Universidade de Aveiro Departamento de Biologia
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CANDIDA ZITA DOS SANTOS COTRIM

Identificação de proteínas neuronais que interagem com PP1γ2

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Cândida Zita dos Santos Cotrim, realizada sob a orientação científica do Dr. Edgar da Cruz e Silva, Professor Associado da Universidade de Aveiro

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agradecimentos

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palavras-chave

Interacção proteína – proteína, Proteína fosfatase 1 (PP1), técnica dois-hibrido em leveduras, PINK1, BRI2 e BRI3.

resumo

A proteína fosfatase 1 (PP1) está envolvida em múltiplos processos de grande relevância fisiológica (por ex. aprendizagem, memória e neurotransmissão) e patológica (envelhecimento, doenças neurodegenerativas). No entanto, restam ainda por estabelecer importantes interacções de relevância fisiológica, bem como a localização intracelular onde estas interacções ocorrem. Esta complexidade é originada pela existência de três isoformas da PP1, organizadas tanto espacialmente como temporalmente e que podem alterar a sua localização intracelular de forma dinâmica.

Este projecto focalizou a identificação de proteínas expressas em cérebro humano que interagem com PP1γ2, através da técnica Dois Híbrido em leveduras. Através desta técnica foram obtidos 317 clones positivos, permitindo a identificação de 298 proteínas que ligam à PP1γ2 e 19 proteínas que ligam PP1γ2end, entre as quais algumas proteínas já conhecidas por interagirem com a PP1, outras nunca antes associadas com a PP1 e várias proteínas não caracterizadas. Foi feito um estudo mais detalhado para três proteínas das mais abundantes, PINK1, BRI2 e BRI3. A ligação destas proteínas com PP1 foi analisada através de várias técnicas e a sua localização subcelular e co-localização com a PP1γ e APP foi estudada por imunocitoquímica. Os resultados aqui apresentados corroboram a localização subcelular destas proteínas e a ligação já descrita entre a APP e BRI2. Permitem acrescentar que PINK1, BRI2 e BRI3 interagem com PP1γ e que não só a proteína BRI2 mas também a BRI3 interagem com APP. Estudos futuros serão necessários para compreender o papel destas interacções no sistema neuronal. Estes resultados também validam o uso do sistema YTH como um meio de estudo para melhor compreender as funções da PP1 e sua regulação em diferentes eventos celulares.

keywords

Protein-protein interaction, Protein phosphatase 1 (PP1), Yeast Two-Hybrid system, PINK1, BRI2, BRI3

abstract

The ubiquitous protein phosphatase 1 (PP1) is involved in multiple processes of great physiological (e.g. learning, memory and neurotransmitter signaling) and pathological (aging, Alzheimer's disease and other neurodegenerative conditions) relevance. However, many physiologically important interactions remain to be established, as well as the exact intracellular locations where these interactions take place. This complexity is provided by the existence of three PP1 isoforms that are organized both spatially and temporally, and can change their intracellular localization dynamically.

This project focused on the identification of the PP1γ2 interacting proteins expressed in human brain using the Yeast Two-Hybrid system. With this technique 317 positive clones were recovered allowing the identification of 298 proteins that bind PP1γ2 and 17 proteins that bind PP1γ2end, among those are some previously known PP1 interacting proteins, other proteins never associated with PP1 before and several uncharacterized proteins. A more detailed study was carried out for three of the most abundant clones in the screen, PINK1, BRI2 e BRI3. The binding to PP1 was analyzed by several techniques and its subcellular localization and co-localization with PP1γ and APP studied by immunocytochemistry. These results corroborate the subcellular localization of this proteins and the interaction of BRI2 with APP already described. These results allow concluding that PINK1, BRI2 and BRI3 interact with PP1 and that BRI2 and BRI3 interact with APP. Further studies are needed to understand the function of the interaction of PP1 and PINK1, BRI2 and BRI3 in the neuronal system. Moreover, our results support the use of the YTH system as a means to study and understand PP1 function and regulation in different cellular events.

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ABBREVIATIONS

Abbreviations

aa	Amino acid (s)
ActD	Activation Domain
AD	Alzheimer's disease
Ade	Adenine
Amp	Ampicillin
APP	Alzheimer's amyloid precursor protein
APS	Ammonium persulfate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
BLAST	Basic Local Alignment Search Tool
cAMP	Cyclic AMP (Adenosine 3',5'-monophosphate)
cDNA	Complementary deoxynucleic acid
CDS	Protein coding sequence
Chr	Chromosome
Cys	Cystein
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxynucleic acid
DNA-BD	DNA-Binding domain
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded deoxynucleic acid
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	Ethylenediaminotetraacetic acid
EST	Expressed sequence tag
GAL4	Gal4 transcription factor
GAL4-AD	GAL4-Activation domain
GAL4-BD	GAL4-Binding domain
His	Histidine

HtrA2	HtrA serine peptidase 2
LB medium	Luria-Bertani Medium (Miller)
Leu	Leucine
LiAc	Lithium acetate
Nt	Nucleotide
OD	Optical density
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PKA	Protein kinase A
PMSF	Phenyl methylsulfoxide
PP1	Protein phosphatase 1
QDO	Quadruple dropout
RNA	Ribonucleic acid
RT	Room Temperature
RT-PCR	Reverse transcriptase - polymerase chain reaction
SD	Supplement dropout medium
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Ser	Serine
STET	Sucrose/Triton/EDTA/Tris (buffer)
TBS	Tris-buffered saline solution
TDO	Triple dropout
TEMED	N,N,N’,N'-tetramethyletylendiamine
Thr	Threonine
Tris	Tris (hydroxymethyl)-aminoethane chloride
Trp	Tryptophan
Tyr	Tyrosine
UAS	Upstream activating sequence
UTR	mRNA Untranslated Region
UV	Ultraviolet
X-α-gal	5-bromo-4-chloro-3-indolyl-alpha-D-galactopyranoside

YPD	Yeast extract, Peptone and Dextrose medium for *S. cerevisiae*
YPDA	YPD with adenine
YTH	Yeast Two-hybrid system

I. INTRODUCTION

I.1. YEAST TWO-HYBRID SYSTEM

Proteins perform most of the work of living cells and this versatile class of macromolecule is involved in virtually every cellular process. Understanding how cells function requires identifying how proteins function and interact (Criekinge and Beyaert., 1999). One of the techniques used to study protein-protein interactions is the Yeast Two-Hybrid (YTH) system. This system take advantage of the fact that for transcribed DNA is requires a protein called a transcriptional activator (TA), as illustrated in the Figure 1. This protein contain at least two distinct functional domains that have specific functions: a DNA-binding domain (BD) that is capable of binding to DNA, and an activation domain (AD) that is capable of activating transcription of the DNA (Phizicky and Fields , 1995; Sobhanifar *et al*., 2003). Because can be separated the BD and the AD two fusion proteins "X" and "Y" of interest can be created. In fact, this is the principle of the Yeast Two-Hybrid system.

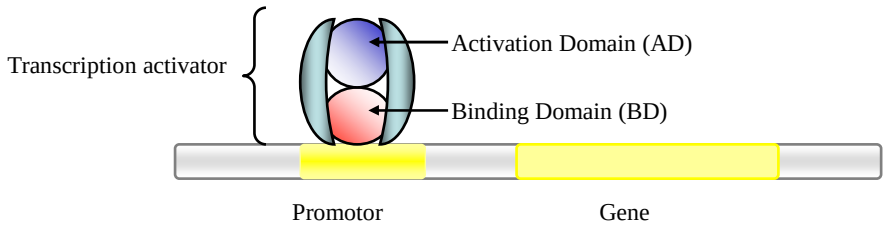

Figure 1: Diagrammatic representation of transcription. Transcription requires both the DNA-binding domain (BD) and the activation domain (AD) of a transcriptional activator (TA). The transcriptional activator binds to the promotor (the region upstream from the gene) and activates transcription via its activation domain.

In 1989, Field and Song (Fields and Song, 1989) used the transcriptional activator GAL4 protein, which is required for the expression of genes encoding enzymes of galactose metabolism in yeast *Saccharomyces cerevisiae*, to create two hybrid proteins containing parts of GAL4: the GAL4 DNA-binding domain fused to a protein 'X' and the GAL4 activating region fused to a protein 'Y'. If protein "X" and protein "Y" formed X-Y complex and thus reconstitute the GAL4 protein, transcription of a gene regulated by the promoter UASG occurs. These initial experiments confirmed the use of this method to

study protein-protein interaction. Once the formation of the protein complex occurs (Figure 2), it allows the selection of the positive clones by the expression of reporter genes. These reporter genes inserted in the yeast DNA immediately after the GAL4 promoter. The most commonly used reporter genes with the GAL4 system included reporter gene for color selection (LacZ gene, an *E. coli* gene whose transcription causes cell to turn blue) and auxotrophic genes for growth selection, such as histidine (*HIS3)*, leucine (*LEU2)*, tryptophan (TRP1) and/or adenine (ADE2).

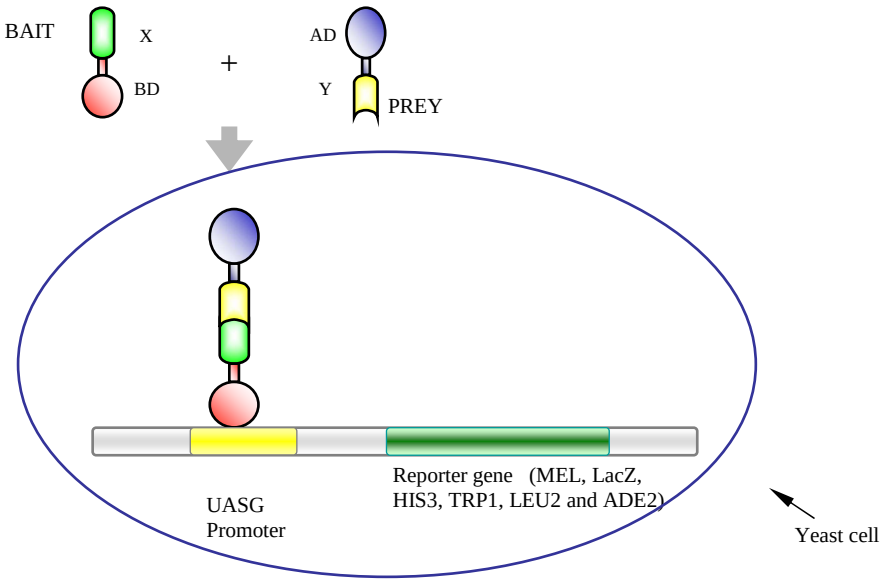

Figure 2: Yeast Two-Hybrid system. Protein "X" is fused to GAL4-BD (bait) which binds to specific DNA sequences (UASG) and protein "Y" is fused with GAL4-AD (prey). If proteins "X" and "Y" interact, then the transcriptional activator will be formed and the reporter genes transcribed.

This technique extensively used to screen libraries of activation domain hybrids to identify proteins that bind to a protein of interest. These screens result in the immediate availability of the cloned gene for any new protein identified (Field and Song., 1989; Phizicky and Fields., 1995; Chien *et al*., 1991; Criekinge and Beyaert., 1999). The Two-Hybrid screen sometimes is referred to as a functional screen, since if one of the interacting proteins has a known function in a known signaling pathway, if will give a functional hint of the other interacting protein's function. Nevertheless, the outcome of a screening needs

to validation by other techniques, such as co-immunoprecipitation and immunocytochemistry analysis (Criekinge and Beyaert., 1999).

I.1.1. Limitations of the YTH System

The YTH makes use of yeast, *S. cerevisiae*, as a host, which implies that the proteins must be able to fold correctly and stably inside the yeast cells. The use of yeast can be an advantage, since yeasts are closer to higher eukaryotic than bacteria. A major disadvantage of assaying protein-protein interaction in yeast is that some interactions depend upon posttranslational modifications that do not, or inappropriately, occur in yeast. Such modifications are frequent and include the formation of disulfide bridges, glycosylation and, most commonly, phosphorylation. Other proteins might proteolyses essential yeast proteins, be capable of activating reporter gene transcription, giving rise to false positives, or even might become toxic upon expression (Field and Song., 1989; Phizicky and Fields., 1995; Chien *et al*., 1991; Criekinge and Beyaert., 1999). It is possible that both proteins, although able to interact, could be expressed in different cell types, be localized in distinct subcellular compartments or be expressed at different times (e.g. at different time points in the cell cycle). Therefore, once identified two interacting partners, the biological relevance of this interaction needs to be confirmed. Other cause which may also lead to false results, are the inability of fusion proteins to migrate to the nucleus or if one of the transcription factor domains occludes the site of interaction (Field and Song., 1989; Phizicky and Fields, 1995; Chien *et al*., 1991; Criekinge and Beyaert, 1999; Nguyen and Goodrich, 2006). Nevertheless, many signaling cascades have been resolved in molecular detail through this method, in considerable number and substantial speed, proving that regardless of the need for confirmation of the protein-protein interaction, the YTH system screen is an advantageous method for protein studies.

I.2. PROTEIN PHOSPHATASES (PPs)

The understanding of protein phosphorylation started when Cori and Green (1943) first described the purification of glycogen phosphorylase. A decade later PP1 (protein phosphatase 1) was identified as phosphorylase phosphatase, which together with the discovery of phosphorylase kinase by Fisher and Krebs (1955), led to the identification of protein phosphorylation/dephosphorylation as the major ubiquitous regulatory mechanism of intracellular protein interaction in eukaryotes (Fisher, *et al.*, 1995). Protein kinases transfer a phosphate from ATP to a specific protein, typically at serine, threonine, or tyrosine residues. Phosphatases remove the phosphoryl group and restore the protein to its original dephosphorylated state (Figure 3). Therefore, it can be consider the phosphorylation-dephosphorylation cycle as a molecular "on-off" switch. In eukaryotic cells up to 30% of proteins, undergo reversible phosphorylation catalyzed by protein kinases and phosphatases, which modulate their biological activity.

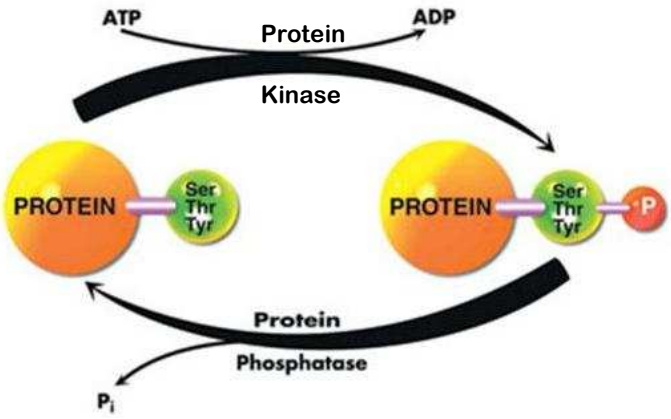

Figure 3: Protein phosphorylation. The balance between protein kinase and phosphatase activities provides the eukaryotic cell with a major control mechanism through the switch of proteins between phosphorylated and dephosphorylated states (adapted from http://www.emdbiosciences.com/html/cbc/protein_phosphatase_spotlight.htm).

I.2.1. Protein Phosphatase classification

Protein phosphatases that catalyze the dephosphorylation of proteins have been classified into three distinct categories: tyrosine-specific, dual-specificity phosphatases, and serine/threonine (Ser/Thr) specific. Protein tyrosine phosphatases (PTPs) remove phosphate groups from phosphorylated tyrosine residues of proteins. PTPs display diverse structural features and play important roles in the regulation of cell proliferation, differentiation, cell adhesion and motility, and cytoskeletal function. They are either transmembrane receptor-like PTPs or cytosolic enzymes. Another category of protein phosphatases is the dual specificity phosphatases (DSPs), which play a key role in the dephosphorylation of MAP kinases. Hence, they are also termed as MAP kinase phosphatases (MKPs). Based on predicted structures, MKPs has been divided into three subgroups: group I (DSP1, DSP2, DSP4 and DSP5); group II (DSP6, DSP7, DSP9 and DSP10); and group III (DSP8 and DSP16). The third category is the Ser/Thr phosphatases, can be divided based on structural homology and biochemical analysis into two families designated PPP and PPM. While PPM comprises Mg^{2+}-dependent phosphatases like PP2C, PP1 together with PP2A, PP4, PP6, PP2B, PP5, and PP7 represent the PPP family. However, PP1 and PP2A holoenzymes represent the major protein phosphatases both in number and in importance that dephosphorylate serine-threonine residues (Ingebritsen and Cohen., 1983). Shown in the Figure 4 are the schematic representations of the PPs classification.

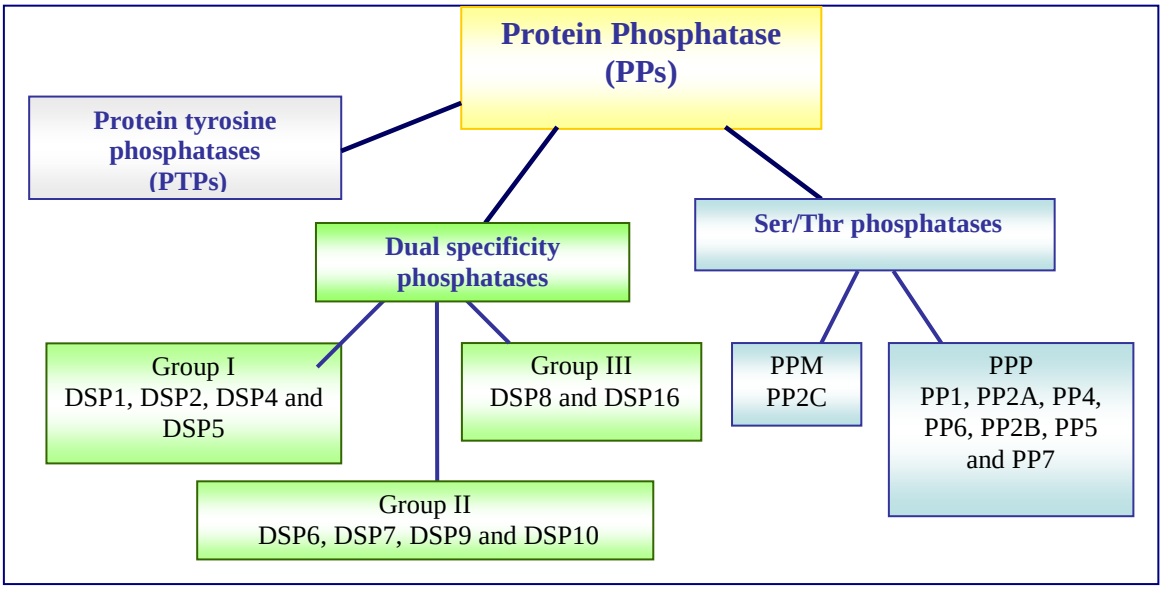

Figure 4: Protein Phosphatase classification.

I.2.2. Protein Phosphatase 1 (PP1)

Protein phosphatase 1 (PP1) is a major protein serine/threonine phosphatase, which is known to regulate an enormous variety of cellular functions. Such as RNA splicing, protein synthesis and recovery from stress, glycogen metabolism, HIV-1 viral transcription, muscle contractility, and is an important regulator of cardiac function it promotes apoptosis when cells are damaged beyond repair, and promotes the exit from mitosis and maintains cells in the G1 or G2 phases of the cell cycle. Beyond this, PP1 is also involved in down regulation of ion pumps and transporters in various tissues (Wakula *et al*., 2003; Meiselbach *et al*., 2006).

In the brain, PP1 is involved in the regulation of proteins such as neurotransmitter receptors and voltage-gated ion channels, a class of transmembrane ion channels that are common in many types of cells but are especially critical in neurons. They are involved in the regulation of neurons and information processing (Allen *et al*., 1997; Strack *et al*., 1999; Croci *et al*., 2003). This phosphatase known to be involved in long-term potentiation (LTP) and long-term depression (LTD), thereby influencing learning and memory. LTP is the persistent increase in synaptic strength following high-frequency stimulation of a chemical synapse. This process is a synaptic mechanism involved in learning and memory. LTD is the opposing process. That is, the weakening of a neuronal synapse that leads to a

decrease in synaptic efficacy, and is important for the clearing of old memory traces, allowing neural networks to store information more effectively. One form of LTD that has been observed in the hippocampus requires activation of postsynaptic PP1. Furthermore, mouse studies suggested that PP1 functions as a suppressor of learning and memory (Ceulemans and Bollen, 2004). Clearly, PP1 is involve in many diseases and dysfunctional states which are associated with the abnormal phosphorylation of key proteins, as shown in Figure 5.

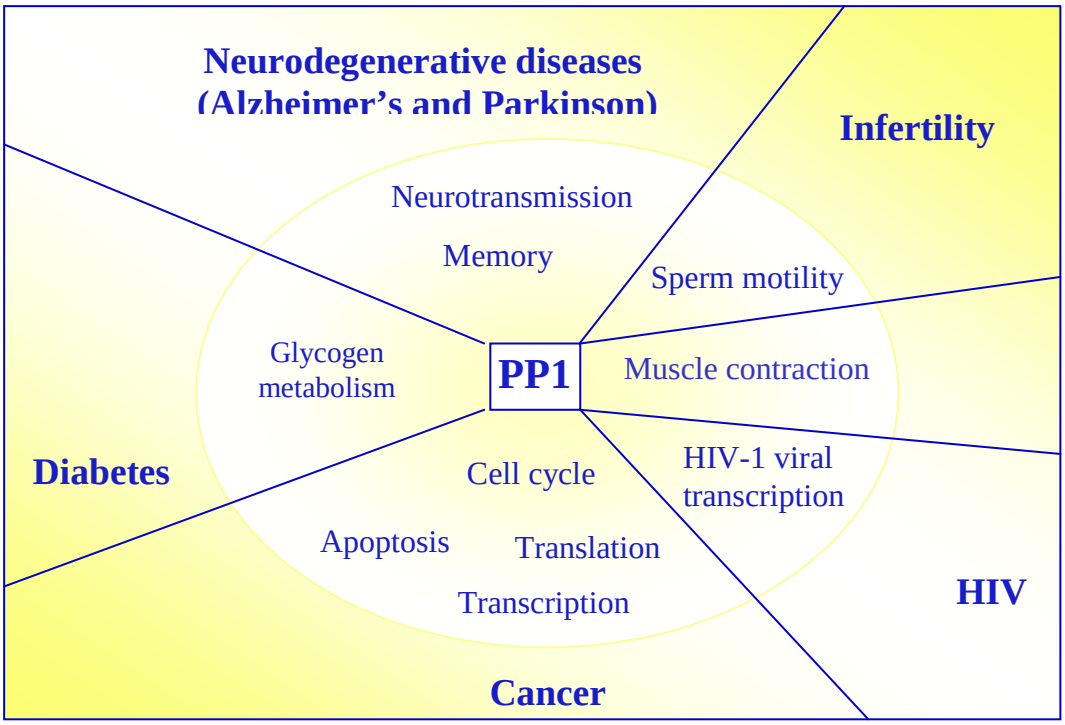

Figure 5: PP1function in relation to various disease states.

Hence, PP1 may a target for the development of therapeutic strategies but because it is involved in many different processes, it may be counterproductive to target the catalytic subunit directly. A better strategy may be to target the specific PP1 complex responsible for a given mechanism. However, in order to achieve this goal must be identified the regulatory proteins present in these complexes and their function elucidated (Fardilha *et al*., 2004).

I.2.2.1. PP1 Binding Motif

The association of the PP1 catalytic subunit with different binding partners regulates PP1 enzymatic activity, substrate specificity, and subcellular localization, forming different PP1 holoenzymes, which are regulated by hormones, growth factors, and metabolites at the molecular level (Meiselbach, *et al*., 2006). These various functions and subcellular localizations of the PP1 holoenzymes or complexes is accomplished the interaction between its catalytic subunit (PP1c) with more them fifty different established or putative regulatory subunits. In fact, it is thought that PP1 does not exist in cells free from such regulatory subunits (Enz *et al*., 2003; Cohen *et al*., 2002; Fardilha *et al*., 2004).

The majority of these achieved interactions by a short conserved binding motif that binds to the hydrophobic groove of the PP1 catalytic subunit, functioning as an anchor (Meiselbach *et al*., 2006). The PP1 binding motif consists of the consensus amino acid sequence (R/K) - $x_{0,1}$ - (V/I) - x_1 - (F/W) motif, where $x_{0,1}$ may be absent (0) or any residue (1) and x_1 is any amino acid except proline. Often is reduced to RVxF or preceded by further basic residues. It also may be found, in some PP1 binding proteins more than one binding motif. Recently two new consensus sequences for PP1 binding were proposed: [RK] - $x_{(0,1)}$ - [V/I/L] – x - [F/W] and F-x-x-[RK]-x-[RK] (Wakula *et al.*, 2003; Meiselbach *et al*., 2006; Wakula, *et al.*, 2006). The Figure 6 presents the matrix with the possible PP1c binding motifs.

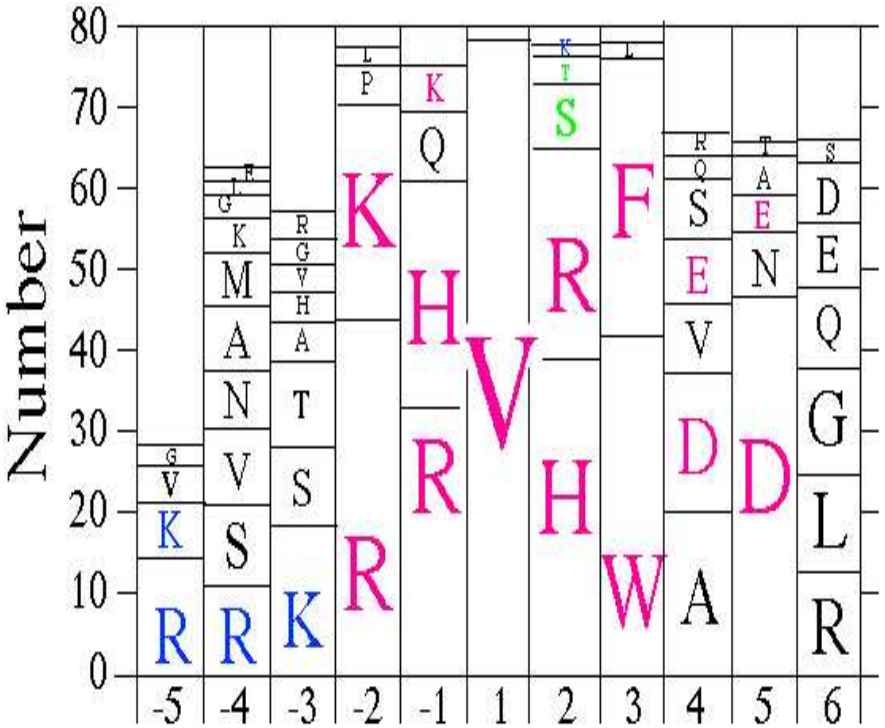

Figure 6: The RVXF PP1 binding motif. Incidence of amino acids among the PP1 binding peptides (http://www.nymc.edu/people/indviewg.asp?which=ernest_lee).

It has been demonstrated that mutations of the hydrophobic V/I/L and/or aromatic F/W/Y residues in this motif is sufficient to disturb or weaken the interaction of the regulatory subunit with PP1c. Conversely, a single amino acid residue substitution in the hydrophobic groove of PP1c is sufficient for disrupting important interactions with inhibitors such as microcystin-LR and okadaic acid (Cohen, *et al.*, 2002; Wakula, *et al.*, 2003; Meiselbach, *et al.*, 2006; Wakula, *et al.*, 2006; http://www.expasy.ch/prosite/; http:/pp1signature.pasteur.fr/general.html).

These motifs appear to be necessary and sufficient for the interaction. Nevertheless, other low affinity interactions have to occur that have regulator-specific effects on PP1 activity and specificity (Egloff *et al.*, 1997; Cohen *et al.*, 2002; Wakula *et al.*, 2003; Croci *et al.*, 2003; Wakula *et al.*, 2006). The existence of common binding sites explains why a relatively small protein such PP1 can interact with numerous different multiple regulatory (R) subunits and why the binding of most R subunits is mutually exclusive.

I.2.2.2. PP1 multiple regulatory subunits

Protein phosphatase 1 catalytic subunit evolved and became an effective catalytic machine but lacks substrate specificity, which it acquires through interactions with a large number of regulatory subunits. These regulatory subunits are generally unrelated and function as targeting subunits, substrate-specifies, and inhibitors and/or substrates for associated PP1. Studies of the catalytic subunit (PP1c) have revealed a complex enzymology, occurring as multiple holoenzyme forms in complexes with different regulatory or targeting proteins (Oguri *et al*, 2006). Amongst the regulatory subunits already known to interact with PP1, a large number are PP1 inhibitors, including: Inhibitor-1 (I-1), I-2, dopamine- and cAMP-regulated phosphoprotein (DARPP-32), nuclear inhibitor of PP1 (NIPP1), C-kinase activated phosphatase inhibitor (CPI17), ribosomal inhibitor of PP1 (RIPP-1) and G-substrate (Connor, *et al*., 2000; Ceulemans and Bollen., 2004). These and other known PP1 interacting proteins are shown in Table 1.

Table 1: Known PP1 interacting proteins. (Adapted from http://pp1signature.pasteur.fr/published.html).

