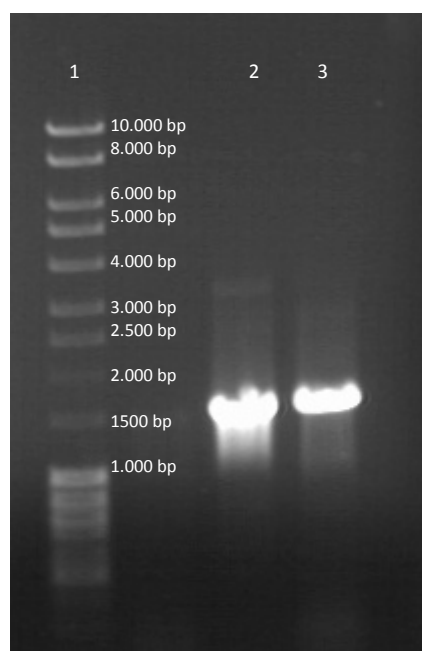
Analysis of sequence results was performed with NCBI Blast program (internet address, <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Highly related sequences matching to sequence query were displayed from nucleotide databank.



### 3. RESULTS

#### 3.1. Bait Plasmid Construction

Katanin p60 cDNA was present as inserted into pEGFP-C1. Primers were designed to specifically amplify p60 cDNA and comprise flanking regions containing restriction enzyme digestion sites of Sall and XmaI. p60 cDNA was sub-cloned into same open reading frame after DNA binding domain of GAL4 transcription factor with the aid of primers. Primers were designed according to *Homo sapiens* katanin p60 mRNA coding sequence, sequence data was taken from [http://www.ncbi.nih.gov/ NM\\_007044](http://www.ncbi.nih.gov/NM_007044). PCR amplification of katanin p60 with designed primers successfully resulted in ~1550 bp product (Fig. 3.1).



**Figure 3.1** PCR was performed to clone p60 (Lane 1: Fermentas Mass Ruler Mix DNA Marker, Lane 2 and 3: p60 PCR products)

Amplified cDNA was designed to be inserted into Clontech pGBKT7 DNA BD vector with restriction enzymes of Sall and XmaI. Therefore cDNA and the vector were restricted with Fermentas Cfr9I(XmaI) and Sall enzymes (Fig. 3.2).

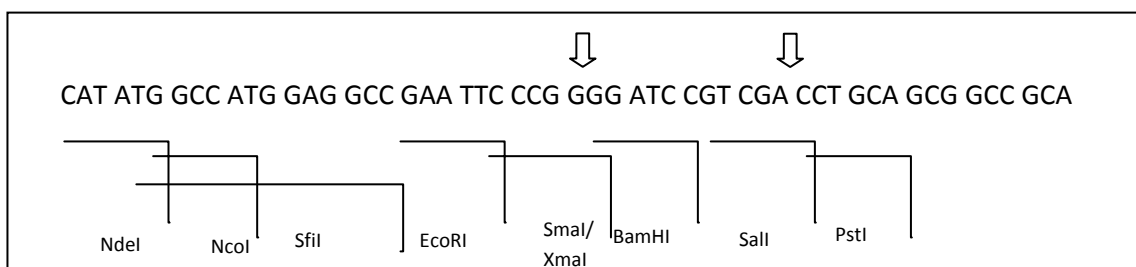
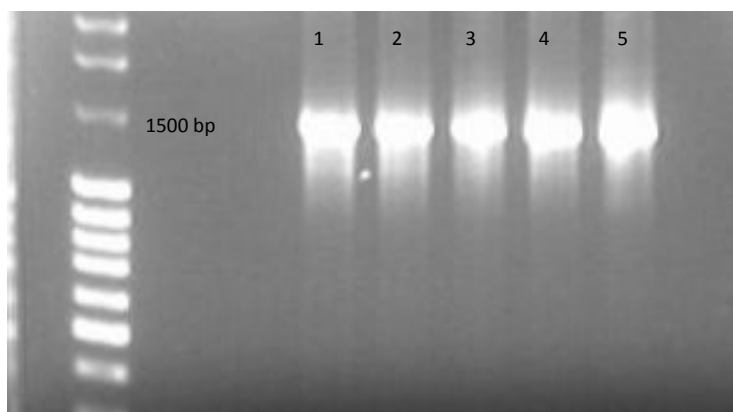


Figure 3.2 Multiple Cloning Site map for pGBKT7 vector, restriction sites are marked with arrows.

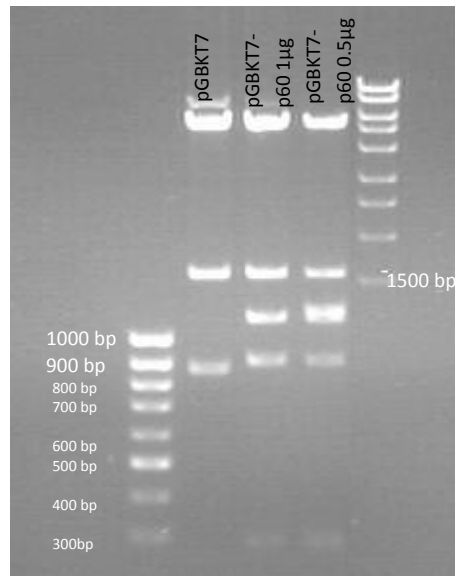
Restricted DNA fragments were purified with Roche High Pure PCR Product Kit and ligation procedure was performed with Roche T4 DNA ligase. Subsequently, ligation mixture was transformed to CaCl<sub>2</sub> treated *E. coli* DH5 $\alpha$  cells. Since plasmid DNA included replication origin of *E. coli*, plasmid DNA was able to replicate in transformed *E. coli* cells. Copy number of plasmid DNA was improved with transformation. In order to verify accuracy, transformed colonies including katanin p60 cDNA were examined with colony PCR using pGBKT7 insert amplification primers. Five colonies were analyzed by PCR, and gel photo (Fig. 3.3) confirmed that all of them contained pGBKT7 with p60 cDNA inserted.



**Figure 3.3** Colony PCR results for five pGBKT7-p60 transformed colonies

After verification of colonies including pGBKT7 with p60 cDNA, one of the colonies was selected and plasmid purification procedure was performed to isolate plasmids. Purified plasmid DNA was subjected to subsequent analysis to identify whether cDNA was inserted into plasmid correctly. Restriction digestion with

Fermentas HindIII was applied and pattern of the restricted bands were as predicted (Fig. 3.4); 4.6 kb, 1.58 kb, 1.3 kb, 870bp, 297bp.



**Figure 3.4** Restriction fragments (Lane 1: Fermentas Mass Ruler Low Range DNA marker, Lane 2: empty pGBKT7, Lane 3: pGBKT7-p60 1µg template, Lane 4: pGBKT7-p 0.5 µg template, Lane 5: Fermentas Mass Ruler High Range marker)

### 3.2. Yeast Mating

After the plasmid construction step was successfully achieved, constructed plasmid was transformed to yeast cells. *S. cerevisiae* Mat a type AH109 strain was subjected to LiAc transformation with constructed plasmid and transformants were selected through nutrient selective medium lacking tryptophan. Transformed yeast cells were mated with Clontech pre-transformed human fetal brain cDNA library. Mating mixture was plated onto 157 synthetic drop-out medium with amino acid mixture lacking histidine, tryptophan, leucine.

Control plates were also plated to analyze the mating (Tab. 3.1). Score in SD –Leu showed the number of yeast cells having pACT2 vector, SD –Trp showed number of pGBKT7-p60 containing yeast cells, and SD –Trp/ -Leu showed the number of diploid cell.

**Table 3.1** Control plate scores

	SD-Leu	SD-Trp	SD-Leu-Trp
1/10000	2	Uncountable	1
1/1000	9	Uncountable	1
1/100	68	Uncountable	7
1/10	890	Uncountable	78

**3.2.1. Mating Calculations**

Since mating results required quantification, control plate scores were used for further calculations. Number of colony forming units (Cfu) for each plate was calculated. Mating efficiency was determined by the ratio of mated diploid clones to limiting mating partner. Considering this mating experiment, limiting partner was cDNA library pACT2 containing yeast cells. Since the other partner pGBKT7-p60 containing cells grew extensively, score was uncountable.

**3.2.1.1. Calculating Number of Cfu per Milliliter**

The formula for calculation of Cfu per milliliter is given below:

$$\text{number of cfu per ml} = \frac{\text{number of cfu} \times 1000 \mu\text{l/ml}}{\text{volume plated} (\mu\text{l}) \times \text{Dilution factor}} \quad (3.1)$$

According to SD – Leu control plate scores, number of cfu/ml of pACT2-cDNA library containing yeast cells was calculated as given below:

$$\frac{2 \times 1000 \mu\text{l/ml}}{100 \mu\text{l} \times 10^{-4}} = 2 \times 10^5$$

$$\frac{9 \times 1000 \mu\text{l/ml}}{100 \mu\text{l} \times 10^{-3}} = 9 \times 10^4$$

$$\frac{68 \times 1000 \mu\text{l/ml}}{100 \mu\text{l} \times 10^{-2}} = 6.8 \times 10^4$$

$$\frac{890 \times 1000 \mu\text{l/ml}}{100 \mu\text{l} \times 10^{-1}} = 8.9 \times 10^4$$

The mean of results was calculated;  $1.12 \times 10^5$  colonies having pACT2 plasmid were present in 1 ml of mating mixture.

According to SD -Leu/ -Trp control plate score, number of cfu/ml of Diploid colonies having both pACT2 and pGBKT7 was calculated as given below:

$$\frac{1 \times 1000 \mu\text{l/ml}}{100 \mu\text{l} \times 10^{-4}} = 10^5$$

$$\frac{1 \times 1000 \mu\text{l/ml}}{100 \mu\text{l} \times 10^{-3}} = 10^4$$

$$\frac{7 \times 1000 \mu\text{l/ml}}{100 \mu\text{l} \times 10^{-2}} = 7 \times 10^3$$

$$\frac{78 \times 1000 \mu\text{l/ml}}{100 \mu\text{l} \times 10^{-1}} = 7.8 \times 10^3$$

The mean of four diploid control plates was calculated to be  $3.12 \times 10^4$ ; therefore,  $3.12 \times 10^4$  colonies present in 1 ml of mating mixture.

### 3.2.1.2. Mating efficiency

$$\text{mating efficiency} = \frac{\text{Number of diploid cells in the mixture}}{\text{number of cells of limiting partner}} \quad (3.2)$$

$$\frac{3.12 \times 10^4}{1.12 \times 10^5} \times 100 = 30 \%$$

30 % percent of mating efficiency was obtained. Mating efficiency should be greater than 5 % as stated in manufacturer manual.

### 3.2.1.3. Number of screened clones

$$\begin{aligned} & \text{number of clones screened} \\ & = \text{number of cfu diploid} \times \text{suspension volume} \quad (3.3) \end{aligned}$$

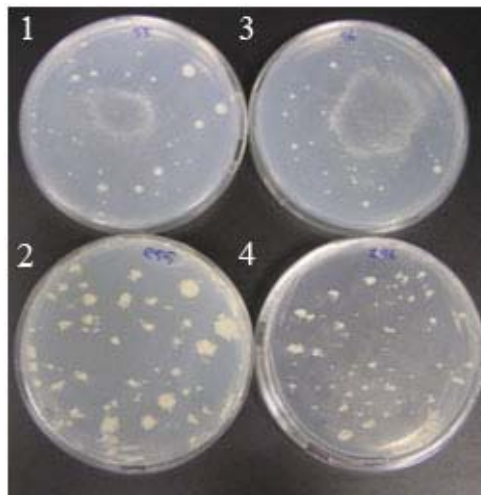
$$23800 = 3.12 \times 10^3 \times 7.5$$

When we calculated the number of clones screened in mating experiment, 23800 clones were shown to be examined.

After evaluation of mating score calculations, we concluded that mating experiment was executed efficiently.

### 3.2.2. Replica Plate

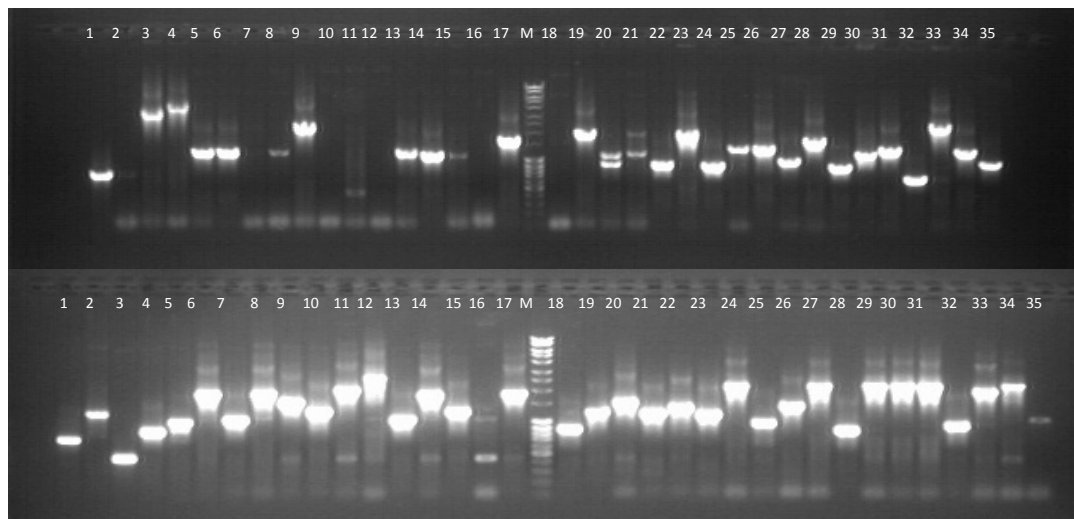
Subsequent to plating mating mixture on SD –His –Leu –Trp plates, the stringency of the experiment was increased one step forward to SD –Ade –His –Leu –Trp plates. SD –His –Leu –Trp plates were replicated onto SD –Ade –His –Leu –Trp plates. In the first stringency level ~3000 clones were present in the SD Triple drop-out plates. ~2000 clones were remained after replica plating.



**Figure 3.5** Representative display of replica plates. (1. Mating Plate 55 SD –His –Leu –Trp, 2. Replica of Mating plate 55 SD –Ade –His –Leu –Trp, 3. Mating Plate 56 SD –His –Leu –Trp, 4. Replica of Mating Plate 56 SD –Ade –His –Leu –Trp)

### 3.2.3. Multiple Streaks and Plasmid Selection

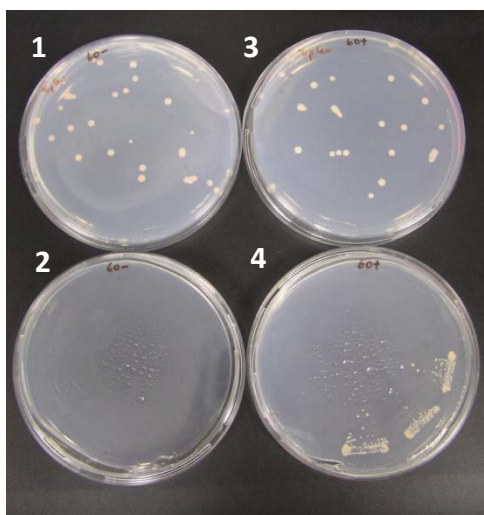
Multiple streaks were performed for 15 times and 121 clones survived at the end of the procedure. Before plasmid DNA was isolated from yeast cells, colony PCR was employed to screen diploid cells to clarify whether they contained more than one activation domain plasmid. 75 of these diploid clones were chosen according to colony PCR results.