Process	Function/cell location	Reference
Glycogen	GM (RGL, R3) Skeletal muscle	Stralfors *et al.*, 1985
	GL (R4) Glycogen metabolism in Liver	Moorhead *et al.*, 1995
	GL LiverPPP1R3B	Doherty *et al.*, 1995
	R5 (Protein Targeting to Glycogen, PTG)	Doherty *et al.*, 1996
	PPP1R3C (PTG) muscle	Printen *et al.*, 1997
	R6 (PPP1R3D)	Armstrong *et al.*,1997
Myofibrillar	M110 (Myosin Phosphatase Targeting -MYPT) Smooth muscle and non-muscle	Alessi *et al.*, 1992
	MYPT1 (MBS, *M130, PPP1R12A*) Smooth muscle	Shimizu *et al.*, 1994
	MYPT2 Skeletal muscle, heart, brain, myofibrils	Fujioka *et al.*, 1998
Nuclear	NIPP1 (Nuclear Inhibitor of PP1/ PPP1R8), Pre-mRNA splicing	Van Eynde *et al.*, 1995
	PSF1 (Polypyrimidine tract-binding protein associated Splicing Factor)	Hirano *et al.*, 1996
	p99 (R111) RNA processing Widely distributed, nucleus	Kreivi *et al.*, 1997
	PNUTS (Phosphatase 1 Nuclear Targeting Subunit) RNA processing	Allen *et al.*, 1998
	Hox11 (homeodomain transcription factor) Cell cycle checkpoint/ Hematopoietic cells nucleus	Kawabe *et al.*, 1997
	HCF (Host Cell Factor) Transcription, cell cycle Widely distributed	Ajuh *et al.*, 2000
	AKAP 149 (A-kinase Anchoring Protein 149) Nuclear envelope	Steen *et al.*, 2000
	Sds22 (PPP1R7) Exit from mitosis	Stone *et al.*, 1993
Plasma membrane/ Cytoskeleton Centrosome/ Microtubule	Neurabin I (PPP1R9A) Neurite outgrowth Neuronal, plasma membrane and actin cytoskeleton	McAvoy *et al.*, 1999
	Spinophilin (neurabin II) + Glutamatergic synaptic Ubiquitous, plasma membrane and actin cytoskeleton	Allen *et al.*, 1997
	NF-L (neurofilament-L) Synaptic transmission, Neuronal, plasma membrane and cytoskeleton	Terry-Lorenzo *et al.*, 2000
	AKAP220 (A-kinase anchoring protein 220) PKA and PP1 signalling Brain, testis, peroxisomes/ cytoskeleton	Schillace *et al.*, 1999
	Yotiao (A-kinase anchoring protein) Synaptic transmission (NMDA Neuronal receptor)	Westphal *et al.*, 1999
	BH-protocadherin. Neuronal cell-cell interactions, Neuronal membrane	Yoshida *et al.*, 1999
	Ryanodyne receptor Calcium ion channel activity? Skeletal and cardiac muscle	Zhao *et al.*, 1998
	NKCC1 (Na-K-Cl Cotransporter) Transcellular chloride ion/ Epitheli, plasma membrane	Darman *et al.*, 2001
	AKAP 350 (CG-NAP, centrosomal and protein)	Takahashi *et al.*, 1999
	Nek2 (NIMA related protein kinase 2) Centrosome separation Cytoplasm	Helps *et al.*, 2000
	Tau. Microtubule stability and Neuronal microtubules	Liao *et al.*, 1998

Process	Function/cell location	Reference
Endoplasmic-Ribosomal	L5 ribosomal protein	Hirano *et al.*, 1995
	RIPP1 (Ribosomal Inhibitor of PP1) Protein synthesis, Ribosome	Beullens *et al.*, 1999
	GADD34 (Growth Arrest and DNA Damage protein/PPP1R15A) Protein synthesis, stress inducible	Novoa *et al.*, 2001
Apoptosis and specific substrates	Bad Pro-apoptotic member of Bcl-2 family.	Ayllón *et al.*, 2000
	Bcl-2 Survival factor. Prototype of a family of survival regulatory proteins/ cytoplasm/ mitochondria	Ayllón *et al.*, 2001
	Bcl-x_L and Bcl-w. Two anti-apoptotic members of Bcl-2 family	Ayllón *et al.*, 2002
	p53BP2 (TP53BP2, p53-Binding Protein 2), cytosol	Helps *et al.*, 1995
	Rb (Retinoblastoma protein) Cell cycle progression, nucleus	Durfee *et al.*, 1993
	PRIP-1 (Phospho-lipase C-Related Inactive Protein, p130, PLC-L1). Ins(1,4,5)P3-mediated Ca^{2+} signalling Brain, cytosol	Yoshimura *et al.*, 2001
	PFK (Phosphofructokinase) Glycolysis Skeletal muscle	Zhao *et al.*, 1997
Inhibitors	I-1 (inhibitor 1 PPP1R1A). Inhibition of PP1c Widely distributed cytosol	Huang and Glinsman, 1976
	DARPP-32 (Dopamine and cAMP-Regulated Phosphoprotein Mr 32000) + Inhibition of PP1c Brain, kidney, cytosol	Hemmings *et al.*, 1984
	I-2 (inhibitor 2) Inhibition of PP1c cytosol and nucleus	Huang *et al.*, 1999
	Inhibitor-3 (HCG V) Inhibition of PP1c	Zhang *et al.*, 1998
	CPI-17 (PKC Potentiated Inhibitor) Inhibition of PP1c Smooth muscle	Eto *et al.*, 1997
	PHI-2 (Phosphatase Holoenzyme Inhibitor) + Inhibition of PP1	Eto *et al.*, 1999
	I-1 PP2A (PHAP1) and I-2 PP2A (SET, PHAPII, TAF1b) inhibition of PP2A but Stimulation of PP1c	Katayose *et al.*, 2000
	G-substrate (cGMP-dependent protein kinase substrate). Inhibition of PP1c in the brain	Aitken *et al.*, 1981
	Grp78 (Glucose-Regulated Protein)	Chun *et al.*, 1994
Enzymes and Unclassified	PP1bp80 Regulation of chaperones, Skeletal muscle	Damer *et al.*, 1998
	MYPT3 Widely distributed microsomes and cytosol	Skinner and Saltiel, 2001
	SARA (Smad Anchor for Receptor Activation)	Bennett and Alphey, 2002
	Aurora-B : mitosis regulatory kinase	Sugiyama *et al.*, 2002

I.2.2.3. PP1 isoforms

In mammals, there are three PP1 genes, encoding the PP1α, PP1β/δ, and PP1γ isoform, also designated as PPP1CA, PPP1CB and PPP1CC, respectively (Figure 7). The PP1γ gene yields PP1γ1 and PP1γ2 isoforms by alternative splicing. The two isoforms possess divergent sequence in their COOH-termimals that allowed the production of isoform-specific antibodies. PP1α, PP1β/δ and PP1γ1 are widely expressed in mammalian tissues particular in brain with a number of important functions (Ouimet *et al*., 1995; da Cruz e Silva *et al*., 1995; Allen *et al.*, 1997; Andreassen, *et al*., 1998; Strack *et al*., 1999).

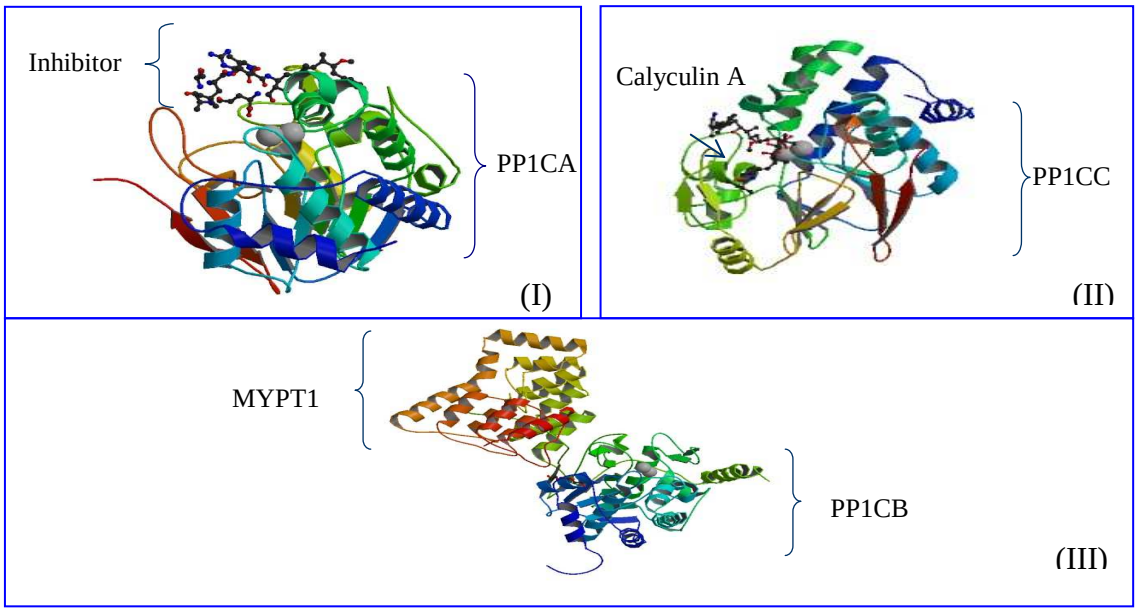

Figure 7: 3-D structures of different PP1 isoform complexes. (I) PP1CA and the inhibitor microcystin-LR toxin (Maynes *et al*., 2004). (II) PP1CC and the inhibitor calyculin A (marine toxin) (Kita *et al.*, 2002). (III) PP1CB and a 34-kDa N-terminal domain of the myosin phosphatase targeting subunit MYPT1 (Terrak *et al*., 2004).

Within the cell, the different PP1 isoforms (α, β/δ, γ) localize in a distinct and characteristic manner. All the isoforms are present both in the cytoplasm and in the nucleus during interphase. Within the nucleus PP1α associates with the nuclear matrix, PP1γ1 concentrates in nucleoli in association with RNA, and PP1β/δ localizes to non-nucleolar

chromatin. During mitosis PP1α localized to the centrosomes, PP1γ1 is associated with microtubules and PP1β/δ associates with chromosomes (Andreassen *et al.*, 1998).

The three PP1 genes are located in three different chromosomes, the PPP1CA gene is located on chromosome 11 (11q13), the PPP1CB gene is located on chromosome 2 (2p23), and PPP1CC gene was mapped to human chromosome 12 (12q24.1-q24.2) (Figure 8). The three genes comprise 7 exons and 8 introns.

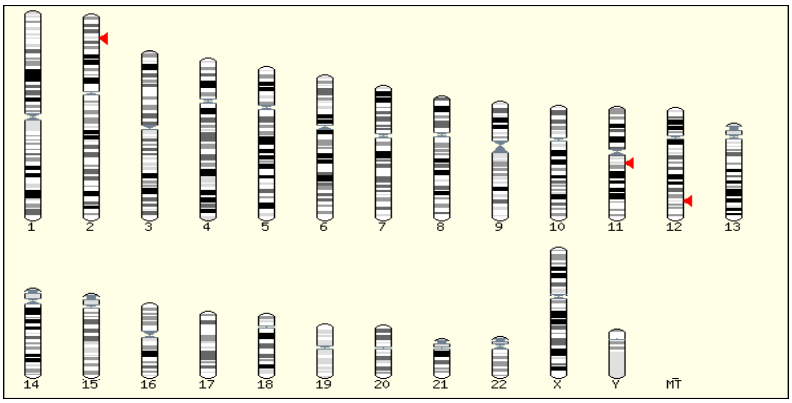

Figure 8: Chromosome localization of the three PP1 genes. The arrowheads indicate the loci of the three catalytic subunits genes.

I.2.2.4. PP1Gamma2

Alternative splicing, occurs when the exons or introns of the primary gene transcript, the pre-mRNA, are separated and reconnected to produce alternative arrangements. These linear combinations then undergo the process of translation where specific and unique sequences of amino acids are specified, resulting in the expression of protein variant. In this way, alternative splicing facilitates the synthesis of a greater variety of proteins allowing many gene products with different functions to be produced from a single coding sequence (Brett *et al.*, 2001). The PP1γ2 isoform is originated by this process, which the sequence alignment PP1γ1 and PP1γ2 is shown in Figure 9.

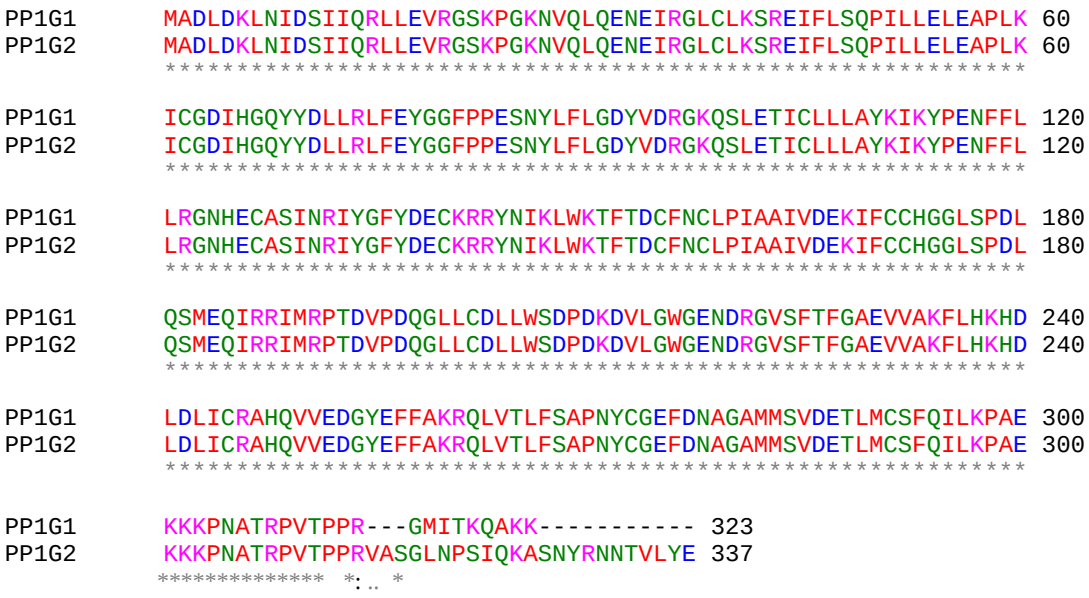

Figure 9: Alignment highlighting the difference between the alternatively spliced PP1CC isoforms, PP1γ1 and PP1γ2. The different colors represent similarities. This alignment was obtained using the bioinformatics tool T-COFFE E.

The PP1γ2 isoform is highly expressed in testis, but also uniformly expressed in most forebrain regions (diencephalon and telencephalon) of the brain, being particularly enriched in the striatum. Low levels of expression were detected in hindbrain (da Cruz e Silva *et al*., 1995; Andreassen *et al*., 1998; Strack *et al*., 1999; Ceulemans and Bollen 2004).

I.3. AIMS

In this study, we propose to identify the proteins expressed in human brain that interact with PP1γ2, through the Yeast Two-Hybrid method that allows the selection and identification of interacting proteins among a large number of clones present in a human brain cDNA library. This identification and elucidation of expression, distribution and biochemical characterization will address novel functions of PP1 isoforms (PP1γ2) in brain and associated diseases. Thus, the specifics aims of this work were:

Aim 1. To isolate the library plasmids present in the positive yeast colonies obtained by screening a human brain cDNA library using the Yeast Two-Hybrid system. The library plasmids from the positive yeast colonies will be extracted, transformed into *E. coli* XL1-Blue, purified, and analysed by restriction enzyme digestion.

Aim 2. To identify the interacting proteins fused to the GAL4-AD by DNA sequencing of the brain library cDNA inserts. The identification of the interacting proteins will be achieved by database comparison of the obtained sequences.

Aim 3. To analyse the deduced amino acid sequences by bioinformatics methods. The interacting proteins identified in Aim 2 will be analyses by bioinformatics methods to obtain further information, such as the occurrence of protein domains, expression pattern, chromosomal location, presence of PP1 binding motifs, and possible cellular functions.

Aim 4. To confirm the interaction with PP1 of selected interacting proteins by co-transformation and co-immunoprecipitation.

Aim 5. To obtain information, on the subcellular location of the selected PP1 interacting proteins by expression in mammalian cell lines.

Aim 6. To develop heterologous recombinant expression systems for selected proteins. Selected interacting proteins will be expressed in *E. coli* and the recombinant protein purified.

II. IDENTIFICATION OF THE ISOLATED POSITIVE CLONES

II.1. INTRODUCTION

The vast majority of the PP1 binding proteins identified to date were discovered using the Yeast Two-Hybrid (YTH) method. In order to select and identify the proteins capable of interact with PP1γ2 and the PP1γ2-specific C-terminus, was performed an YTH screen of a human brain cDNA library in our laboratory by Margarida Fardilha and Sara Esteves. The YTH method take advantage of the fact that most eukaryotic transcription activators acquire two functionally independent domains, a DNA-binding domain (BD) that recognizes a specific DNA sequence in the promoter regions and a DNA-activation domain (AD) that brings the transcriptional machinery to the vicinity of the promoter, directing expression of the gene. In this screen the reporter gene were: the MEL gene encoding α-galactosidase that is secreted into the culture medium, the LacZ gene encoding β-galactosidase, and the auxotrophic HIS3, TRP1, ADE and LEU2, which allow the yeast cells to grow in medium lacking Histidine, Tryptophan, Adenine and Leucine. These three reporter genes allow the elimination of false positives that result from the activation of the reporter gene even when the bait (GAL4-BD) and the prey (GAL4-AD) do not interact. These false positives might occur from preys that interact with DNA upstream of the reporter genes or with proteins that interact with promoter sequences.

In this screen, the PP1γ2 and PP1γ2end clones (Figure 10) were integrated in the pAS-2 vector and expressed as a fusion with the GAL4-BD. The brain cDNA library was inserted in the pACT-2 vector and expressed as a fusion with GAL4-AD. The results for the interactions with PP1γ2 were the identification 333 positives and for PP1γ2-specific C-terminus 27 positives. We decided to analyze all the positive clones in order to identify not only the most abundant clones but also the more interesting ones. Even though some may have been detected only once or twice in the screen, this does not mean that they are not important but may simply reflect the low abundance of the mRNA in the library used or its low abundance in the tissue from which the library was made. The positive clones were partially sequenced to allows the identification of the correspond protein and some of the clones were further analyzed.

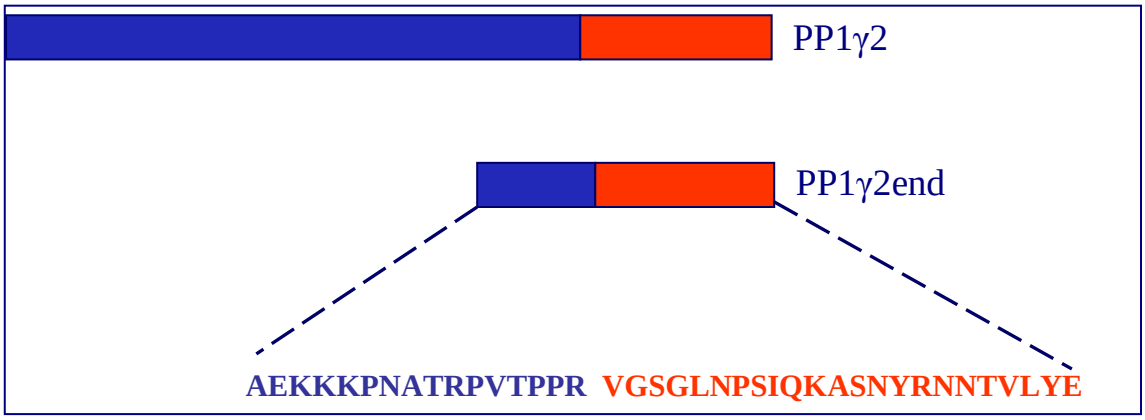

Figure 10: Diagrammatic composer of PP1γ2 with the PP1γ2end fragment used as bait. The alternatively spliced PP1γ2 specific C-terminal sequence is indicated in red.

II.2. MATERIALS AND METHODS

For the complete composition of all reagents, media and solutions used, see Appendix I. All reagents were cell culture grade or ultrapure.

II.2.1. Yeast culture and plasmid extraction by the boiling method

A single yeast colony was grown in SD/QDO medium and 3 ml of yeast cells were pelleted for 3 min at 14000 rpm and resuspended in 100 µl of STET solution. About 300 µl of 0.5 mm acid-washed glass beads (Sigma) were added and vortexed on high for 6-8 min. After adding 100 µl of STET solution the tubes were boiled for 3 min. After chill on ice, the tubes were centrifuged for 10 min at 14000 rpm at 4º C. The supernatant was transferred into a new microtube, 500 µl of ammonium acetate 7.5 M was added and mixed briefly and the tubes were incubated for 1-2 hrs at -20º C. The tubes were, then, centrifuged for 20 min at 14000 rpm and 4º C and the supernatant transferred to new tubes containing 200 µl of ice cold ethanol. After spinning down for 10 min at 14000 rpm and 4º C, the supernatant was removed and the tubes rinsed with 200 µl of 70% ethanol. Another 5 min centrifugation was performed and the pellet was dried in a vacuum device. The pellet was resuspended in 15 µl of sterile 20 µg/ml RNase solution.

II.2.2. Preparation of competent cells

A single colony of *E. coli* XL1-Blue was incubated overnight in 10 ml SOB medium at 37ºC. Then, 1 ml of this culture was used to incubate 50 ml SOB medium until OD_{550} =0.3. The culture was incubated on ice for 15 min and centrifuged at 4,000 rpm and 4ºC for 5 min. The supernatant was discarded and 15 ml of Solution I were added. After 15 min on ice, the cells were centrifuged at 4000 rpm for 5 min at 4ºC and 3 ml of Solution II were added to the cell pellet. The cells were immediately divided in 0.1 ml aliquots and stored at –80ºC

II.2.3. Transformation of *E. coli* XL-1 Blue

For transformation with plasmid DNA competent *E. coli* XL-1 Blue was used. To 50 µl of competent cells was added 15 µl of plasmid DNA recovered from the yeast positive colonies. The microtube was incubated on ice for 20 min and heat shocked at 42ºC for 90 sec. After incubation on ice for 30 min, 900 µl of SOC medium was added and incubated for 60 min at 37°C with shaking at 220 rpm. The cells were then plated on LB/Amp plates and incubated overnight at 37ºC to select the transformants.

II.2.4. Isolation of plasmid DNA by the alkaline lysis method

A single bacterial colony (transformants) was transferred into 3 ml of LB/amp medium and incubated overnight at 37ºC with shaking at 220 rpm. Then, 1.5 ml were transferred into a microtube and centrifuged at 8,000 rpm for 2 min at room temperature. The bacterial pellet was resuspended in 100 µl of solution I (50 mM glucose/ 25 mM Tris.HCl (pH 8.0)/ 10 mM EDTA) by vortexing. Then, 200 µl of solution II (0.2 N NaOH/ 1%SDS) were added to the microtube and mixed by inverting several times before adding 150 ml of solution III (3 M potassium acetate/ 2 M glacial acetic acid) and mixing inverted the microtube gently. After centrifugation at 14,000 rpm for 10 min at 4ºC the supernatant was transferred to a clean microtube. The plasmid DNA was precipitated by adding two volumes of ethanol at room temperature. The mixture was incubated for 10 min at -20°C. After centrifugation at 14,000 rpm for 5 min at 4ºC the supernatant was completely removed and the pellet washed with 70% ethanol. After centrifugation the pellet was drying in Speedvac system for 5 min. The plasmid DNA was resuspended in 50 µl of H_2O containing 20 mg/ml of RNase A and stored at -20ºC.

II.2.5. Restriction fragment analysis by agarose gel electrophoresis

The restriction enzyme *Hind* III was used for analysis the transformants. After added 4 µl of *Hind* III to 5 µl of DNA and to the appropriate volume of buffer and H_2O, the mix

was incubated for 2 hr at 37ºC. During this time the electrophoresis apparatus was prepared and a 1% agarose gel was prepared in 1X TAE buffer (40 mM Tris-base/ 20 mM acetic acid/ 1 mM EDTA). After the gel was completely set the comb was carefully removed and the gel mounted in the tank. The electrophoresis tank was filled with enough 0.25 X TAE to cover the agarose gel. After the restriction reaction, was added loading buffer on the samples and the mix was loaded into the slots of the submerged gel using a micropipette. Marker DNA (1kb ladder) of known size was also loaded into the gel. The gel was run at 120 V for 20 min and examined under UV light and photographed or analyzed using a Molecular Imager (Biorad).

II.2.6. DNA Sequencing

II.2.6.1. Plasmid DNA purification

DNA samples to be sequenced were purified with a QIAquick kit (QIAGEN), according to the manufacturer's instructions. In a microtube 1 volume of DNA sample was mixed with 5 volumes of PB buffer, transfered to a QIAquick column, and centrifuged for 1min at 14,000 rpm. The eluate was discarded and 750 µl of PE buffer added to wash the column, centrifuged for 1min at 14,000rpm, discarded again the eluate, and centrifuged for 1 min at 14,000 rpm to remove traces of washing buffer. The purified DNA was eluted into a clean microtube through the addition of 50 µl of H_2O and centrifuged for 1 min at 14,000 rpm. The DNA was stored at –20ºC.

II.2.6.2. Sequencing PCR reaction

The DNA samples were all sequenced following the same protocol. In a microtube were added 300 ng to 500 ng of DNA, 1 pmol of primer, 4 µl of Ready Reaction Mix (Applied Biosystems), and H_2O to a final volume of 20 µl. The reaction mixture was vortexed and centrifuged for a few seconds. The PCR was then performed, 1 cycles of 96ºC/1 min, 25 cycles of 96ºC/1 sec, 42ºC/30 sec, and 60 ºC/15 sec.

II.2.6.3. DNA purification by ethanol precipitation

All samples were purified by ethanol precipitation. This method was used to concentrate nucleic acids, as well as to purify them. Approximately 1/10 volume of 3 M sodium acetate (pH 5.2) was added to the DNA solution to adjust the salt concentration, followed by 2 volumes of ethanol. The solution was mixed and left at room temperature for 15 min to allow the DNA to precipitate. The DNA was recovered by centrifugation 20 min at 14,000 rpm and 20ºC. The supernatant was carefully removed without disturbing the pellet. The pellet was washed with 250 µl of 70% ethanol and centrifuged using the same conditions as above for 5 min. The supernatant was again removed and the pellet was dried in the Speedvac system.

II.2.6.4. Automatic DNA Sequencing

The DNA samples to be sequenced were resuspended in 20 µl of formamide, boiled for 20 min, and then introduce in the well tray of the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Analysis under standard conditions yielded chromatograms for each sample that were then converted by the manufactures software into the corresponding nucleotide sequence.

II.2.6.5. Sequence analysis

The nucleotide sequences were converted to FASTA format, and the vector and sequence linker removed. Through bioinformatics tools as BLAST from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) was possible to acquired information regarding different aspects. The information obtained allowed grouping the positives into different types depending on whether they encodes a known PP1 binding protein, other known proteins, proteins of unknown function or genomic clones. It was also possible to determine if each clone contained the full the coding sequence (CDS), and if it was in frame with the ATG from the gene. The protein sequence obtained was analyzed with

ELM (Eukaryotic Linear Motif, http://elm.eu.org/), with the purpose of determining the presence of the PP1 binding motif [R/K]-$X_{0,\ 1}$-[V/L/I/M]-X-[F/W], and/or other protein domains, which might give a hint to the protein function.

II.3. RESULTS

II.3.1. Isolation of the cDNA library plasmids from the positives clones

After selection of the positives by the YTH method, the plasmid DNA must be isolated from yeast. Since each yeast colonies cell can incorporate more than one plasmid, a mixture of different plasmid DNAs can be isolated from a single yeast clone. Including the library plasmids (Figure 11, lanes 1 -11) and the library plasmids (Figure 11, lane 12).

In order to obtain single plasmids the yeast positive were grown in SD/QDO (media without Adenine, Histidine, Leucine and Tryptophan), overnight to 72 hr, at 30°C and then extract and purified by boiling method (see chapter II.2). The plasmid DNA isolated from yeast was transformed in *E. coli* XL1-Blue, which was then grown on LB plates with Ampilicin (100 μg/ml).

The alkaline lysis method the plasmid DNA obtained from the resulting transformants was analyzed by restriction digestion with endonuclease *HindIII*. Knowing the enzyme restriction pattern of the empty pACT2-1 plasmid (7.4 kb and 0.7 kb), it is possible to deduce the size of the insert by subtracting 0.7 Kb from the observed size of the smaller fragment(s). This allows estimating if the isolated plasmid possesses estimate if the full coding sequence when compared with the results from the BLASTn.

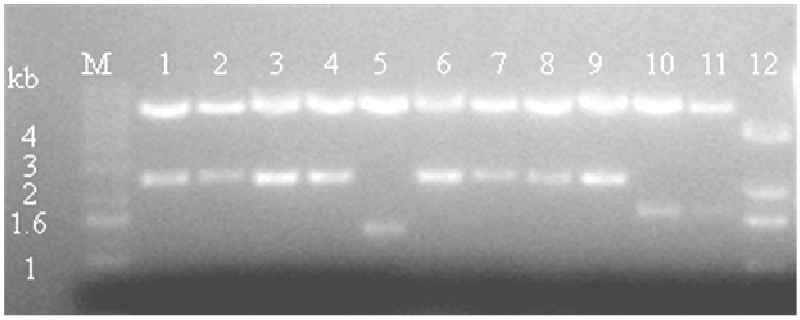

Figure 11: *HindIII* restriction analysis of the plasmids isolated from the transformed *E. coli* XL1-Blue. From each plate two colonies were purified and analysed. Most colonies contained pACT library plasmids, (eg. 1 - 11) with a vector fragment of proximally 7.4 Kb and 0.7 Kb + the cDNA library insert. Some colonies, like 12, contained the bait plasmid and yielded fragment of 4.6 kb, 2.2 kb, 1.5 kb and 0.9 kb. M stands for 1Kb Ladder marker.

II.3.2. Identification of the positive clones

The procedure described was carried for a total of 317 library cDNA insert selected by both YTH screens carried out. The plasmid DNAs were purified as described and sequenced by PCR using the GAL4-AD primer (see Appendix I).

A chromatogram obtained for each positive that was visualized with Chromas software which (Figure 12) was provides the nucleotide sequence, as well as sample information like the positive in the well tray, the number of the clone, and the data that the sample was introduce in the sequence machine.

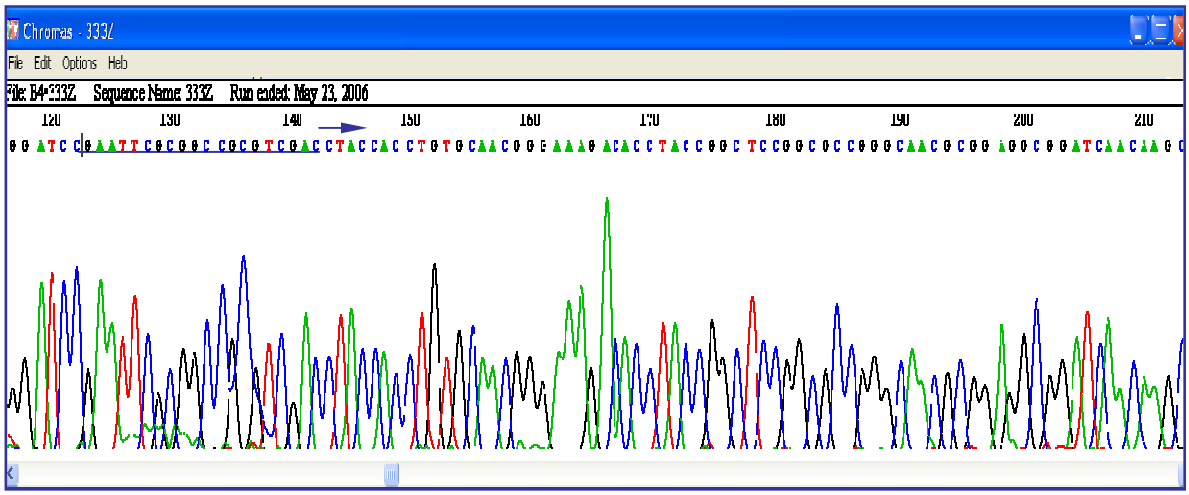

Figure 12: Example of chromatogram. Partial chromatogram obtained by the positive 333 from the two-hybrid screen for PP1γ2 YTH screen. The blue line marks the linker sequence and the arrow mark the beginning of the nucleotide sequence of the clone 333.

The sequence obtained was then converted to FASTA format, where in the first line the signal > precedes a name or additional information of the sample sequenced, and on the sequence starts second line the (Figure 13).

>B4•333Z sequence exported from chromatogram file

ACNTCATTCGTGATGAAGAACCCACCAAACCCAAAAAAAGTAGATCTCTA
TGGCTTACCCATACGATGTTCCAGATTACGCTAGCTTGGGTGGTCATATGG
CCATGGAGGCCCNGGGGATCC **GAATTCGCGGCCGCGTCGAC**

CTACCACCTGTGCAACGGGAAAGACACCTACCGGCTCCGGCGCCGGGCAA
CGCGGAGGCGGATCAACAAGCGTGGGGCCAAGAACTGCAATGCCATCCGC
CACTTCGAGAACACCTTCGTGGTGGAGACGCTCATCTGCGGGGTGGTGTG
AGGCCCTCCTCCCCCAGAACCCCCTGCCGTGTTCCTCTTTTCTTCTTTCCAG
CTGCTCTCTGGCCCTCCTCCTTCCCCCTGCTTAGCTTGTACTTTGGACGCGT
TTCTATAGAGGTGACATGTCTCTCCATTCCTCTCCAANCCTG

Figure 13: Example of a nucleotide sequence in FASTA format. Nucleotide sequence in FASTA format for clone 333 derived from the previous chromatogram. The sequence in bold is the linker.

Analyses of the FASTA format sequence and the chromatogram, allowed the identification of the linker region, as well as the vector derived sequence. Both were removed before further analysis. The sequence was then compared with the GenBank Database of nucleotide sequences using BLAST software. A representative example is on Figure 14 (http://www.ncbi.nlm.nih.gov/BLAST/).

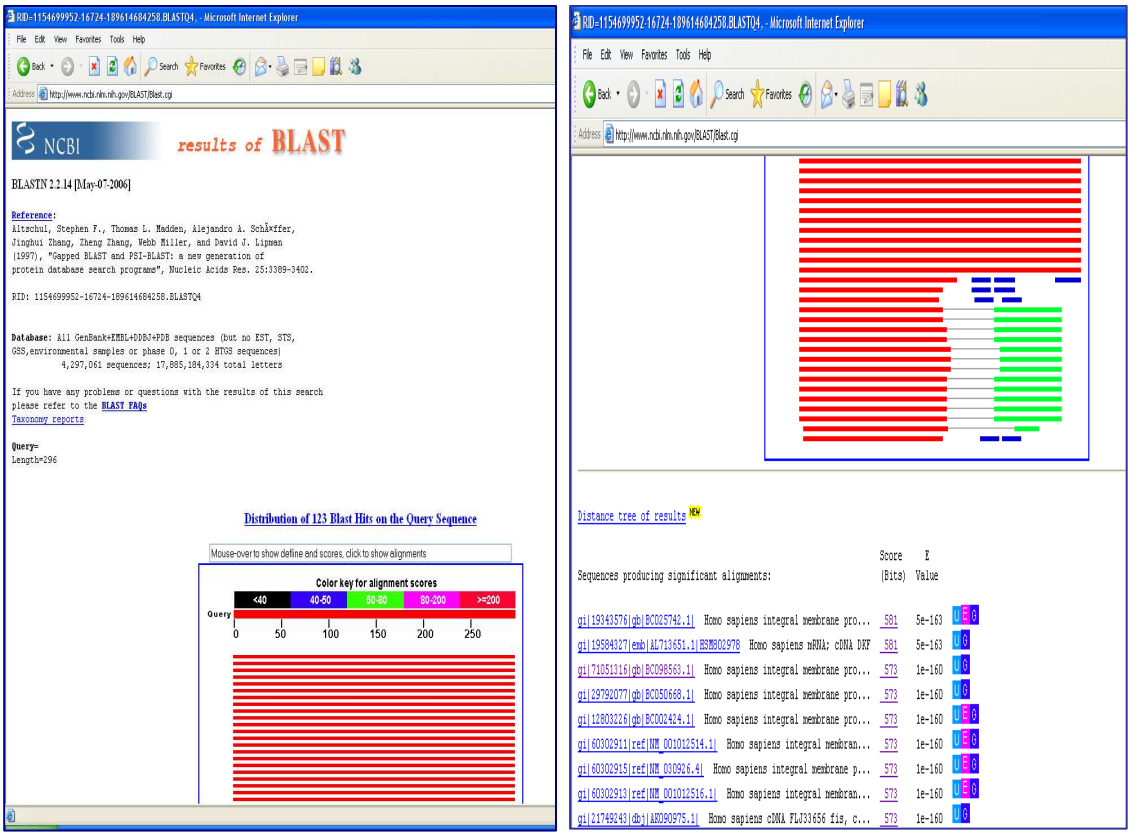

Figure 14: Example of results obtained with the bioinformatics tool Blastn. This example was obtained for clone 333 after removing the vector and the linker sequences.

The results obtained from Genbank homology search allowed the unambiguous identification of the positive brain library cDNA and the proteins they encode. The NCBI nucleotide database provides information regarding the size of gene, the coding sequence (CDS), the protein amino acids, the chromosome, and the organism. Thus, it was possible to determine if the sequence clone encoded known PP1 binding protein, another known proteins, or proteins of unknown function or genomic clones. It was also feasible to establish exactly where the sequence the clone obtained from the YTH screen started and if it was in frame with the ATG start codon.

The ELM software (Eukaryotic Linear Motif, http://elm.eu.org/) from the ExPASy (Expert Protein Analysis System) server of the Swiss Institute of Bioinformatics (http://us.expasy.org/tools/scanprosite/) was used. To verify if the encoded proteins contained a [R/K]-$X_{0,1}$-[V/L/I/M]-X-[F/W] PP1 binding motif, and other protein domains that might elucidate the function of the protein.

II.3.3. Identification of positive clones obtained with the PP1γ2 specific C-terminus

Table 2 shows the results obtained from the human brain cDNA library YTH screen with PP1γ2-specific C-terminus. Of the 19 positives clones obtained, 15 were found to encode the protein, RANBP9, a known PP1 binding protein (violet) and three correspond to known proteins: GOLGA7 -Golgi autoantigen 7, IL3RA -interleukin 3 receptor alpha (light blue), and a hypothetic new splice variant of BMPR2 -Bone morphogenetic protein receptor type II (dark blue). This screen also yielded one positive clone identified as the hypothetical protein LOC100129112 (light green).

Table 2: List of identified positives obtained with the PP1γ2end bait by YTH screening of a human brain cDNA library.

Clone ID	YTHG2end Positive nº	PP1 BM	chr	Data base ID	Total nº of positives	Function
RANBP9	2;4;5,7;8;9;10;12;14;15;16;18;21;25;26	RMIHF	6	gi:39812377	15	Signaling
GOLGA7 -golgi autoantigen, golgin subfamily a, 7	29	n	8	gi:50541949	1	Transport
IL3RA -interleukin 3 receptor alpha	13	KFVVF	X	gi:54781360	1	Signaling
BMPR2 -Bone morphogenetic protein receptor, type II	24	RVPW; RLKF	2	gi:72376969	1	Receptor
hypothetical protein LOC100129112	20	N	17	gi:169211920	1	Unknown

II.3.4. Identification of positive clones obtained with PP1γ2

The screen of the human brain cDNA library for proteins that interact with PP1γ2 resulted in the identification of 46 positives encoding previously known PP1 interacting proteins, and 226 encoding other potential PP1 binding proteins. Of these 226 positive clones, 210 encode known proteins (191 known proteins and 19 hypothetical new splice

variants) and 16 encode unknown proteins. This screen also identified 21 genomic clones and 5 mitochondrion clones, for a total of 298 blastn positives identified (Figure 15 and Table 3). Thus, this screen resulted in the identification of 85.9% known proteins and 14.1% unknown proteins, genomic and mitochondrial clones. This includes 15.4% that are known PP1 binding proteins and 70.5% that are proteins previously not shown to bind PP1, of which 6.4% are hypothetical new splice variants. Therefore, the YTH approach allowed the identification of a large number of potential new PP1 binding proteins that need further confirmation of the interaction. The set of proteins thus identified constitute the human brain PP1γ2 interactome.

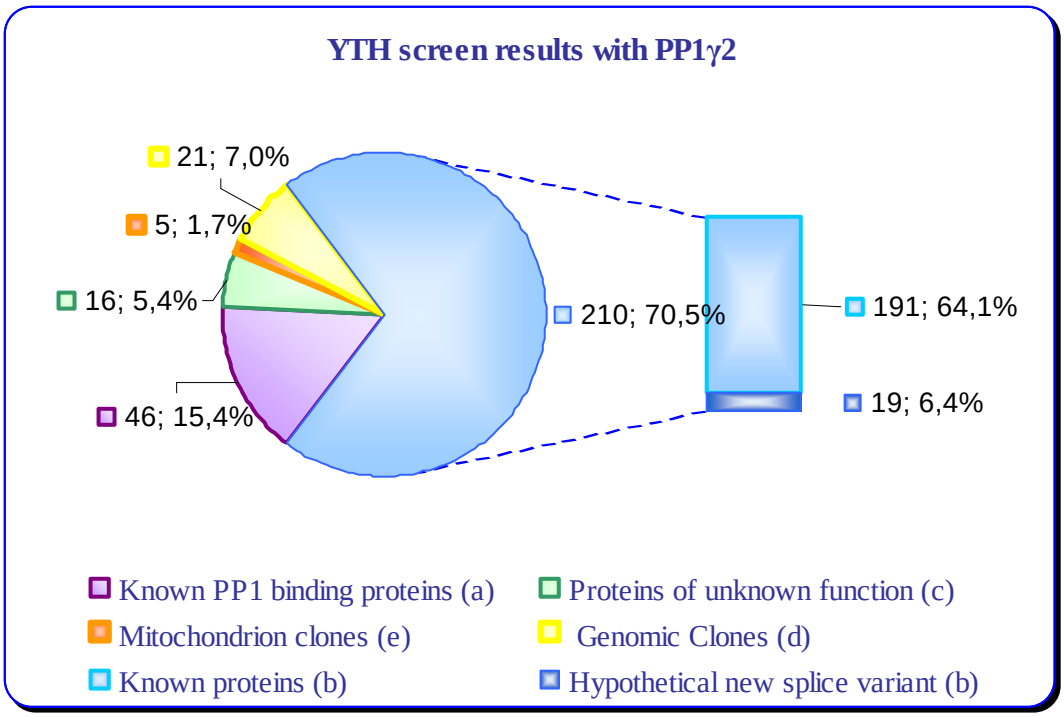

Figure 15: Schematic representation of the number and percentage of positives clones identified by YTH screening of a human cDNA library using PP1γ2 as bait.

A striking observation is that the 210 positives comprising the major group, corresponding to known proteins, encode 134 different proteins. In fact, 99 of these were detected only once (Table 3), possibly reflecting their low abundance in human brain or in the cDNA library used.

Table 3: Frequency list of the human brain cDNA library positives identified with PP1γ2.

Nº of Positive clones	Nº of Protein	Classification
17	1	a
12	1	a
9	1	a
7	1	b
6	2	b
5	4	b
4	3	b
4	1	c
4	1	d
3	10	b
2	3	a
2	15	b
2	2	c
2	1	d
2	2	e
1	2	a
1	99	b
1	8	c
1	15	d
1	1	e
298	173	Total

(a) - Known PP1 binding protein (violet); (b) – other previously Known proteins (ice blue); (c) - Protein of unknown function (green); (d) - Genomic sequence (yellow) and (e) - Mitochondrion clones (orange).

Nevertheless, it is interesting to note that with the exception of one protein of unknown function and one genomic sequence all other positives detected more than twice encoded either known PP1 binding proteins (a) or other known proteins (b) (the most abundant group) (Table 3). This is exemplified by the most abundant ´hit`, 17 clones recognized as protein RANBP9, by the 12 positives identified as Beta Tubulin (TUBB2A), and by the 9 positives identified as Kinesin Family member 1A (KIF1A), among others. A total of 25 proteins were detected more than twice. In contrast, the group of proteins detected only once or twice comprises known PP1 interacting proteins, plus 124 other proteins and 19 genomic and mitochondrial clones. It is interesting to note that this group

includes 99 known proteins not previously related to PP1, corresponding to 57.2% of the total number of interacting proteins identified. There is a striking variety of different proteins in this group. Further than the frequency analysis of the results, the positive clones were also categorized according to the function of each protein (Figure 16).

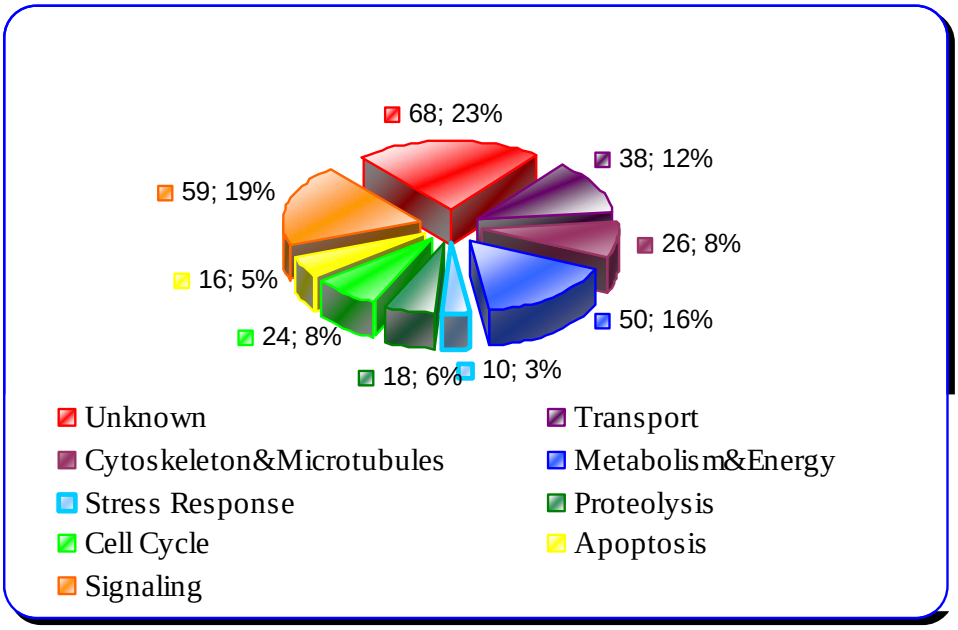

Figure 16: Functional distribution of the interacting proteins identified for PP1γ2.

Accordingly, since some proteins could belong to more than one functional group, this explains why the total number of proteins (309) is higher than the total number of positive clones analyzed (298). With the exception of 68 proteins of unknown function (also including genomic clones), the most abundant group of interacting proteins were found to be related to signaling pathways (19%), closely followed by 16% involved in metabolism and energy and 12% related to transport. All other functional categories comprised less than 10% of the identified proteins, with the less abundant group being that of proteins associated with stress response (3%). These results are shown in more detail below in Tables 4-8. Table 4 lists the known PP1 binding proteins obtained, Table 5 lists other proteins with known functions including possible new splice variants (in dark blue), Table 6 lists proteins of unknown function, Table 7 lists the genomic clones identified (in yellow), and Table 8 lists mitochondrial protein. All tables have information on the name of each protein, chromosome, the database ID, the number of the clone in the two-hybrid

screen, the binding motif for PP1, the number of positives obtained, and the possible function. For the genomic clones it is also indicated the exact location of the identified homology. Tables are arranged in order of frequency of occurrence for each protein. The clone 152 resulted in the identification of two different plasmids (a and b).

Table 4: List of known PP1 binding proteins identified.

Known PP1 binding proteins (a)						
Clone ID	**YTH PP1γ2 positives nº**	**PP1 BM**	**Chr**	**Data base ID**	**Total of nº positives**	**Function**
RANBP9	1;2;21;23;32;53; 69;135;154;263; 173.1;192,1;214; 218;290;296;297	RMIHF	6	gi:39812377	17	Signaling
TUBB2A beta tubulin	28;64;158;166; 195;207;240;257; 314;319;334;341	RLHF	6	gi:68299771	12	Cytoskeleton & Microtubules
KIF1A kinesin family member 1A	10;40;41;51;73;94 ;95;140;225	RILF; RDLLW; KVQF; KISF	2	gi:41327743	9	Transport
RIF1- RAP1 interacting factor homolog	141; 151	KKIAF; RVSF	2	gi:56676334	2	Cell cycle
TP53BP2	62; 119	RLKF; RVKF; KLLPF	1	gi:4885642	2	Apoptosis
C1orf71	66; 83	RRVRF;	1	gi:22749246	2	Unknown
1STAU1- staufen, RNA binding protein, homolog	152.b	RKVTF	20	gi:82659088	1	Cell cycle
PPP1R9A- protein phosphatase 1, regulatory subunit 9A	122	RKMF; RKIKF; KELDF; KVRW	7	gi:93204860	1	Cytoskeleton & Microtubules

Table 5: List of known proteins not previously related to PP1.

Other previously known proteins (b)

Clone ID	YTH PP1γ2 positives nº	PP1 BM	chr	Data base ID	Total of positives nº	Function
COP9 -Constitutive photomorphogenic homolog subunit 5 (COPS5)	33;34;45;48; 98;219;258	None	8	gi:38027922	7	Metabolism & Energy; Proteolysis
COX11-Cytochrome c oxidase	78;92;149;16 5;196;227	RGLRW; KISF	17	gi:17921983	6	Metabolism & Energy
PINK1- PTEN induced putative kinase 1	**68;96;199;20 0;256;269**	**RLRF; KMLF;**	**1**	**gi:14165271**	**6**	**Apoptosis; Stress response**
AATK - Apoptosis-associated tyrosine kinase	61;117;123; 163;221	RGLNF; KAVSF;	17	gi:89066851	5	Apoptosis
ALDOC - Aldolase C fructose-bisphosphate	12;89;91;245 246	RQVLF	17	gi:68303552	5	Metabolism & Energy
FTL -Ferritin light polypeptide	110;136;138; 167;254	None	19	gi:56682960	5	Transport
LRP3- Low density lipoprotein receptor-related 3	99;103;126; 243;268	None	19	gi:167555124	5	Signaling
ITM2C- Integral membrane protein 2C	**24;76;80;333**	**KISF**	**2**	**gi:60302915**	**4**	**Unknown**
SLC36A1 - Solute carrier family 36 member 1	114;156;233; 250,	None	5	gi:41352720	4	Transport
KLHL21 - Kelch-like 21	170;173;174; 182	RLPF	1	gi:55925607	4	Proteolysis
STMN3- Stathmin-like 3	130;134;186	None	20	gi:14670374	3	Signaling
GPR56 G - Coupled receptor 56	38;67;284	None	16	gi:41584201	3	Signaling
TYRO3 - Tyrosine kinase	42;159;271	KLSW	15	gi:30704372	3	Signaling
CREB5 - cAMP responsive element binding 5	105;252;289	None	7	gi:59938771	3	Cell cycle
CDC23 - cell division cycle 23	213;247;263	None	5	gi:16554575	3	Cell cycle
TOR1AIP1 - Torsin A interacting protein 1	124;164;169	REVRF; KVNF; KVKF	1	gi:39753956	3	Cytoskeleton & Microtubules
ENTPD6 - Ectonucleoside triphosphate diphosphohydrolase 6	15;27;285	None	20	gi:4557422	3	Metabolism & Energy
ITSN1- Intersectin 1	226;229;299	RQLEW; RQIGW; KTLEF; KRQLEW	21	gi:47717124	3	Transport
Smoothelin	75;145;155	RVGF; RILW	22	gi:89059027	3	Cytoskeleton & Microtubules
glutamate receptor	325; 337;340	None	4	NW_00183891 5	3	Signaling

Other previously known proteins (b)						
Clone ID	**YTH PP1γ2 positives nº**	**PP1 BM**	**chr**	**Data base ID**	**Total of positives nº**	**Function**
GABA – gamma-aminobutyric acid B	31;220	RVGW; KIWW	6	gi:167000239	2	Signaling
LPXN –Leupaxin	4;9	None	11	gi:4758669	2	Signaling
ATP6V1H - ATPase H+ transporting lysosomal V1subunit H	242;259	KVNW; RLEW	8	gi:47717099	2	Transport
ATP2A2 - ATPase Ca++ transporting, cardiac muscle	143;294	RVSF	12	gi:161377445	2	Transport
DBNDD2 -Dystrobrevin binding protein 1	157;201	None	20	gi:56676381	2	Transport
ARHGEF17 - Rho guanine nucleotide exchange factor 17	118;224	RSVSF;RLGF	11	gi:21361457	2	Metabolism & Energy
WSB1 -WD repeat and SOCS box-containing 1	264;335	RDLTF; RSVSF; KLLEF	17	gi:58331181	2	Proteolysis
COL4A2 -Collagen type IV alpha 2	56;171	RGLGF	13	gi:116256353	2	Cytoskeleton & Microtubules
SDHB -Succinate dehydrogenase complex subunit B iron sulfur (Ip)	93;175	None	1	gi:115387093	2	Metabolism & Energy
DDO- D aspartate oxidase	39;57	None	6	gi:40217814	2	Metabolism & Energy
TNS1 -Tensin 1	161;302	KLVF	2	gi:156142195	2	Cytoskeleton & Microtubules
SMPD1 -Sphingomyelin phosphodiesterase 1, acid lysosomal	30;313	RILF	11	gi:56117841	2	Metabolism & Energy
GPM6B - Glycoprotein M6B	146;61	None	X	gi:50263049	2	Unknown
RAB6A - Member RAS oncogene family	116;1116	KLVF	11	gi:38679887	2	Transport
RAB5C- Member RAS oncogene family	307	RAVEF	17	gi:41393544	1	Transport
RAB1B - Member RAS oncogene family	311	None	11	gi:116014337	1	Transport
APBB1- Fe65 -Amyloid beta (A4) precursor protein-binding, family B, member 1	44	RVRF;RVGW	11	gi:22035553	1	Transport
PTPRN - protein tyrosine phosphatase receptor type N	87	None	2	gi:18860905	1	Transport
ARF3 –ADP-ribosylation factor 3	142	KNISF	12	gi:186910284	1	Transport
ATP6V0A1 - ATPase H+ transporting lysosomal	300	KVQF; RKLRF;RVW; KLTF	17	gi:77539781	1	Transport

Other previously known proteins (b)						
Clone ID	**YTH PP1γ2 positives nº**	**PP1 BM**	**chr**	**Data base ID**	**Total of positives nº**	**Function**
GPX1 - glutathione peroxidase 1	13	None	3	gi:41406083	1	Metabolism & Energy; Stress response
MBP -Myelin basic protein	332	None	18	gi:68509939	1	Cytoskeleton & Microtubules
EPB49 -Erythrocyte membrane protein band 49	273	None	8	gi:166706874	1	Cytoskeleton & Microtubules
M-RIP - Myosin phosphatase-Rho interacting protein	249	None	17	gi:50980306	1	Cytoskeleton & Microtubules
PSMD8 -Proteasome 26S subunit non-ATPase 8	217	KILF	19	gi:156631004	1	Proteolysis
ARIH2-Ariadne homolog 2	16	KLILV	3	gi:83776589	1	Proteolysis
CTSH -Cathepsin H	222	KAIGF	15	gi:148536856	1	Proteolysis
SUMO2 -SMT3 suppressor of mif two 3 homolog 2	346	RQIRF	17	gi:54792068	1	Proteolysis
UBE1L -Ubiquitin - activating enzyme E1-like	250	KLCF; KPLMF	3	gi:38045947	1	Proteolysis
NFAT5 - Nuclear factor of activated T-cells 5, tonicity-responsive	184;238	KVIF	16	gi:27886521	2	Cell Cycle; Stress response
LMNB2 -Lamin B2	282	None	19	gi:27436950	1	Cell Cycle
ADAR -Adenosine deaminase, RNA-specific	206	RMGF; KKTVSF	1	gi:70167112	1	Cell Cycle
EXDL2 - exonuclease 3'-5' domain-like 2	162	None	14	gi:8922630	1	Cell Cycle
MD13L -Mediator complex subunit 13-like	303	None	12	gi:47575843	1	Cell Cycle
TSPAN7 -Tetraspanin 7	11	None	X	gi:183396766	1	Cell Cycle
PURA -Purine-rich element binding protein A	191	None	5	gi:62530389	1	Cell Cycle
TCFL5 - Transcription factor-like 5	279	None	20	gi:38505158	1	Cell Cycle
PEX16 -Peroxisomal biogenesis factor 16	18	RILF	11	gi:17136079	1	Cell Cycle
CHD5 -Chromodomain helicase DNA 5	79	RVLIF	1	gi:24308088	1	Cell Cycle
NK2 -Transcription factor related locus 2	230	RVLF; KIWF	20	gi:32307133	1	Cell Cycle
NEUROD2 -Neurogenic differentiation 2	106	None	17	gi:98986460	1	Cell Cycle
OLFM1 -Olfactomedin 1	292	None	9	gi:34335281	1	Cell Cycle

Other previously known proteins (b)						
Clone ID	**YTH PP1γ2 positives nº**	**PP1 BM**	**chr**	**Data base ID**	**Total of positives nº**	**Function**
TEGT -Testis enhanced gene transcript (BAX inhibitor 1)	60	RKINF	12	gi:148746208	1	Apoptosis
FAIM2 -Fas apoptotic inhibitory molecule 2	331	None	12	gi:34101289	1	Apoptosis
LRDD -Leucine-rich repeats and death domain containing	172	RLQF; KVIF; RICF	11	gi:61742785	1	Apoptosis
GLUL - Glutamate-ammonia ligase	58	RRLTGF	1	gi:74271836	1	Metabolism & Energy
ENO2 -Enolase 2 (gamma, neuronal)	81	None	12	gi:16507966	1	Metabolism & Energy
ACAD8 -Acyl-Coenzyme A dehydrogenase family, member 8	202	KVAF; KVGW	11	gi:111120326	1	Metabolism & Energy
CYB561D2 -Cytochrome b-561 domain containing 2	321	None	3	gi:31541779	1	Metabolism & Energy
ADCY1 -Adenylate cyclase 1 (brain)	251	RMKF; RILF	7	gi:168480138	1	Metabolism & Energy
PTPN5 -Tyrosine phosphatase	281	None	11	gi:90652860	1	Metabolism & Energy
PGD -Phosphogluconate dehydrogenase	152a	KGILF	1	gi:40068517	1	Metabolism & Energy
PKM2 -Pyruvate kinase, muscle	82	RLNF	15	gi:33286417	1	Metabolism & Energy
GAA - Lysosomal alpha-glucosidase	126	RLHF	17	gi:119393890	1	Metabolism & Energy
SCD5 -Stearoyl-CoA desaturase 5	131	KIPF	4	gi:148596960	1	Metabolism & Energy
RASGRF2 -Ras protein-specific guanine nucleotide-releasing factor 2	129	KSLEF	5	gi:38505169	1	Metabolism & Energy
NDUFB7-Dehydrogenase (ubiquinone) 1 beta	54	None	19	gi:33519471	1	Metabolism & Energy
NAD(P)H - Dehydrogenase, quinone 2 (NQO2),	228	None	6	gi:156564356	1	Metabolism & Energy; Stress response
PTGDS -Prostaglandin D2 synthase (brain))	132	None	9	gi:38505192	1	Metabolism & Energy
DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (DDX5)	235	RLIDF; KTIVF	17	gi:13514826	1	Metabolism & Energy
BCR -Breakpoint cluster region	102	KLYF	22	gi:82546842	1	Metabolism & Energy
B3GNT1 -UDP-GlcNAc betaGal beta-1,3-N-acetylglucosaminyltransferase 1	29	None	11	gi:92091577	1	Metabolism & Energy
DGKZ -Diacylglycerol kinase, zeta	43	KINF	11	gi:41872521	1	Metabolism & Energy
DGKE -Diacylglycerol kinase, epsilon 64kDa	192	None	17	gi:4503312	1	Metabolism & Energy

Other previously known proteins (b)						
Clone ID	**YTH PP1γ2 positives nº**	**PP1 BM**	**chr**	**Data base ID**	**Total of positive s nº**	**Function**
SCARB2 -Scavenger receptor class B, member 2	160	KIVEW	4	gi:33598925	1	Signaling
PTGER4 -Prostaglandin E receptor 4 (subtype EP4)	210	None	5	gi:38505196	1	Signaling
BRCA1 -Associated protein-1 (ubiquitin carboxy-terminal hydrolase) (BAP1),	216	KVLF	3	gi:19718752	1	Signaling
DTX3 -Deltex 3 homolog	120	RLTF	12	gi:31341899	1	Signaling
FGFR3 - fibroblast growth factor receptor 3	8	None	4	gi:169808429	1	Signaling
KCNQ2- Potassium voltage-gated channel, KQT-like subfamily, member 2,	20	None	20	gi:26051261	1	Signaling
OPRL1 -Opiate receptor-like 1	315	None	20	gi:33286425	1	Signaling
NCAM1 -Neural cell adhesion molecule	153	KLMF	11	gi:117320541	1	Signaling
RTN4R -Reticulon 4 receptor	176	RLLAW	22	gi:47519383	1	Signaling
ADRA1A -Adrenergic, alpha1A	194	RLLKF; KIVF;	8	gi:111118985	1	Signaling
THY1 -Thy-1 cell surface antigen	310	KHVLF	11	gi:19923361	1	Signaling
p35, CDK5R - Cyclin-dependent kinase 5	301	None	17	gi:34304373	1	Signaling
SRPR -Signal recognition particle receptor	246	KISF	11	gi:23308696	1	Signaling
SYNGAP1- Synaptic Ras GTPase activating protein 1	287	None	6	gi:54607095	1	Signaling
Sec61 -Alpha 1 subunit	248	RKIQF	3	gi:60218911	1	Transport
Sec23 Homolog	112	None	14	gi:38202213	1	Transport
ATP2B3 -ATPase ca2+ transporting plasma membrane 3	74	None	X	gi:48255952	1	Transport
ST3GAL2- ST3 beta-galactoside alpha-2,3 sialyltransferase 2	291	None	16	gi:151101481	1	Metabolism & Energy
PKIA -Protein Kinase cAMP-dependen catalytic inhibitor alpha	309	None	8	gi:32483387	1	Metabolism & Energy
RBBP9 -Retinoblastoma binding protein 9	266	None	20	gi:24119165	1	Metabolism & Energy
PIGG -Phosphatidylinositol glycan anchor biosynthesis	85	None	4	gi:187608437	1	Metabolism & Energy
HDGFRP3 -Hepatoma derived growth factor relator protein 3	26	None	15	gi:21359902	1	Unknown
TTYH3 -Tweety homolog 3	50	None	7	gi:53831992	1	Signaling
RANBP10 -RAN binding protein 10	327	None	16	gi:40538735	1	Signaling

Other previously known proteins (b)						
Clone ID	**YTH PP1γ2 positives nº**	**PP1 BM**	**chr**	**Data base ID**	**Total of positives nº**	**Function**
RBPJ - Recombination signal binding protein immunoglobulin kappa J	190	None	4	gi:42560224	1	Signaling
PRKCB1 -Protein kinase Cbeta1	25	None	16	gi:51472974	1	Signaling
KCNJ6 -Potassium inwardly-rectifying channel	101	None	21	gi:51475294	1	Signaling
ATF7IP -Activating transcription factor 7 interacting	308	None	12	gi:38261961	1	Cell Cycle
GIPC1- GIPC PDZ domain containing family1	317	RLVF	19	gi:42544147	1	Unknown
MPPED1 -Metallophosphoesterase domain containing 1	193	None	22	gi:113204626	1	Unknown
GBAS -Glioblastoma amplified sequence	322	KLQF	7	gi:4503936	1	Unknown
FAM127A -family with sequence similarity 127	305	None	X	gi:118200334	1	Unknown
POLDIP3 -Polymerase delta interacting protein 3	49	None	22	gi:30089917	1	Unknown
RNF187- Ring finger protein 187	17	None	1	gi:40555764	1	Unknown
MEGF8 - Multiple EGF-like-domains 8	144	RTVLF	19	gi:145701024	1	Unknown
FXYD6 -FXYD domain containing ion transport 6	223	None	11	gi:11612654	1	Unknown
NICN1 -Nicolin 1	35	None	3	gi:91992150	1	Unknown
PKNOX2 -PBX/knotted 1 homeobox 2	237	None	11	gi:116812643	1	Unknown
PWP2 -Periodic tryptophan protein homolog	204	RVLF; KVLEF	21	gi:48762925	1	Unknown
ARPP-21 -Cyclic AMP-regulated phosphoprotein	127	None	3	gi:68161521	1	Unknown
MTMR6 -Motubularin protein 6	212	RTVHF	13	gi:134142347	1	Unknown
RHBDD2 -Rhomboid domain containing 2	88	RALVF	7	gi:94818789	1	Unknown
DAZAP2 -DAZ associated protein 2	198	None	12	gi:41281480	1	Unknown
WDR42A -WD repeat domain 42A	108	KGVNF; KVVVW	1	gi:30089953	1	Unknown
HRNBP3 -Hexaribonucleotide binding protein 3	59	None	17	NM_001082575	1	Unknown
ATG2 -Autophagy related 2 homolog B	181	None	14	NM_018036	1	Unknown
POM121C -POM121 membrane glycoprotein C	189	None	7	NM_001099415	1	Unknown
KIAA0513	323	None	16	gi:57242775	1	Unknown
MIAT -Myocardial infarction associated transcript	312	None	22	NR_003491	1	Unknown
DENND4B -DENN/MADD domain containing 4B	97	REILF; RLRW	1	NM_014856	1	Unknown

Proteins highlighted in red were selected for further study. The darker blue color indicates possible new splice variants.