**Figure 3.6** Colony PCR results (Lanes were loaded in order, except Fermantas Mass Ruler Mix was loaded in the middle of each row)

### **3.3. Co-transformation**

Plasmid DNA of activation domain plasmids was isolated from diploid cells. The next step was confirming whether the interaction between bait and cDNA protein was genuine. Co-transformation eliminated the false positives results from yeast two hybrid screens. 38 diploids were proven to be genuine positives from 75 plasmids. These genuine positive plasmids were later sequenced to identify cDNA in pACT2 vector.



**Figure 3.7** Representative genuine positive co-transformation result (1. Colony # 60 co-transformed with empty pGBKT7 on SD –Leu –Trp plate, 2. Colony co-transformed with empty pGBKT7 on SD –Ade –His –Leu –Trp, 3. Colony # 60 co-transformed with pGBKT7-p60 on SD –Leu –Trp, 4. Colony # 60 co-transformed with pGBKT7-p60 on SD –Ade –His –Leu –Trp)

### 3.4. Sequence Alignment Analysis

#### 3.4.1. Sequence Data Mining

cDNA sequences of genuine positive plasmids were aligned via NCBI Blast Tool. Results are given in Appendix.

#### 3.4.2. Sequence Alignment Results

Sequence alignment results indicated following proteins. Brief descriptions of proteins are given below:

- NADH dehydrogenase (ubiquinone) 1 beta complex 6 is a polypeptide that constitutes in first enzyme complex in electron transport chain of mitochondria (Smeitink et al., 1998).
- Rho-related Broad Complex, Tramtrack, and Bric-a-brac (BTB) domain containing 3 is a member of the rho BTB subfamily of Rho-GTPase which is abundant in neural system (Ramos et al., 2002).
- Chondroitin polymerizing factor (CHPF) is a transmembrane glycosyltransferase enzyme (Kitagawa et al., 2003).



- Rho-guanine nucleotide exchange factor stimulates the exchange of guanyl nucleotides by GTPase of Rho family. This protein is prevalently present in neural system and its intracellular localization is neurite outgrowth zones and nucleus (Meyer et al., 1999).
- Glucose-6-phosphate dehydrogenase is a cytosolic protein takes role in pentose phosphate pathway and it is a housekeeping gene (Luzzatto and Afolayan, 1968).
- Myeloid leukemia factor 2 : Myeloid leukemias with various cytogenetic abnormalities, failed to reveal MLF2 gene rearrangements (Kuefer et al., 1996)
- Leukocyte receptor cluster member 8 interacts with telomeric motor neuron survival factor 1 (Liu and Dreyfuss, 1996) and homeo box protein PEPP subfamily 2, a transcription factor (Rual et al., 2005) where both related proteins distinctively interacting with nuclear RNA activating complexes.
- Small nuclear RNA activating complex, SNAPC4 recognizes RNA polymerase II and III snRNA promoters and initiate basal levels of transcription and functions as transcription factor (Wong et al., 1998)
- Src homology 3 (SH3) domain growth factor receptor bound protein 2 (GRB2)-like endophilin B2 is hypothesized as link between Bax and cytoskeleton elements (Benoit et al., 2001).
- Septin 3 takes role in neuronal differentiation (Methner et al., 2001).
- Tubulin beta is constituent of microtubules. Microtubules are extensively described in section 1.1.2.
- Meteorin plays important roles in both glial cell differentiation and axonal network formation during neurogenesis (Nishino et al. 2004).
- Actin beta is a cytoskeletal protein. Polymerization of actin beta monomers generates actin filament (F-actin) (Alberts et al., 2003).

- Zinc finger family member 767 is a zinc finger motif containing protein (Strausberg et al., 2002).
- Solute carrier family 44, member 2 : Choline transporter like protein 2 (CTL2) contains multi-trans-membrane domain. This protein takes role in activation of NfkB cascade (Matsuda et al., 2003) and hearing (Nair et al., 2004).
- KIAA1853 protein; is a medullablastoma tumor antigen(Behrends et al., 2003).
- Wiskott-Aldrich syndrome (WAS) protein family member 2 involved in Actin cytoskeleton organization cascade under rho GTPases (Bear et al., 1998).
- Carboydrate sulfotransferase is a membrane integrated protein and it possesses a role in central nervous system in pituitary gland (Hiraoka et al., 2001) and interacts with chondroitin modifying enzymes.
- Calcium binding protein 22 pseudogene (Calcineurin B homologous protein, CHP) is EF-hand Calcium binding protein binds microtubule (Timm et al., 1999) and takes role in neuron growth cone pathway.
- Regulator of G protein signaling 11 is a GTPase activator in intracellular signaling cascade so controls the activity and fidelity of G protein  $\beta$  (Snaw et al., 1998).
- Chromosome 6 contig fragment
- Chromosome 9 open reading frame 127

## **4. DISCUSSION**

### **4.1. Yeast cultivation**

Cultivation of yeast cells was performed with appropriate selection medium at 30 °C. Initially, incubation of yeast cultures was performed in falcon tubes as stated in Clontech Pre-transformed cDNA library manual. Apparently, cultures did not grow even after 3 days of incubation at 30 °C.

Since aged cultures are possibly lost their plasmids, our diploid cell inoculums might also have lost their plasmids. In order to detect whether inoculums used in yeast cultivation caused this problem, renewal of diploid cells was performed via streaking on SD QDO and renewed diploid cells were inoculated into appropriate selection medium. However, this troubleshooting trial did not work; cell growth was not observed with the renewed inoculums either. The second trial was done by culturing yeast cells in glass tubes, and actually the cultivation problem was solved with this approach. One can conclude that yeast cells prefer to grow in glass, rather than polypropylene falcon tubes.

### **4.2. Transformation of DNA isolated from yeast**

Following the isolation of pACT2 activation domain vector with interacting cDNA from yeast, transformation of *E. coli* DH5 $\alpha$  with newly isolated DNA was performed in order to increase the amount of plasmid. However, efficiency of these transformations was inadequate; for instance 1 or 2 colonies per transformation was observed.

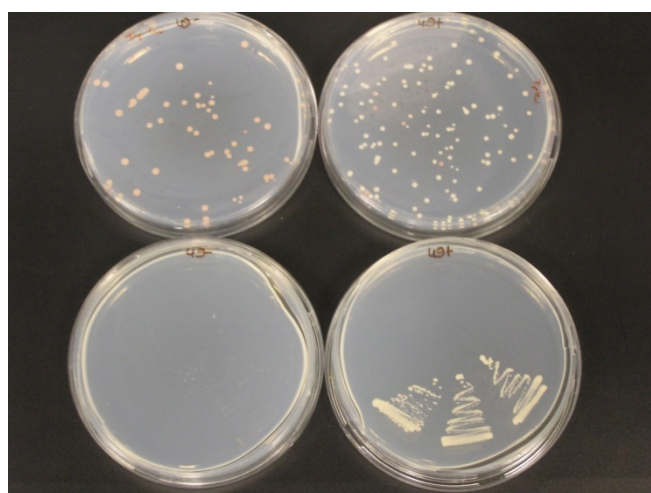
For this impairment, transformation efficiency of *E. coli* XL 1 blue cells was also examined besides *E. coli* DH5 $\alpha$ . Yet, this approach did not solve the problem. The efficiency was in the same range as previous experiments, and also the organism was a slow growing *E. coli* strain. Consequently, study continued with the previous organism, *E. coli* DH5 $\alpha$ .

Since competent cells lose their viability up to some extent while freezing; preparing fresh batch of competent cells for each transformation was considered. Preparation of fresh competent cell worked efficiently.

### 4.3. Co-transformation

Co-transformation experiments were performed to eliminate false positive results. Candidate plasmid was co-transformed with empty and bait containing DNA binding domain vector and each co-transformation was examined separately in synthetic medium lacking tryptophan, leucine (SD DDO) and lacking adenine, histidine, tryptophan, leucine (SD QDO). Experimentally, although growth of SD DDO plate was present extensively, growth in SD QDO plate was not observed. This pattern was present on large number of plates; as a result co-transformation was not working properly.

After examination of co-transformation protocol, plating yeast cells on SD QDO after co-transformation was thought to be exceptionally harsh. The cells were plated immediately after co-transformation where their stability and integrity were changed. In addition, yeast cells had to express reporter gene products on SD QDO medium. Probably, cells did not survive during the time required for reporter gene expression. The resultant colonies of SD DDO co-transformation was subsequently streaked on SD QDO plate, and finally this procedure solved co-transformation problem.



**Figure 4.1** Co-transformation representative streak (2 plates in top are SD DDO plates and streaked on SD QDO respectively)

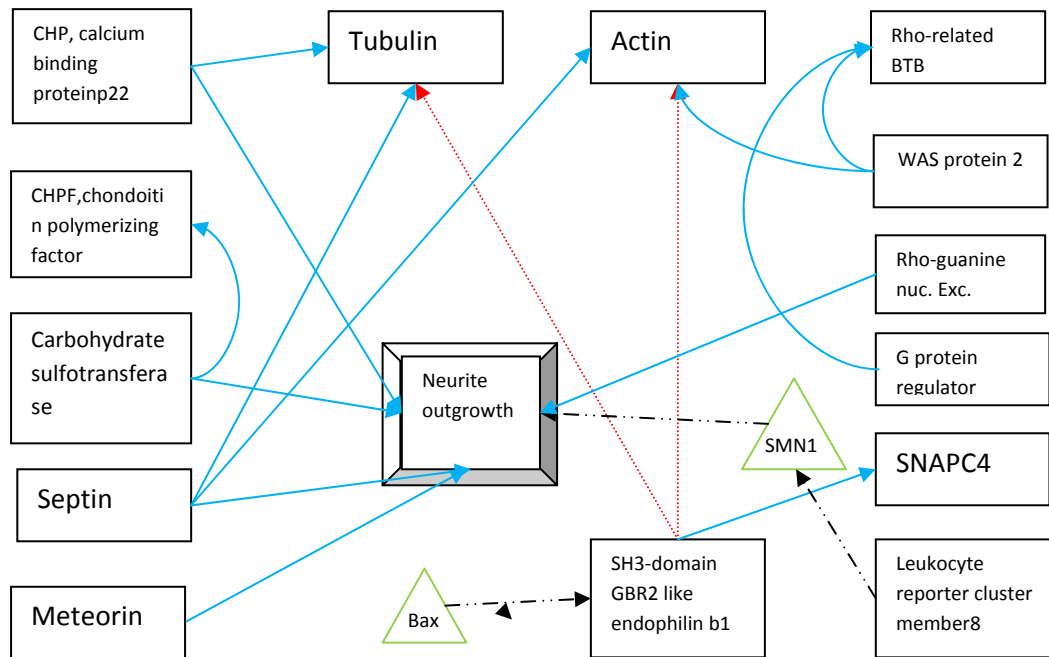
In addition, cells could be cultivated in nutrient rich medium for a short time after co-transformation and then these cells could be plated onto an appropriate nutrient selective media as frequently used in *E.coli* heat transformation protocols.

#### **4.4. Sequencing Results**

22 sequences were identified, where some sequence results were eliminated due to poor resolution in sequencing. There are 19 novel interactions found for katanin p60. Although the interactions should be proved with methods besides Yeast Two Hybrid technique, the results are highly promising.

1 of 22 results was tubulin which was shown to interact with katanin p60 (detailed information is given in the next page). 2 of 22 results did not comprise any product. 1 was a part of chromosome 6 genomic contig. While producing cDNA libraries, it is possible to end up with a small portion of genomic DNA contamination. Since Yeast Two Hybrid is a fishing experiment, this genomic contig could be pulled from such a genomic DNA contamination. The other result was part of an open reading frame in chromosome 9, which does not express any protein or product.

Brief information about interacting proteins has already given in the result section. A schematic representation (Fig. 4.2) of their interaction with each other is given below:



**Figure 4.2** Schematic display of interface between katanin p60 interactions. Rectangles are katanin interacting proteins and triangles are secondary protein interactions. Blue arrows illustrate direct interactions, red arrows illustrate interpreted interactions. Dotted arrows illustrate interaction with secondary proteins.

Tubulin beta is a subunit of tubulin heterodimer which builds microtubules thus its interaction with microtubules is expected. However, in the experiment only beta subunit of tubulin heterodimer was identified as interacting with katanin p60. This can lead us to a conclusion that katanin p60 possibly binds microtubules via tubulin beta subunits. However, tubulin alpha may not be selected because of elimination during selection processes or this protein is a false negative. Additionally, only one third of cDNA clones are integrated accurately into activation domain vector hence employed cDNA library may not contain tubulin alpha. Nevertheless, tubulin beta and katanin p60 interaction also confirmed accuracy of the Yeast Two Hybrid technique which was used for the first time in our lab.

Actin beta is a monomer in actin cytoskeleton. Previously, katanin has not been found to interact with actin filament. However, interaction of katanin with actin filaments is possible since the katanin is present in axonal outgrowth regions (Yu et al., 2008) and actin filaments are also enriched in newly forming axon branches.

Septin 3 is a brain specific protein, and it forms filaments in non-mitotic cells associated with strongly either with tubulin (Surka et al., 2002) or actin network

(Kinoshita et al., 1997). Moreover, in some cases it is associated with both networks (Surka et al., 2002). Septin 3 takes role in vesicle trafficking, neurite outgrowth and neurofibrillary tangle formation (Methner et al. 2001). According to the properties of septin 3, its interaction with katanin p60 seems to be possible. Since katanin p60 provides motile microtubules to newly forming axon branches via microtubule severing (Karabay et al., 2004), interaction between these two proteins may regulate the microtubule severing signal of katanin p60. Septin 3 is also considered to be involved in leukemia, since its genomic orientation is located at a chromosomal hot spot for translocations in leukemia (Methner et al., 2001). A microtubule destabilizing protein op18/stathmin was also shown to be overexpressed in leukemia (Hanash et al., 1988). Microtubule severing by katanin p60 could also destruct microtubule organization in cell cycle dynamics of leukocyte.

WAS protein family member 2 is a downstream effector in actin cytoskeleton regulatory pathway. This protein takes signal from tyrosine kinase or Rho small GTPases and conducts it to actin cytoskeleton. In this study, we have also shown that actin was interacting with katanin p60, WASF2 might also interact with katanin p60 in order to conduct received signal to actin cytoskeleton elements.

Meteorin is a secreted protein that acts as autocrine activator of glial cell differentiation and axonal outgrowth (Nishino et al., 2002). Since it is a secreted protein, interaction with katanin p60 could seem to be irrelevant. However, this protein might be internalized in neurons by endocytosis. Moreover, there might be different mRNA splice forms displacing the secretion signal and meteorin could act as an intracellular axonal outgrowth agent.

Calcium binding protein 22 (CHP) is an EF-hand calcium binding protein and interacts directly with tubulin in the presence of N-myristoylation (Timms et al., 1999). It also interacts with Kif1Bbeta2 motor protein (Nakamura et al., 2002). Moreover, this protein has a role in axon guidance in neuron growth cone formation. Interaction of katanin p60 with this protein could be due to the tubulin binding property of CHP. CHP might conduct the growth cone formation signal to katanin p60, and this interaction might result in microtubule severing. Additionally, this interaction might enhance the access of katanin p60 to microtubules.

Rho related BTB domain containing 3, is a small Rho GTPase. Rho GTPases are responsible for converting and amplifying the external signals into cellular effects, they are responsible for F-actin polymerization, cell proliferation, survival, differentiation, adhesion, secretion (Boureaux et al., 2007). Rho related BTB3 is abundant in neuronal cells (Ramos et al., 2002). Small Rho GTPases are conductors of extracellular signal, and katanin might be one of their effector proteins. Furthermore, this protein could be responsible for GTP turnover in catalytic site of katanin p60.

Rho-guanine exchange factor is a neuronal abundant exchanger protein. It was shown that this protein is abundant in neurites (Meyer et al., 1999). Thus, interaction between katanin and rho-guanine exchange factor seems to be relevant. This protein also could be involved in GTP turnover in neurites where katanin p60 functions.

Choline transporter like protein 2 is also an interesting match for katanin. This protein is involved in NF $\kappa$ B signaling cascade. Since a katanin p60 ortholog, lipotransin, is specified as functioning in hormonal regulated lipolysis (Syu and Saltiel, 1999), this interaction could be interpreted as involvement of katanin p60 in lipid metabolism. Moreover, this match might be pulled due to the homology between lipotransin and katanin since AAA proteins tend to form multimeric structures.

Carbohydrate (N-acetylglucosamine 4-O) sulfotransferase 8 is a membrane integrated protein executing the sulphate transfer activity to N-acetylglucosamine. This protein is characterized in modification of hormones pituitary gland of central nervous system (Xia et al., 2000). Interaction with chondroitin modifying enzymes was already verified (Hiraoka et al., 2001). Katanin p60 was also demonstrated to interact with Chondroitin polymerization factor which is a trans-membrane glycosyltransferase. In the light of these two interactions, it can be speculated that katanin p60 may be modified by these proteins.

SH3-domain GBR2 like endophilin B1 was found via Yeast Two Hybrid assay with bait Bax, an apoptotic effector. Researchers interpreted that this protein could be link between Bax protein and cytoskeletal proteins (Benoit et al., 2001). Bax protein might conduct its apoptotic signal to microtubules via interaction of SH3-domain



GBR2 like endophilin B1 with katanin p60. Hence, p60 might be responsible of microtubule rearrangements in apoptosis.

Regulator of G protein Signaling 11, is a GTPase activator functioning in intracellular signaling cascade. This protein is responsible from the G protein  $\beta$  fidelity via GTPase activation property (Snow et al., 1999). Katanin may be involved in G protein signaling cascade. However, this interaction may be fished due to homology between GTPase domain and AAA domain, since katanin p60 has an ATP hydrolysis functioning AAA domain.

NADH dehydrogenase (ubiquinone) complex 1 polypeptide 6 is a nuclear coded mitochondrial protein which functions in the first enzyme complex in electron transport chain. This protein might interact with other AAA superfamily members and interaction with katanin p60 could take place due to its homology with AAA super family. Hence, this interaction is considered to be false positive.

Glucose-6 phosphate dehydrogenase is a cytosolic housekeeper protein, and takes a role in pentose phosphate pathway. Interaction with katanin p60 can occur due to tendency of AAA superfamily members to form multimeric structure. Hence, this interaction may be also considered to be false positive.

SNAPC4 is a transcription factor and required for transcription of snRNA by RNA polymerase I and III (Henry et al 1995). Since they are present in different compartments of the cell, interaction does not occur normally. Hence, this interaction might be considered as false positive due to AAA superfamily homology.

Leukocyte receptor cluster member 8 is a transmembrane protein and function of protein is unknown. On the other hand, this protein is shown to interact with survival of motor neuron factor 1 (Rual et al., 2005) which has a role in neurogenesis. Myeloid leukemia factor 2 is involved in leukemia. These two proteins could be fished from myeloid ancestral glial cells. Since we identified interaction of katanin p60 with 3 different leukemia related proteins (with septin 3), maybe katanin has a function in microtubule rearrangements of disease mechanism of leukemia.

As mentioned above, many interesting interactions of katanin are discovered. These interactions may be classified under six categories:

- Structural interactions are with tubulin and actin monomers which constitute cytoskeleton in neurons.
- Interactions modulating axonal growth are with tubulin, actin, septin3, WAS protein family member 2, Rho GTPase BTB3, and Calcium binding protein 22. These interactions are candidate for participating in katanin microtubule severing cascade.
- Modification interactions of katanin are found to be with chondroitin polymerase and carbohydrate sulfotransferase. These two proteins have also been found to be interacting with each other. Since oligomerization pattern of katanin is still unknown, it is possible that katanin p60 is modified to generate hexameric structure.
- Another category is committed to GTP turnover containing Rho guanine exchange factor and RhoGTPase BTB3. Since many tubulin-GDP heterodimers are generated in the action site of katanin, turnover of tubulin monomer may be fulfilled by these proteins. Also, since katanin p60 requires ATP for its activity, these proteins may participate in acquiring nucleoside triphosphate to its activity site.
- Another interesting category is leukemia related proteins. This category could be emerged due to presence of myeloid ancestral glial cells in whole fetal brain. Additionally, op18 which is another microtubule destabilizing protein was discovered to be involved in leukemia. It could be speculated that microtubule severing also involves in cell cycle dynamics of leukocytes.
- Finally, there is apoptosis category constituted by SH3 domain GRB2-like endophilin B2. This protein was discovered while Bax interacting proteins were screened with Yeast Two Hybrid method. It is speculated that this protein is responsible from conducting the Bax apoptosis signal to microtubules and actin filaments. Katanin p60 is also candidate for transduction of apoptosis signal because of its microtubule severing feature.

## **5. CONCLUSION AND FURTHER COMMENTS**

Yeast Two Hybrid stage of katanin p60 interaction study was thoroughly completed. 22 protein interactions with katanin p60 were discovered by exploiting Yeast Two Hybrid Method. Most of the interacting proteins are promising in order to explain action cascade of katanin.

However, mammalian cells have different post transcriptional modification system than yeast cells. Therefore, these promising interactions should be confirmed by further analysis with mammalian expression.

For this purpose, mammalian expression and coimmunoprecipitation experiments are designed and are being conducted in our laboratory.

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## APPENDIX A: SEQUENCE ALIGNMENTS

Sequence alignments of fetal human cDNA library are given below:

### 1. Meteorin, glial cell differentiation regulator

Score = 1360 bits (736), Expect = 0.0 Identities = 761/773 (98%), Gaps = 1/773 (0%)  
Strand=Plus/Plus

```
Query 123 CCAAACACTTCTGCAAACACTAGAAAAGGAGGTAGCCCGTGTGCCCTCCTGCACCGCCTC 182
          |||
Metrn 2713 CCAAACACTTCTGCAAATACTAGAAAAGGAGGTAACCCGTGTGCCCTCCTGCACCGCCTC 2772

Query 183 CTCCACTCCTGTCTTGGAGAGGCTTTCCCGTGGGCCGAGCAACCCCTTACCTTTGGCAC 242
          |||
Metrn 2773 CTCCACTCCTGTCTTGGAGAGGCTTTCCCGTGGGCCGAGCAACCCCTTACCTTTGGCAC 2832

Query 243 TTGCCCTGATCTCTGGCCAGCTTCTACAACCTCTCCTTTTCAGCCACACCCAAGTCCA 302
          |||
Metrn 2833 TTGCCCTGATCTCTGGCCAGCTTCTACAACCTCTCCTTTTCAGCCACACCCAAGTCCA 2892

Query 303 CCTGTTACAAAACACCTGGGCTGGGCCCTGGCCGAACCTGGCTTCTACCTACCTGTCT 362
          |||
Metrn 2893 CCTGTTACAAAACACCTGGGCTGGGCCCTGGCAGAACCCTGGCTTCTACCCACCTGTCT 2952

Query 363 CAGGAAGTGCCTGAGCCTTGACCTACAGACCCTCCAGAGAAAGCCCTGGCTTTGCAAC 422
          |||
Metrn 2953 CAGGAAGTGCCTGAGCCTTGACCTACAGACCCTCCAGAGAAAGCCCTGGCTTTGCAAC 3012

Query 423 CAGCTGTCTTCTCATGAGACACAGGGCCACAGGAGCACCACGGTGCCCAAATGGGTCTTG 482
          |||
Metrn 3013 CAGCTGTCTTCTCATGAGACACAGGGCCACAGGAGCACCACGGTGCCCAAATGGGTCTTG 3072

Query 483 GAGCATTAGACCAGGGGCTTTCATGCCTGCCCTGGGGACAGGTGGCCAAAGTGGCATGGGA 542
          |||
Metrn 3073 GAGCATTAGACCAGGGGCTTTCATGCCTGCCCTGGGGACAGGTGGCCAAAGTGGCATGGGA 3132

Query 543 GATAGGGAGACAGTGTGGGTGAGCAGGTGGGCAGGAGCTACAGGAGCTGGGGCCTGTGG 602
          |||
Metrn 3133 GATAGGGAGACAGTGTGGGTGAGCAGGTGGGCAGGAGCTACAGGAGCTGGGGCCTGTGG 3192

Query 603 ATCACAGACACCGCCAGGCAAGTGCCAGTTCACACAGGTACAGGTGGCTGCAGAGTGCCC 662
          |||
Metrn 3193 ATCACAGACACCGTCAGGCAAGTGCCAGTTCACACAGGTACAGGTGGCTGCAGAGTGCCC 3252

Query 663 AAAGGCCAAGCAGCAGAGTGCTGGTGTGGACGGGAGAGGAGTGGGGAGGTTGGGCGCAG 722
          |||
Metrn 3253 AAAGGCCAAGAAGCAGAGTGCTGGTGTGGACGGGAGAGGAGTGGGGAGGTTGGGCGCAG 3312

Query 723 TGGCTCAGCCTGTAATCCCAACACTTTGGGAGGCCAGGCAAGCAGATCATGAGGTCAG 782
          |||
Metrn 3313 TGGCTCATGCCTGTAATCCCAACACTTTGGGAGGCCAGGCAAGCAGATCATGAGGTCAG 3372

Query 783 GAGATGGAGACCATCTGGCCAACATGGTGAACCGTCTCTAATAAACAACAAAAATTA 842
          |||
Metrn 3373 GAGATGGAGACCATCTGGCCAACATGGTGAACCGTCTCTAATAAACAACAAAAATTA 3432

Query 843 GCCAGACCTTGGTGGCATGCATCTGTAATCCAGCTACTCAAAGGCTGAGGC 895
          |||
Metrn 3433 GCCAGACGT-GGTGGCATGCATCTGTAATCCAGCTACTCAAAGGCTGAGGC 3484
```

2. SH3-domain GRB2-like endophilin B2 [Homo sapiens]

Score = 1511 bits (818), Expect = 0.0 Identities = 868/888 (97%), Gaps = 19/888 (2%)  
Strand=Plus/Plus

```

Query 122 CCTCCGCGCTCTGGAATGATGAAGTGGACAAGGCCGAGCAGGAGCTCCGCGTGGCCCAGA 181
          |||
Sh3 763 CCTCCGCGCTCTGGAATGATGAAGTGGACAAGGCCGAGCAGGAGCTCCGCGTGGCCCAGA 822

Query 182 CAGAGTTTGACCGGCAAGCAGAAGTGACCCGTCTCTTGCTGGAGGGAATCAGTAGCACTC 241
          |||
Sh3 823 CAGAGTTTGACCGGCAAGCAGAAGTGACCCGTCTCTTGCTGGAGGGAATCAGTAGCACTC 882

Query 242 ACGTGAACCACCTGCGCTGCCTCCACGAGTTCGTCAAGTCTCAGACAACCTACTACGCAC 301
          |||
Sh3 883 ACGTGAACCACCTGCGCTGCCTCCACGAGTTCGTCAAGTCTCAGACAACCTACTACGCAC 942

Query 302 AGTGTACCGCCACATGCTGGACTTGCGAGAAGCAGCTGGGCAGCTCCCAGGGTGCCATAT 361
          |||
Sh3 943 AGTGTACCGCCACATGCTGGACTTGCGAGAAGCAGCT-GG--G----CA----G----AT 987

Query 362 TTCCCGGCACCTTCGTGGGCACCACAGAGCCCGCTCCCACCCCTGAGCAGCACCTCAC 421
          |||
Sh3 988 TTCCCGGCACCTTCGTGGGCACCACAGAGCCCGCTCCCACCCCTGAGCAGCACCTCAC 1047

Query 422 CCACCCTGCTGCGGCCACTATGCCTGTGGTGCCTCTGTGGCCAGCCTGGCCCTCCGG 481
          |||
Sh3 1048 CCACCCTGCTGCGGCCACTATGCCTGTGGTGCCTCTGTGGCCAGCCTGGCCCTCCAG 1107

Query 482 GGGAGGCCTCGCTCTGCCTGGAAGAGGTGGccccccTGCCAGTGGGACCCGCAAAGCTC 541
          |||
Sh3 1108 GGGAGGCCTCGCTCTGCCTGGAAGAGGTGGCCCCCCTGCCAGTGGGACCCGCAAAGCTC 1167

Query 542 GGGTGTCTATGACTACGAGGAGCCGACAGCAGTGTAGCTGGCCCTGCTGGCTGATGAGC 601
          |||
Sh3 1168 GGGTGTCTATGACTACGAGGAGCCGACAGCAGTGTAGCTGGCCCTGCTGGCTGATGAGC 1227

Query 602 TCATCACTGTCTACAGCCTGCCTGGCATGGACCCTGACTGGCTCATTGGCGAGAGAGGCA 661
          |||
Sh3 1228 TCATCACTGTCTACAGCCTGCCTGGCATGGACCCTGACTGGCTCATTGGCGAGAGAGGCA 1287

Query 662 ACAAGAAGGGCAAGGTCCCTGTCACCTACTTGGAACTGCTCAGCTAGGCAGGTGccccca 721
          |||
Sh3 1288 ACAAGAAGGGCAAGGTCCCTGTCACCTACTTGGAACTGCTCAGCTAGGCAGGTGCCCCCA 1347

Query 722 tccccccGCATTCTGGCCTAGGCAGGAGAGGATGGGCGCAGCCCTGCCACTTAACCTGT 781
          |||
Sh3 1348 TCCCCCGCATTCTGGCCTAGGCAGGAGAGGATGGGCGCAGCCCTGCCACTTAACCTGT 1407

Query 782 TTGTTGGTGACACAGTTGTTCA-AGTGGGGGAGAATTACCCCATTCTGTCCCTGCC 840
          |||
Sh3 1408 TTGTTGGTGACACAGTTGTTCAAG-AGTGGGGGAGAATTACCCCATTCTGTCCCTGCC 1464

Query 841 CTAGTCACCTAGCTGTGAGGGTGCCCTGAGGCTGAATGGCTCCAccccctccccagcctg 900
          |||
Sh3 1465 CTAGTCACCTAGCTGTGAGGGTGCCCTGAGGCTGAATGGCTCCACCCCTCCCCAGCCCTG 1524

Query 901 cttctgacctgtggctctggagccccctgcccctgacctgcatccccGAGCACCCACCTC 960
          |||
Sh3 1525 CTTCTGACCTGTGGCTCTGGAGCCCCCTGCCCTGCCTGCATCCCCGAGCACCCACCTC 1584

Query 961 CAGGCTCCACTAAGGAGGGAGGGGCTGTCTGCAGCAGCTGCACTCAGC 1008
          |||
Sh3 1585 CAGGCTCCACTAAGGAGGGAGGGGCTGTCTGCAGCAGCTGCACTCAGC 1632
    
```