Table 6: List of proteins of unknown function.

Proteins of unknown function (c)						
Clone ID	**YTH PP1γ2 positives nº**	**PP1 BM**	**chr**	**Data base ID**	**Total of positives nº**	**Function**
DKFZP434B0335	203;211;215; 225	KVWF; RAVYF; RGVVW	7	gi:32698703	4	Unknown
FLJ45445	179;270	None	19	gi:51972221	2	
C20orf117	72;84	None	20	NW_001838665	2	
C6orf136	183	None	6	gi:21450766	1	
C14orf83	330	RLRF	14	gi:109627660	1	
C9orf164	125	None	9	gi:32699075	1	
C9 orf 127,	128	None	9	gi:28175551	1	
hypothetical protein LOC653319	139	None	16	gi:106507203	1	
hypothetical protein LOC51149	234	None	5	gi:65508249	1	
p20 hypothetical protein LOC130074	22	None	2	NM_001009993	1	
hypothetical protein LOC100129965	298	None	11	XM_001724519	1	

Table 7 lists the sequences obtained for identified homologies detected at the mRNA/cDNA level in the database. They may represent new proteins not yet identified. Between all the clones identified as genomic sequences, it is interesting to observe that the five clones identified in chromosome 18 are similar; all start between nucleotide 294140 and 294235, and finished between 294515 and 294595. Further studies are required to determine if the sequences identified encode novel proteins not yet annotated in the database.

Table 7: Lists of genomic clones**.**

Genomic clones (d)							
Database ID	YTH PP1γ2 positives nº	chr	PP1 BM	Total of positives nº	Identified Homology		Function
					Start	Stop	
NW_001838467	3	18	unknown	5	294231	294515	Unknown
	187		unknown		294231	294651	
	107		unknown		294235	294595	
	205		unknown		294140	294538	
	150		unknown		294233	294560	
NW_001838111	231	14	unknown	2	13755462	13755004	
	232		unknown		13755462	13755162	
gi:37547157	288	1	unknown	1	79121	79556	
gi:157812171	253	10	unknown	1	20410909	20410523	
gi:51460741	267	2	unknown	1	284269	284641	
NW_001838476	293	19	unknown	1	3444952	3444586	
gi:15131325	90	9	unknown	1	19839696	19840046	
NW_001842414.1	7	X	unknown	1	118718	119039	
gi:51460714	177	2	unknown	1	51979010	51979347	
NW_001839072	295	7	unknown	1	7903476	7903151	
NT_033927	115	11	unknown	1	13127568	13127886	
NW_001838236	328	16	unknown	1	683515	683218	
NT_077812	178	19	unknown	1	1072831	1073201	
NW_001838339	168	16	unknown	1	1942902	1942613	
NW_001839071	6	7	unknown	1	3635765	3636178	
gi:51467153	309	8	unknown	1	31369594	31369992	

Table 8: List of mitochondrial clones.

Mitochondrial clones (e)				
Clone ID	YTH PP1γ2 positives nº	PP1 BM	Data base ID	Total of positives nº
F165 (PS72) mitochondrion,	100; 304	None	gi:32894605	2
IND23 mitochondrion	104;180	None	gi:78499271	2
NADH dehydrogenase subunit 4	111	KHMIW	gi:17981852	1

II.4. DISCUSSION

A variety of methods for detecting protein interactions have been developed, including the very common YTH System, or Affinity Purification coupled to Mass Spectrometry and, on a smaller scale, proteome chips (Auerbach *et al.*, 2002). They have yielded vast amounts of data that can be exploited to understand protein function and regulation. These methods together with the availability of sophisticated databases help to decode complex interactions among proteins and to integrate interacting proteins in complex cellular pathways. In this study to detect protein-protein interaction between PP1γ2, PP1γ2end, and protein encoded in a human brain library cDNA was used the YTH system.

All detection methods may yield false positive results. The reason for such results with the YTH system lies mainly in the principle of the screen; the assay investigates the interaction between overexpressed fusion proteins in the yeast nucleus. For example, overexpression can result in non-specific interactions. Also the inability of fusion proteins to migrate to the nucleus or if one of the transcription factor domains may occlude the site of interaction, may also lead to misleading results. Moreover, a mammalian protein is sometimes not correctly modified (e.g. glycosylated or phosphorylation), or folded in yeast calls, which can also lead to false results. Finally, some proteins might specifically interact when they are co-expressed in the yeast, but not in a physiological context, for example if they never were present in the same cell or the same subcellular environment at the same time. Therefore, once are identified two interacting partners by YTH method, the biological relevance of this interaction needs to be confirmed. Nevertheless, this method can provide an important first hint for the identification of interaction partners, as numerous studies to data have proven.

In the present two YTH screens were performed, one with the PP1γ2end as bait, and the other with the full length PP1γ2. Given the short sequence of the PP1γ2end, the results obtained were a small number of positives (19 positive clones), of which 15 encoded a known PP1 binding protein (RANBP9). The screening of human brain cDNA library with PP1γ2 resulted in 298 positive; of which 85.9% of the identified clones as known protein and 70.5% are potential new binding partners for PP1. May of these potential PP1 binding proteins have unclear function, others are involved in signaling pathways, transport, stress response, cytoskeleton, microtubules, metabolism, cell cycle,

apoptosis, and proteolysis. In this study is observable a vast interacting proteins each posses a variety of function, this could indicate that PP1 is involved in a variety of neuronal process, what is in agreement whit previous studies. Nevertheless, of the PP1 known neuronal binding proteins, were undetected probably because PP1γ2 it isn't the most abundant isoform in the brain and the expression pattern is very specific. However, some of the known PP1 binding proteins were detected what validate the results. Curiously the RANBP9 was the boost abundant protein detected, probably because was the most abundant in the library or because posses more affinity. The majority of the proteins identified posses the PP1 binding motif what validate the YTH.

In this screen, we also identified some cDNA clones that may represent new splice variants or new hypothetical proteins, but these results need further confirmation (not only the interaction with PP1γ2 but also the veracity of the hypothetical new splice proteins).

One possible approach to confirm the interaction result is to use the recombinant proteins and verify binding *in vitro* through a variety of biochemical assays such, as blot overlay. Another approach is to express both proteins in cells by transfection and analyse the interaction by immunoprecipitation studies. However, even if immunoprecipitation is successful, there is a possibility that the proteins only interact under the conditions used. Therefore, a crucial validation of the YTH results is to prove that the two proteins exist endogenously in the same subcellular environment by co-immunoprecipitation from tissue of interest. This means that all interactions med confirmation by a high confidence assay like co-immunoprecipitation of the endogenous proteins. However, this is a difficult task, especially for large-scale protein-protein interaction data.

Given the need to confirm the interaction of the proteins detected with the YTH assay with PP1 and since a rather large number of interacting proteins were detected, it was necessary to restrict our initial analysis to a couple of proteins of interest. To this end, the chose was made on the basis that the target proteins not having been previously related to PP1 and their involvements or relation to number of pathologies of interest. Thus, the proteins chosen for confirmation of interaction with PP1 2 were PINK1 (PTEN induced putative kinase 1) and ITM2C or BRI3 (Integral membrane protein 2C). Described in the next chapter are the analyses of the interactions of these proteins with PP1.

III. PINK1, BRI2 AND BRI3: NOVEL PP1 INTERACTING PROTEINS

III.1. INTRODUCTION

In these studies two of the identified PP1 interacting protein were chosen, for further analysis, namely PINK1 (PTEN induced putative kinase 1) and ITM2C or BRI3 (Integral membrane protein 2C). These were the chosen proteins because of their relative abundance among the identified positives and both contain a PP1 consensus RVXF binding motif. Beyond this, the decision was strengthened the by likely involvement of these proteins with related brain pathology, as shown for PINK1 with Parkinson disease. Although the BRI3 protein predominantly expressed in the brain, it is unknown to be involved with brain disease. However, BRI3 is highly homologous to BRI2, a protein that when mutated has been show to be the cause of two distinct pathologies: familial British (FBD) and Danish dementias (FDD). Thus, for this reason, and since BRI2 also shares the known consensus PP1 binding motif, BRI2 was also added to the current studies.

III.1.1 PINK 1

From the analyses by Unoki and Nakamura (2001) of the expression profiles of cancer cells after the introduction of exogenous PTEN, a tumor suppressor, an EST clone, was found derived from a gene encoding a serine/threonine protein kinase they named PINK1 (PTEN induced putative kinase 1). Further studies demonstrated that *PINK1* encodes a putative serine-threonine protein kinase, highly conserved in evolution, with proximately 75%-85% identity to the mammalian orthologues (Rogaeva *et al*, 2004). The Homo sapiens *PINK1* gene was mapped to chromosome 1 as the (Figure 17).

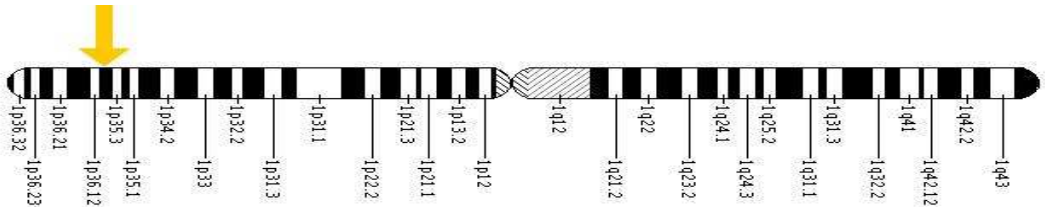

Figure 17: Cytogenetic location of the PINK1 gene. The PINK1 gene mapped to chr1p32-p36. (http://ghr.nlm.nih.gov/dynamicImages/chromomap/PINK1.jpeg).

Interns of genomic organization of the *PINK1* gene consist of eight exons and seven introns, with a transcript length of 2,660 bps (Figure 18).

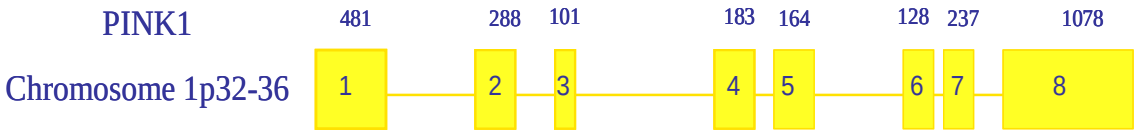

Figure 18: Genomic structure of the *PINK1* gene. The diagram illustrates the relative position of introns represented as a horizontal line and exons represented as solid boxes. The length of each exon is presented on top of the solid boxes.

The *PINK1* gene, also known as BRPK or PARK6, encodes a highly conserved putative serine/threonine protein kinase member of a small family of novel kinases. This gene encodes a 581 amino acid protein with a predicted isoelectric point of 9.46 and a high

value of hydrophobicity (Valente *et al*., 2004(a); Silvestri *et al*., 2005). The predicted molecular mass is 62.8 kDa, but several proteolysed forms of PINK1 with molecular masses ranging from 30 to 50 kDa also been reported. In fact, in human brain extracts and upon cellular overexpression two major PINK1 isoforms were detected with apparent molecular weights of 66 kDa and 55 kDa. The 55 kDa appears to be the mature form derived from 66 kDa form (Weihofen *et al*., 2008). The difference between the two isoforms is explained by cleavage at amino acid 77, giving rise to a smaller fragment by about 10 kDa at the N-terminus (Silvestri *et al*., 2005; Sim *et al*., 2006; Gandhi *et al*., 2006).

Some studies (Silvestri *et al*., 2005; Sim *et al*., 2006) classify PINK1 as an integral membrane protein due to the presence of a transmembrane domain at the position 101-107. However, Weihofen and colleagues detected that PINK1 is soluble in detergent-free buffer, giving rise to a still remaining controversy (Weihofen *et al*., 2008). On the N-terminus, the PINK1 protein has a 34 amino acid mitochondrial targeting motif (MTS). The C-terminus possesses a highly conserved protein kinase domain from residues 156 to 509, which shows a high degree of homology to the serine/threonine kinases of the calcium/calmodulin family. It is possible that the C-terminus has a role in the regulation of the autophosphorylation process, binding to specific sequences in a protein substrate and in turn directs the kinase domain to phosphorylate a specific site in the substrate (Valente *et al*., 2004 (a); Silvestri *et al*., 2005; Sim *et al*., 2006; Kitada *et al*., 2007). Figure 19 shows a schematic representation of the PINK1 protein.

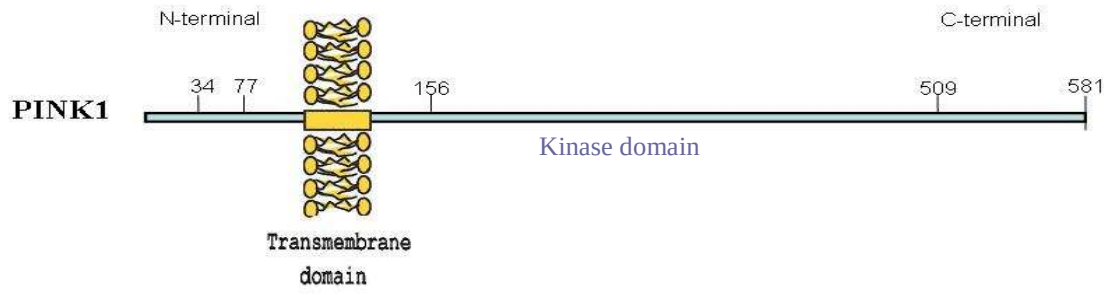

Figure 19: Topology of the PINK1 protein. The protein comprises a with the N-terminus mitochondrial signal of 34 amino acids (MTS-mitochondrial targeting sequence), a transmembrane domain at position 101 to 107 and a C-terminal serine/threonine kinase domain at position 156-509.

Since is thought that PINK1 as a mitochondrial protein encoded by nuclear genes, it is likely to be synthesized in the cytosol and then imported into the mitochondria in an unfolded configuration (Sim *et al*., 2006).

III.1.1.1. PINK1 expression pattern

It is generally accepted that PINK1 is located in the mitochondria, the energy-producing centers that provide power for cellular activities (Valente *et al*., 2004 (b); Silvestri *et al*., 2005; Beilina *et al*., 2005; Sim *et al.*, 2006). Further studies indicate that this protein is in the mitochondrial cristae, that the N-terminus of the PINK1 protein is sufficient for its mitochondrial targeting and that the C-terminus is probably exposed to the intermembrane space (Silvestri *et al*., 2005; Gandhi *et al*., 2006; Pridgeon *et al*., 2007). Other authors have demonstrated that the 66 KDa and the processed 55 KDa PINK1 forms are localized in the mitochondria, mainly to the inner and outer membrane fractions (Petit *et al*., 2005). However, PINK1 was also detected in cytoplasmic microsome-rich fractions, indicating that it is also present in other cellular organelles (Gandhi *et al*., 2006). It is unknown how both PINK1 isoforms end up in microsome-rich fractions that normally consist of fragments of plasma membrane and the endoplasmic reticulum (Gandhi *et al*., 2006; Weihofen *et al*., 2008). PINK1 is ubiquitously expressed, with highest expression levels in heart, skeletal muscle, and testis, and lower levels in brain, placenta, liver, kidney, pancreas, prostate, ovary and small intestine. If is also present in the embryonic testis from an early stage of development (Unoki and Nakamura, 2001). In the brain, PINK1 protein is detected in many brain regions including frontal cortex, temporal cortex, hippocampus, caudate, thalamus, hypothalamus, striatum, substantia nigra and cerebellum, with the level of expression of the cleaved PINK1 protein being about equal across these regions (Gandhi *et al.*, 2006).

III.1.1.2. PINK1 function

It is thought that PINK1 protein promotes cell survival, either by protecting against mitochondrial dysfunction during cellular stress, presumably by phosphorylating specific cellular proteins in the mitochondria, or by having an anti-apoptotic function (Valente *et al*., 2004(b); Petit *et al*., 2005; Pridgeon *et al*., 2007). Downregulation of PINK1 expression by small interfering RNA (siRNA) revealed decreased viability of SH-SY5Y cell and increased apoptosis (Bogaerts, *et al*., 2008). siRNA is widely used to silence gene expression in the study of gene functions and in the development of therapeutic strategies.

In spite of lower levels of expression in the brain, PINK1 protein is detected in many brain regions. Studies in neuronal cells treated with neurotoxins, such as rotenone and *N*-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), suggest that PINK1 can protect neuronal cells from undergoing apoptosis through an unknown mechanism (Petit *et al*., 2005; Sim *et al.,* 2006). These data suggest that PINK1 is neuroprotective since it protects neuronal cells against neurotoxins and stress-induced cell death. Other report verified that PINK1 has an essential and selective role in dopaminergic synaptic transmission in striatum. This is also supported by other study in Drosophila, which concludes that PINK1 has an important role in the survival of DA neurons (Wang, *et al* 2006). These results reveal a critical role for PINK1 in dopamine (DA) release, since in its absence impaired release of dopamine and consequent defects in both LTP and LTD were observed (Ibáñez *et al*., 2006; Kitada *et al*., 2007; Bogaerts *et al*., 2008).

The interactome of PINK1 is poorly known, but has been shown to include DJ-1, a protein that is similarly involved in sensing and protecting against oxidative stress (Tang *et al*., 2006). However, the significance of this interaction is unclear, since PINK1 and DJ-1 are normally localized to different cellular compartments within living cells. It was demonstrated that Cdc37 and Hsp90 also interact with PINK1 but the physiological relevance of this interaction remains to be determined. Cdc37 is a molecular co-chaperone that functions with Hsp90 to promote folding of many kinases, forming the Hsp90/Cdc37 chaperone system (Weihofen *et al*., 2008). Furthermore, it was shown that PINK1 binds and colocalizes in mitochondria of HeLa and PC12 cells, with TRAP1 (Pridgeon *et al*., 2007). This tumor necrosis factor receptor–associated protein 1 is a mitochondrial protein

of the family of molecular chaperones that facilitate protein folding, regulate a number of cellular processes including cell survival and apoptosis, and is involved in the maturation of a subset of protein involved in signal transduction. This cellular substrate contributes to strengthen the function of PINK1 as a protector against oxidative-stress-induced apoptosis and cell death. It seems likely that more mitochondrial proteins are regulated by phosphorylation by PINK1 and that, in the absence of functional PINK1, mitochondrial dysfunction leads to degeneration of neurons and subsequent clinical symptoms of Parkinson´s disease (PD) (Pridgeon *et al*., 2007). Recently, PINK1 was demonstrated to interact with HtrA2 and it seems that both proteins are components of the same stress-sensing pathway (Plun-Favreau *et al*., 2007).

In the search for elucidating how PINK1 mutation and/or loss-of-function contribute to Parkinson`s disease, several studies in Drosophila and mice (*in vivo*) have been carried out. In Drosophila, inactivation of PINK1 it leads to a Parkinson's-like phenotype, including shortened lifespan, apoptotic muscle degeneration, male sterility and defects in mitochondrial morphology (Bogaerts *et al*., 2008). Genetic experiments in Drosophila strongly suggest that PINK1 and Parkin (ubiquitin ligase protein encode by the PARK2 gene) are related at a biochemical level (Weihofen *et al*., 2008). On the other hand, studies with RNAi, silencing *PINK1* gene expression in mice did not appear to cause obvious neuronal death and did not induce Parkinson's disease phenotypes (Kitada *et al*. 2007; Zhou *et al*., 2007). Further studies might uncover novel protective roles for PINK1 in dopaminergic neurons, or provide insights into why these cells are more highly dependent upon PINK1 function in the mitochondria. However, PD-related mutations and an artificial kinase-dead mutant abolished the protective effect of PINK1 (Petit *et al*. 2005). Nevertheless, the *in vivo* substrate(s) and biochemical function of PINK1 remain largely unclear, and no animal models have yet been reported with PINK1 mutations, but PINK1-deficient animals and cells should be valuable for identifying the cellular targets of PINK1 and mapping their functional pathways.

III.1.1.3. PINK1 related disease

Mitochondrial dysfunction have been related to several neurodegenerative conditions in the last decade; such as late-onset sporadic Parkinson`s disease (PD) (Silvestri *et al*., 2005; Abahuni *et al*., 2007). PINK1 provides a direct molecular link between mitochondria and the pathogenesis of PD being the only gene product directly targeted to the mitochondria to have a protective effect on the cell. Protecting against mitochondrial dysfunction and anti-apoptotic function, suggests that altered phosphorylation of one or more target proteins by PINK1 might be involved in the neuronal degeneration of PD (Petit *et al*., 2005; Silvestri *et al*., 2005). In fact, loss-of-function associated with mutations of the PINK1 protein (Figure 20) are the cause of autosomal recessive early-onset Parkinson disease 6 (PARK6) (Valente, *et al.*, 2004 (b); Hoepken, *et al*., 2007). Homozygous mutations have been unequivocally associated with early-onset Parkinson disease, and heterozygous mutations are rather common among early-onset (<50) PD patients. Genetic analysis revealed that heterozygous mutations, affecting only one PINK1 allele is associated with late-onset PD or might increase the risk of developing Parkinson's disease (Schlitter *et al*., 2005; Hedrich *et al*., 2006; Kitada *et al*., 2007). After the nonsense W437X and missense G309D mutations, the first mutations detected in PINK1 in familial PARK6 cases, more than twenty *PINK1* mutations have now been identified that cause early-onset Parkinson (EOP) disease, with or without family history (Valente *et al*., 2004 (b); Sim *et al.*, 2006).

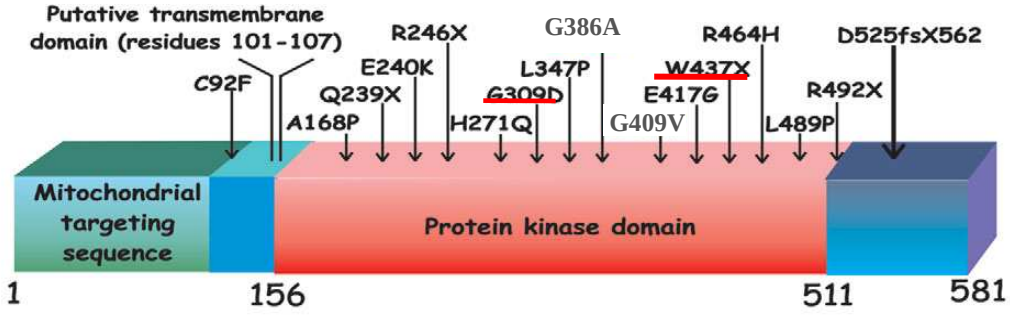

Figure 20: Localization of PINK1 mutation. The positions of missense mutations (single-letter code) associated with familial forms of PD are indicated by each arrow. Non-sense mutations are indicated by X, which denotes the introduction of a premature stop codon. Frame shift mutation is indicated by fsX.

Underlines in red are the two first detected mutations. All but two of the PD associated mutations map to the protein kinase domain of PINK1 (Sim *et al.*, 2006).

Many of these mutations alter or eliminate the kinase domain, probably affecting either kinase activity or substrate recognition, and leading to a loss of protein function. At least one mutation affects the mitochondrial-targeting motif and may disrupt delivery of PINK1 to the mitochondria. With reduced or absent PINK1 activity, mitochondria may malfunction, particularly when cells are stressed (Klein *et al*., 2006; Sim *et al.,* 2006; Kitada *et al*., 2007). The discovery that mutations in the PINK1 gene could result in Parkinsonism (Valente *et al*., 2004 (b); Rogaeva *et al*., 2004; Hedrich *et al*., 2006) was the first evidence that a kinase signalling pathway may be important in the pathogenesis of dopaminergic nigral cell death (Gandhi *et al.*, 2006). Until now it is unclear how *PINK1* mutations cause the selective death of nerve cells that characterizes Parkinson disease, and the pathogenic mechanisms by which PINK1 mutations lead to neurodegeneration are still unknown.

Parkinson disease (PD) is a complex, multifactorial disorder that typically manifests after the age of 50 years, although early-onset cases (before 50 years) are known. PD generally arises as a sporadic condition but is occasionally inherited as a simple Mendelian trait. Although sporadic and familial PD is very similar, inherited forms of the disease usually begin at earlier ages and are associated with atypical clinical features. PD is characterized by bradykinesia, resting tremor, muscular rigidity and postural instability, as well as by a clinically significant response to treatment with levodopa. The pathology involves the loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies (intraneuronal accumulations of aggregated proteins), in surviving neurons in various areas of the brain. Table 9 list the genes and proteins implicated in PD.

Table 9: Chromosomal loci and specific genes implicated in inherited Parkinson´s disease.

Locus	Chr	Protein	Protein function	PD inheritance
PARK1	4	α-Synuclein	?	Dominant
PARK2	6	Parkin	Ubiquitin ligase	Recessive
PARK3	2	?	?	Dominant
PARK4	4	A –Synuclein	?	Dominant
PARK5	4	UCH-L	Ubiquitin ligase, ubiquitin hydrolase	Dominant
PARK6	**1**	**PINK1**	**Mitochondrial kinase**	**Recessive**
PARK7	1	DJ-1	Chaperone? Mitochondrial kinase?	Recessive
PARK8	12	LRRK2	?	Dominant
PARK9	1	?	?	Recessive
PARK10	1	?	?	?
PARK11	2	?	?	?

UCH-L1, ubiquitin C-terminal hydrolase–L1; PINK1, PTEN-induced putative kinase–1; LRRK2, leucine-rich repeat kinase–2; and "?" nknown.

III.1.2 BRI family

The *BRI2* and *BRI3* genes belong to a multigene family of type II transmembrane glycoproteins, with an extracellular C-terminus and a cytosolic N-terminus. This family includes at least three different members, *BRI1*, *BRI2* and *BRI3*, commonly denominated as *ITM2A*, *ITM2B* and *ITM2C* or *E25A*, *E25B* and *E25C*, respectively (Deleersnijder *et al*., 1996; Pittois *et al*., 1999; Rissoan *et al*., 2002; Choi *et al*., 2001; Vidal *et al*., 2001; Rostagno *et al*., 2005). Homology searches of expressed sequence tags (EST) indicate the existence of BRI_{1-3} genes in rat, monkey, chicken, rabbit, pig and horse, and in non vertebrate animals; including flies and worms. The three genes are homologues in mouse and humans, with similar genomic organization, in fact mouse *BRI3* gene shares 92.9% amino acid sequence identity with the human gene. These observations suggest that the BRI_{1-3} genes are part of an evolutionarily conserved gene family (Vidal *et al*., 2001; Rostagno *et al*., 2005). The genomic organization of the BRI family consists of six exons and five introns as represented in Figure 21. The first intron is remarkably longer in

comparison with the other four, and may contain regulatory sequences important for transcription (Pittois *et al*., 1999; Vidal *et al*., 2001; Rostagno *et al*., 2005). The BRI_{1-3} genes were mapped by fluorescence *in situ* hybridization (FISH), to different chromosome. The human *BRI1* gene is located in chromosome X (Pittois *et al*., 1999), *BRI2* in the long arm of chromosome 13 (Vidal *et al*., 1999) and *BRI3* in chromosome 2 (Vidal *et al*., 2001).

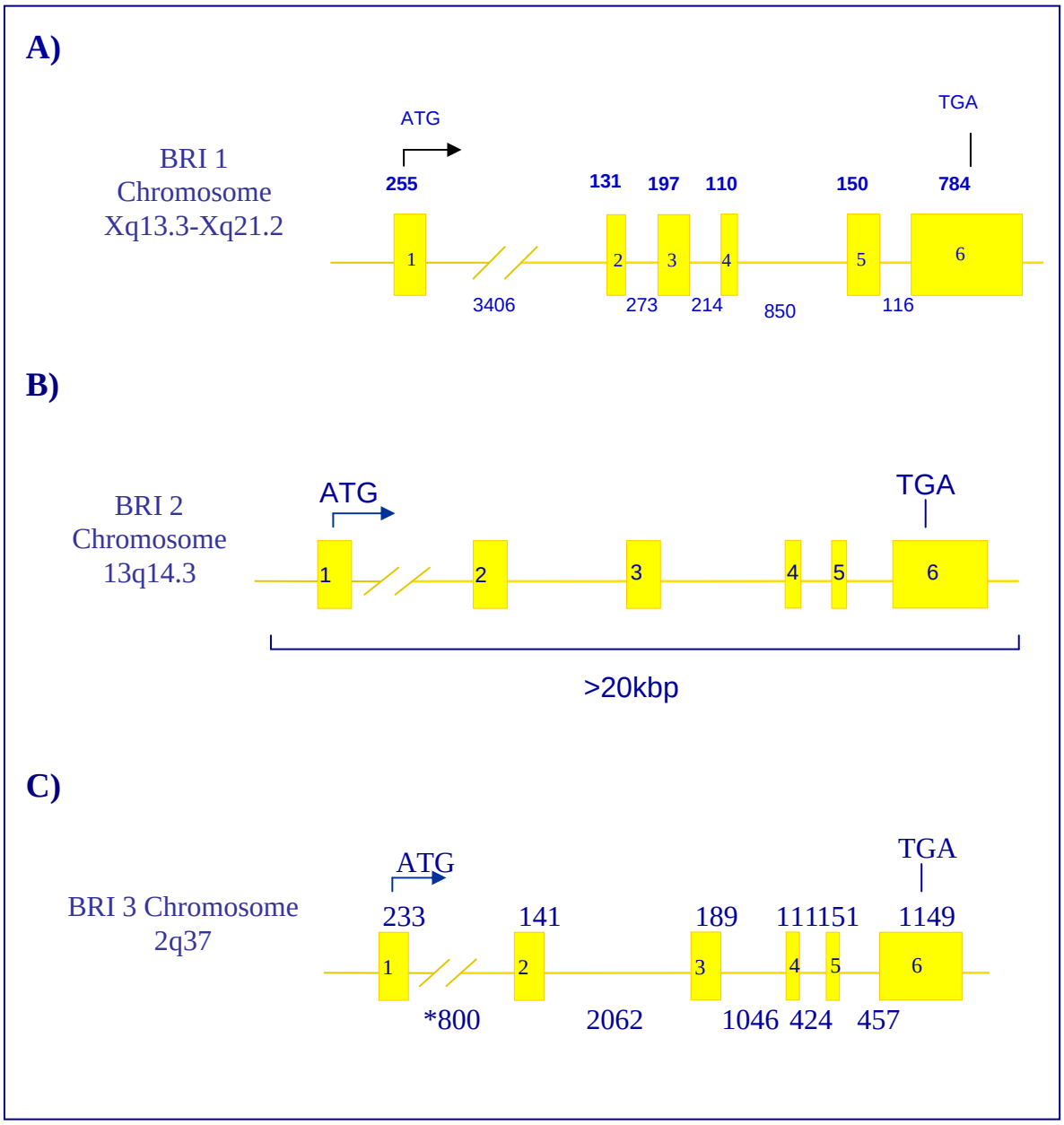

Figure 21: Genomic structures of the human BRI_{1-3} genes. A) *BRI1* gene, B) BRI2 gene and C) *BRI3* gene. Introns and exons are represented in black line and solid boxes, respectively. Exons sizes as base pairs are indicated above the number of the exons and the intron lengths are denoted below. *Approximate length.