3. zinc finger family member 767 [Homo sapiens]

Score = 959 bits (519), Expect = 0.0, Identities = 541/552 (98%), Gaps = 0/552 (0%),  
Strand=Plus/Plus

```

Query 135 CTGAAAAGACGCACAAAGAATGGCCATAAAAGATATGAGCAACCCAGCTTTCCAGACAG 194
          |||
Zincf 1183 CTGAAAAGACGCACAAAGAATGGCCATAAAAGATATGAGCAACCCAGCTTTCCAGACAG 1242
    
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Query 195 TCACTTTTCCAGTGGTCATACCTGGTCTGGAAGATTCCCCATCATCTCGAATAAAGCTG 254
      |||
Zincf 1243 TCACTTTTCCAGTGGTCATACCTGGTCTGGAAGATTCCCCATCATCTCGAATAAAGCTG 1302

Query 255 TTGTTGCTTTTAACTCCATGGAGAGACCGAATGGAGTGAGCCCAGCAGGGCATGCTGGGC 314
      |||
Zincf 1303 TTGTTGCTTTTAACTCCATGGAGAGACCGAATGGAGTGAGCCCAGCAGGGCATGCTGGGC 1362

Query 315 AAGAGAGGTCCCCGAGTCCCAAATAAGAATTTCAACTAGTATAAAACGAGGCAGCGAAC 374
      |||
Zincf 1363 AAGAGAGGTCCCCGAGTCCCAAATAAGAATTTCAACTAGTATAAAACGAGGCAGCGAAC 1422

Query 375 CCACACGTGGAAGTCTGATACCGCTTGCAAGGAATTGAATAGATGTCTCCCTATG 434
      |||
Zincf 1423 CCACACGTGGAAGTCTGATACCGCTTGCAAGGAATTGAATAGATGTCTCCCTATG 1482

Query 435 TAAGGATGTGGTTTTCTTGACTTGAATTAACAAGCCCGCAAGCAACATCTGATCATCCG 494
      |||
Zincf 1483 TAAGGATGTGGTTTTATTGACTTGAATAACAAGCCCGCAAGCAACACTGATCATCCG 1542

Query 495 CGGGATGCTGCCACAAGGAATAATTGAGCACTCATTAGACACAGGGGAAACCACTGCCT 554
      |||
Zincf 1543 CGGGATGCTGCCACAAGGAATAATTGAGCACTCATTAGACACAGGGGAAACCACTGCCT 1602

Query 555 CTTTTAGTCTTTCTCCAGATFCCAACAGTCATTGTTACAGCATTTACCTTGTTCACCT 614
      |||
Zincf 1603 CTTTCAGTCTTTCTCCAGATFCCAACAGTCAGTGTACAGCATTTACCTTGTTCACCT 1662

Query 615 CTCTGAGAAGACGTTGTTGCTCACTCTCCCTAGTGGGCACTGGGAGCCTCTGCTCAGGTG 674
      |||
Zincf 1663 CCCTGAGAAGACGTTGCTGAGCTCACTCACCCAGTGGGCACTGGGAGCCTCTGCTCAGGTG 1722

Query 675 GGAGATAGATGC 686
      |||
Zincf 1723 GGAGACAGATGC 1734

```

4. solute carrier family 44, member 2 [Homo sapiens]

Score = 459 bits (248), Expect = 1e-126  
 Identities = 248/248 (100%), Gaps = 0/248 (0%)  
 Strand=Plus/Plus

```

Query 108 GGGAACTGGCTTCCCAGTTGCCCTTTGCCATATTCCAAGTCCCCCTCAGACTTCATGTC 167
      |||
CTL2 3142 GGGAACTGGCTTCCCAGTTGCCCTTTGCCATATTCCAAGTCCCCCTCAGACTTCATGTC 3201

Query 168 TCTGCTCATCAGCACTGTCCCAGGATCCTGGAGAGGGAGAACCCTGGCCCCAGGGGAAA 227
      |||
CTL2 3202 TCTGCTCATCAGCACTGTCCCAGGATCCTGGAGAGGGAGAACCCTGGCCCCAGGGGAAA 3261

Query 228 GAggggggggTCTCCGTTTCTGTGCTGCACCAGCCCTGCCCCATTGCGTCTGCACA 287
      |||
CTL2 3262 GAGGGGGGGTCTCCGTTTCTGTGCTGCACCAGCCCTGCCCCATTGCGTCTGCACA 3321

Query 288 CCCCTGCGTGTAACTGCATTCCAACCACTAATAAAGTGCCTATTGTACAGGTAAAAAAA 347
      |||
CTL2 3322 CCCCTGCGTGTAACTGCATTCCAACCACTAATAAAGTGCCTATTGTACAGGTAAAAAAA 3381

Query 348 AAAAAAAA 355
      |||
CTL2 3382 AAAAAAAA 3389

```

5. KIAA1853 [Homo sapiens]

Score = 675 bits (365), Expect = 0.0  
 Identities = 369/371 (99%), Gaps = 0/371 (0%)  
 Strand=Plus/Plus

```

Query 123 CTTTTGCAAAAGGGTCTGCTTGGAGAGGCCAAAATTCAGGGTGCTCTCAAAGGCAAAGAA 182
      |||
KIAA1 6382 CTTTTGCAAAAGGGTCTGCTTGGAGAGGCCAAAATTCAGGGTGCTCTCAAAGGCAAAGAA 6441

```

```

Query 183 AGCACATTGttttcctttctccagttccaactttcatcttttcttctgctgtttttctttttcc 242
          |||
KIAA1 6442 AGCACATTGTTTTCTTCTCCAGTCCAACCTTCATCTTTTCTTCTGCTGTTTTCTTTTCC 6501

Query 243 cctctttcttttttcACAAATGTTCAAATGGTCTCATGCGCATGTGTCTTGCCCCACTTTC 302
          |||
KIAA1 6502 CCTCTTCTTTTTCACAAATGTTCAAATGGTCTCATGCGCATGTGTCTTGCCCCACTTTC 6561

Query 303 CCCTTTAGCTGAACAGAAAATTTTGTCTCAGTAAAACGAAGTCAAAAAACAGGATTCTC 362
          |||
KIAA1 6562 CCCTTTAGCTGAACAGAAAATTTTGTCTCAGTAAAACGAAGTCAAAAAACAGGATTCTC 6621

Query 363 CAAACATGCCTCCTCCCGCACTGGCCAGCCGATTCCATCTGAGAACTTATGCTAGATTC 422
          |||
KIAA1 6622 CAAACATGCCTCCTCCCGCACTGGCCAGCCGAGTCCAGCTGAGAACTTATGCTAGATTC 6681

Query 423 AATGTCATTGAGCAATGCTTTTATTGAAGTCTCGTTCTTCTCACTTCTGCACCAGTGAGCC 482
          |||
KIAA1 6682 AATGTCATTGAGCAATGCTTTTATTGAAGTCTCGTTCTTCTCACTTCTGCACCAGTGAGCC 6741

Query 483 AATGATACTGA 493
          |||
KIAA1 6742 AATGATACTGA 6752

```

6. WAS protein family, member 2 [Homo sapiens]

Score = 1530 bits (828), Expect = 0.0  
Identities = 847/855 (99%), Gaps = 5/855 (0%)  
Strand=Plus/Plus

```

Query 124 CCTCTCTGGGGGACCAGAAAGTAGTGAGAGAAGGAAGGGGATAGGCAGCTTTGACAGGTGC 183
          |||
WAS 2905 CCTCTCTGGGGGACCAGAAAGTAGTGAGAGAAGGAAGGGGATAGGCAGCTTTGACAGGTGC 2964

Query 184 TGCTTTCAATTCCTCTGCAACTCCTCCCCCTTTTATTTCCTCAATTTAAACAAAGATTCT 243
          |||
WAS 2965 TGCTTTCAATTCCTCTGCAACTCCTCCCCCTTTTATTTCCTCAATTTAAACAAAGATTCT 3024

Query 244 GCCAACTGTGAAACTTCAGTCCCTCAGGCTGGCAGCCATGCCAGTACCTGCCTgggggt 303
          |||
WAS 3025 GCCAACTGTGAAACTTCAGTCCCTCAGGCTGGCAGCCATGCCAGTACCTGCCTGGGGGT 3084

Query 304 ggggggTGCCTGGCAGCCATGAAGCAGGCTGAAAGGCAGAGGGGCTCCAGGTCTGTTC 363
          |||
WAS 3085 GGGGGGTGCCTGGCAGCCATGAAGCAGGCTGAAAGGCAGAGGGGCTCCAGGTCTGTTC 3144

Query 364 CAGCTCCCCTCACTGCACATGGTGAAGCTCGCTCCCTCCCTCCCTCCCTTCCCGCTTTTC 423
          |||
WAS 3145 CAGCTCCCCTCACTGCACATGGTGAAGCTCGCTCCCTCCCTCCCTCCCTTCCCGCTTTTC 3204

Query 424 CCAGAGCTAATACACAGGTGCTATTATTTCAGaaaaaaCTGGTCAGCTCTAGCCAACAGT 483
          |||
WAS 3205 CCAGAGCTAATACACAGGTGCTATTATTTCAGAAAAAACTGGTCAGCTCTAGCCAACAGT 3264

Query 484 GAGGTTTCTTTTCTTCTGCCCTAACTATTGTGTAGCCTCTTATGCTGAAATCGGCTTCTG 543
          |||
WAS 3265 GAGGTTTCTTTTCTTCTGCCCTAACTATTGTGTAGCCTCTTATGCTGAAATCGGCTTCTG 3324

Query 544 CTGGCTTCTCCGGCTTTCAGAGCCCTGAAACAAAGAGAAACAGGATCTGTCCCTACCCAG 603
          |||
WAS 3325 CTGGCTTCTCCGGCTTTCAGAGCCCTGAAACAAAGAGAAACAGGATCTGTCCCTACCCAG 3384

Query 604 CACAGCAAATGGTTGTAGTAATTCGCAAAGCCCTCATAAAGCCCTCCGGCTTGAGGAGAG 663
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WAS 3385 CACAGCAAATGGTTGTAGTAATTCGCAAAGCCCTCATAAAGCCCTCCGGCTTGAGGAGAG 3444

Query 664 AGTGTATAGTCATGGGTTCTGCCTCTGTGCCCTTGCTGGCCGCTTCTCCTCTGCCTTCTT 723
          |||
WAS 3445 AGTGTATAGTCATGGGTTCTGCCTCTGTGCCCTTGCTGGCCGCTTCTCCTCTGCCTTCTT 3504

Query 724 TCCTGGAACCTCAGGGTGTGGGGACTGAGCCTGTAGGGGACAGCATGCCGTCTTGCTGTGG 783
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WAS 3505 TCCTGGAACCTCAGGGTGTGGGGACTGAGCCTGTAGGGGACAGCATGCCGTCTTGCTGTGG 3564

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```

Query 784  CCACTCCCAAGTGTGCCCTCTTCCCTCTTTACACATCATGTGTCTCTGGCACAGGACTTG 843
          |||
WAS 3565  CCACTCCCAAGTGTGCCCTCTTCCCTCTTTACACATCAGGTGTCTCTGGCACAGGACTTG 3624

Query 844  GCACTAAGCTCCCATGCTGAGACACCAGGCTATGTGGGGCCCCACCTTGTTTCCCAGCC 903
          |||
WAS 3625  GCACTAAGCTCC-ATGCTGAGACACCAGGCTATGTGGG-CCCCACCTTGTTTCCCAGCC 3682

Query 904  TGCACCTTAAAAGCCGAAAGGTGCTTTCATCAGAACCCTAAAAGGTCGTTGAAGGCGC- 962
          |||
WAS 3683  TGCACCTTAGAAGCCGAA-GGTGCTTTCATCAGAACCCTAAAATGGTCGTTGAAGGCGCC 3741

Query 963  TGGGGCCGCAGCCCA 977
          |||
WAS 3742  TGGG-CCGCAGCCCA 3755

```

7. Homo sapiens actin, beta (ACTB), mRNA

Score = 1016 bits (550), Expect = 0.0  
Identities = 550/550 (100%), Gaps = 0/550 (0%)  
Strand=Plus/Plus

```

Query 124  GCCACGGCTGCTTCCAGCTCCTCCCTGGAGAAGAGCTACGAGCTGCCTGACGGCCAGGTC 183
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ACTB 766  GCCACGGCTGCTTCCAGCTCCTCCCTGGAGAAGAGCTACGAGCTGCCTGACGGCCAGGTC 825

Query 184  ATCACCATTGGCAATGAGCGGTTCCGCTGCCCTGAGGCACTCTCCAGCCTTCCTTCCTG 243
          |||
ACTB 826  ATCACCATTGGCAATGAGCGGTTCCGCTGCCCTGAGGCACTCTCCAGCCTTCCTTCCTG 885

Query 244  GGCATGGAGTCTGTGGCATCCACGAAACTACCTTCAACTCCATCATGAAGTGTGACGTG 303
          |||
ACTB 886  GGCATGGAGTCTGTGGCATCCACGAAACTACCTTCAACTCCATCATGAAGTGTGACGTG 945

Query 304  GACATCCGCAAAGACCTGTACGCCAACACAGTGCTGTCTGGCGGCACCACCATGTACCCT 363
          |||
ACTB 946  GACATCCGCAAAGACCTGTACGCCAACACAGTGCTGTCTGGCGGCACCACCATGTACCCT 1005

Query 364  GGCATGCGCAGAGGATGCAGAAGGAGATCACTGCCCTGGCACCCAGCACAATGAAGATC 423
          |||
ACTB 1006  GGCATGCGCAGAGGATGCAGAAGGAGATCACTGCCCTGGCACCCAGCACAATGAAGATC 1065

Query 424  AAGATCATTGCTCCTCCTGAGCGCAAGTACTCCGTGTGGATCGGCGGCTCCATCCTGGCC 483
          |||
ACTB 1066  AAGATCATTGCTCCTCCTGAGCGCAAGTACTCCGTGTGGATCGGCGGCTCCATCCTGGCC 1125

Query 484  TCGCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTATGACGAGTCCGGCCCC 543
          |||
ACTB 1126  TCGCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTATGACGAGTCCGGCCCC 1185

Query 544  TCCATCGTCCACCGCAAATGCTTCTAGGCGGACTATGACTTAGTTGCGTTACACCCTTTC 603
          |||
ACTB 1186  TCCATCGTCCACCGCAAATGCTTCTAGGCGGACTATGACTTAGTTGCGTTACACCCTTTC 1245

Query 604  TTGACAAAACCTAACTTGCGCAGAAAACAAGATGAGATTGGCATGGCTTTATTTGttttt 663
          |||
ACTB 1246  TTGACAAAACCTAACTTGCGCAGAAAACAAGATGAGATTGGCATGGCTTTATTTGTTTTT 1305

Query 664  tttGTTTTGT 673
          |||
ACTB 1306  TTTGTTTTGT 1315

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8. chromosome 9 open reading frame 127 [Homo sapiens]

Score = 1674 bits (906), Expect = 0.0  
Identities = 906/906 (100%), Gaps = 0/906 (0%)  
Strand=Plus/Plus

```

Query 120  ACCACCAGGGTTGCCAGGCTGCGAATCCCATTCCTCCGACACGGGGACCTGGTTCTGGCC 179
          |||
ORF127 1697  ACCACCAGGGTTGCCAGGCTGCGAATCCCATTCCTCCGACACGGGGACCTGGTTCTGGCC 1756

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Query 180 CTCCGCTCCCTGTGCGGGGTGGGGCCTCGGTTTCGTGCGGTGCCGCAACGCGACGGCCGAG 239
          |||
ORF127 1757 CTCCGCTCCCTGTGCGGGGTGGGGCCTCGGTTTCGTGCGGTGCCGCAACGCGACGGCCGAG 1816

Query 240 GTGCGGATGCGCACCTTCCTGTCCCCATGCGTGGACGACTGCGGGCCCTACGGCCAGTGC 299
          |||
ORF127 1817 GTGCGGATGCGCACCTTCCTGTCCCCATGCGTGGACGACTGCGGGCCCTACGGCCAGTGC 1876

Query 300 AAGCTGCTGCGCACACACAATATCTGTACGCAGCCTGCGAGTGCAAGCCGGGTGGAGA 359
          |||
ORF127 1877 AAGCTGCTGCGCACACACAATATCTGTACGCAGCCTGCGAGTGCAAGCCGGGTGGAGA 1936

Query 360 GGCTGGGGCTGCACCGACAGTGCAGATGCGCTCACCTATGGATTCCAGCTGTGTCCACA 419
          |||
ORF127 1937 GGCTGGGGCTGCACCGACAGTGCAGATGCGCTCACCTATGGATTCCAGCTGTGTCCACA 1996

Query 420 CTCCTGCTCTGCCTGAGCAACCTCATGTTTCTGCCACCTGTGGTCCCTGGCCATTTCGGAGT 479
          |||
ORF127 1997 CTCCTGCTCTGCCTGAGCAACCTCATGTTTCTGCCACCTGTGGTCCCTGGCCATTTCGGAGT 2056

Query 480 CGATATGTGCTGGAAGCTGCAGTCTACACCTTCACCATGTTCTTCTCCACGTTCTATCAT 539
          |||
ORF127 2057 CGATATGTGCTGGAAGCTGCAGTCTACACCTTCACCATGTTCTTCTCCACGTTCTATCAT 2116

Query 540 GCCTGTGACCAGCCAGGCATCGTGGTFTTCTGCATCATGGACTACGATGTGCTGCAGTTC 599
          |||
ORF127 2117 GCCTGTGACCAGCCAGGCATCGTGGTFTTCTGCATCATGGACTACGATGTGCTGCAGTTC 2176

Query 600 TGTGATTTCTGGGCTCCTTAATGTCCGTGTGGGTCACTGTGATTGCCATGGCTCGTTTA 659
          |||
ORF127 2177 TGTGATTTCTGGGCTCCTTAATGTCCGTGTGGGTCACTGTGATTGCCATGGCTCGTTTA 2236

Query 660 CAGCCCGTGGTCAAGCAGGTGCTGTATTTGCTGGGAGCTATGCTGTGTCCATGGCTCTG 719
          |||
ORF127 2237 CAGCCCGTGGTCAAGCAGGTGCTGTATTTGCTGGGAGCTATGCTGTGTCCATGGCTCTG 2296

Query 720 CAGCTTGACCACATGGACTCTGGAACCTGCTTGGACCCAGTCTCTTCGCCCTGGGGATC 779
          |||
ORF127 2297 CAGCTTGACCACATGGACTCTGGAACCTGCTTGGACCCAGTCTCTTCGCCCTGGGGATC 2356

Query 780 TTGGCCACAGCCTGGACAGTACGCAGCGTCCGCCCGGCACTGCTACCCACCCACGTGG 839
          |||
ORF127 2357 TTGGCCACAGCCTGGACAGTACGCAGCGTCCGCCCGGCACTGCTACCCACCCACGTGG 2416

Query 840 CGCCGCTGGCTTTTCTACTTGTGCCCTGGCAGCCTTATTGCAGGCAGTGCCTGCTGCTT 899
          |||
ORF127 2417 CGCCGCTGGCTTTTCTACTTGTGCCCTGGCAGCCTTATTGCAGGCAGTGCCTGCTGCTT 2476

Query 900 TATGCTTTTGTGGAGACCCGGGACAACCTACTTCTACATTACAGCATTGGCATATGCTC 959
          |||
ORF127 2477 TATGCTTTTGTGGAGACCCGGGACAACCTACTTCTACATTACAGCATTGGCATATGCTC 2536

Query 960 ATTGCGGGCAGTGTGGGCTTCTGCTGCCCCCTCGTGCCAAGACTGACCACGGGTCCCA 1019
          |||
ORF127 2537 ATTGCGGGCAGTGTGGGCTTCTGCTGCCCCCTCGTGCCAAGACTGACCACGGGTCCCA 2596

Query 1020 TCTGGA 1025
          |||
ORF127 2597 TCTGGA 2602

```

9. carbohydrate (N-acetylgalactosamine 4-0)sulfotransferase 8  
[Homo sapiens]

Score = 1138 bits (616), Expect = 0.0  
Identities = 616/616 (100%), Gaps = 0/616 (0%)  
Strand=Plus/Plus

```

Query 119 CCTCATCCGCGCGCCGCGGAACCTGACCTTCCCCCGGTTCAAGGACCGGCACTCGCAGGA 178
          |||
CHST8 1838 CCTCATCCGCGCGCCGCGGAACCTGACCTTCCCCCGGTTCAAGGACCGGCACTCGCAGGA 1897

Query 179 GGCGCGGACCACAGCGAGGATCGCCACCAGTACTTCGCCCAACTCTCGGCCCTGCAAAG 238
          |||
CHST8 1898 GGCGCGGACCACAGCGAGGATCGCCACCAGTACTTCGCCCAACTCTCGGCCCTGCAAAG 1957

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Query 239 GCAGCGCACCTACGACTTCTACTACATGGATTACCTGATGTTCAACTATTCCAAGCCCTT 298
          |||
CHST8 1958 GCAGCGCACCTACGACTTCTACTACATGGATTACCTGATGTTCAACTATTCCAAGCCCTT 2017

Query 299 TGCAGATCTGTACTGAGGGGCGCCGAGCTGGCCGGGGCCGCCCTGCCCCGGTCACTCAC 358
          |||
CHST8 2018 TGCAGATCTGTACTGAGGGGCGCCGAGCTGGCCGGGGCCGCCCTGCCCCGGTCACTCAC 2077

Query 359 CTGTGCTCCCGGGCATCCTCCTGTCCCTGGCTCCTCATCCTGGGAGCAACAGGGCTCTGA 418
          |||
CHST8 2078 CTGTGCTCCCGGGCATCCTCCTGTCCCTGGCTCCTCATCCTGGGAGCAACAGGGCTCTGA 2137

Query 419 GGACGTGAGGAGCCATCGCTGTGGGAGGCAGCAGGCCCCGGGTGGGGGGCAGAGGCGCC 478
          |||
CHST8 2138 GGACGTGAGGAGCCATCGCTGTGGGAGGCAGCAGGCCCCGGGTGGGGGGCAGAGGCGCC 2197

Query 479 AGCCTTGATGGGGACCCAGCCCTGGCCTGTACCTGTTTCTCATTCTTGGCTGAGG 538
          |||
CHST8 2198 AGCCTTGATGGGGACCCAGCCCTGGCCTGTACCTGTTTCTCATTCTTGGCTGAGG 2257

Query 539 GAGAGGCTGAGAAGTGGGCAGACACCCTGGAGCTCAGCCGACAGTTTGTATGAGCAGGG 598
          |||
CHST8 2258 GAGAGGCTGAGAAGTGGGCAGACACCCTGGAGCTCAGCCGACAGTTTGTATGAGCAGGG 2317

Query 599 AAGTCTGAGGCCAGAGGACGGGGGCCAGCGGTAAGGGATGTCCCGCACTCCCTTAGC 658
          |||
CHST8 2318 AAGTCTGAGGCCAGAGGACGGGGGCCAGCGGTAAGGGATGTCCCGCACTCCCTTAGC 2377

Query 659 CATTGCCTTGACCAAACCACGTGGTTTGCAGCTTTTCTACGAGCCAGGGGGAGGTTCC 718
          |||
CHST8 2378 CATTGCCTTGACCAAACCACGTGGTTTGCAGCTTTTCTACGAGCCAGGGGGAGGTTCC 2437

Query 719 CTTGGATTAAGGTTCC 734
          |||
CHST8 2438 CTTGGATTAAGGTTCC 2453

```

10. Calcium binding protein P22 pseudogene[Homo sapiens]

Score = 346 bits (187), Expect = 9e-93  
Identities = 232/254 (91%), Gaps = 2/254 (0%)  
Strand=Plus/Minus

```

Query 1 TGGAGGGTAGGGGCACGATCTTGGCTCACTGCAAGCTCCACCTCCCAGGTTACACCATT 60
          |||
CHP 1748 TGGAGTGTAGTGGCAGCATCTTGGCTCACTGCAAGCTCTGCCTCCCAGGTTACAGCCATT 1689

Query 61 CTCCTGCCTCAGCCTCCC-ATGTAGCTGGGACTACAGGCACCTGCCACCACGCCCGGCTA 119
          |||
CHP 1688 CTCCTGCCTCAGCCTCCCAG-ATGTAGCTGGGACTACAGGCACCCGCCACCACGCCCGGCTA 1630

Query 120 ATTTTTTGTGTATTTAGCAGAGATGGGGTTTACC CGGTTAGCCAGGATGGTCTCGATCT 179
          |||
CHP 1629 ATTTTTTGTATTTTAGTAAAGACGGGGTTTACC CGTGTAGCCAGGATGGTCTCGATCT 1570

Query 180 CCTGACCTCGTGATCCGCCCACTTCGGCCTCCCAAAGTGCTGGGATTACAGGTGTGAGTC 239
          |||
CHP 1569 CCTGACCTCGTGATCTGCCACCTCAGCCTCCACAGTGCTGGGATTACAGGCGTGAGCC 1510

Query 240 ACCATGCCCGGCTC 253
          |||
CHP 1509 ACCACGCCAGCTC 1496

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11. NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa

Score = 1208 bits (654), Expect = 0.0  
Identities = 666/672 (99%), Gaps = 0/672 (0%)  
Strand=Plus/Plus

```

Query 1 GACCCGACGGAGGTAGAGGCCAGGGCAGCGCTCCGGGAGCGGAGTCCGCGCCCGCCGA 60
NADHDH 46 GACCCGACGGAGGTAGAGGCCAGGGCAGCGCTCCGGGAGCGGAGTCCGCGCCCGCCGC 105

Query 61 AGCCAGGCCGGACAGCTGGGACAAGGATGTGTACCCCTGAGCCCCGCGCCGACGCCGGT 120
NADHDH 106 CGCCATGCCGGACAGCTGGGACAAGGATGTGTACCCCTGAGCCCCGCGCCGACGCCGGT 165

Query 121 GCAGCCCAATCCCATCGTCTACATGATGAAAGCGTTCGACCTCATCGTGGACCGACCCGT 180
NADHDH 166 GCAGCCCAATCCCATCGTCTACATGATGAAAGCGTTCGACCTCATCGTGGACCGACCCGT 225

Query 181 GACCCTCGTGAGAGAATTTATAGAGCGGCAGCACGCAAGAAGCAGGTATTACTACTACCA 240
NADHDH 226 GACCCTCGTGAGAGAATTTATAGAGCGGCAGCACGCAAGAAGCAGGTATTACTACTACCA 285

Query 241 CCGGCAGTACCGCCGCGTGCCAGACATCACTGAGTGCAAGGAGGAGGACATCATGTGCAT 300
NADHDH 286 CCGGCAGTACCGCCGCGTGCCAGACATCACTGAGTGCAAGGAGGAGGACATCATGTGCAT 345

Query 301 GTATGAAGCCGAAATGCAGTGGAGAGGGACTACAAAGTCGACCAAGAAATTATCAACAT 360
NADHDH 346 GTATGAAGCCGAAATGCAGTGGAGAGGGACTACAAAGTCGACCAAGAAATTATCAACAT 405

Query 361 TATGCAGGATCGGCTCAAAGCCTGTGTCAGCAGAGGGAAGGACAGAACTACCAGCAGAACTG 420
NADHDH 406 TATGCAGGATCGGCTCAAAGCCTGTGTCAGCAGAGGGAAGGACAGAACTACCAGCAGAACTG 465

Query 421 TATCAAGGAAGTGGAGCAGTTCACCCAGGTGGCCAAGGCCTACCAGGACCGCTATCAGGA 480
NADHDH 466 TATCAAGGAAGTGGAGCAGTTCACCCAGGTGGCCAAGGCCTACCAGGACCGCTATCAGGA 525

Query 481 CCTGGGGGCCTACAGTTCTGCCAGGAAGTGCCTGGCCAAACAGAGGCAGAGGATGCTGCA 540
NADHDH 526 CCTGGGGGCCTACAGTTCTGCCAGGAAGTGCCTGGCCAAACAGAGGCAGAGGATGCTGCA 585

Query 541 AGAGAGAAAAGCTGCAAAGAGGCCGCGCTGCCACCTCCTGAGGCAGCTGTGGGTGCC 600
NADHDH 586 AGAGAGAAAAGCTGCAAAGAGGCCGCGCTGCCACCTCCTGAGGCAGCTGTGGGTGCC 645

Query 601 CTGCTGTGTGGCTCTGTATGACTGTTGCTGAAATATAAAGTCCTGCATTCTGaaaaaaaa 660
NADHDH 646 CTGCTGTGTGGCTCTGTATGACTGTTGCTGAAATATAAAGTCCTGCACCTGAAAAAAAA 705

Query 661 aaaaaaaaaaaaaa 672
NADHDH 706 AAAAAAAAAAAAAA 717