The BRI_{1-3} genes encode homologous, as shown by sequence alignment in the (Figure 22).

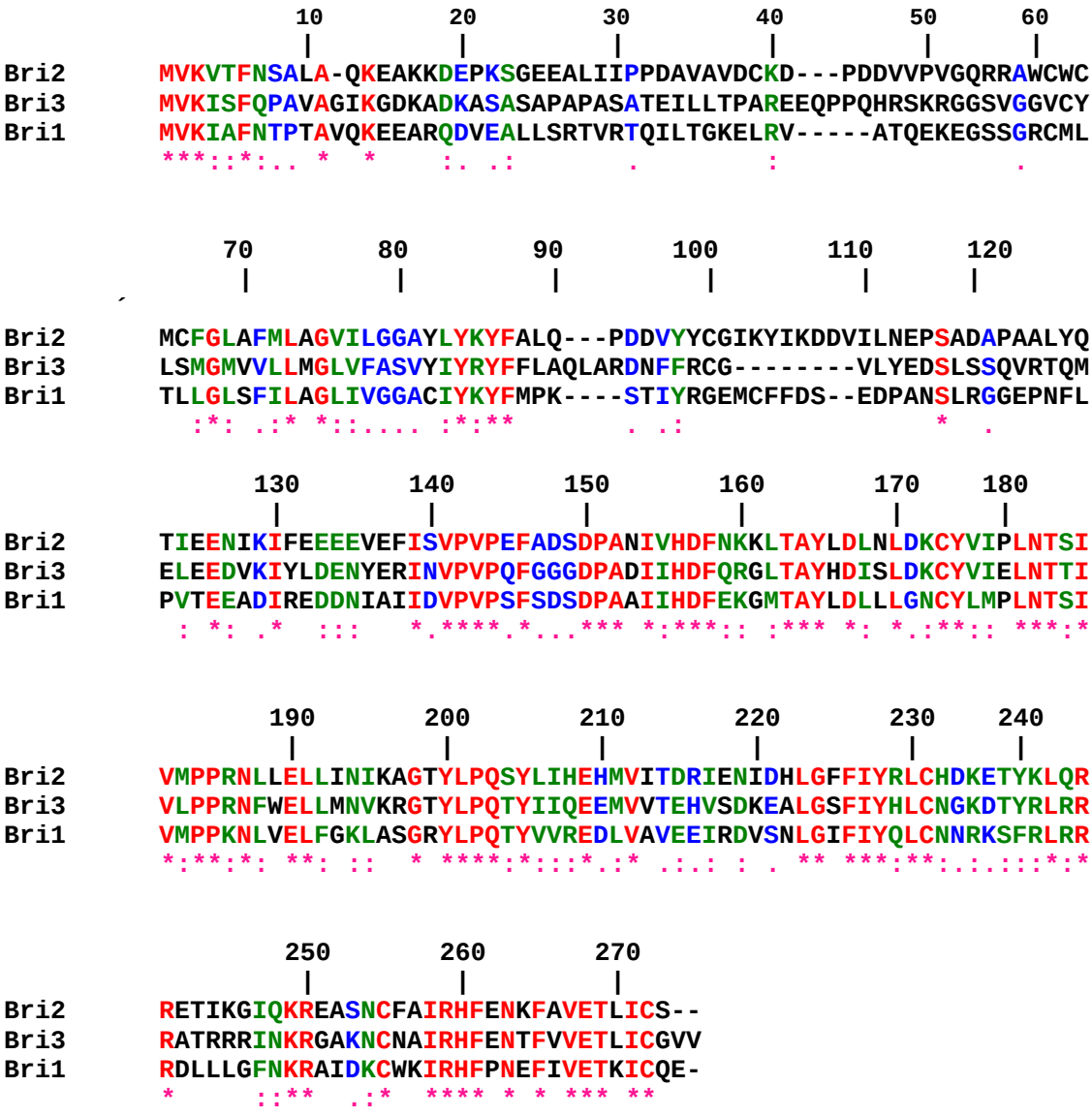

Figure 22: Amino acid sequence alignment of the three BRI proteins. Bri2 has 266 residues, Bri3 has 267 residues and Bri1 has 263 residues. This alignment was obtained through the bioinformatics tool clusterW2 (http://npsa-pbil.ibcp.fr/cgi-bin/align_clustalw.pl). (*) amino acid identify; (:), conservative amino acid change; (.) eanty conservative change.

III.1.2.1. THE BRI2 PROTEIN

The protein product of the *BRI2* gene is a polypeptide of 266 amino acids, with a molecular mass of 30.3 KDa and a theoretical isoelectric point (pI) of 4.86. It contains nine cysteine residues and is highly rich in leucine (9.02%) and isoleucine (9.02%) (Vidal *et al*., 2000). The BRI2 protein has a predicted structure of a type II transmembrane glycoprotein since it possess an intracellular N-terminal followed by a 53-75 amino acids transmembrane domain and an extracellular C-terminus of 192 amino acids (Vidal *et al*., 2001). Studies demonstrated a single N-glycosylation site in the C-terminus at the position 170 is and, at position 244 (…KGIQKR^{243} ↓ EA..) the BRI2 protein is processed by pro-protein convertase furin, with a consequential release of a short C-terminal peptide (Figure 23) (Vidal *et al*., 2001; Wickham *et al*., 2005; Rostagno *et al*., 2005).

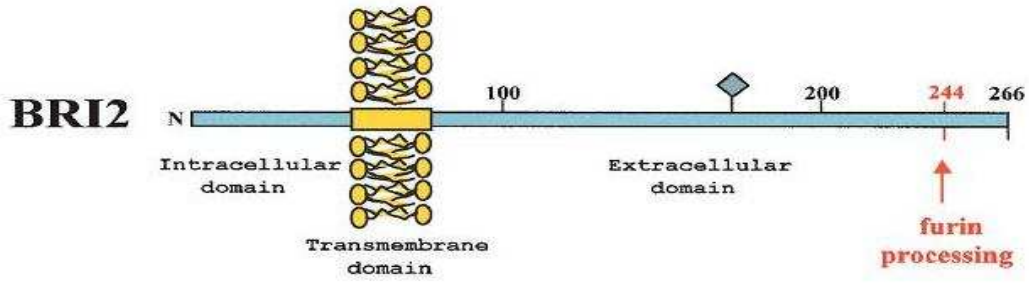

Figure 23: Membrane topology of the protein BRI2. The intracellular N-terminus, a transmembrane domain and the extracellular C-terminus are shown. The diamond at position 170 stands for N-glycosylation site. In position 244 is marked the furin-like pro-protein convertase site.

III.2.1.2.1. BRI2 expression pattern

Interns of their expression patter, *BRI2* is highly ubiquitous, while *BRI1* and *BRI3* show a limited expression profile. *BRI1* is expressed in chondrogenesis, osteogenesis and T-cell development in mouse (Wickham *et al*., 2005) and is highly expressed in the muscle (Choi *et al*., 2001). The *BRI2* gene is expressed in peripheral organs, with high levels of

expression in heart, kidney, pancreas, liver, placenta, and in the brain where it is ubiquitously present in white and gray matter but shows more abundant distribution in the hippocampus and cerebellum compared with the cerebral cortex (Vidal *et al*., 2001; Choi *et al*., 2001). Studies demonstrated that in cultured human brain cells the *BRI2* mRNA is widely distributed in different cerebral cell populations, neurons, astrocytes and microglial cells, as well as in smooth muscle and cerebral endothelial cells (Rostagno *et al*., 2005).

III.2.1.2.2. BRI2 function

The function of *BRI2* remains largely unknown. Even so, it is possible to associate *BRI2* with evidence that might give an indication of its function. For example, the gene locus in the chromosome 13q14 is frequently associated with tumor-related mutations in primary prostate cancer. It is one of the only six genes 13q14 that show significantly reduced levels in prostate tumors compared with normal tissues and low expression in prostate tumor cell lines as well as evidence of allelic loss at the same locus in other cancers. Consequently, *BRI2* has been proposed to be a tumor suppressor gene (Vidal *et al*., 2004; Rostagno *et al*., 2005). Its presence in various neurodegenerative conditions, along with its axonal localization in transfected neurons suggest a role in transport along neuronal processes, making BRI2 a potential candidate for protein-protein interaction with other molecules related to neurodegeneration, including membrane proteins like the amyloid precursor protein (APP). In fact BRI2 interacts with APP, forming a molecular complex in cellular membranes and it is thought that BRI2 plays a strong regulatory role in the processing of APP (Matsuda *et al*., 2008; Fotinopoulou *et al,* 2005; Tsachaki *et al*., 2008b). Fleischer and colleagues identified alternatively spliced variants that originate long and short BRI2, the short form lacking exon 1 and is associated with the mitochondria (Fleischer *et al*., 2002a). They demonstrated that the alternatively spliced short BRI2 short form acts as proapoptotic protein and interacts with Bcl-2, whereas the long BRI2 form does not induce apoptosis (Fleischer *et al*., 2002b).

III.2.1.2.3. BRI2 related disease

Until now, it was demonstrated that BRI2 interacts with APP, (known to be involved in AD) and regulates its processing, and two cases of *BRI2* mutations have been reported, which cause familial British (FBD) and Danish dementias (FDD) that share clinical and pathological features with AD (Matsuda *et al.*, 2005; Matsuda *et al.*, 2008; Ghiso *et al*., 2006; Tsachaki *et al*., 2008b). The first description of FBD occurred in 1933 but its classification remained uncertain until 1999 when it was considered an example of atypical AD (Vidal *et al*., 2004; Tomidokoro *et al*., 2005). FBD is an autosomal dominant disease characterized by progressive dementia, spasticity and cerebellar ataxia, with onset at around the fifth decade of life (Vidal *et al*., 2000; Vidal *et al*., 2001; Vidal *et al*., 2004; Rostagno *et al*., 2005). Familial Danish dementia is an early-onset autosomal dominant disorder originating in the Djursland peninsula in Denmark, identified in nine cases spanning three generations of a single family. Cataracts seem to be the early manifestation of the disease, starting before the age of 30. FDD is characterized by cataracts, deafness, progressive ataxia, and dementia (Vidal *et al*., 2004). FBD patients have a point mutation TGA to AGA at stop-codon 267 of the *BRI2* gene, resulting in a longer reading frame of 277 amino acids (Vidal *et al*., 2000; Vidal *et al*., 2001; Vidal *et al*., 2004; Rostagno *et al*., 2005). The amyloid protein ABri is encoded by the last 34 amino acid of the mutated BRI2 gene (Figure 24) The FDD patients have a ten nucleotide duplication 795-796 insertion TTTAATTTGT between codons 265 and 266, just before the normal stop-codon 267 (Figure 24 and 25). The decamer duplication mutation produces a frame-shift in the BRI2 sequence generating a larger-than-normal precursor protein of 277 amino acids, of which the amyloid ADan comprises the last 34 C-terminal amino acids (Vidal *et al*., 2000; Vidal *et al*., 2001; Vidal *et al*., 2004; Rostagno *et al*., 2005).

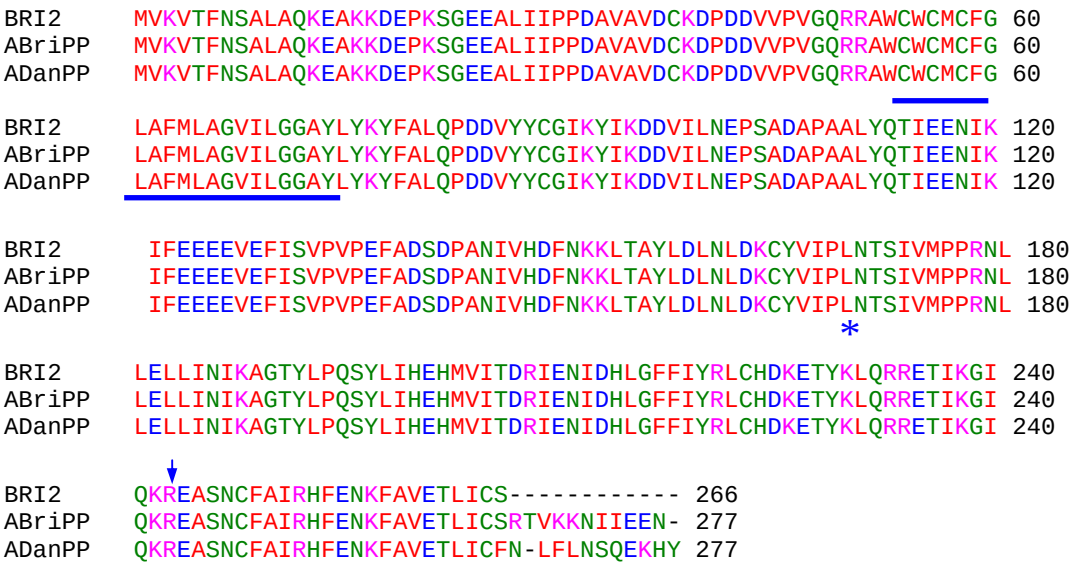

Figure 24: Alignment of the amino acid sequence of wild-type and mutant BRI2 proteins. The sequence of the transmembrane domain (TM) is underlined. The single N-linked glycosylation site at position 170 is indicated by an asterisk (*). The arrow indicates the cleavage site between amino acids 243 and 244 that releases the carboxyl-terminal peptides. This alignment was obtained through the bioinformatics tool clusterW2.

ABri and ADan are cleavage products of the mutated forms of BRI2 protein cleave by furin, a ubiquitous endopeptidase, immediately before the amyloid peptide N-terminus. The amyloid peptide cause neuronal dysfunction and dementia (Vidal *et al*., 2001; Vidal *et al*., 2004; Rostagno *et al*., 2005). The two amyloid peptides have similar characteristic. The amyloid precursor proteins in the British kindred (ABriPP) and in the Danish kindred (ADanPP) have the same length of 277 amino acids. ABri and ADan peptides also have the same length and share identical N-terminal amino acid sequence (first 22 residues) both have secretory pathway endoprotease and, exhibit heterogeneity at the N- and C-terminal ends as shown in Figure 25. The brain lesions in FDD are neuropathologically similar but not identical to the lesions observed in patients with FBD.

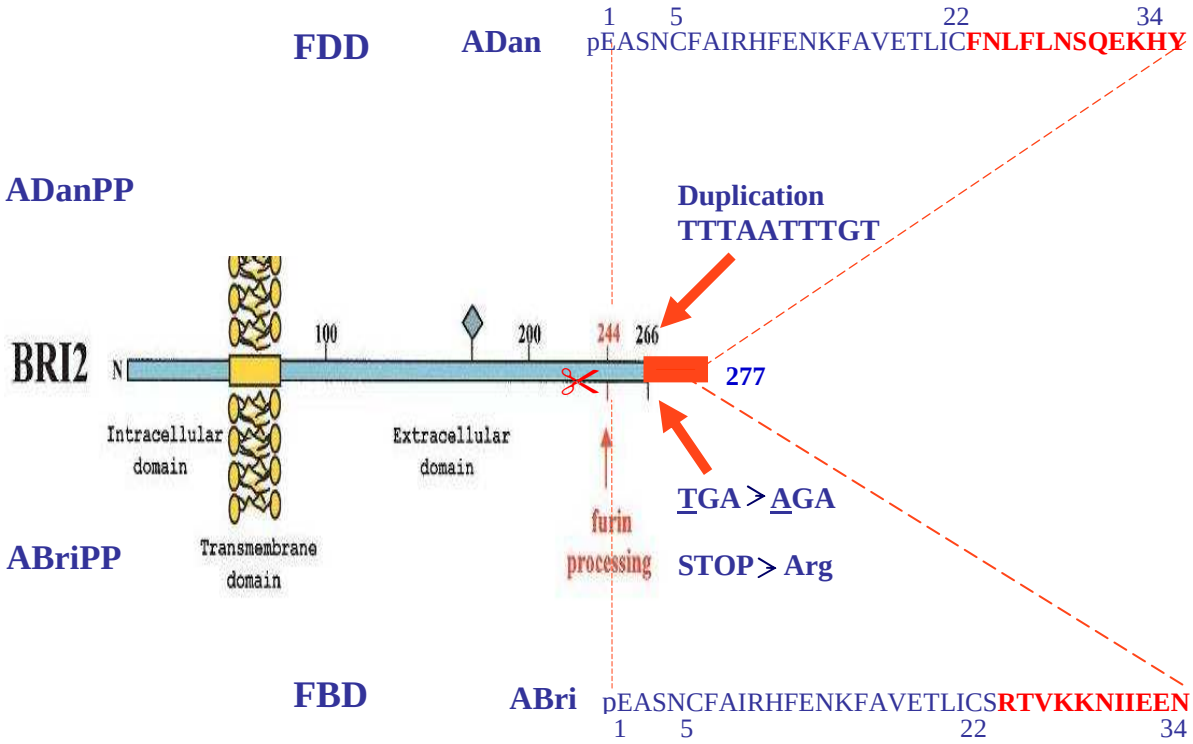

Figure 25: Genetic defects in *BRI2* and genesis of the ABri and ADan precursors in patients with FBD and FDD. Schematic representation of genetic defects in the *BRI2* gene: stop-to-Arg at codon 267 in FBD (bottom) and a 10-nucleotide duplication insertion after codon 265 (top) generate longer-than-normal precursors ABriPP and ADanPP (positions 266–277). Furin-like pro-convertase activity proteolytically processes both extended precursors at peptide bond 243–244, generating ABri and ADan C-terminal fragments composing amyloid deposits in FBD and FDD. ABri and ADan are both 34 amino acids long, identical in their first 22 residues and completely different in their 12 C-terminal amino acids (bold). They contain a single disulphide bond between cysteine residues 5 and 22 (Gibson *et al*., 2005).

However, ABri and ADan peptides are generated by the same proteolytic mechanism and their precursor proteins share identical patterns of expression. Possibly, it is the carboxyl-terminal amino acid sequence that determines the biochemical properties of the peptides, their ability to aggregate, deposit, and cause neuronal dysfunction (Vidal *et al*., 2004).

III.1.2.2. THE BRI3 PROTEIN

The human *BRI3* gene encodes a polypeptide of 267 amino acids, 43.7% identical to the sequence of human BRI2 and 38.3% identical to human BRI1, with the highest percentage of amino acid identity being concentrated on the C-terminal half of the molecules. The polypeptide encode by the *BRI3* gene has a molecular weight of 30,482KDa and a theoretical pI of 8.47, and it is rich in leucine (8.92%), arginine (8.55%), valine (8.17%), and alanine (7.43%) residues (Pittois *et al*., 1999; Vidal *et al*., 2001; Rostagno *et al*., 2005). As previously mentioned, the BRI3 protein is a type II integral transmembrane protein, with a short N-terminal cytoplasmic tail of 60 amino acids, followed by a 23 amino acids transmembrane domain and a 186 amino acid large extracellular C-terminus (Figure 26).

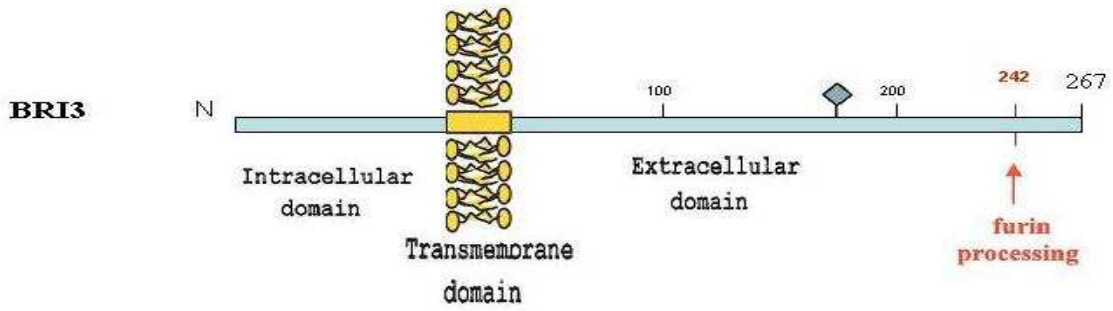

Figure 26: Membrane topology of the BRI3 protein. The intracellular N-terminus, a transmembrane domain and the extracellular C-terminus are shown. The diamond at position 170 under is the N-glycosylation site and amino acid 242 marks the cleavage by furin.

Analogous to the BRI2 cleavage by furin, BRI3 is also processed by furin and less so by other members of the pro-protein convertase family. This cleavage occurs within the sequence …**R**RIN**KR**^{242}↓GA… which contains P1 (**R**), P2 (**K**) and P6 (**R**) basic residues characteristic for cleavage by furin-like enzymes. The furin processing occurs in the TGN (*Trans* Golgi network) or post-TGN compartments, where furin is active, generating N- and C-terminal polypeptides that might be disulfide linked (Wickham *et al*., 2005).

III.2.1.2.4. BRI3 expression pattern

BRI3 is localized predominantly in the brain, with the highest levels of expression in cerebral cortex, medulla, amygdale, hippocampus, thalamus, striatum, caudate nucleus and spinal cord (Wickham *et al*., 2005; Rostagno *et al*., 2005). *BRI3* is also expressed in plasmacytoid dendritic cells, granulocytes, bone marrow and fetal liver (Rissoan *et al*., 2002).

III.2.1.2.5. BRI3 function

The function of BRI3 remains largely unknown. To date no mutations have been identified and little is known concerning interacting proteins. BRI3 protein colocalizes and co-immunoprecipitates with the membrane associated *b*-amyloid converting enzyme 1 (BACE 1), verified in neurons from normal human brain and samples from brain of patients with AD. This interaction is between the N-terminal cytosolic tail of the type II membrane protein BRI3 and the C-terminal cytosolic tail of the type I membrane protein BACE1 (Wickham *et al*., 2005). BACE1 participates in the amyloidogenic pathway that generates the Alzheimer's A*β* peptide, and is a substrate for furin. It seems that furin generated N- and C-terminal fragments of BRI3, may have their own independent biological functions (Wickham *et al*., 2005; Gong *et al*., 2008). Further studies are needed to improve our understanding and knowledge of the BRI3 protein.

III.2.1.2.6. BRI3 related disease

BRI3 interacts with BACE 1 but whether this has any implications in disease pathogenesis remains unknown (Wickham *et al*., 2005). Contrary to BRI2, no similar mutation of the stop codon or any form of mutation has yet been reported for BRI3. Database scans for possible functional roles of BRI proteins revealed that they shared BRICHOS domain as mention before, which is found in a variety of proteins implicated in dementia, respiratory distress and cancer, but further studies are needed to verify the involvement of BRI3 in any of these disease (Sánchez-Pulido *et al*., 2002).

III.2. MATERIALS AND METHODS

III.2.1. Construction of recombinant expression vectors for PINK1, BRI2 and BRI3

Three proteins (PINK1, BRI2 and BRI3) were selected for further confirmation of the interaction with PP1γ2. Since none of the positive clones identified contained the full coding sequence, the necessary cDNAs were obtained from the Geneservice- I.M.A.G.E. consortium. The IMAGE PINK1, BRI2 and BRI3 cDNAs were totally sequenced, to ensure the correctness of the expressed sequence. To confirm the interaction with PP1γ2, the coding sequences were amplified with a forward primer including an EcoRI sequence, and a reverse primer with an XhoI site (see appendix). These enzymes were preferred because they do not digest the coding sequence and could be used to insert all three in the different cloning vectors: pACT2, pCMVMyc and pET28C. To confirm the correctness of the sequences all constructs were fully sequenced. The constructs pACT-2PINK1, pACT-2BRI2 and pACT-2BRI3 were used to co-transform yeast, thus allowing a first confirmation of the interaction between the proteins and PP1γ2. The pCMVMyc construct were used for co-immunoprecipitation and immunocytochemistry analysis. The pET28C constructs were used to express the recombinant proteins in *E.coli Rosetta* cells (Table 10).

Table 10: List of vectors and restriction enzymes used the antibiotic resistance and the corresponding application.

Vector	PINK1	BRI2	BRI3	Antibiotic Resistance	Application
pACT2	EcoRI /XhoI	EcoRI /XhoI	EcoRI /XhoI	Ampicillin	Co-transformation
pET28C	EcoRI /XhoI	EcoRI /XhoI	EcoRI /XhoI	Kanamycin	Expression in Rosetta cells
pCMV Myc	EcoRI /XhoI	EcoRI /XhoI	EcoRI /XhoI	Ampicillin	co-immunoprecipitation, immunocytochemistry analysis

III.2.1.1. Growth and purification of the IMAGE PINK1, BRI2 and BRI3 plasmids

The IMAGE cDNA were transformed into *E. coli* and plated according the suppliers' instructions. A single bacterial colony for each clone (PINK1, BRI2 and BRI3), was transferred into 3 ml of LB with Ampicillin or Kanamycin medium according to the antibiotic resistance, and incubated overnight at 37ºC with shaking at 220 rpm. Then, 0.25 ml of this culture was used to innoculate 250 ml of medium LB with Ampicillin or Kanamycin and grown overnight (16 hr) at 37ºC with shaking at 220 rpm. The cell culture were centrifuged at 10,000 × *g* for 10 min, the supernatant was discarded and the pellet was resuspend in 6 ml cell resuspension solution by gently inverting the tube several times. After incubation for 3 min at room temperature 6 ml neutralization solution was added to the lysed cells, and mixed by gently inverting the tube.

The prepared lysate was transferred into a Pure Yield Clearing Column in a 50 ml disposable Falcon, left for 2 min to allow the cellular debris to rise to the top, and centrifuged at 1,500 × *g* for 5 min. To bind the DNA, the eluate was transferred to a Pure Yield Binding Column in a new 50 ml disposable Falcon and centrifuged at 1,500 × *g* for 3 min. Then, 5.0 ml of endotoxin removal wash solution was added and centrifuged at 1,500 × *g* for 3 min. The flow-through was discarded and 20 ml column wash solution was added and centrifuged at 1,500 × *g* for 5 min. The eluate was discarded and the column centrifuged at 1,500 × *g* for an additional 10 min to ensure the complete removal of ethanol. To elute the DNA, the binding column was placed in a new 50 ml Falcon tube, 600 µl of nuclease-free water were added, and centrifuged at 1,500 × *g* for 5 min. The eluate was then transfered to a 1.5 ml microtube. The DNA concentration was measured by measuring its OD at 260 nm in a spectrophotometer and stored at -20ºC.

III.2.1.2. Subcloning of the coding sequence for PINK1, BRI2 and BRI3 in different vectors

III.2.1.2.7. PCR Amplification

The PINK1, BRI2 and BRI3 coding sequences were amplified by PCR in 0.2 ml microtubes by adding 10X reaction buffer, a mixture of the four dNTPs, two specific

primers (see appendix III-Primers) and the corresponding DNA template (Table 11). The high fidelity Pfu DNA polymerase was used in order to minimize the introduction of nucleotide changes.

Table 11: PCR amplification conditions.

Components	**Concentration**
Template	100 ng/µl
FW primer	10 pmol
RV primer	10 pmol
10x Pfu buffer	5µl
dNTP's	2 µl (10 mM)
Pfu DNA polymerase	1 µl
H_2O to final volume of	50 µl

Depending on the DNA template and the specific primers used, the PCR temperatures were adjusted (eg. Table 12). To verify the results following amplification, 5 µl of sample were added to 2 µl loading buffer and the mix was loaded onto 1% agarose gel containing eithidiun bromide. Marker DNA (1 kb ladder) of known size was also loaded on the gel. The gel was run at 100 V for 20 min and examined by UV light and photographed or analysed on a Molecular Imager (Biorad).

Table 12: Example of amplification conditions for the different plasmid DNA. These conditions were optimizing for each plasmid DNA.

Temperature	Extent	Number of cycle
95ºC	2min	1
95ºC	30sec	25 to 35
64ºC	20sec	
72ºC	3min	
72ºC	10min	1
4ºC	∞	1

III.2.1.2.8. DNA digestion with restriction enzymes

For a typical DNA digestion the restriction enzymes manufacturer's instructions were followed. In a microtube were mixed 100 µg/ml DNA, 1x reaction buffer (specific for each restriction enzyme), and 1 U/µg DNA of restriction enzyme. The mixture was incubated at the appropriate temperature for a few hours (or overnight if convenient). The restriction enzymes contribute less than 10% of the final volume. When sequential digestions with different enzymes were carried out the DNA was purified between the two reactions.

III.2.1.2.9. DNA purification

To purify DNA after restriction digestion, the mixtures were run on Agarose Ultra Pure gels. After localizing the desired DNA band on the gel using a long-wavelength UV lamp, it was excised using a sharp scalpel and transferred to a clean, pre-weighted microtube. To extract the DNA from the agarose a DNA Purification kit (QIAGEN) was used.

III.2.1.2.10. Ligation of vector and insert DNA

The vectors and the corresponding DNA fragment to be inserted were digested with the appropriate restriction enzymes (Table 10). Digested fragments were separated by gel electrophoresis and purified from ultra pure agarose, as described. Then, 0.04 µg of vector DNA were transferred to a microtube with an equimolar amount of insert DNA, 2 µl of T4 DNA ligase buffer (10x), 1 µl T4 DNA ligase and H_2O to a total volume of 20 µl. Two additional control reactions were set up that contained the plasmid vector alone with or without T4 DNA ligase. The microtubes were incubated overnight at 16ºC to allow the ligations to occur. The procedures to confirm the correctness of the insert DNA was then carried out by transformation of *E. coli XL1-Blue*, isolation of the plasmid DNA, restriction fragment analysis and complete sequencing of each plasmid (methods II).

III.2.2. Yeast Co-Transformation Procedure by Lithium Acetate

A single colony of the yeast PJ694A was inoculated in 1 ml of YPD and grown overnight at 30˚C and 250 rpm. Then, the overnight culture was transferred to a flask containing 50 ml of YPD and incubated at 30°C for 16–18 hr with shaking at 250 rpm to stationary phase (OD600>1.5). From the overnight culture, 15 ml were transferred to a new flask containing 300 ml of YPD and incubated at 30°C for 3 hr with shaking at 230 rpm, until the OD_{600} was between 0.4- 0.6. The cells were pelleted by centrifugation at 1000 x g for 5 min at room temperature, the supernatants discarded and the pelleted was thoroughly resuspended in 25 ml sterile distilled H_2O, and centrifuged at 1,000 x g for 5 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in 1.5 ml of freshly prepared, sterile 1x TE/1x LiAc. For the co-transformation assays we used 0.1 µg of pACT2PINK1 or pACT2BRI2 or pACT2BRI3 and pAS2PP1γ1 or pAS2PP1γ2 or pAS2PP1γ2end together with 0.1 mg of carrier DNA (salmon sperm). 100 µl of competent yeast cells were added to each tube and vortexed. Then, 0.6 ml of sterile PEG/LiAc solution was added to each tube and vortexed at high speed for 10 sec to mix. The cells were incubated at 30°C for 30 min with shaking at 200 rpm. Afterwards, 70 µl of DMSO were added and mixed by inverting. The cells were transformed by heat shock for 15 min

at 42˚C, chilled on ice for 2 min and centrifuged for 30 sec at 14 000 rpm at room temperature. The supernatant was removed, and the cells were resuspend in 500 µl of sterile 1x TE. The cells were plated on SD/QDO plates and incubated at 30˚C from 2- 4 days. A single colony was picked from each plate and streaked on a SD/QDO/X-α-GAL (80 mg/l) plate. A positive control with pTDT and pVA3 (two plasmids encoding known binding proteins) and a negative control with two empty vectors were also included.