```

12. Rho-guanine nucleotide exchange factor [Homo sapiens]

Score = 985 bits (533), Expect = 0.0  
Identities = 600/632 (94%), Gaps = 6/632 (0%)  
Strand=Plus/Plus

```

Query 78 CATGCACCAAGAAATTC AAGAGAAATATAACAAGAACAACCACAGACCATCCTTGGaa 137
RhoGEF 2269 CATGCACCAAGAAATTC AAGAGAAATATAACAAGAACAACCACAGACCATCCTTGG-A 2327

Query 138 aaaaaaaGA-GAAGGAGACATCCCACTTTCCTGGTCTCTCCTTGACCCCTTCTTCTCCG 196
RhoGEF 2328 AATTC TTCATTTA-GAGACATCCAC-AGCCTGGTCTCTCCTTGACCCCTTCTTCTCCG 2385

Query 197 TGCCTGTTGGATTGCCGACTGGAAGGAGGGATACTGTGGGACAGGTCCATCCATTGTCCA 256
RhoGEF 2386 TGCCTGTTGGATTGCCGACTGGAAGGAGGGAGACTGTGGGACAGGTCCATCCATTGTCCA 2445

Query 257 GAAGTGTTCAGGTACCACCTTGGAAATGTTTCAGGAGGTGAGCCACATCCTTGGAGTCTG 316
RhoGEF 2446 GAAGTGTTCAGGTACCACCTTGGAAAGCTTCAGGAGGTGAGCCACATCCTTGGAGTCTG 2505

Query 317 AGAGTGACAATAACAGCTGCAGAAGCAGGTCTCATTCTttttttatttttttACAGTCCAT 376

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RhoGEF 2506 AGAGTGACCATAACAGCTGCAGAAGCAGGTCTCATTCTGATGA-GCTGCTACAGTCCAT 2563  
Query 377 GGGCTCTTCTCCCTCTTTTGTAGTCTTTCATAATGGAAGATGTTGTGGATTCTTCTCTGTG 436  
||||| 750  
RhoGEF 2564 GGGCTCTTCTCCCTCTACAGAGTCTTTCATAATGGAAGATGTTGTGGATTCTTCTCTGTG 2623  
Query 437 GAGTGACCTCAGCAGTGATGCCAGGAGTTTGAAGCAGAATCTGGAGTCTTGTGGTGGA 496  
||||| 750  
RhoGEF 2624 GAGTGACCTCAGCAGTGATGCCAGGAGTTTGAAGCAGAATCTGGAGTCTTGTGGTGGA 2683  
Query 497 TCCCTCATTGTAATAGGCAGGAGAAGGATGTCATCAAAAGACAGGATGTCATTTTTGA 556  
||||| 750  
RhoGEF 2684 TCCCTCATTGTAATAGGCAGGAGAAGGATGTCATCAAAAGACAGGATGTCATTTTTGA 2743  
Query 557 GCTAATGCAAACAGAGATGCATCACATCCAGACCCGTTCATCATGCTGAGATCTTCAG 616  
||||| 750  
RhoGEF 2744 GCTAATGCAAACAGAGATGCATCACATCCAGACCCGTTCATCATGCTGAGATCTTCAG 2803  
Query 617 GAAAGGCATGAAAGAGGAGCTGCAGCTGGACCACAGCACCCGTGGATAAAAATTTCCCTG 676  
||||| 750  
RhoGEF 2804 GAAAGGCATGAAAGAGGAGCTGCAGCTGGACCACAGCACCCGTGGATAAAAATTTCCCTG 2863  
Query 677 TTTAAATGAGTTGCTTGAAATCCACAGGCATT 708  
||||| 750  
RhoGEF 2864 TTTAGATGAGTTGCTTGAAATCCACAGGCATT 2895

13. glucose-6-phosphate dehydrogenase [Homo sapiens]

Score = 424 bits (229), Expect = 2e-115  
Identities = 324/370 (87%), Gaps = 6/370 (1%)  
Strand=Plus/Plus

Query 33 GCGAGCAGCCCACTAGGCCGTGTACACCAAGATGATGACCAAGAAGCCGGGCATGTTCT 92  
||| 750  
G6PDH 1242 GCGTGACAGCCCAACGAGGCCGTGTACACCAAGATGATGACCAAGAAGCCGGGCATGTTCT 1301  
Query 93 aaaaaaaaaGGTGAAG-CGGAGCTGGTCTGACCTACGGCAACAGATAACAAGAACGTGAAG 151  
||| 750  
G6PDH 1302 TCAACCCCGAG-GAGTCGGAGCTGGACCTGACCTACGGCAACAGATAACAAGAACGTGAAG 1360  
Query 152 CTCCCTGACGCCTACGAGCGCCTCATCCTTTCCCTCTTCTGCGGAAGCCAGATGCACTT 211  
||||| 750  
G6PDH 1361 CTCCCTGACGCCTACGAGCGCCTCATCCTGGAGC-TCTTCTGCGGGAGCCAGATGCACTT 1419  
Query 212 CGTGCGCAGCGACGAGCTCCGTGAGTTTTTGTGATTTTTCACCCCACTGCTGCACCAGA 271  
||||| 750  
G6PDH 1420 CGTGCGCAGCGACGAGCTCCGTGAGGCTGG-CGTATTTTCACCCCACTGCTGCACCAGA 1478  
Query 272 TTGAGCTGGAGAAGCCCAAGCCCATCCCTATATTTATGTTTTTCTTTTCCACCGGAG 331  
||||| 750  
G6PDH 1479 TTGAGCTGGAGAAGCCCAAGCCCATCCCTATATTTATGGCAG-CCGAGGCCCCACGGAG 1537  
Query 332 GCAGACGAGCTGAttttttttttGGGTTTCCAGTATTAGGGCTCCTACAAGTGGGTGAA 391  
||||| 750  
G6PDH 1538 GCAGACGAGCTGATGAAGAGAGT-GGGTTTCCAGTATGAGGGCACCTACAAGTGGGTGAA 1596  
Query 392 CCCCCACAAG 401  
||||| 750  
G6PDH 1597 CCCCCACAAG 1606

14. Homo sapiens chondroitin polymerizing factor (CHPF), mRNA

Score = 1452 bits (786), Expect = 0.0  
Identities = 806/815 (98%), Gaps = 4/815 (0%)  
Strand=Plus/Plus

Query	38	GGAGCCAGTGCAGGAGGGGGACCCTCATTTCCGAAGTGCCCTGACAGCCCACCCCTGTGCG	97
CHPF	1151	GGAGCCAGTGCAGGAGGGGGACCCTCATTTCCGAAGTGCCCTGACAGCCCACCCCTGTGCG	1210
Query	98	AGA-ACTGTGCACATGTACCAGTTGCACAAAGCTTTCGCCCCGAGCTGAACTGGAACGCAC	156
CHPF	1211	TGACCCTGTGCACATGTACCAGCTGCACAAAGCTTTCGCCCCGAGCTGAACTGGAACGCAC	1270
Query	157	GTACCAGGAGATCCAGGAGTTACAGTGGGAGATCCAGAATACCAGCCATCTGGCCGTGA	216
CHPF	1271	GTACCAGGAGATCCAGGAGTTACAGTGGGAGATCCAGAATACCAGCCATCTGGCCGTGA	1330
Query	217	TGGGGACCGGGCAGCTGCTTGGCCCCGTTGGGTATTCCAGCACCATCCCGCCCGCCTCCCG	276
CHPF	1331	TGGGGACCGGGCAGCTGCTTGGCCCCGTTGGGTATTCCAGCACCATCCCGCCCGCCTCCCG	1390
Query	277	CTTTGAGGTGCTGCGCTGGGACTACTTCACGGAGCAGCATGCTTTCCTGCGCCGATGG	336
CHPF	1391	CTTTGAGGTGCTGCGCTGGGACTACTTCACGGAGCAGCACGCTTTCCTGCGCCGATGG	1450
Query	337	CTCACCCCGCTGCCACTGCGTGGGGCTGACCGGGCTGATGTGGCCGATGTTCTGGGGAC	396
CHPF	1451	CTCACCCCGCTGCCACTGCGTGGGGCTGACCGGGCTGATGTGGCCGATGTTCTGGGGAC	1510
Query	397	AGCTCTAGAGGAGCTGAACCGCCGCTACCACCCGGCCTTGGCGCTCCAGAAGCAGCAGCT	456
CHPF	1511	AGCTCTAGAGGAGCTGAACCGCCGCTACCACCCGGCCTTGGCGCTCCAGAAGCAGCAGCT	1570
Query	457	GGTGAATGGCTACCGACGCTTTGATCCGGCCCGGGGTATGGAATACACGCTGGACTTGCA	516
CHPF	1571	GGTGAATGGCTACCGACGCTTTGATCCGGCCCGGGGTATGGAATACACGCTGGACTTGCA	1630
Query	517	GCTGGAGGCACTGACCCCCAGGGAGGCCGCGGCCCTCACTCGCCGAGTGCAGCTGCT	576
CHPF	1631	GCTGGAGGCACTGACCCCCAGGGAGGCCGCGGCCCTCACTCGCCGAGTGCAGCTGCT	1690
Query	577	CCGGCCGCTGAGCCGCTGGAGATCTTGCCCTGTGCCCTATGTCACTGAGGCCTCACGTCT	636
CHPF	1691	CCGGCCGCTGAGCCGCTGGAGATCTTGCCCTGTGCCCTATGTCACTGAGGCCTCACGTCT	1750
Query	637	CACTGTGCTGCTGCCTCTAGCTGCGGCTGAGCGTGACCTGGCCCTGGCTTCTTGGAGGC	696
CHPF	1751	CACTGTGCTGCTGCCTCTAGCTGCGGCTGAGCGTGACCTGGCCCTGGCTTCTTGGAGGC	1810
Query	697	CTTTGCCACTGCAGCACTGGAGCCTGGTGTGCTGCGGCAGCCCTGACCCTGTGCTACT	756
CHPF	1811	CTTTGCCACTGCAGCACTGGAGCCTGGTGTGCTGCGGCAGCCCTGACCCTGTGCTACT	1870
Query	757	GTATGAGCCGCGCCAGGCCAGCGCTGGCCATGCAGATGTCTTC-CACCTGTCAAGGC	815
CHPF	1871	GTATGAGCCGCGCCAGGCCAGCGCTGGCCATGCAGATGTCTTCGCACCTGTCAAGGC	1930
Query	816	CCATTGTGGCAGAGCTGGAGCGCGTTTTTCCCCGG 850	
CHPF	1931	CCAC-GTGGCAGAGCTGGAGCGCGTTTT-CCCCGG 1963	

15. myeloid leukemia factor 2 [Homo sapiens]

Score = 1277 bits (691), Expect = 0.0  
 Identities = 696/698 (99%), Gaps = 1/698 (0%)  
 Strand=Plus/Plus

Query	38	AGACACGGAGGACTGTTCCGGGATTCAGACAGTGGACTGGAGCAGATGTCCATTGGGCATC	97
MFL2	540	AGACACGGAGGACTGTTCCGGGATTCAGACAGTGGACTGGAGCAGATGTCCATTGGGCATC	599
Query	98	ACATCCGGGACAGGGCTCACATCCTCCAGCGCTCCCGAAACCATCGCACGGGGGACCAGG	157
MFL2	600	ACATCCGGGACAGGGCTCACATCCTCCAGCGCTCCCGAAACCATCGCACGGGGGACCAGG	659
Query	158	AGGAGCGGCAGGACTATATCAACCTGGATGAGAGTGAGGCCGAGCGTTTGTATGACGAGT	217
MFL2	660	AGGAGCGGCAGGACTATATCAACCTGGATGAGAGTGAGGCCGAGCGTTTGTATGACGAGT	719

Query	218	GGCGGCGGGAGACCTCCCATTCCGGCAGCAGCGTCCCCTGGAGTTTCGGCGGCTTGAGT	277
MFL2	720	GGCGGCGGGAGACCTCCCATTCCGGCAGCAGCGTCCCCTGGAGTTTCGGCGGCTTGAGT	779
Query	278	CCTCAGGGGCTGGGGGACGAAGGGCGGAGGGGCCTCCCCGCCTGGCCATCCAGGGACCTG	337
MFL2	780	CCTCAGGGGCTGGGGGACGAAGGGCGGAGGGGCCTCCCCGCCTGGCCATCCAGGGACCTG	839
Query	338	AGGACTCCCCTTCCCAGAGTCCCAGCGCTATGACTGGTGAGGGCCCCGGGCCCTCAGCC	397
MFL2	840	AGGACTCCCCTTCCCAGAGTCCCAGCGCTATGACTGGTGAGGGCCCCGGGCCCTCAGCC	899
Query	398	TCTCTGTACAGGCTGAGAGGCTGAGAAATCATCCCCTGAATAACCTTTTCTCTCGATT	457
MFL2	900	TCTCTGTACAGGCTGAGAGGCTGAGAAATCATCCCCTGAATAACCTTTTCTCTCGATT	959
Query	458	CCCATCCCCAATTTAATATTAATAAACAGGCAAGCCGGCCCCACCTCTCCCTGGGGGT	517
MFL2	960	CCCATCCCCAATTTAATATTAATAAACAGGCAAGCCGGCCCCACCTCTCCCTGGGGGT	1019
Query	518	CTCAGGGAGAACCTTTCACGGCACCTTTCCTACCTTTTCCTTCTTAATCTCCTGGTT	577
MFL2	1020	CTCAGGGAGAACCTTTCACGGCACCTTTCCTACCTTTTCCTTCTTAATCTCCTGGTT	1079
Query	578	TACCATTGATGACTTCGCCTCTGCATCTACTGACTTGATTTTTCATTCTGCCACTCCATC	637
MFL2	1080	TACCATTGATGACTTCGCCTCTGCATCTACTGACTTGATTTTTCATTCTGCCACTCCATC	1139
Query	638	TTCAAACCCCTCACCTTTCATCTACTCCTGCCATGCATTGAAGGGTCAATGCATT	697
MFL2	1140	TTCAAACCCCTCACCTTTCATCTACTCCTGCCATGCATTGAAGGGTCAATGCATT	1199
Query	698	TGGGGGTGAGCTCTGGGTTTAGGGGCCCCCTCCATCCC 735	
MFL2	1200	TGGGG-TGAGCTCTGGGTTTAGGGGCCCCCTCCATCCC 1236	

16. leukocyte receptor cluster (LRC) member 8[Homo sapiens]

Score = 1408 bits (762), Expect = 0.0  
 Identities = 805/824 (97%), Gaps = 10/824 (1%)  
 Strand=Plus/Plus

Query	104	gtttttatTTTTGCCTCAgagggatgggattggggaggaggggatgggcagcggagggt	163
LENG8	2736	GTTTTTATTTTTGCCTCAGAGGGATGGGATTGGGGAGGAGGGGATGGGCAGCGGAGGGT	2795
Query	164	tgggggCATGGTCTGCAGGCTCATCTGTGTCCGCTTTCCTCCACTAATGCTGTCTCAG	223
LENG8	2796	TGGGGGCATGGTCTGCAGGCTCATCTGTGTCCGCTTTCCTCCACTAATGCTGTCTCAG	2855
Query	224	TGTTTTtctctctctctctctTTTCGAGCTTGCCTCCGGTACCCGACCCGGCGCCTGGCCCA	283
LENG8	2856	TGTTTTCTCTCTCTCTTTTCGAGCTTGCCTCCGGTACCCGACCCGGCGCCTGGCCCA	2915
Query	284	TCCCATGCGGGGGGGCCAGTGGAAAGAAGACAGGCCGTCCAGCCCGTGCCCGCCTGCGGC	343
LENG8	2916	TCCCATGCGGGGGGGCCAGTGGAAAGAAGACAGGCCGTCCAGCCCGTGCCCGCCTGCGGC	2975
Query	344	GGGGGCACCCAGCAAGCCCGCCACCGCCCGCTGCCTCACCTGCTTCGCCACAGACTCTT	403
LENG8	2976	GGGGGCACCCAGCAAGCCCGCCACCGCCCGCTGCCTCACCTGCTTCGCCACAGACTCTT	3035
Query	404	GTTCCAGCCCTTGGGGCCTCCGTGTTTGGGGTGGGGGAGCTGCTTAGAGACTGTGCC	463
LENG8	3036	GTTCCAGCCCTTGGGGCCTCCGTGTTTGGGGTGGGGGAGCTGCTTAGAGACTGTGCC	3095
Query	464	GTCTCGGCCCCCACCTGAAGTGCCAGCACCACCAGCACCAGATCCTCCGCCGCCACA	523
LENG8	3096	GTCTCGGCCCCCACCTGAAGTGCCAGCACCACCAGCACCAGATCCTCCGCCGCCACA	3155
Query	524	CCGCACTGAGGACACGCCGGCCGGCCCGCTCGTCTCAAGTTGTATAAAGTTGTCTCCGT	583
LENG8	3156	CCGCACTGAGGACACGCCGGCCGGCCCGCTCGTCTCAAGTTGTATAAAGTTGTCTCCGT	3215

Query	584	GTCCCCCTCCTCCCTCTGCCCCAGTGTTCCTCTGA	tttttttttccccctttccctccct	643	
LENG8	3216	GTCCCCCTCCTCCCTCTGCCCCAGTGTTCCTCTGA	TTTTTTTTTccccctttccctccct	3275	
Query	644	CCCTCTCCGCATTCTTCCCTTGGTTGAGCACAGG	AAAAACGGTTCCCCTCCCTCCCTGCC	703	
LENG8	3276	CCCTCTCCGCATTCTTCCCTTGGTTGAGCACAGG	AAAAACGGTTCCCCTCCCTCCCTGCC	3335	
Query	704	TTCATGGATCACCAGCTCACGTCATGTTGCCTT	TCTCTTTTCTTTGTGTGTGT-TT-AFTT	761	
LENG8	3336	TTCATGGATCACCAGCTCACGTCATGTTGCCTT	TCTCTTTTCTTTGTGTGTGTGTTTATTT	3395	
Query	762	TAA	tttttttttcttccctcctctccctttttcttttggg	ccctccctccctcttc	821
LENG8	3396	-AAGTTATTTTCTTCC-TCCTCTCCCTTTT-CT	TTTTTGGCCCTCCCTCCCTCCCTCTTC	3452	
Query	822	tGCCATGTA	ACTGGAGGATGTGCTATGA-TT	TGCAAACAGTTGGGTTGTCAGGGTGcttt	880
LENG8	3453	TGCCATGTA	ACTGGAGGATGTGCTATGAGTT	TGCAAACAGCTGGACTGTCAGGCTGCTTT	3512
Query	881	ttttt	CCAAAGGT-CTFCCCTCTGCCTFCCCTFCCCTCC-CTC	922	
LENG8	3513	TTTT-CCAGATGTTCTCTCC-TCTGCCTFCCCTFCCCTCCTCTC		3554	

17. small nuclear RNA activating complex, polypeptide 4, 190kDa [Homo sapiens]

Score = 1489 bits (806), Expect = 0.0  
 Identities = 815/819 (99%), Gaps = 2/819 (0%)  
 Strand=Plus/Plus

Query	78	CTGTCCCAGGAGGGCGAGGCGCCACACAGCAGTGG	CTGGGGGCCAGCGGGGGTGCCT	137
SNAPC4	216	CTGTCCCAGGAGGGCGAGGCGCCACACAGCAGTGG	CTGGGGGCCAGCGGGGGTGCCT	275
Query	138	GTGCCTTCTTGGGCAGCAGACTGCCCTATCAGCCCC	AGCCCTGTGCAGCCTGCGAGCT	197
SNAPC4	276	GTGCCTTCTTGGGCAGCAGACTGCCCTATCAGCCCC	AGCCCTGTGCAGCCTGCGAGCT	335
Query	198	CTGTCCGGTCTCCTACTCCACAAGAAGGCCCTGG	GAGCACAAGGCCACCTCCCTGGTGGTG	257
SNAPC4	336	CTGTCCGGTCTCCTACTCCACAAGAAGGCCCTGG	GAGCACAAGGCCACCTCCCTGGTGGTG	395
Query	258	GGGGGCGAGGCTGAGCGGCCGCGGAGCACTGCA	AAGCCTCACTGGGGCTGGTGGGGGG	317
SNAPC4	396	GGGGGCGAGGCTGAGCGGCCGCGGAGCACTGCA	AAGCCTCACTGGGGCTGGTGGGGGG	455
Query	318	CAGCTCCAGGACAACCCGGCCTACCTCCTGTTG	CGGGCGCGGTTTCTGGCAGCCTTACC	377
SNAPC4	456	CAGCTCCAGGACAACCCGGCCTACCTCCTGTTG	CGGGCGCGGTTTCTGGCAGCCTTACC	515
Query	378	CTCCCTGCGCTCCTGGCCACCCTGGCCCCCAAG	GCGTCCGCACCACCTCTCAGTACCT	437
SNAPC4	516	CTCCCTGCGCTCCTGGCCACCCTGGCCCCCAAG	GCGTCCGCACCACCTCTCAGTACCT	575
Query	438	TCGAGGGTGGGCTCTGAGAGTGAGGATGAAGAC	CCTCCTGAGCGAGCTGGAACCTGCAGAC	497
SNAPC4	576	TCGAGGGTGGGCTCTGAGAGTGAGGATGAAGAC	CCTCCTGAGCGAGCTGGAACCTGCAGAC	635
Query	498	AGGGACGGGCAGCCGGGCTGCACGACAGCCACAT	GCCCCATTACAGGGAGCCCCAGACTCT	557
SNAPC4	636	AGGGACGGGCAGCCGGGCTGCACGACAGCCACAT	GCCCCATTACAGGGAGCCCCAGACTCT	695
Query	558	GGTAAATGCTCTGCTTCTCTCTGCCTGGATACT	TCTAATGACTCTGACGACCTGGACGTG	617
SNAPC4	696	GGTAAATGCTCTGCTTCTCTCTGCCTGGATACT	TCTAATGACTCTGACGACCTGGACGTG	755
Query	618	CTCAGAACCCGGCATGCCAGGCACACCCGGAAG	CGGAGGCGGCTGGTGTGAGCAGCAGGA	677
SNAPC4	756	CTCAGAACCCGGCATGCCAGGCACACCCGGAAG	CGGAGGCGGCTGGTGTGAGCAGCAGGA	815
Query	678	CAAGCACTTTCTCAGAAGCCCGTGGCCCTGCTG	ACGAGAGACAGCCCACCCCCAGCCTGC	737
SNAPC4	816	CAAGCACTTTCTCAGAAGCCCGTGGCCCTGCTG	ACGAGAGACAGCCCACCCCCAGCCTGC	875

```

Query 738 AAGAGAGGCAGCCAGAGGCCAGGTATCTGCTGGCCGCTGACCGACCAGGTCCGCATTGAA 797
          |||
SNAPC4 876 AAGAGAGGCAGCCAGAGGCCAGGTATCTGCTGGCCGCTGACCGACCAGGTCCGCAGTGAA 935

Query 798 CAGAGGCGGGCCTGCCGACTGACTGTGTGGCATGGAGCATGGCTGTCCCCAAGGTGCAC 857
          |||
SNAPC4 936 CAGAGGCGGGCCTGCCGACTGACTGTGTGGCATGGAGCATGGCTGTCCCCAAG-TGCAC 994

Query 858 ACCTGAACACTTGGAGGAATAAAGTTCTTGTTTTAATT 896
          |||
SNAPC4 995 ACCTGAACACTTGGAGGAATAAAGTTCT-TTTTAATT 1032

```

18. septin 3 [Homo sapiens]

Score = 547 bits (296), Expect = 2e-152  
 Identities = 305/309 (98%), Gaps = 1/309 (0%)  
 Strand=Plus/Plus

```

Query 1 CCGTTTGCAGGGGCCGCTCGGCCGGGAAGCCCGCCCCGCTCAGCCTTGCAGCCCC 60
        |||
SEPT3 34 CCGTTTGCAGGGGCCGCTCGGCCGGGAAGCCCGCCCCGCTCAGCCTTGCAGCCCC 93

Query 61 GCGCCCGAAGCATCTCCCTGGAGGAACGGAGACAAAGGAGGATTCATGTCCAAAGGGCTC 120
        |||
SEPT3 94 GCGCCCGGAGCATCTCCCTGGAGGAACGGAGACAAAGGAGGATTCATGTCCAAAGGGCTC 153

Query 121 CCAGAGACCAGGACGGACGCGAGCCATGTCAGAGCTGGTGCCTGAGCCCAGGCCTAAGCCA 180
        |||
SEPT3 154 CCAGAGACCAGGACGGACGCGAGCCATGTCAGAGCTGGTGCCTGAGCCCAGGCCTAAGCCA 213

Query 181 GCGGTGCCCATGAAGCCCATGAGCATCAACTCCAACCTGCTGGGCTACATCGGCATCGAC 240
        |||
SEPT3 214 GCGGTGCCCATGAAGCCCATGAGCATCAACTCCAACCTGCTGGGCTACATCGGCATCGAC 273

Query 241 ACCATCATCGAGCAGATGCGCAAGAAGACCATGATTTACCGTTTCGACTTCAACATCAT 300
        |||
SEPT3 274 ACCATCATCGAGCAGATGCGCAAGAAGACCATGAAG-ACCGTTTCGACTTCAACATCAT 332

Query 301 GTCGTTGG 309
        |||
SEPT3 333 GTCGTTGG 341

```

19. Homo sapiens chromosome 6 clone RP11-20F2, complete sequence

Score = 957 bits (518), Expect = 0.0  
 Identities = 522/524 (99%), Gaps = 0/524 (0%)  
 Strand=Plus/Plus

```

Query 36 AGCTCCTGACCTCAGGTGATCTGCCTGCCTTAGCCTCCCAAAGTGCTGGGACTACAGGCG 95
          |||
Sbjct 130379 AGCTCCTGACCTCAGGTGATCTGCCTGCCTTAGCCTCCCAAAGTGCTGGGACTACAGGCG 130438

Query 96 TGAGCCACCACGCCTGGCTGCTCCACCATCACTTTATCCTGCCTGTATTCCCCAGTTCTC 155
          |||
Sbjct 130439 TGAGCCACCACGCCTGGCTGCTCCACCATCACTTTATCCTGCCTGTATTCCCCAGTTCTC 130498

Query 156 CTCCACCAGTGTTCAGATCCAGAGCTGTTTAGACATAGGGTATTCAGAAAGTTGTGGT 215
          |||
Sbjct 130499 CTCCACCAGTGTTCAGATCCAGAGCTGTTTAGACATAGGGTATTCAGAAAGTTGTGGT 130558

Query 216 TTTGTTATTTAAGATTTTAAAGTTCACCTCAATGTAAGGAAGTTAGTAAATGTCTGTGC 275
          |||
Sbjct 130559 TTTGTTATTTAAGATTTTAAAGTTCACCTCAATGTAAGGAAGTTAGTAAATGTCTGTGC 130618

Query 276 TATGAGGAAAAGTTGATTCTGAATGGGTTGGTTTAACTCCTACAGTCTTAGATAAGAACT 335
          |||
Sbjct 130619 TATGAGGAAAAGTTGATTCTGAATGGGTTGGTTTAACTCCTACAGTCTTAGATAAGAACT 130678

Query 336 TGTTAATCGTATAAACATACTGTTTCAAAGAAAAGCCAATTATAATTACTTTTATACAT 395
          |||
Sbjct 130679 TGTTAATCGTATAAACATACTGTTTCAAAGAAAAGCCAATTATAATTACTTTTATACAT 130738

Query 396 ACATAAATCTATGAGTAAGATGAAGAAGTCAAATCAAAGGATTTTATTTCTATTCTGAGA 455

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Sbjct 130739 |||
ACATAAATCTATGAGTAAGATGAAGAAGTCAAATCAAAGGATTTTATTTTCATTTCTGAGA 130798
Query 456 ATAAATTTGTATCTCACTTGCTGTTTCTCAATAATATTGTATAATTTTATTTCTGATTTT 515
Sbjct 130799 |||
ATAAATTTGTATCTCACTTGCTGTTTCTCAATAATATTGTATAATTTTATTTCTGATTTT 130858
Query 516 TAAAAATAAGCAAACCTGCCCTTGACCTCAAAAAAAAAAAAAAAAAA 559
Sbjct 130859 TAAAAATAAGCAAACCTGCCCTTGACCTCAAAGAGAAAAAAAAAAAAA 130902

```

20. regulator of G-protein signaling 11 [Homo sapiens]

Score = 1376 bits (745), Expect = 0.0  
Identities = 745/745 (100%), Gaps = 0/745 (0%)  
Strand=Plus/Plus