III.2.3. Expression and analysis of recombinant PINK1, BRI2 and BRI3 in eukaryotic cells

III.2.3.1. Preparation and Lysis of Bacterial Cell Cultures

A single bacterial colony transformed with pCMVMycPINK1, or pCMVMycBRI2, or pCMVMycBRI3, was transferred into 3 ml of LB/amp medium and incubated overnight at 37ºC with shaking at 220 rpm. Then, 0.5 ml of this culture was used to innoculate 500 ml LB/amp medium and incubated overnight (16 hours) at 37ºC with shaking at 220 rpm. The cultures were centrifuged at 5,000 × *g* for 10 min in a room temperature rotor. The supernatant was discarded and the pellet resuspended in 15 ml of Cell Resuspension Solution, 15 ml of Cell Lysis Solution was added and gently mixed by inverting. After adding 15 ml Neutralization Solution and immediately mixing by gently inverting the centrifuge bottle several times, the mix was centrifuged at 14,000 × *g* for 15 min at room temperature and the cleared supernatant was filtered and measured. Finally, 0.5 volumes of isopropanol were added, mixed by inversion and centrifuge at 14,000 × *g* for 15 min at room temperature. The supernatant was discarded and the DNA pellet resuspended in 2 ml of TE buffer.

III.2.3.2. Purification

The DNA solution was mixed with 10 ml of Wizard® Maxiprep DNA Purification Resin. The resin/DNA mix was them introduce in the Maxicolumn and vacuum applied to

pull the resin/DNA mix into the Maxicolumn. Column Wash Solution (25 ml) was added to the Maxicolumn and passed through the Maxicolumn by applying vacuum. To rinse the resin, 5 ml of 80% ethanol were added and through the Maxicolumn as before. Then, the Maxicolumn was placed in a 50 ml Falcon tube and centrifuge at 2,500 rpm for 5 min. The tube and the liquid were discarded and vacuum was applied to the Maxicolumn to dry the resin for 5 min. The Maxicolumn was placed in a new Falcon and 1.5 ml of preheated (65°C to 70°C) nuclease-free water added. After one min the DNA was eluted by centrifuging at 2,500 rpm for 5 min. The eluate was transferred to a 1.5 ml microtube and centrifuged at 14,000 ×*g* for 1 min to remove the resin fines that might be present in the final eluate. The supernatant (DNA) was transfered to a new microtube, its concentration was determined by measuring its OD at 260 nm in a spectrophotometer and it was stored at -80 ºC.

III.2.3.3. Cell Culture Conditions

The COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen). Cells were grown in 10 cm plates or 6-well plates (Table 13) in a 5% CO_2 humidified incubator at 37ºC and subcultured every 2-3 days when they were 90% to 100% confluent.

A cervical cancer cell line (HeLa cells) was also used in this study. HeLa cells were grown in Minimal Essential Medium with Earle's salts and GlutaMAX (MEM, Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% MEM Non-Essential amino acids (Gibco, Invitrogen) and 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). Cultures were maintained at 37º C and 5% CO_2. Cells were subcultured whenever 95% confluence was reached.

III.2.3.4. Cell transfection method

For expression in mammalian cells, the coding sequences of PINK1, BRI2 and BRI3 were amplified by PCR using high fidelity Pfu polymerase (Stratagene) and

subcloned the pCMVMyc vector. Lipofectamine was used for transfection of COS-7 and Hela cells. Lipofectamine 2000 (Invitrogen) is a cationic liposome formulation that functions by complexing with nucleic acid molecules, allowing them to overcome the electrostatic repulsion of the cell membrane and to be taken by the cell. This method of DNA delivery in culture cell lines is well described.

One day before transfection, the COS-7 cells were plated on to 6-well plates in DMEM with 10% fetal fetal calf serum so they were 90% confluent at the time of transfection. For each transfection sample, the DNA/Lipofectamine complexes were prepared as follows: 10 µl of Lipofectamine were diluted in 250 µl DMEM without serum or antibiotics and added to 1 µg, 2 µg or 5 µg of the different DNA (pCMVMycPINK1, or pCMVMycBRI2, or pCMVMycBRI3) previously diluted in 250 µl of the same medium. The mixture was allowed to complex for 30 min at RT and then the 500 µl sample was added to each well and gently mixed by rocking the plate back and forth. The cells were grown at 37ºC for 20 hr.

HeLa cells were grown in complete MEM until 85 - 95% confluence and on the transfection day the culture medium was replaced with complete medium (antibiotic/antimycotic-free). The appropriate amount of DNA (pCMVMycPINK1, or pCMVMycBRI2, or pCMVMycBRI3) for each plate/well was diluted in Opti-MEM (serum- and antibiotic/antymicotic-free). The Lipofectamine 2000 reagent was diluted appropriately in the same medium, and the tubes were left to rest for 5 min. The DNA solution was added to the Lipofectamine solution drop by drop, and the solution was mixed by gentle bubbling with the pipette. In order to form the DNA-lipid complexes, the tube was allowed to rest for 25 - 30 min at RT. Then, the solution was directly added into the cell medium, drop by drop and with gentle rocking of the plate. The cells were further incubated at 37º C/5% CO_2 for the indicated transfection time, prior to cell collection or fixation.

Table 13: Cells transfection reagents.

Culture Plates	Medium w/o serum (plate)	DNA (µg)	Lipofectamine 2000 (µl)	Medium w/o serum (tube)
100mmplate	15 ml	8	50	1.5 ml
35mm 6-wells	2.0 ml	2	10	250 ml

III.2.3.5. Total protein quantification by the BCA method

The principle of the bicinchoninic acid (BCA) assay is the formation of a Cu^{2+}-protein complex under alkaline conditions. BCA forms a purple-blue complex with Cu^{+} with an absorbance maximum at 562 nm. This provides a basis to monitor the reduction of alkaline Cu^{2+} to Cu^{+} by proteins. The amount of reduction is proportional to the protein present and the absorbance is directly proportional to protein concentration. Thus, the cells were collected after 20 hr of transfection in 400 µl pre-warmed 1% SDS at 100ºC. The samples were boiled for 10 min and cooled on ice for 2 min. After, samples were sonicated, at maximum power for 20 sec and cooled on ice. BCA reagent was prepared by mixing reagent A with reagent B in the proportion of 50:1. Then the samples were prepared as follow: 25 µl of 1% SDS + 25 µl of sample + 1 ml of BCA reagent. A standard curve was prepared as described below (Table 14). All samples were incubat for 30 min at 37ºC and their absorbence measured with spectrophotometer at 562 nm. The standard curve was prepared by plotting the absorbance measured vs. the corresponding BSA concentration. The total protein concentration of each sample was then correlated from the standard curve.

Table 14: Standards curve values used in the BCA protein assay method

BSA (µl) (2 mg/ml)	SDS 10% (µl)	H_2O (µl)	BCA Reagent (ml)	Protein mass (µg)
-	5	45	1	0
1	5	44	1	2
2	5	43	1	4
5	5	40	1	10
10	5	35	1	20
20	5	25	1	40
30	5	5	1	80

III.2.3.6. SDS-PAGE analysis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using well established methods (Laemmli, 1970). This method consists in the migration of proteins

determined by molecular weight, since sodium dodecylsulfate (SDS) is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone and confers a net negative charge to the protein in proportion to its length. This way the polypeptide becomes a rod of negative charges. The percentage and size of the gel used depended on the molecular weight of the proteins being separated in the gel (Table15 and 16). The SDS-PAGE gels were prepared in a Bio-Rad apparatus. After setting up the apparatus the running gel solution was introduced and allowed to set. Water was added on top of the running gel, and after approximately 45 min the polymerization was finished.

Table 15: Running gel compostion for SDS-PAGE.

Running gel	Component volume(ml) per gel mold volume			
	5 ml	10 ml	40 ml	60 ml
Solution components	10%	12%	12%,	12%
H_2O	1.6	3.45	13.8	20.7
30% Acrylamide/0,8% Bisacrylamide	2.0	4.0	16	24
4*LGD	1.3	2.5	10	15
10% APS	0.05	0.05	0.200	0.3
TEMED	0.002	0.004	0.020	0.03

The different concentration of acrylamide in the small gel (5 ml per gel), was used to verify the expression in Rosetta cells of recombinant PINK1 proteins in 10% acrylamide, and BRI2 and BRI3 in 12% acrylamide. The 40 ml gel was use to verify expression of the proteins subcloned in pET28C in *E.coli* Rosseta and 60 ml gel was used to verify the expression of the proteins subcloned in pCMVMyc in mammalian cells (COS-7).

The water was poured off and the stacking gel was prepared (Table16).

Table 16: Stacking gel composition for SDS-PAGE.

Stacking gel	Component volume(ml) per gel mold	
Solution components	4 ml	10 ml
H_2O	2.7	6.6
30% Acrylamide/0.8% Bisacrylamide	0.670	1.2
4*UGB	0.500	2
10%SDS	0.040	0.1
10% APS	0.040	0.1
TEMED	0.004	0.01

After polymerization with an appropriate in placed, the gels were transferred to the electrophoresis tank and 1x running buffer was poured in the tanks. The small gels run for 2 hours at 100V and the two large gels for 3h at 90mA.

III.2.3.7. Western transfer conditions

The sample proteins along with the molecular weight markers were separated on by SDS-PAGE as describe above, and the transferred to a nitrocellulose membrane using the Tank Transfer System. The proteins are negatively charged due to the bound SDS and consequently they will transfer from the cathode (-) to the anode (+). Therefore, the stack is oriented so that the negative charged molecules migrate towards the anode. Transfer was allowed to proceed for 4h at 100mA, for mini gels, or overnight at 200mA for larger gels. Afterwords, the transfer cassettes were disassembled, the membrane carefully removed and allowed to air dry prior to further manipulations.

III.2.3.8. Immunodetection by Enhanced Chemiluminescence (ECL)

First, the membrane was washed for 10 min in 1x TBS under shaking. Non-specific binding sites were blocked by immersing the membrane in 5% (w/v) milk in 1x TBS-T for 5 hrs. The solution was removed and the primary antibody was added (Myc antibody diluted 1:2500 in 3% milk) and incubated for 4 hr under continuous shaking. The membrane was washed for 3 times 10 min in 1x TBS-T, then the secondary antibody was added and incubated for 2 hr. Depending on the origin of the primary antibody (mouse, rabbit or sheep), the secondary antibody was anti-rabbit or anti-mouse, diluted 1:5000, or anti-sheep, diluted 1:1000, all conjugated to peroxidase. The membrane was washed, three times for 10min each in 1x TBS-T, and incubated for 1min at room temperature with the ECL detection solution (a mixture of equal volumes of solution 1 and solution 2 from the ECL kit, approximately 0.125 ml/cm2 membrane). Excess solution was discarded the membrane was gently wrapped with cling film, eliminating all air bubbles. In the dark room, the membrane was placed in a film cassette and an autoradiography film (XAR-5 film, KODAK) was placed on the top. The cassette was closed and the blot exposed over a certain period of time. The film was then removed and developed in developing solution, washed in water and fixed in fixing solution. If needed, a second film was exposed more or less time according to the first result. To reuse the same membrane with another antibody, the membrane was sometimes stripped with stripping solution for 45 min at 50ºC, washed with 1X TBST for 15 min three times and then dried until needed. ECL^{TM} is a light emitting non-radioactive method for the detection of immobilized antigens, conjugated directly or indirectly with horseradish, peroxidase (HRP) labelled antibodies.

III.2.3.9. Co-immunoprecipitation analysis

Transfected cells were collected in 100 µl of lysis buffer in the presence of proteases inhibitors cocktail. The cells were sonicated at max speed for 3 times 10 sec, and cooled on ice. After pre-clearance with 10 mg (25 µl) of protein A Sepharose for 1 hour at 4°C and centrifugation at 10,000 g for 1 min at 4°C, the pellet was removed. Then 25 µl of primary antibody (anti-myc) and 10 mg (25 µl) of protein A Sepharose were added, and incubated overnight at 4°C with orbital agitation. After incubation, the solution was centrifuged at

10000 g for 1 min at 4°C, and followed by 4 times washing with lysis buffer without 4% CHAPS, and proteases inhibitors cocktail. The supernatant was removed and 100 µl of electrophoresis buffer was added. The samples was stored at -20°C or directly used.

III.2.3.10. Immunocytochemistry analysis

Mammalian cells were grown in pre-treated glass coverslips pre-coated with 100 µl/ml poly-L-ornithine. After the experimental procedures, cells were washed 3 times with 1 ml of serum-free DMEM, after which 1 ml of a 1:1 DMEM/4% paraformaldehyde fixative solution was gently added and allowed to stand for 1-2 min. Subsequently, 1 ml of fixative solution was gently added for 25 min. Finally, cells were washed 3 times with 1x PBS for 10 min. The cells were permeabilized with methanol for 2 min at RT. The cells were immediately washed 5 times with 1x PBS. Afterward, cells were incubated with the blocking solution, FBS (BSA + 3% PBS) for 1 hr. Then the primary antibody (anti-myc 1:2000) was added and incubated for 1 hr 30 min, followed by, 3 times washing for 10 min with PBS. The secondary antibody (1:50) was added for 1 hr 30 min followed by 3 times washing for 10 min with PBS. The coverslip was mounted with one drop of antifading reagent or for DAPI staining Mounting medium for fluorescence with DAPI (Vector laboratories), on a glass slide.

III.2.4. Expression and purification of recombinant PINK1 and BRI3 in bacterial cells

The coding sequences of interest were sub-cloned into the pET28C vector and fully sequenced. The correct clone and one empty pET28C vector as a negative control were transformed into *E. coli* Rosetta (DE3) strain. Inoculation in LB with kanamycin (30 mg/ml) was followed by incubation overnight at 37˚C at 250rpm. After, 30 µl of the overnight culture was added to 3 ml of LB with kanamycin, and incubated until the OD_{600} was between 0.4 and 0.8. Induction was started by adding of 1 mM or 5 mM IPTG (final concentration). The samples were incubated at 37˚C and 250 rpm for three different times

of culture (3 hr, 6 hr and overnight). Then, 1 ml of each sample was centrifuged for 2 min at 8000 rpm and the cells were resuspended in 100 µl of water. After, the samples were separated by SDS-PAGE in small gel. Loading buffer 4 µl was added to 15 µl of each sample. The samples were first boiled for 10 min before loading. The gel was run for 2 hours at 100 V.

III.2.4.1. Coomassie blue staining

After electrophoresis the gel was transferred to Coomassie blue staining solution and stained for 10 min. Then, the gel was washed with water and destained with destain buffer for 1 hr. After the first hour of destaining, fresh destaining buffer was added and the gel was destained overnight. The gel was transferred to gel drying solution and left with shaking for 30 min. Finally, the gel was placed between two cellophane sheets and dried for 1 hour at 80ºC under vacuum.

III.3. RESULTS

III.3.1. Identification of binding motifs and protein domains

With bioinformatics software such as ELM (Eukaryotic Linear Motif, http://elm.eu.org/) from the ExPASy (Expert Protein Analysis System) server of the Swiss Institute of Bioinformatics (http://us.expasy.org/tools/scanprosite/) it was possible to acquire information regarding the occurrence of specific features in a chosen protein, such as PP1 binding motifs and functional domains. For PINK1, besides the identification of two PP1 binding motifs (Figure 27), the bioinformatics analysis suggested that residues Gly-193 to Leu-507 comprise the catalytic domain, and that residues Gly-193 to Lys-219 form the ATP binding domain.

Bioinformatics analyses of the BRI2 amino acids sequence resulted in the identification of several putative motifs and protein domains, including a PP1 binding motif (Figure 28), and BH3 domain Bcl-2-family members. Regarding the domains present in the BRI2 protein, it contain a transmembrane domain from amino acids 53-75 and a BRICHOS domain from 137-231. The proteins usually described as possessing BRICHOS domain are divergent and associate with a diverse range of phenotypes, varying from dementia to cancer and respiratory distress, but they share two common characteristics: they are type II transmembrane proteins and are processed by furin-like endoproteases. In several of these proteins, the conserved BRICHOS domain is located within the propeptide region that is removed after proteolytic processing by pro-protein convertases. The BRICHOS domain includes a pair of conserved cysteine residues that probably form a disulfide bridge (Sánchez-Pulido *et al*., 2002; Rostagno *et al*., 2005). The BRI family may be the oldest with the conserved BRICHOS domain, as it has members from the most remote species (fly and worm), as well as the largest sequence diversity, encompassing at least three subfamilies (BRI1, BRI2 and BRI3) (Sánchez-Pulido *et al*., 2002).

A possible PP1 binding motif was also identified near the N-terminus of BRI3. It possesses a single N-glycosylation site that is conserved in the three mouse and human isoforms, and multiple consensus sites for phosphorylation. Furthermore, the analysis

shows the presence of a transmembrane domain from amino acids 58-80 and a BRICHOS domain from 136-230.

III.3.2. Construction of the different cDNA expression vectors

Since none of the positives clones possessed the complete coding sequence, as shown in Table 17, and given the addition of BRI2 to these studies, the cDNA encoding the proteins PINK1, BRI2 and BRI3 were acquired commercially.

Table 17: Positive clones obtained for PINK1 and BRI3.

Protein	Nº of positive clone	Start nt	CDS	Data base ID
PINK1	68	1010	95 - 1840	NM_032409
	96	1010		
	199	1010		
	200	1010		
	256	1001		
	269	938		
BRI3	24	921	121 - 924	NM_030926
	76	882		
	80	840		
	333	774		

The complete cDNA and corresponding amino acid sequences of PINK1, BRI2 and BRI3 are shown in Figures 27-29, and the respective PP1 binding motifs are indicated.

PINK1

```
   1 cgcagaggcaccgccccaagtttgttgtgaccggcgggggacgccggtgg   50
  51 tggcggcagcggcggctgcgggggcaccgggccgcggcgccaccatggcg  100
   1 ---------------------------------------------M--A-    2
 101 gtgcgacaggcgctgggccgcggcctgcagctgggtcgagcgctgctgct  150
   3 -V--R--Q--A--L--G--R--G--L--Q--L--G--R--A--L--L--L   19
 151 gcgcttcacgggcaagcccggccgggcctacggcttggggcggccgggcc  200
  20 --R--F--T--G--K--P--G--R--A--Y--G--L--G--R--P--G--   35
 201 cggcggcgggctgtgtccgcggggagcgtccaggctgggccgcaggaccg  250
  36 P--A--A--G--C--V--R--G--E--R--P--G--W--A--A--G--P-   52
 251 ggcgcggagcctcgcagggtcgggctcgggctccctaaccgtctccgctt  300
  53 -G--A--E--P--R--R--V--G--L--G--L--P--N--R--L--R--F   69
 301 cttccgccagtcggtggccgggctggcggcgcggttgcagcggcagttcg  350
  70 --F--R--Q--S--V--A--G--L--A--A--R--L--Q--R--Q--F--   85
 351 tggtgcgggcctggggctgcgcgggcccttgcggccgggcagtctttctg  400
  86 V--V--R--A--W--G--C--A--G--P--C--G--R--A--V--F--L-  102
 401 gccttcgggctagggctgggcctcatcgaggaaaaacaggcggagagccg  450
 103 -A--F--G--L--G--L--G--L--I--E--E--K--Q--A--E--S--R  119
 451 gcgggcggtctcggcctgtcaggagatccaggcaatttttacccagaaaa  500
 120 --R--A--V--S--A--C--Q--E--I--Q--A--I--F--T--Q--K--  135
 501 gcaagccggggcctgacccgttggacacgagacgcttgcagggctttcgg  550
 136 S--K--P--G--P--D--P--L--D--T--R--R--L--Q--G--F--R-  152
 551 ctggaggagtatctgatagggcagtccattggtaagggctgcagtgctgc  600
 153 -L--E--E--Y--L--I--G--Q--S--I--G--K--G--C--S--A--A  169
 601 tgtgtatgaagccaccatgcctacattgccccagaacctggaggtgacaa  650
 170 --V--Y--E--A--T--M--P--T--L--P--Q--N--L--E--V--T--  185
 651 agagcaccgggttgcttccagggagaggcccaggtaccagtgcaccagga  700
 186 K--S--T--G--L--L--P--G--R--G--P--G--T--S--A--P--G-  202
 701 gaagggcaggagcgagctccgggggcccctgccttcccccttggccatcaa  750
 203 -E--G--Q--E--R--A--P--G--A--P--A--F--P--L--A--I--K  219
 751 gatgatgtggaacatctcggcaggttcctccagcgaagccatcttgaaca  800
 220 --M--M--W--N--I--S--A--G--S--S--S--E--A--I--L--N--  235
 801 caatgagccaggagctggtcccagcgagccgagtggccttggctggggag  850
 236 T--M--S--Q--E--L--V--P--A--S--R--V--A--L--A--G--E-  252
 851 tatggagcagtcacttacagaaaatccaagagaggtcccaagcaactagc  900
 253 -Y--G--A--V--T--Y--R--K--S--K--R--G--P--K--Q--L--A  269
 901 ccctcaccccaacatcatccgggttctccgcgccttcacctcttccgtgc  950
 270 --P--H--P--N--I--I--R--V--L--R--A--P--T--S--S--V--  285
 951 cgctgctgccaggggccctggtcgactaccctgatgtgctgccctcacgc 1000
 286 P--L--L--P--G--A--L--V--D--Y--P--D--V--L--P--S--R-  302
1001 ctccaccctgaaggcctgggccatggccggacgctgttcctcgttatgaa 1050
 303 -L--H--P--E--G--L--G--H--G--R--T--L--F--L--V--M--K  319
1051 gaactatccctgtaccctgcgccagtacctttgtgtgaacacacccagcc 1100
 320 --N--Y--P--C--T--L--R--Q--Y--L--C--V--N--T--P--S--  335
1101 cccgcctcgccgccatgatgctgctgcagctgctggaaggcgtggaccat 1150
 336 P--R--L--A--A--M--M--L--L--Q--L--L--E--G--V--D--H-  352
1151 ctggttcaacagggcatcgcgcacagagacctgaaatccgacaacatcct 1200
 353 -L--V--Q--Q--G--I--A--H--R--D--L--K--S--D--N--I--L  369
1201 tgtggagctggacccagacggctgcccctggctggtgatcgcagattttg 1250
 370 --V--E--L--D--P--D--G--C--P--W--L--V--I--A--D--F--  385
1251 gctgctgcctggctgatgagagcatcggcctgcagttgcccttcagcagc 1300
 386 G--C--C--L--A--D--E--S--I--G--L--Q--L--P--F--S--S-  402
1301 tggtacgtggatcggggcggaaacggctgtctgatggccccagaggtgtc 1350
 403 -W--Y--V--D--R--G--G--N--G--C--L--M--A--P--E--V--S  419
1351 cacggcccgtcctggccccagggcagtgattgactacagcaaggctgatg 1400
 420 --T--A--R--P--G--P--R--A--V--I--D--Y--S--K--A--D--  435
1401 cctgggcagtgggagccatcgcctatgaaatcttcgggcttgtcaatccc 1450
 436 A--W--A--V--G--A--I--A--Y--E--I--F--G--L--V--N--P-  452
```

```
1451 ttctacggccagggcaaggcccaccttgaaagccgcagctaccaagaggc 1500
 453 -F--Y--G--Q--G--K--A--H--L--E--S--R--S--Y--Q--E--A  469
1501 tcagctacctgcactgcccgagtcagtgcctccagacgtgagacagttgg 1550
 470 --Q--L--P--A--L--P--E--S--V--P--P--D--V--R--Q--L--  485
1551 tgagggcactgctccagcgagaggccagcaagagaccatctgcccgagta 1600
 486 V--R--A--L--L--Q--R--E--A--S--K--R--P--S--A--R--V-  502
1601 gccgcaaatgtgcttcatctaagcctctggggtgaacatattctagccct 1650
 503 -A--A--N--V--L--H--L--S--L--W--G--E--H--I--L--A--L  519
1651 gaagaatctgaagttagacaagatggttggctggctcctccaacaatcgg 1700
 520 --K--N--L--K--L--D--K--M--V--G--W--L--L--Q--Q--S--  535
1701 ccgccactttgttggccaacaggctcacagagaagtgttgtgtggaaaca 1750
 536 A--A--T--L--L--A--N--R--L--T--E--K--C--C--V--E--T-  552
1751 aaaatgaagatgctctttctggctaacctggagtgtgaaacgctctgcca 1800
 553 -K--M--K--M--L--F--L--A--N--L--E--C--E--T--L--C--Q  569
1801 ggcagccctcctcctctgctcatggagggcagccctgtgatgtccctgca 1850
 570 --A--A--L--L--L--C--S--W--R--A--A--L--#----------  582
1851 tggagctggtgaattactaaaagaacttggcatcctctgtgtcgtgatgg 1900
1901 tctgtgaatggtgagggtgggagtcaggagacaagacagcgcagagaggg 1950
1951 ctggttagccggaaaaggcctcgggcttggcaaatggaagaacttgagtg 2000
2001 agagttcagtctgcagtcctgtgctcacagacatctgaaaagtgaatggc 2050
2051 caagctggtctagtagatgaggctggactgaggaggggtaggcctgcatc 2100
2101 cacagagaggatccaggccaaggcactggctgtcagtggcagagtttggc 2150
2151 tgtgacctttgcccctaacacgaggaactcgtttgaaggggcagcgtag 2200
2201 catgtctgatttgccacctggatgaaggcagacatcaacatgggtcagca 2250
2251 cgttcagttacgggagtgggaaattacatgaggcctgggcctctgcgttc 2300
2301 ccaagctgtgcgttctggaccagctactgaattattaatctcacttagcg 2350
2351 aaagtgacggatgagcagtaagtaagtaagtgtggggatttaaacttgag 2400
2401 ggtttccctcctgactagcctctcttacaggaattgtgaaatattaaatg 2450
2451 caaatttacaactgcagatgacgtatgtgccttgaactgaatatttggct 2500
2501 ttaagaatgattcttatactctgaaggtgagaatattttgtgggcaggta 2550
2551 tcaacattggggaagagatttcatgtctaactaactaactttatacatga 2600
2601 tttttaggaagctatgcctaaatcagcgtcaacatgcagtaaaggttgtc 2650
2651 ttcaactgaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 2700
```

Figure 27: Full-length nucleotide sequence and predicted amino acid sequence of the cDNA encoding human PINK1 protein. The putative PP1 binding motifs are indicated in red. The arrows mark the start of the positives clones obtained in the YTH screen as shown in Table 17.

BRI2

```
  1 cccacgcgtccgcggacgcgtgggcgcgcgggagctgggaggctgcgaga   50
 51 tccctaccgcagtagccgcctctgccgccgcggacttcccgaacctcttc  100
101 agccgcccggagccgctcccggagcccggccgtagaggctgcaatcgcag  150
151 cgggagcccgcagcccgcgccccgagcccgccgccgcccttcgagggcgc  200
201 ccaggccgcgccatggtgaaggtgacgttcaactccgctctggcccagaa  250
 1 -------------M--V--K--V--T--F--N--S--A--L--A--Q--K   13
251 ggaggccaagaaggacgagcccaagagcggcgaggaggcgctcatcatcc  300
 14 --E--A--K--K--D--E--P--K--S--G--E--E--A--L--I--I--   29
301 cccccgacgccgtcgcggtggactgcaaggacccagatgatgtggtacca  350
 30 P--P--D--A--V--A--V--D--C--K--D--P--D--D--V--V--P-   46
351 gttggccaagaagagcctggtgttggtgcatgtgctttggactagcatt  400
 47 -V--G--Q--R--R--A--W--C--W--C--M--C--F--G--L--A--F   63
401 tatgcttgcaggtgttattctaggaggagcatacttgtacaaatattttg  450
```

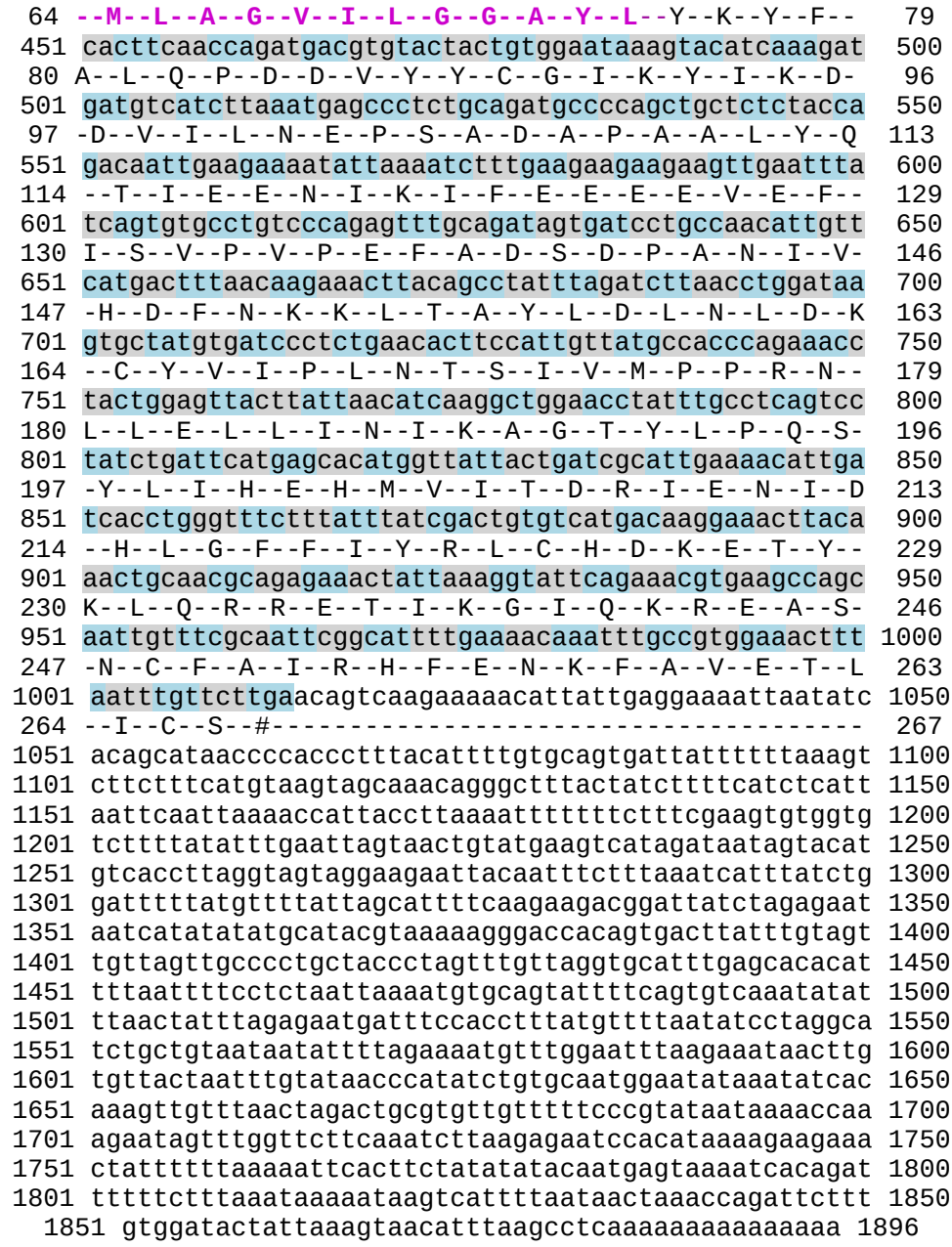

Figure 28: Full-length nucleotide sequence and predicted amino acid sequence of the cDNA encoding human BRI2 protein. The PP1 binding motif is indicated in red. The intracellular N-terminus is shown in green, the transmembrane domain is shown in purple, and the extracellular region in the C-terminus is shown in black.