```

Query 122 CTCATGACCAAGAGTGCAGATTTCCATAAGCGGGAGATCGAGTACTTCAGGAAAGCGCTG 181
RGP 666 CTCATGACCAAGAGTGCAGATTTCCATAAGCGGGAGATCGAGTACTTCAGGAAAGCGCTG 725
Query 182 GGCAGACCCGAGTGAAGTCTCCGTCTGCCTTGAGGCGTACCTGAGTTTCTGCGGCCAG 241
RGP 726 GGCAGACCCGAGTGAAGTCTCCGTCTGCCTTGAGGCGTACCTGAGTTTCTGCGGCCAG 785
Query 242 CGTGGACCCACGATCCCCTCGTGTGCGGGTGCCTGCCAGCAATCCCTGGATCTCAGAC 301
RGP 786 CGTGGACCCACGATCCCCTCGTGTGCGGGTGCCTGCCAGCAATCCCTGGATCTCAGAC 845
Query 302 AATGACGCCTACTGGGTCATGAATGCCCCACGGTGGCTGCCCCACGAAGCTCCGTGTG 361
RGP 846 AATGACGCCTACTGGGTCATGAATGCCCCACGGTGGCTGCCCCACGAAGCTCCGTGTG 905
Query 362 GAGAGATGGGGCTTTCAGTTCCGGGAGCTCCTGGAGACCCCGTGGGGCGGGCCACTTC 421
RGP 906 GAGAGATGGGGCTTTCAGTTCCGGGAGCTCCTGGAGACCCCGTGGGGCGGGCCACTTC 965
Query 422 ATGGACTTTCTGGGAAAGGAGTTTCAGTGGAGAAAACCTCAGTTCTGGGAGGCATGTGAG 481
RGP 966 ATGGACTTTCTGGGAAAGGAGTTTCAGTGGAGAAAACCTCAGTTCTGGGAGGCATGTGAG 1025
Query 482 GAGCTTCGATATGGAGCGCAGGCCAGGTCCCACCCCTGGTGGATGCCGTGTACGAGCAG 541
RGP 1026 GAGCTTCGATATGGAGCGCAGGCCAGGTCCCACCCCTGGTGGATGCCGTGTACGAGCAG 1085
Query 542 TTCCTGGCCCCGGAGCTGCCACTGGGTCAACATCGACAGCCGACCATGGAGCAGACC 601
RGP 1086 TTCCTGGCCCCGGAGCTGCCACTGGGTCAACATCGACAGCCGACCATGGAGCAGACC 1145
Query 602 CTGGAGGGGCTGCGCCAGCCCCACCGCTATGTCCTGGATGACGCCAGCTGCACATATAC 661
RGP 1146 CTGGAGGGGCTGCGCCAGCCCCACCGCTATGTCCTGGATGACGCCAGCTGCACATATAC 1205
Query 662 ATGCTCATGAAGAAGGACTCCTACCCAAGGTTCTGAAAGTCTGACATGTACAAGGCCCTC 721
RGP 1206 ATGCTCATGAAGAAGGACTCCTACCCAAGGTTCTGAAAGTCTGACATGTACAAGGCCCTC 1265
Query 722 CTGGCAGAGGCTGGGATCCCGCTGGAGATGAAGAGACGCGTGTCCCGTTTACGTGGAGG 781
RGP 1266 CTGGCAGAGGCTGGGATCCCGCTGGAGATGAAGAGACGCGTGTCCCGTTTACGTGGAGG 1325
Query 782 CCACGGCACTCGAGCCCCAGCCCTGCACTCCTTCCCACCCCTGTGGAGCCCACAGCGGCT 841
RGP 1326 CCACGGCACTCGAGCCCCAGCCCTGCACTCCTTCCCACCCCTGTGGAGCCCACAGCGGCT 1385
Query 842 TGTGGCCCTGGGGGTGGAGATGGGG 866
RGP 1386 TGTGGCCCTGGGGGTGGAGATGGGG 1410

```



21. tubulin, beta 2A [Homo sapiens]

Score = 1417 bits (767), Expect = 0.0  
 Identities = 776/780 (99%), Gaps = 2/780 (0%)  
 Strand=Plus/Plus

```

Query 79   CCCGCCGGTCCACGCCGCGCACCGCTCCGAGGGCCAGCGCCACCCGCTCCGCAGCCGGCA 138
          |||
BTub 25   CCCGCCGGTCCACGCCGCGCACCGCTCCGAGGGCCAGCGCCACCCGCTCCGCAGCCGGCA 84

Query 139  CCAAGCGCGAGATCGTGCACATCCAGGCGGGCCAGTGCGGCAACCAGATCGGCGCCAAGT 198
          |||
BTub 85   CCATGCGCGAGATCGTGCACATCCAGGCGGGCCAGTGCGGCAACCAGATCGGCGCCAAGT 144

Query 199  TTTGGGAGGTTCATCAGCGATGAGCATGGGATCGACCCACAGGCAGTTACCATGGAGACA 258
          |||
BTub 145  TTTGGGAGGTTCATCAGCGATGAGCATGGGATCGACCCACAGGCAGTTACCATGGAGACA 204

Query 259  GTGACTTGCAGCTGGAGAGAATCAACGTGTACTACAATGAGGCTGCTGGTAACAAATATG 318
          |||
BTub 205  GTGACTTGCAGCTGGAGAGAATCAACGTGTACTACAATGAGGCTGCTGGTAACAAATATG 264

Query 319  TACCTCGGGCCATCCTGGTGGATCTGGAGCCTGGCACCATGGACTCTGTCAGGTCTGGAC 378
          |||
BTub 265  TACCTCGGGCCATCCTGGTGGATCTGGAGCCTGGCACCATGGACTCTGTCAGGTCTGGAC 324

Query 379  CCTTCGGCCAGATCTTCAGACCAGACAACCTTCGTGTTTCGGCCAGAGTGGAGCCGGGAATA 438
          |||
BTub 325  CCTTCGGCCAGATCTTCAGACCAGACAACCTTCGTGTTTCGGCCAGAGTGGAGCCGGGAATA 384

Query 439  ACTGGGCCAAGGGCCACTACACAGAGGGAGCCGAGCTGGTTCGACTCGGTCCTGGATGTGG 498
          |||
BTub 385  ACTGGGCCAAGGGCCACTACACAGAGGGAGCCGAGCTGGTTCGACTCGGTCCTGGATGTGG 444

Query 499  TGAGGAAGGAGTCAGAGAGCTGTGACTGTCTCCAGGGCTTCAGCTGACCCACTCTCTGG 558
          |||
BTub 445  TGAGGAAGGAGTCAGAGAGCTGTGACTGTCTCCAGGGCTTCAGCTGACCCACTCTCTGG 504

Query 559  GGGGCGGCACGGGGTCCGGGATGGGCACCCTGCTCATCAGCAAGATCCGGGAAGAGTACC 618
          |||
BTub 505  GGGGCGGCACGGGGTCCGGGATGGGCACCCTGCTCATCAGCAAGATCCGGGAAGAGTACC 564

Query 619  CAGACCGCATCATGAACACCTTCAGCGTCATGCCCTCACCAAGGTGTCAGACACGGTGG 678
          |||
BTub 565  CAGACCGCATCATGAACACCTTCAGCGTCATGCCCTCACCAAGGTGTCAGACACGGTGG 624

Query 679  TGGAGCCCTACAACGCCACCCTCTCGGTCCACCAGCTGGTGGAAAACACAGATGAAACCT 738
          |||
BTub 625  TGGAGCCCTACAACGCCACCCTCTCGGTCCACCAGCTGGTGGAAAACACAGATGAAACCT 684

Query 739  ACTCCATTGATAACGAGGCCCTGTATGACATCTGCTTCCGCACCCTGAAGCTGACCACCC 798
          |||
BTub 685  ACTCCATTGATAACGAGGCCCTGTATGACATCTGCTTCCGCACCCTGAAGCTGACCACCC 744

Query 799  CCCACCTACGGGGACCTCAACCACCTGGTGTGGC-ACCATGAGCGGGGTACCCACCTGC 857
          |||
BTub 745  CC-ACCTACGGGGACCTCAACCACCTGGTGTGGCACCATGAGCGGGGTACCCACCTGC 803
  
```

22. rho-related BTB domain containing 3

Score = 1219 bits (660), Expect = 0.0  
 Identities = 671/676 (99%), Gaps = 2/676 (0%)  
 Strand=Plus/Minus

```

Query 37   GCAGAGGTAGCTTTGTCTTCTCCTCTTTGTGTCTTCAATTCCAAATGCAATGATTGGCA 96
          |||
RhoGTP   GCAGAGGTAGCTTTGTCTTCTCCTCTTTGTGTCTTCAATTCCAAATGCAATGATTGGCA
43671340
  
```

Query 97	CAAGTTGATTCTCGGTAATAATCTGTTGAAGTGATCCAGGCTGAACTGAAGGCTGGCAGT	156
RhoGTP 43671339 43671280	 CATGCTGATTCTCGGTAATAATCTGTTGAAGTGATCCAGGCTGAACTGAAGGCTGGCAGT	
Query 157	GTGTATTCGTGGTTTTCTAAGGTGGTGGAAAGTAGAGAGCCCTGGCCACTCACGGCCCTAT	216
RhoGTP 43671279 43671220	 GTGTATTCGTGGTTTTCTAAGGTGGTGGAAAGTAGAGAGCCCTGGCCACTCACGGCCCTAT	
Query 217	CCAGCGCTGCCTTTGTGTGGTGCAGCAGTAGCTAGAGAGCCTAGCAAGCTTTTCTAGAG	276
RhoGTP 43671219 43671160	 CCAGCGCTGCCTTTGTGTGGTGCAGCAGTAGCTAGAGAGCCTAGCAAGCTTTTCTAGAG	
Query 277	AGTTAATTCAGTTCGGTGAGTTCAGTGAGCAGTTTGGGCTGAGAGAGCCCTCAAAATTCA	336
RhoGTP 43671159 43671100	 AGTTAATTCAGTTCGGTGAGTTCAGTGAGCAGTATGGGCTGAGAGAGCCCTCAAAATTCA	
Query 337	ACGTAGCACTGGCTCTCCCAGCAGCAGGCATTGTTTCAGGTGAGACAGAATGGTTGCCTGA	396
RhoGTP 43671099 43671040	 ACGTAGCACTGGCTCTCCCAGCAGCAGGCATTGTTTCAGGTGAGACAGAATGGTTGCCTGA	
Query 397	GGAAGTAGTGTGCTGGGGAGACTGCAGGGATTAAGGTCATCCTCTTCCAGATTTTCATTA	456
RhoGTP 43671039 43670980	 GGAAGTAGTGTGCTGGGGAGACTGCAGGGATTAAGGTCATCCTCTTCCAGATTTTCATTA	
Query 457	AGTCTGAAATGCTCTCCTTGGCTCCACAAGAGATCCTCTTGCTATTCTACTGTGGTGTA	516
RhoGTP 43670979 43670920	 AGTCTGAAATGCTCTCCTTGGCTCCACAAGAGATCCTCTTGCTATTCTACTGTGGTGTA	
Query 517	CGGACAGCTTATGGTTTACTTCTTACAGTTTCTGTTTCAGATAAAGGGGAAGCATGGCCA	576
RhoGTP 43670919 43670860	 CGGACAGCTTATGGTTTACTTCTTACAGTTTCTGTTTCAGATAAAGGGGAAGCATGGCCA	
Query 577	AGATGGTTCGTGAGTCAGGCCAGAGGGTAATGGGGTTGTTTCTTGGGAGGCACATG	636
RhoGTP 43670859 43670800	 AGATGGTTCGTGAGTCAGGCCAGAGGGTAATGGGGTTGTTTCTTGGGAGGCACATG	
Query 637	GGCACGGGTAGTATTATTAA-ATTTCAAGCCAATTGGAAAAGGATTGATGTTTCATTATA	695
RhoGTP 43670799 43670741	 GGCACGGGTAGTATTATTAAATATTTCAAGCCAATTGGAAA-GGATTGATGTTTCATTATA	
Query 696	AAAATATGAAGCAAAA 711	
RhoGTP 43670740	 AAAATATGAAGCAAAA 43670725	

## **APPENDIX B: CLONTECH TECHNICAL SUPPORT**

From: Duygu Esen [mailto:duygu.esen@yahoo.com]  
Sent: jeudi 24 avril 2008 13:07  
To: CLONTECH TECH SUPPORT  
Subject: Re: non-coding sequences after screening of pretransformed human fetal MM library

Dear Klaus,

My bait is a microtubule severing protein, katanin. it is a cytosolic protein so i can't understand why it could interact with non-coding sequences. I blasted my results first to human genome, where they are not matching any coding sequence of a gene, then i investigated for their ORF. but nothing present as i mentioned.

The other results (the coding ones) seem to be relevant by the way...  
Regards

Duygu

From: CLONTECH TECH SUPPORT <tech@clontech-europe.com>  
To: Duygu Esen <duygu.esen@yahoo.com>  
Cc: "tez@tez.com.tr" <tez@tez.com.tr>  
Sent: Monday, April 28, 2008 5:11:30 PM  
Subject: RE: non-coding sequences after screening of pretransformed human fetal MM library

Dear Duygu

So, I'm wondering where do these clones map to human genomic DNA. Are these intronic sequences? I suspect they might stem from incompletely spliced mRNAs.

Unfortunately it is not always predictable which clones a yeast two-hybrid screen will pull out. 60 clones is a perfectly reasonable number of hits though. If you found several interaction partners that activate all reporters, that are not self-activating and "make sense" after analyzing the protein sequences, then I would say that this was a successful screen!

You are certainly aware that candidate interactions from a yeast two-hybrid screen need to be confirmed with other methods. At this point I would move on to an IP assay as soon as possible, e.g. with the Matchmaker Chemiluminescent Co-IP System or pCMV-Myc & pCMV-HA Vector Set.

Hope this helps  
Best regards  
Klaus

From: Duygu Esen [mailto:duygu.esen@yahoo.com]  
Sent: mercredi 18 juin 2008 14:37  
To: CLONTECH TECH SUPPORT  
Subject: construction of pACT2 library

In our lab, pretransformed cDNA library is being used. Now we are in the step of interchanging vector from yeast expression (pACT2) to mammalian expression (c-myc). While trying to insert the cDNA fragments in frame with c-myc epitope, one question came to my mind.

mRNA pool is primed with oligo dt-XhoI and after cDNA amplified EcoRI adaptor sequence is ligated (Cat no 638831, certificate of analysis). Adapter sequence is in frame with pACT2, after adaptor sequence cDNA should start either with ATG or a sequence which is in frame with ATG. How can you achieve to obtain cDNA to be in frame with pACT2.

Regards,

Duygu Esen

Research Assistant  
Molecular Biology Genetics and  
Biotechnology Research Center  
Istanbul Technical University

From: CLONTECH TECH SUPPORT <tech@clontech-europe.com>  
To: Duygu Esen <duygu.esen@yahoo.com>  
Sent: Wednesday, June 18, 2008 4:00:25 PM  
Subject: RE: construction of pACT2 library

Dear Duygu

Thank you very much for contacting Clontech.

The Matchmaker libraries are cloned directionally but randomly in all 3 reading frames, so that 1/3 of all clones will be in-frame with the Gal4 AD.

Hope this answers your question.

Best regards

Klaus

**RESUME:**

Duygu Esen was born on September 11 1983, in Adana. She went Elementary School in Seyhan, Adana. She graduated from ATO Anatolian High School in Adana. She attended to Middle East Technical University, Molecular Biology and Genetics Department at 2001 and earned her Bachelor of Science degree from the department at 2005. She pursued her academic studies in Istanbul Technical University, Molecular Biology-Genetics and Biotechnology master program at 2005. She is still carrying her studies in this department.