BRI3

```
   1 gggggacgcgagcgggatccaaacttccggtgcctgcagagctcggagc   50
  51 ggcggaggcagagaccgaggctgcaccggcagaggctgcggggcggacgc  100

 101 gcgggccggcgcagccatggtgaagattagcttccagcccgccgtggctg  150
   1 -----------------M--V--K--I--S--F--Q--P--A--V--A--   11
 151 gcatcaagggcgacaaggctgacaaggcgtcggcgtcggcccctgcgccg  200
  12 G--I--K--G--D--K--A--D--K--A--S--A--S--A--P--A--P-   28
 201 gcctcggccaccgagatcctgctgacgccggctagggaggagcagccccc  250
  29 -A--S--A--T--E--I--L--L--T--P--A--R--E--E--Q--P--P   45
 251 acaacatcgatccaagagggggggctcagtgggcggcgtgtgctacctgt  300
  46 --Q--H--R--S--K--R--G--G--S--V--G--G--V--C--Y--L--   61
 301 cgatgggcatggtcgtgctgctcatgggcctcgtgttcgcctctgtctac  350
  62 S--M--G--M--V--V--L--L--M--G--L--V--F--A--S--V--Y-   78
 351 atctacagatacttcttccttgcgcagctggcccgagataacttcttccg  400
  79 -I--Y--R--Y--F--F--L--A--Q--L--A--R--D--N--F--F--R   95
 401 ctgtggtgtgctgtatgaggactccctgtcctcccaggtccggactcaga  450
  96 --C--G--V--L--Y--E--D--S--L--S--S--Q--V--R--T--Q--  111
 451 tggagctggaagaggatgtgaaaatctacctcgacgagaactacgagcgc  500
 112 M--E--L--E--E--D--V--K--I--Y--L--D--E--N--Y--E--R-  128
 501 atcaacgtgcctgtgccccagtttggcggcggtgaccctgcagacatcat  550
 129 -I--N--V--P--V--P--Q--F--G--G--G--D--P--A--D--I--I  145
 551 ccatgacttccagcggggtctgactgcgtaccatgatatctccctggaca  600
 146 --H--D--F--Q--R--G--L--T--A--Y--H--D--I--S--L--D--  161
 601 agtgctatgtcatcgaactcaacaccaccattgtgctgccccctcgcaac  650
 162 K--C--Y--V--I--E--L--N--T--T--I--V--L--P--P--R--N-  178
 651 ttctgggagctcctcatgaacgtgaagaggggacctacctgccgcagac  700
 179 -F--W--E--L--L--M--N--V--K--R--G--T--Y--L--P--Q--T  195
 701 gtacatcatccaggaggagatggtggtcacggagcatgtcagtgacaagg  750
 196 --Y--I--I--Q--E--E--M--V--V--T--E--H--V--S--D--K--  211
 751 aggccctggggtccttcatctaccacctgtgcaacgggaaagacacctac  800
 212 E--A--L--G--S--F--I--Y--H--L--C--N--G--K--D--T--Y-  228
 801 cggctccggcgccgggcaacgcggaggcggatcaacaagcgtggggccaa  850
 229 -R--L--R--R--R--A--T--R--R--R--I--N--K--R--G--A--K  245
 851 gaactgcaatgccatccgccacttcgagaacaccttcgtggtggagacgc  900
 246 --N--C--N--A--I--R--H--F--E--N--T--F--V--V--E--T--  261
 901 tcatctgcggggtggtgtgaggccctcctcccccagaaccccctgccgtg  950
 262 L--I--C--G--V--V--#------------------------------  268
 951 ttcctcttttcttctttccggctgctctctggccctcctccttcccccctg 1000
1001 cttagcttgtactttggacgcgtttctatagaggtgacatgtctctccat 1050
1051 tcctctccaaccctgcccacctccctgtaccagagctgtgatctctcggt 1100
1101 gggggggcccatctctgctgacctgggtgtggcggagggagaggcgatgct 1150
1151 gcaaagtgttttctgtgtcccactgtcttgaagctgggcctgccaaagcc 1200
1201 tgggcccacagctgcaccggcagcccaaggggaaggaccggttgggggag 1250
1251 ccgggcatgtgaggccctgggcaaggggatggggctgtgggggcggggcg 1300
1301 gcatgggcttcagaagtatctgcacaattagaaaagtcctcagaagcttt 1350
1351 ttcttggagggtacactttcttcactgtccctattcctagacctggggct 1400
1401 tgagctgaggatgggacgatgtgcccagggagggacccaccagagcacaa 1450
1451 gagaaggtggctacctgggggtgtcccagggactctgtcagtgccttcag 1500
1501 cccaccagcaggagcttggagtttggggagtggggatgagtccgtcaagc 1550
1551 acaactgttctctgagtggaaccaaagaagcaaggagctaggacccccag 1600
1601 tcctgccccccaggagcacaagcagggtcccctcagtcaaggcagtggga 1650
1651 tgggcggctgaggaacggggcaggcaaggtcactgctcagtcacgtccac 1700
1701 gggggacgagccgtgggttctgctgagtaggtggagctcattgctttctc 1750
1751 caagcttggaactgttttgaaagataacacagagggaaagggagagccac 1800
1801 ctggtacttgtccaccctgcctcctctgttctgaaattccatcccccctca 1850
```

```
1851 gcttaggggaatgcacctttttccctttccttctcacttttgcatgtttt 1900
1901 tactgatcattcgatatgctaaccgttctcagccctgagccttggagagg 1950
1951 agggctgtaacgccttcagtcagtctctggggatgaaactcttaaatgct 2000
2001 ttgtatattttctcaattagatctcttttcagaagtgtctatagaacaat 2050
2051 aaaaatcttttacttctgaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 2100
                2101 aaaaaaaaaaaaaaaaaa 2119
```

Figure 29: Full-length nucleotide sequence and predicted amino acid sequence of the cDNA encoding human BRI3 protein. The PP1 binding motif is indicated in red. The arrows mark the start of the positives clones shown in Table 17. The intracellular N-terminus is shown in green, the transmembrane domain is shown in purple, and the extracellular region in the C-terminus is shown in black.

After PCR amplification of the coding sequence for each of the three proteins, the amplified cDNAs were digested with appropriate restriction enzymes, according to the characteristics of the cloning vectors: pACT2, pCMVMyc and pET28C. The digested plasmid vectors were purified by agarose gel electrophoresis, as exemplified in Figure 30.

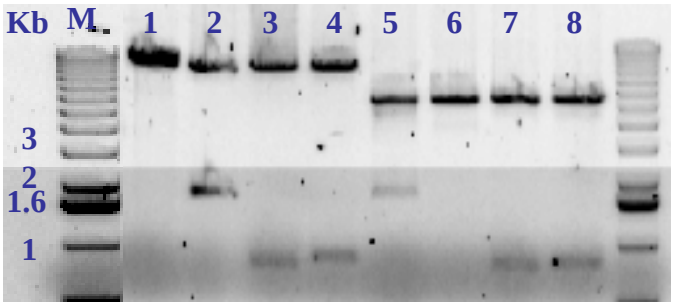

Figure 30: *EcoRI/XhoI* restriction analysis of 1- pACT2 vector, 2- pACT2PINK1, 3- pACT2BRI2, 4- pACT2BRI3, 5- pET28CPINK1, 6- pET28C vector, 7- pET28CBRI2, 8- pET28CBRI3. M, 1Kb Ladder DNA marker.

The chosen plasmids were isolated, purified and completely sequenced as shown in Figure 31 for pCMVMycBRI2, thus confirming the absence of mutations and the correctness of the reading frame.

Figure 31: Confirmation of the correctness of the BRI2 sequence cloned in pCMVMyc by comparison to the database sequence.

III.3.3. Verification of interactions by co-transformation

Confirmation of interactions through co-transformation of yeast with the cDNA obtained in the YTH screen (a), or the acquired cDNA (b) and the prey was performed in order to confirm the YTH interactions. Following co-transformation single colonies were transformed to SD/QDO/X-α-GAL medium, where the development of a blue color indicates positive interaction. Both positive (with two plasmids encoding known binding protein) and negative controls (with two empty pACT-2 and pAS2 vectors) were used. The results obtained are shown below in Figure 32.

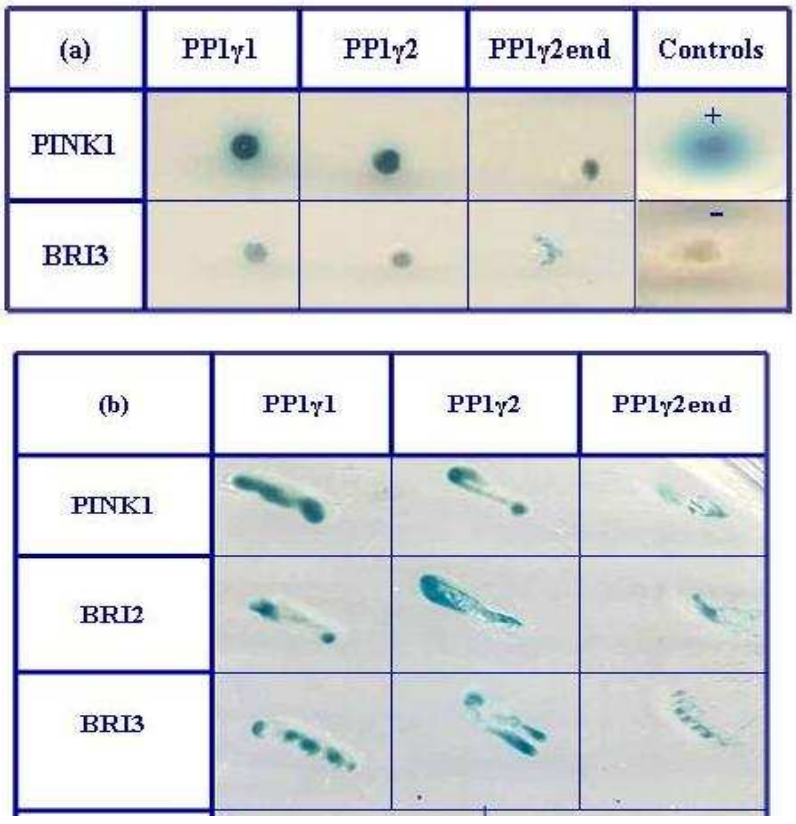

Figure 32: Co-transformation of yeast AH109 with a bait plasmid (either pAS2PP1γ1, or pAS2PP1γ2 or pAS2PP1γ2end) and the prey plasmid (pACT2PINK1, pACT2BRI2 or pACT2BRI3). (a) Co-transformation with the incomplete cDNA obtained from the YTH screen; (b) co-transformation with cDNA encoding the complete protein (acquired from Geneservice).

The results obtained indicate that PINK1, BRI2 and BRI3 interact with PP1γ1 and PP1γ2. They also appear to interact with the PP1γ2 C-terminal, although the interaction is weaker since the blue color takes longer to develop. This was expected since the C-terminal PP1 peptide expressed did include the sequence thought to be responsible for binding to the consensus binding motifs in other proteins. The results obtained for PINK1 indicate that of the two possible PP1 binding motifs identified (Figure 32) it is the C-terminus motif that is likely to be physiologically relevant since the construct used in Figure 32 was strongly positive.

III.3.4. PINK1, BRI2 and BRI3 expression in COS-7 cells

The mammalian cell line COS-7 was transfected with the pCMVmycPINK1, pCMVmycBRI2 or pCMVmycBRI3 construct, in order to verify the expression of the corresponding proteins. Increasing DNA concentrations yielded the results shown in the Figure 33.

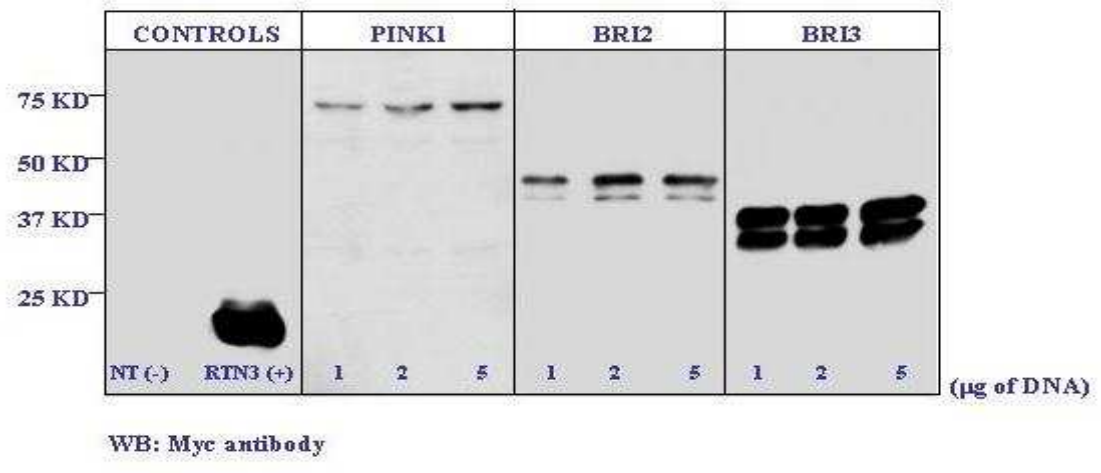

Figure 33: Expression of recombinant myc-tagged proteins in COS-7 cells with pCMVmycPINK1, pCMVmycBRI2 and pCMVmycBRI3. NT, non-transfected cells, negative control; RTN3, positive control transfection with Reticulon 3 (approximately 20 kDa). Immunoblot analysis of transfected Cos-7 cells lysates using an antibody against the myc tag, showing increasing protein expression with increasing DNA concentration.

The expression of PINK1, BRI2 and BRI3 in COS-7 was successful as shown in Figure 33. The immunoreactive PINK1 protein appears to have a molecular mass around 70 KDa, the BRI2 protein migrated as two bands of molecular mass between 40-45 KDa, and BRI3 also migrated as two bands between 30-37 KDa. These results are consistent with the theoretical molecular mass of PINK1, BRI2 and BRI3, although not exactly coincident with the exact theoretical value. The BRI2 and BRI3 proteins are thought to migrate as two bands because they undergo proteolytic cleavage near the C-terminus (at amino acids 244 and amino acids 242, respectively). Since the myc tag is present in the N-terminus, both large fragments were detected with the myc antibody. The PINK1 protein is also cleaved near the N-terminus, at amino acid 77, but the fragment of 10 KDa was not visible and the larger resultant fragment does not have the Myc epitope tag, and therefore could not be detected.

III.3.5. Co-immunoprecipitation of PINK1, BRI2 and BRI3 with PP1

With the purpose of confirming and to provide *ex vivo* evidence for the interaction of PINK1, BRI2 and BRI3 with PP1, immunoprecipitation (IP) of protein extracts obtained from COS-7 cells transfected with pCMVmycPINK1, pCMVmycBRI2 or pCMVmycBRI3 was performed, using a highly specific anti-PP1γ antibody. Proteins from cells lysates and IPs were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and immunobloted with an anti-Myc antibody. The results obtained are shown in the Figure 34, and confirm the interaction of PP1 with PINK1, BRI2 and BRI3.

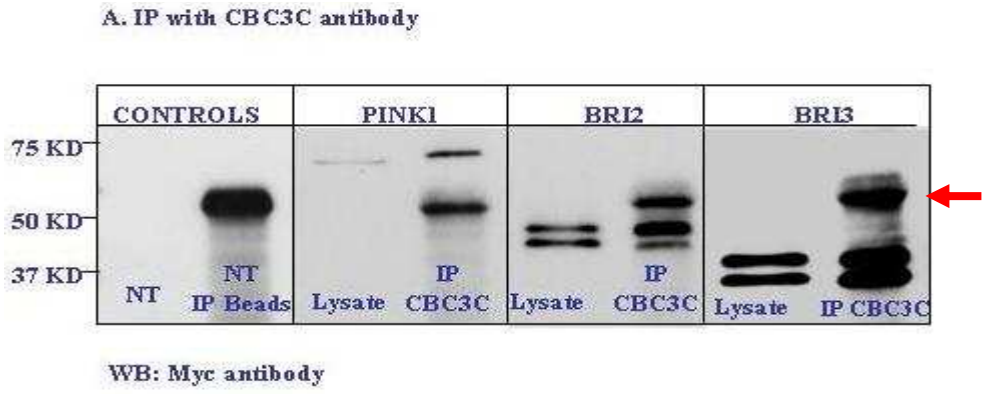

Figure 34: Co-immunoprecipitation of PINK1, BRI2 and BRI3 with PP1. Immunoblot analysis of COS-7 cells transfected with pCMVmycPINK1, pCMVmycBRI2 and pCMVmycBRI3 and immunoprecipitated with anti-PP1γ (CBC3C) antibody. The immunoblot was performed with an anti-myc antibody. NT, non-transfected control. The arrows indicate a non-specific immunoreactive band that originates from the IP beads (NT IP Beads).

III.3.6. Co-immunoprecipitation of PINK1, BRI2 and BRI3 with APP

Although the interaction between the BRI2 and APP (Alzhemer's amyloid pecursor protein) has been previously described (Matsuda *et al.*, 2005; Matsuda *et al.*, 2008; Fotinopoulou *et al*, 2005), the interaction of PINK1 or BRI3 with APP has not been explored. An immunoprecipitation (IP) of protein extracts obtained from COS-7 transfected with pCMVmycPINK1, pCMVmycBRI2 or pCMVmycBRI3 was performed, using the highly specific 22C11 (anti-APP) antibody. Proteins from cells lysates and immunoprecipitates (IPs) were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and immunobloted with an anti-Myc antibody. The results indicated that both BRI2 and BRI3 interact with of APP, as shown in Figure 35.

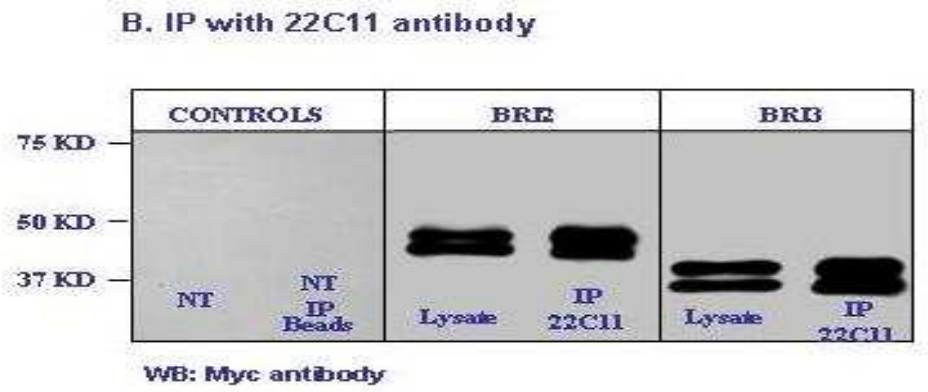

Figure 35: Co-immunoprecipitation of BRI2 and BRI3 with APP. Immunoblot analysis of COS-7 cells transfected with pCMVmycBRI2 and pCMVmycBRI3 and immunoprecipitated with an anti-APP antibody (22C11). The immunoblot was performed with an anti-myc antibody. NT, non-transfected control.

From the analysis of Figure 34 and 35, it is clear that PINK1, BRI2 and BRI3 co-immunoprecipitate with PP1, and that BRI2 and BRI3 co-immunoprecipitate with APP. However, in our experiments PINK1 did not co-immunoprecipitate with APP. In agreement with Figure 34, a single band was observed for mycPINK1 and two bands were again observed for mycBRI2 and mycBRI3. As explained before, this is due to the fact that both proteins are cleaved in the C-terminus but the PP1 binding motif (KVTF for BRI2 and KISF for BRI3) is located in the N-terminus, thus the interaction occurs also with the smaller cleaved fragment.

III.3.7. Subcellular localization of PINK1, BRI2 and BRI3 in COS7 and HeLa cells

With the purpose of studying the location of the recombinant proteins, COS7 and HeLa cells were co-transfected with expression vectors designed for fluorescent labeling of mitochondria and pCMVmycPINK1, or Golgi and pCMvmycBRI2 or pCMVmycBRI3, and subjected to immunocytochemistry with an anti-myc antibody and an anti-mouse Texas Red fluorescent secondary antibody. Nuclear DNA was stained with DAPI. Our results show the localization of PINK1 protein in the mitochondria (Figure 36), as indicated also by other studies (Valente *et al.*, 2004 (b); Silvestri *et al.*, 2005; Beilina *et al.*, 2005; Sim *et al.*, 2006).

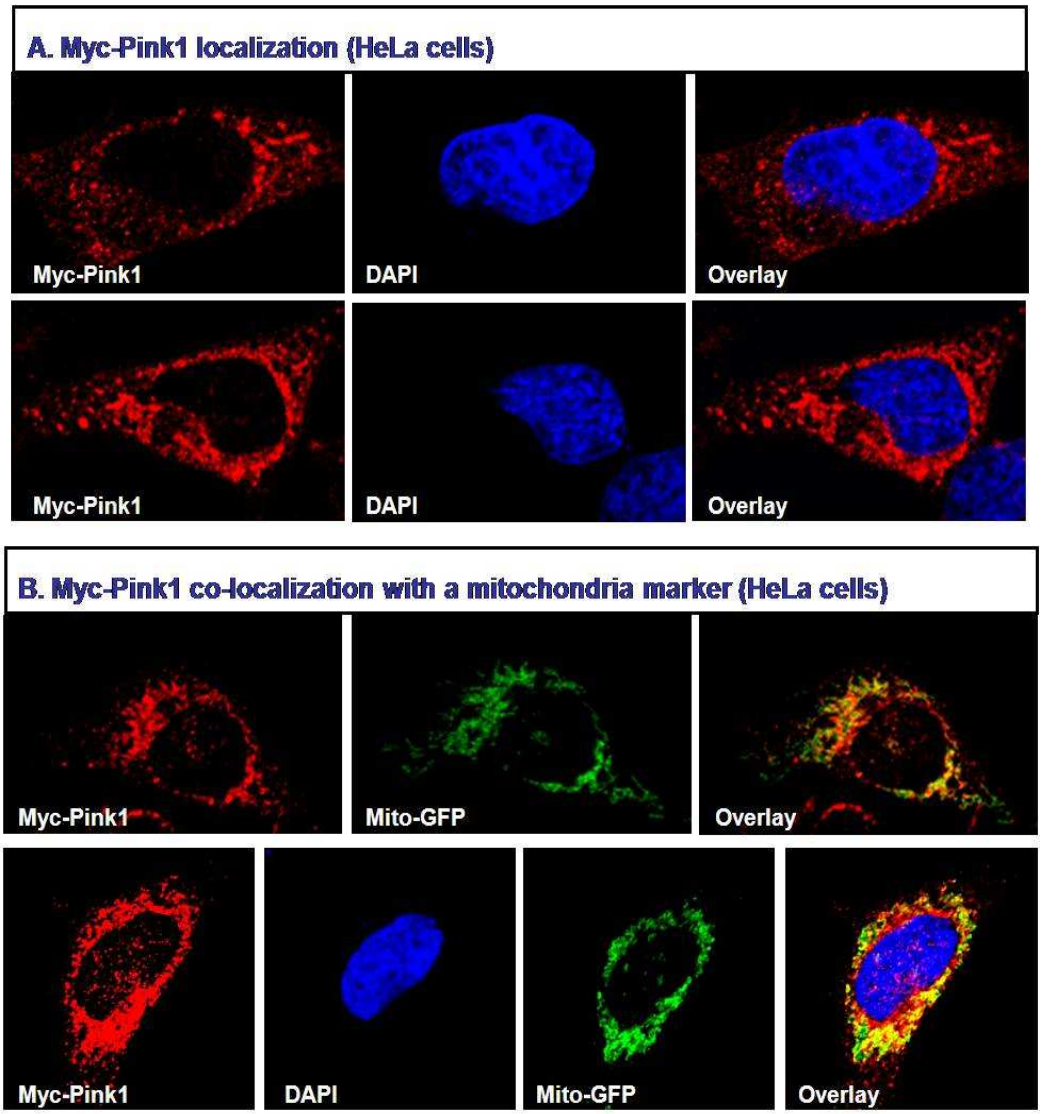

Figure 36: Subcellular localization of recombinant myc-PINK1 protein in HeLa cells (A), and co-localization of Myc-PINK1 with a mitochondria marker (B). DNA was stained with DAPI (in blue). PINK1 is shown in red (Myc-PINK1), Mito-GFP marks the mitochondria in green and the overlay image shows co-localization of Myc-PINK1 with the mitochondria (orange/yellow).

Further studies are needed to determine if other proteolysed forms of PINK1, such as the 55 KDa PINK1 fragment is also in the mitochondria and if all or any PINK1 protein are present in the cytoplasm, as some reports indicated.

In our studies the BRI2 and BRI3 proteins appears to be localized in the Golgi apparatus, as indicated by co-localization with a Golgi marker (CFP-Golgi), shown in Figure 37 and 38.

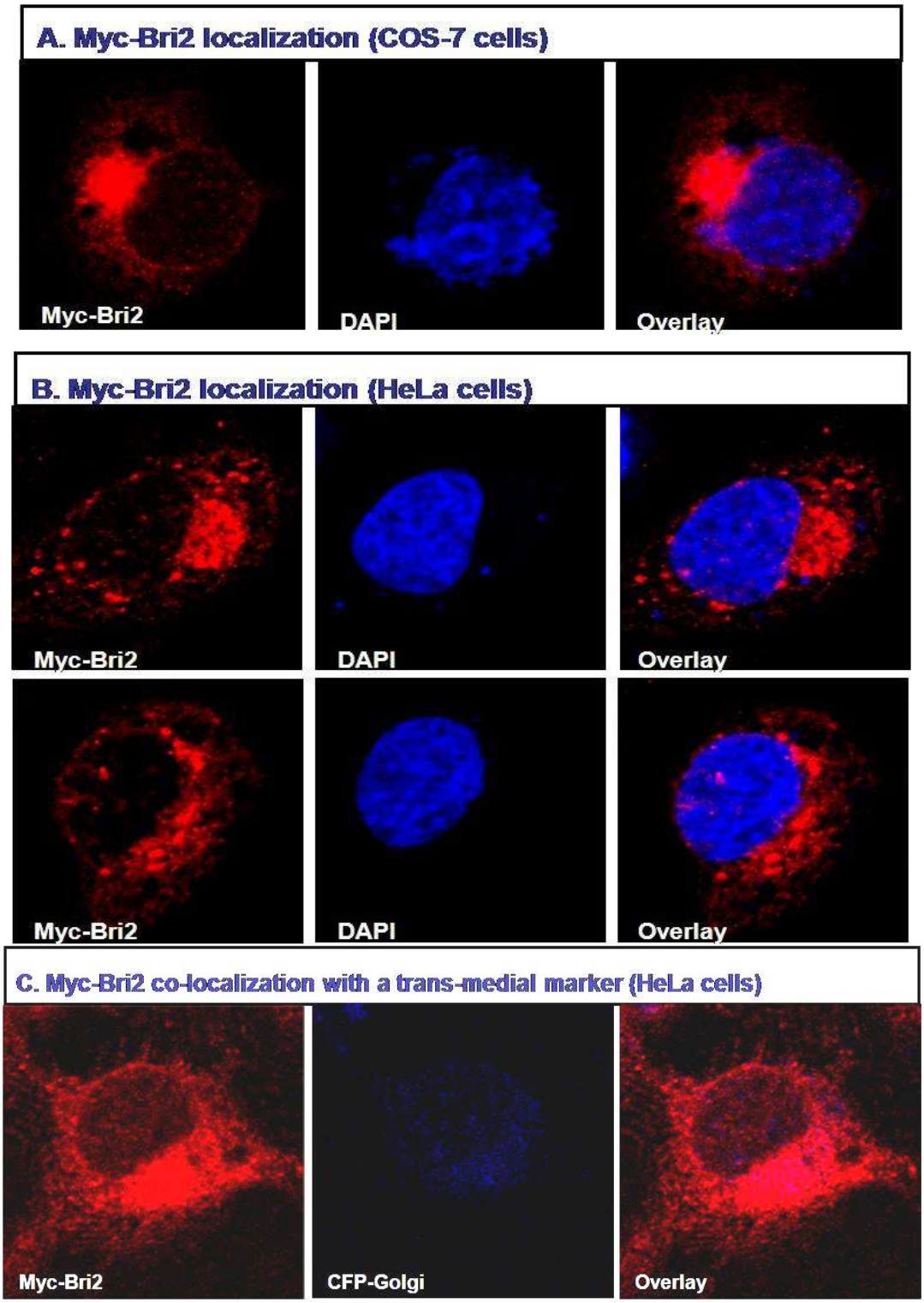

Figure 37: Subcellular localization of BRI2 protein. A) Localization of Myc-BRI2 in COS 7 cells: Myc-BRI2 is shown in red, DNA staining with DAPI in blue and the overlay. B) Localization in Hela Cells of recombinant BRI2 in red, DNA staining with DAPI in blue and the overlay. C) Co-localization of recombinant BRI2 in red, a CFP-Golgi marker and the merged image.

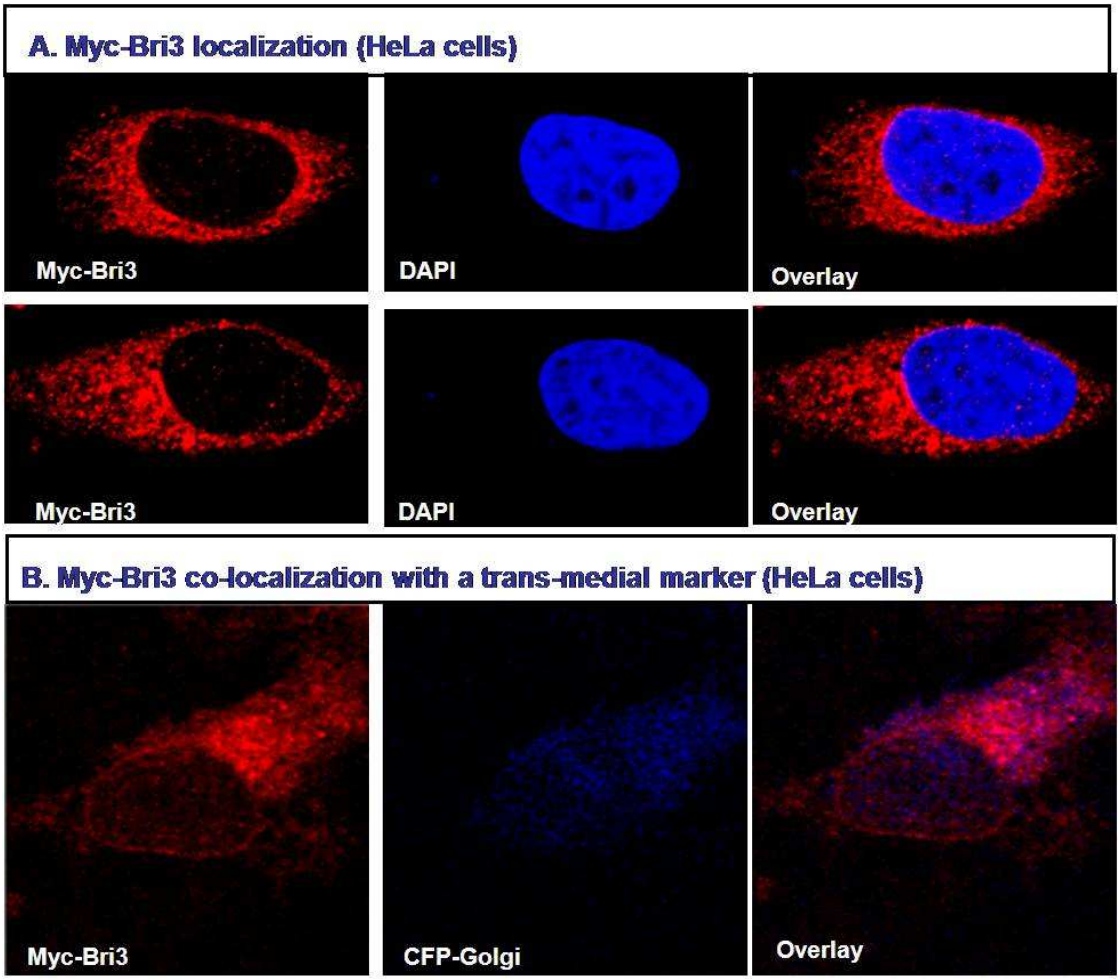

Figure 38: Subcellular localization of BRI3 protein. A) Localization in Hela Cells of recombinant BRI3 in red, DNA staining with DAPI in blue and the overlay. B) Co-localization of Myc-BRI3 in red, a CFP-Golgi marker and the merged image.

III.3.8. Co-localization of PINK1, BRI2 and BRI3 with PP1 in HeLa cells

For co-localization analysis with PP1, HeLa cells were transfected with pCMVmycPINK1, pCMvmycBRI2 or pCMVmycBRI3 and subjected to immunocytochemistry with an anti-myc antibody and an anti-PP1γ antibody. As shown in Figure 39 in the merged image (in orange), some degree of co-localization was observed between PP1 and PINK1, BRI2 and BRI3.

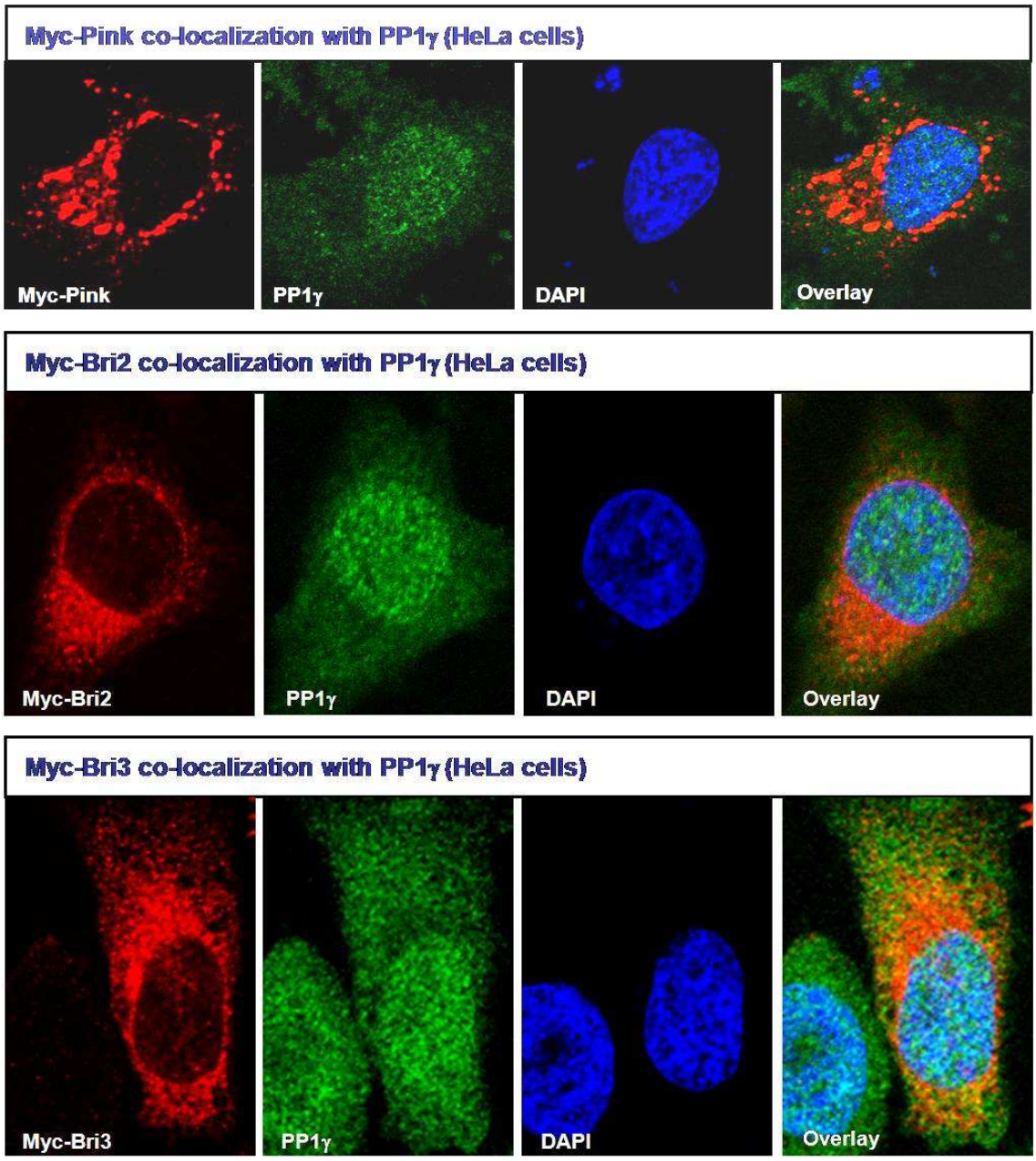

Figure 39: Co-localization of Myc-PINK1, Myc-BRI2 and Myc-BRI3 with PP1γ. Immunodetection of the myc tag of the different proteins used a fluorescent Texas Red secondary antibody, immunodetection of endogenous PP1γ used a green fluorescent secondary antibody, DNA was stained with DAPI (in blue) and the merged images show the degree of co-localization in orange.

III.3.9. Co-localization of BRI2 and BRI3 with APP in HeLa cells

Co-localization analysis in HeLa cells transfected with pCMvmycBRI2 or pCMVmycBRI3 and subjected to immunocytochemistry with an anti-myc antibody and a C-terminal anti-APP antibody. As shown in Figure 40 in the merged image (in orange), the proteins BRI2 and BRI3 co-localize with APP to some extent.

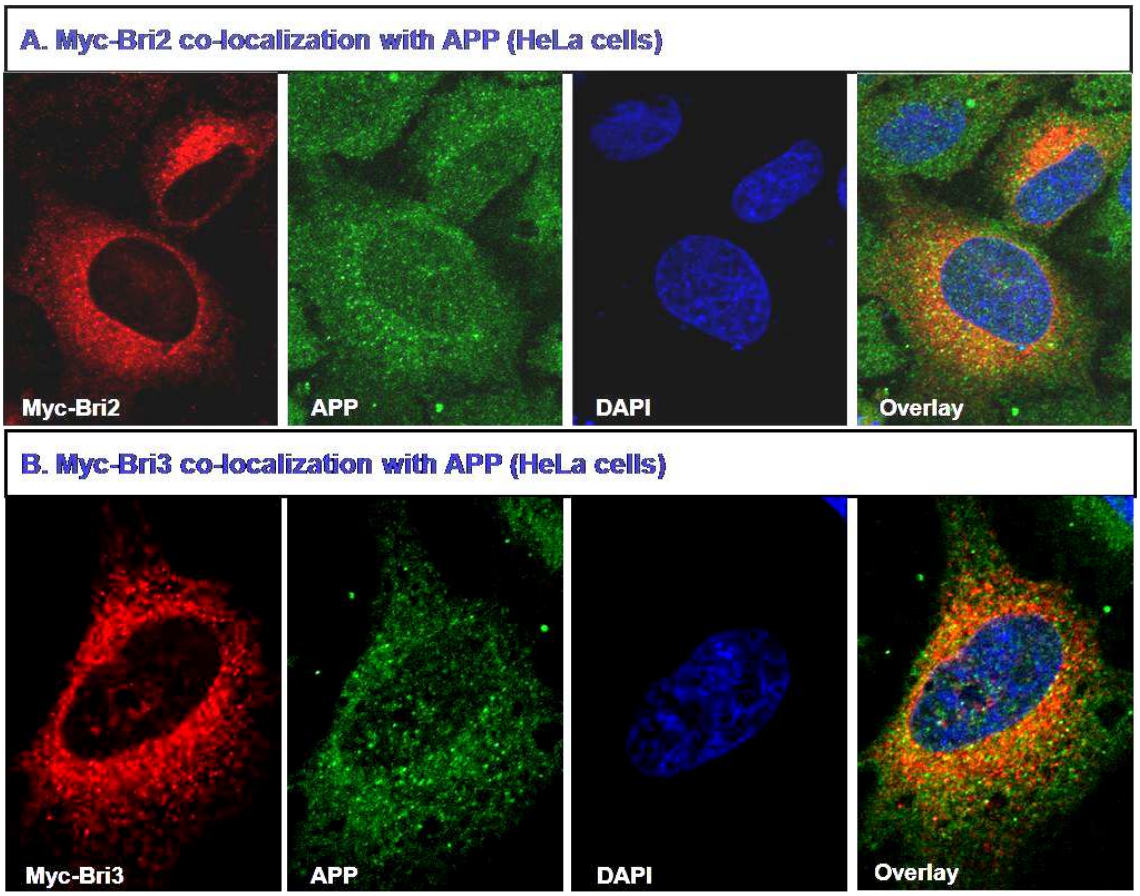

Figure 40: Co-localization of Myc-BRI2 and Myc-BRI3 with APP. Immunodetection of the myc tag used a fluorescent Texas Red secondary antibody, immunodetection of endogenous APP used a green fluorescent secondary antibody, DNA was stained with DAPI (in blue) and the merged image shows the degree of co-localization in orange.

III.3.10. Expression of recombinant PINK1, BRI2 and BRI3 in bacteria

The *E. coli* Rosetta strain was transformed with the pET28c vector or with pET28cPINK1, or pET28cBRI2, or pET28cBRI3 expression vectors and incubated on LB with kanamycin. The Rosetta strain was designed to enhance expression of eukaryotic proteins that contain codons rarely used in *E.coli*, and hereby provides "universal" translation. A single colony was picked for each transformation and recombinant protein expression was induces using IPTG. In order to optimize the expression conditions, a time course analysis was performed as shown in Figure 41.

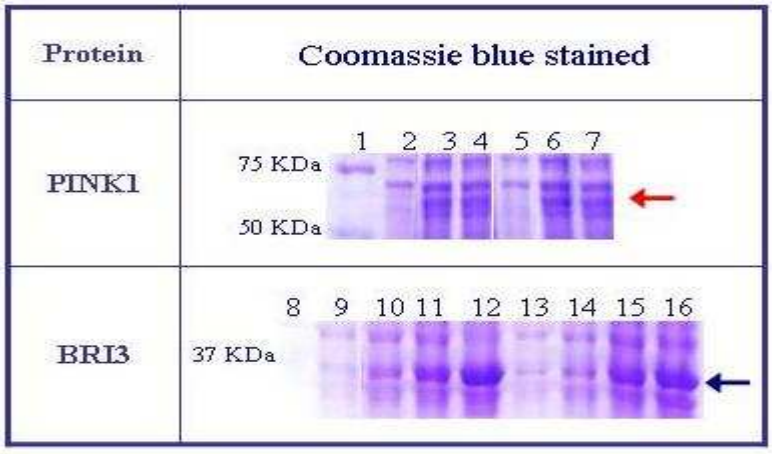

Figure 41: Time course analysis of expression of recombinant pET28cPINK1and pET28cBRI3 in *E.coli*. Lane 1, Prestained SDS-PAGE Standards, Broad range (Biorad); lanes 2-4 and 9-12, induction with 1mM IPTG; lanes 5-7 and 13-16, induction with 5mM IPTG; Lanes 2, 5, 9 and 13 show the empty vector as control; Lanes 3, 6, 10 and 14 show the expression after 3 hrs; Lanes 4, 7, 11 and 15 show the expression after 6 hrs; Lane 12 and 16 shows the expression after overnight incubation. The arrows indicate the recombinant proteins (around 63.5 kDa for PINK1 and 30.2 kDa BRI3).

Based on the results obtained, the highest amounts of recombinant protein were obtained after incubation for 3hr (PINK1) or overnight (BRI3). Since the recombinant proteins contained a His-tag, immunoblot analysis with anti-His antibody confirmed the expression of these proteins (Figure 42). Expression of recombinant BRI2 proved to be more challengeing and requires further experimentation and optimization.

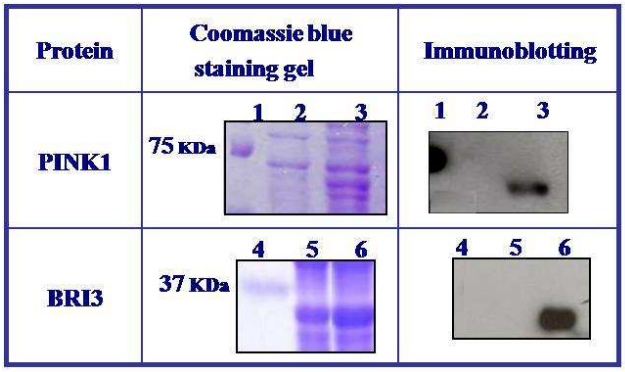

Figure 42: Immunoblot analysis of recombinant PINK1 and BRI3 expression in *E.Coli*. Samples were separated in duplicate by SDS-PAGE and the gel was divided in two parts: one for staining with Coomassie blue and the other for immunobloting with anti-His antibody. Lanes 1 and 4, Prestained SDS-PAGE Standards; Lanes 2 and 5, empty vector control; Lane 3, PINK1 expression; Lane 6, BRI3 expression.

The expression of recombinant PINK1 and BRI3 in *E.coli* was relatively successful. PINK1 appears too migrated as a band of around 63KDa, as expected, and BRI3 appears as a single band around 30 KDa.

Recombinant proteins, such as BRI2, with hydrophobic regions often have a toxic effect on host cells, most likely due to the association of the protein with or incorporation into vital membrane systems; this may explain the observed difficulty to express BRI2. This effect could be reduced by limiting the level of basal transcription that occurs in the absence of induction, by reducing the number of generations before induction, and by reducing the growth temperature from 37ºC to 25°C before induction. The IPTG concentration could be also optimized, to lower or higher concentrations. Nevertheless, for future uses the expression of PINK1, BRI2 and BRI3 needs to be optimized.

III.4. DISCUSSION

In this study we report three novel interacting proteins for PP1γ2, expressed in human brain: PINK1, BRI2 and BRI3. These interactions are supported by different techniques, such as yeast co-transformation and co-immunoprecipitation from mammalian cells. The yeast co-transformation indicates that PINK1, BRI2 and BRI3 interact with PP1γ. However, regarding PINK1, it will be interesting in the future, to construct a clone without the C-terminus binding motif, to determine if the N-terminus and/or C-terminus binding motifs interact preferentially with PP1. Other crucial validation of the two-hybrid results is to prove that the two proteins exist in the same subcellular environment through co-immunoprecipitation. As demonstrated, ours result show that a PP1 isoform (PP1γ) interacts with PINK1, BRI2 and BRI3. The BRI2 and BRI3 proteins appear as two distinct bands in the IP, probably because they are cleaved by furin at amino acid 244 for BRI2 and amino acid 242 for BRI3.

Another important validation of the putative interactions discovered with the YTH system is the confirmation of co-expression of the two binding partners in either the same cell-type or the same subcellular compartment. A condition for *in vivo* interaction of two proteins is their simultaneous presence in the same subcellular compartment. It was, therefore, important to confirm the subcellular localization of these proteins. PINK1 is generally accepted to be a mitochondrial protein encoded by nuclear genes, it is likely to be synthesized in the cytosol and then imported into the mitochondria (Sim *et al*., 2006). Several reports have suggested PINK1 might also reside in the cytoplasm at low levels, particularly in the context of proteosomal inhibition (Weinhofen *et al*., 2008). Ours result are in overall agreement with such studies. PP1 and PINK1 may interact in the cytoplasm were PP1 is present before PINK1 is targeted to the mitochondria. The BRI2 and BRI3 protein are both cleaved, by furin in the cis- or medial-Golgi compartment and appears at the cell surface (Vidal, *et al*., 2004; Tsachaki *et al*., 2008). For BRI3 it was described that this processing occurs in the TGN (*Trans* Golgi network) or post-TGN compartments, where furin is active (Wickham *et al*., 2005). Our co-localization data further indicates that the PP1γ is in the same subcellular environment as PINK1, BRI2 and BRI3.

PINK1, BRI2 and BRI3 are expressed in forebrain regions (diencephalon and telencephalon) such as the striatum where these PP1 is highly abundant. This similarity of expression pattern strengthens the physiological context of the interaction between PP1

and PINK1, BRI2 and BRI3, since is possible that these proteins could be expressed in the same cells. The subcellular locations of these proteins also strengthen the interaction, given that is well established that PP1 isoforms are ubiquitously present in much cellular localization (Andreassen, *et al*., 1998; Lesage, *et al*., 2005).

Further data provide biological relevance to these interactions, since the novel PP1 interacting proteins are involved in neurodegenerative conditions. For example, autosomal recessive early-onset Parkinson and late-onset Parkinsonism caused by mutations in the PINK1 gene (Valente *et al*., 2004 (b); Rogaeva *et al*., 2004; Hedrich *et al*., 2006). Studies suggest PINK1 maybe a neuroprotective protein, and maybe involved with LTP and LTD, processed also known to involve PP1 (Ibáñez *et al*., 2006; Kitada *et al*., 2007; Bogaerts *et al*., 2008). The BRI2 protein is associated with various neurodegenerative conditions, such as FBD and FDD, which are clinically and pathologically similar to Alzheimer disease (Matsuda *et al*., 2005). Alzheimer´s disease is caused by abnormal phosphorylation of Tau protein that results in neurofibrillary degeneration (Delobel *et al*. 2002). It was also proposed that the Tau gene is implicated in the pathogenesis of PD (Healy *et al*., 2004) and it is known that PP1 is involved in the dephosphorylation of hyperphosphorylated tau protein (Rahman *et al*., 2005). Furthermore, BRI2 was shown to interact with APP, which is recognized as one of the primary neurotoxic agents in Alzheimer's disease (Fotinopoulou *et al*, 2005 ; Kim *et al*., 2008), and these is evidence that the proteolytic cleavage of APP involves PP1 (Knobloch *et al*., 2007). BRI3 colocalizes in neurons with *b*-amyloid converting enzyme 1 (BACE 1), which participates in the amyloidogenic pathway that generates the Alzheimer's A*b* peptide (Wickham *et al*., 2005; Rogaeva *et al*., 2004). Whether this colocalization has any implications in disease pathogenesis remains unknown.

IV. CONCLUSION

PP1 is involved in several important physiological processes, such as cell cycle control, apoptosis, transcription, motility, metabolism and memory, regulating them through the dephosphorylation of multiple key substrates. This plasticity of PP1 is due to interaction with diverse regulators and targeting subunits that function as inhibitors, substrate specifies and substrate targeting proteins. Therefore, the identification of its binding proteins and regulators is required in order to understand the function and regulation of PP1 in eukaryotic cells. Several PP1 interacting proteins have been identified through Yeast Two-Hybrid screens.

Our results identified several previously known PP1 regulators, such as p53BP2. This validates our approach and the results obtained. Of particular interest was the identification of a great number of potential new PP1 binding proteins, possibly providing new perspectives on PP1 function in the human brain and in neuronal processes, and validating the Yeast Two-Hybrid System as a useful tool to explore the role of PP1 in cellular regulation. Among the new interacting proteins identified are PINK1, BRI2 and BRI3.

Although PP1γ2 (the bait used in our screen) has been shown to be particularly enriched in human testis, its expression in mammalian brain has been previously described. Thus, it will be interesting to compare the PP1γ2 brain interactome described here with the PP1γ2 interactome previously determined in the laboratory. Of course, given the high degree of similarly between all PP1 isoforms, the nature of the YTH methodology may not discriminate sufficiently between the different PP1 isoforms. Consequently, the interaction of the proteins identified in our screen with PP1γ2 (or with other PP1 isoforms) needs to be confirmed using more physiologically relevant techniques. In this respect, given the lack of information regarding the function of PP1γ2 in the brain, our results may provide novel insights into the physiological relevance of PP1γ2 in human brain.

Having identified BRI3 in our screen as a PP1 interacting protein, it was inevitable to address the question of whether BRI2 might also interact with PP1, given the high degree of homology between BRI2 and BRI3. Indeed, our results indicate that both proteins interact with PP1 *in vitro* and *in vivo.* The other protein chosen for further characterization was PINK1. Given the well-characterized role of these proteins in different neurodegenerative conditions, their identification as PP1 interactors/regulators is

particularly interesting. The possible involvement of PP1 in those corresponding conditions deserves to be further investigated in the future.

PP1α, PP1β and PP1γ1, are widely expressed in mammalian brain, particularly in dopaminergic neurons of the basal ganglia. Indeed, PP1α and PP1γ1 were show to be exquisitely enriched in the dendritic spines, a critical site for neuronal signal transduction. However, much less is known regarding PP1γ2 expression and localization in mammalian brain. Our results, using a variety of different techniques, indicate that PINK1, BRI2 and BRI3 interact with PP1γ1 and PP1γ2. However, further work is required to investigate their interaction in neuronal systems. The possible involvement of PP1 isoforms in Parkinson Disease and Familial British/Danish Dementia, as suggested by our work is a novel and interesting finding, highlighting the central role for PP1 in mammalian brain. The large number of interacting proteins identified as comprising the PP1γ2 brain interactome precluded a systematic investigation of all of them. Here we chose to focus on a selected few known to be involved in dysfunctional states. However, the PP1γ2 human brain interactome revealed here should provide unforeseen clues to the yet unknown roles of PP1 in human health and disease.

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VI. APPENDICES

APPENDIX I - MATERIALS

- **Bacterial media**

LB (Luria-Bertani) Medium

To 950 mL of deionised H_2O add:

LB 25 g

Agar 20 g (for plates only)

Shake until the solutes have dissolved. Adjust the volume of the solution to 1 liter with deionised H_2O. Sterilize by autoclaving.

SOB Medium

SOB 25 g/l

KCL 10 mM

To 950 mL of deionised H_2O add:

25,5 g SOB Broth

Shake until the solutes have dissolved. Add 10mL of a 250mM KCl (prepared by dissolving 1.86g of KCl in 100 mL of deionised H_2O). Adjust the pH to 7.0 with 5N NaOH. Adjust the volume of the solution to 1 liter with deionised H_2O. Sterilize by autoclaving. Just prior to use add 5 mL of a sterile solution of 2M $MgCl_2$ (prepared by dissolving 19 g of $MgCl_2$ in 90 mL of deionised H_2O; adjust the volume of the solution to 1000 mL with deionised H_2O and sterilize by autoclaving).

SOC Medium

SOC is identical to SOB except that it contains 20 mM glucose. After the SOB medium has been autoclaved, allow it to cool to 60ºC and add 20mL of a sterile 1M glucose (this solution is made by dissolving 18 g of glucose in 90 mL of deionised H_2O; after the sugar has dissolved, adjust the volume of the solution to 1 L with deionised H_2O and sterilize by filtration through a 0.22-micron filter).

- **Antibiotics (1000x)**

100 mg/ml of Ampicilin

30 mg/ml of Kanamycin

- **Yeast Media**

SD synthetic medium/ agar

SD 6.8 g/l

Agar-Agar 10 g/l

Sterilize by autoclaving, after autoclaving add 50 ml of sterile Glucose 40% (final concentration 2%) and 100 ml of the appropriate 10x dropout solution.

10x dropout solution

This solution contains all but one or more of these nutrients. Serine, aspartic acid, and glutamic acid are not included because they make the medium to acid and because yeast can synthesize these amino acids endogenously.

	10x concentration (mg/L)
L-Isoleucine	300
L-Valine	1500
L-Adenine hemisulfate salt	200
L-Arginine HCL	200
L-Arginine HCL monohydrate	200
L-Leucine	1000
L-Lysine HCL	300
L-Methionine	200
L- Phenylalanine	500
L-Threonine	2000
L-Tryptophan	200
L-Tyrosine	300
L-Uracil	200

10x dropout supplements were autoclaved and stored up to one year

Double DropOut; DO/-Leu/-Trp

Triple Drop Out; DO/-Leu/-Trp/-His
Quadruple Drop Out DO/-Leu/-Trp/-His/-Ade

- **Yeast medium- YPD medium**

To 950mL of deionised H_2O add:
50 g YPD
20 g Agar (for plates only)
Shake until the solutes have dissolved. Adjust the volume to 1 L with deionised H_2O and sterilize by autoclaving. Allow medium to cool to 60ºC and add glucose to 2% (50mL of a sterile 40% stock solution).

- **Plasmid extraction of yeast by boiling method**

STET
8% Sucrose
5% Triton X-100
50 mM Tris pH 8.0 (Stock 1 M)
50 mM EDTA pH 8.0 (Stock 0.5 M)

- **Miniprep Solutions:**

Solution I
50 mM glucose
25 mM Tris.HCl (pH 8.0)
10 mM EDTA

Solution II
0.2 N NaOH
1% SDS

Solution III
3 M potassium acetate

2 M glacial acetic acid

- **Midiprep Solutions**

Cell Resuspension Solution
50 mM Tris-HCl (pH 7.5)
10 mM EDTA
100 µg/ml RNAase A

Cell Lysis Solution
0.2 M NaOH
1% SDS

Neutralization Solution
4.09 M Guanidine hydrochloride (pH 4.8)
759 mM potassium acetate
2.12 M Glacial acetic acid

Column Wash Solution
60 mM potassium acetate
8.3 mM Tris-HCl (pH 7.5)
0.04 mM EDTA
60 % ethanol

- **DNA Electrophoresis**

50x TAE
2M Tris-acetate
0.05 M EDTA
0.9 M Glacial acetic acid

Ethidium bromide (10 mg/ml)

Loading Buffer

3.7 mM bromophenol blue, 30% glycerol

- **Preparation of heat shock competent *E. coli* XL1-blue cells**

Buffer 1

Glycerol	15%
$MnCl2 \cdot 4 H_20$	50 mM
$CaCl2 \cdot 2H_20$	10 mM
Potassium acetate (1M)	30 mM

Adjust the pH to 5.8 with acetic acid

Use filter 0.2 μm for sterilisation, and store at 4ºC

Buffer 2

Glycerol	15%
CaCl2.2H2O	75 mM
RbCl	10 mM
MOPS (0.5 M pH 6.3)	10 mM

Use filter, 0.2 μm for sterilisation, and store at 4ºC

- **SDS-PAGE**

4x LGB (Lower Gel Buffer)

1M Tris

14 mM SDS

Adjust the pH to 8.9 and adjust volume to 1 l with deionized water.

5x UGB (Upper Gel Buffer)

0.6M Tris

Adjust pH to 6.8 with HCl

10x Running Buffer

250 mM Tris-HCl (pH8.3)

250 mM Glycine

1% SDS

10x Transfer Buffer

250 mM Tris-HCl (pH8.3)

1.92 M Glycine

4x Protein Loading Buffer

250 mM Tris HCl (pH 6.8)

8 % SDS

40 % Glycerol

2 % β-Mercaptoethanol

0,01 % Bromophenol blue

30%Acrylamide/0.8% Bisacrylamide

To 70 mL of deionised H_2O add:

29.2 g Acrylamide

0.8 g Bisacrylamide

Mix until the solutes have dissolved. Adjust the volume to 100mL with deionised H_2O. Store at 4ºC.

- **Coomassie blue staining solutions**

Staining solution

0.1% Coomassie Brilliant Blue

25% Methanol

10% Acetic Acid

Destaining solution

50% Methanol

5% Acetic Acid

- **Immunoblotting**

10X TBS

10mM Tris-HCl (pH 8.0)

150mM NaCl

10x TBST

10mM Tris-HCl (pH 8.0)

150mM NaCl

0.05% Tween20

- **Antibody stripping solution**

62.5 mM Tris-HCl (pH 6.7)

2% SDS

100mM 2-mercaptoethanol 98% (Sigma)

- **Binding assay by yeast co-transformation**

10x TE

10mM Tris-HCl (pH7.5)

1mM EDTA (pH 8.0)

1x LiAc/ 1x TE

PEG4000/LiAc/TE

Final concentration: 40% PEG, 1xLiAc, 1xTE

- **Mammalian cell expression**

1xPBS

For a final volume of 500ml, dissolve one pack of BupH Modified Dulbecco´s Phosphate Buffered Saline Pack (Pierce) in deionised water.

Final composition:

8 mM Sodium phosphate

2 mM Potassium phosphate

140 mM Sodium chloride

10 mM Potassium chloride

1 mg/mL Poly-L-ornithine solution (10x)

To a final volume of 100 mL, dissolve in deionised H_2O 100 mg of poly-L-ornithine (Sigma-Aldrich, Portugal).

4% Paraformaldehyde Fixative solution

For a final volume of 100 mL, add 4 g of paraformaldehyde to 25 mL deionised H_2O. Dissolve by heating the mixture at 58 ºC while stirring. Add 1-2 drops of 1 M NaOH to clarify the solution and filter (0.2 μm).

Add 50 mL of 2X PBS and adjust the volume to 100 mL with deionised H_2O.

Mammalian cell culture medium (COS7 cells)

Complete DMEM medium, for maintenance of COS7 cells. For a final volume of 1 l, dissolve one pack of DMEM powder (with L-glutamine and 4500 mg glucose/l, Sigma) in deionised water and add: 44 mM NaHCO3 (Sigma-Aldrich) and 100 ml/l Fetal Bovine Serum (FBS) (Gibco BRL, Invitrogen). Adjust pH to 7.4 and add 10 ml of a Stretomycin/ Penicilin/ Amphotericin solution (Gibo BRL, Invitrogen). Sterilize by filtration through a 0.2μm filter and store at 4°C.

Transfection medium (DMEM) for COS7 cells

For a final volume of 1 l, dissolve one pack of DMEM powder (with L-glutamine and 4500 mg glucose/l, Sigma) in deionised water and add: 44 mM NaHCO3 (Sigma-Aldrich). Adjust pH to 7.4

Transfection medium (complete MEM + GLUTAMAX) for HeLa cells

For a final volume of 500 mL, add:

50 mL (10% v/v) Fetal Bovine Serum (FBS) (Gibco BRL, Invitrogen)

5 mL Non-Essential aminoacids (100x)

100 U/mL penicillin

100 mg/mL streptomycin 5 mL

FBS is heat-inactivated for 30 min at 56 ºC. For cells maintenance, prior to pH adjustment add 100 U/mL penicillin and 100 mg/mL streptomycin [10 mL Streptomycin/ Penicilin/Amphotericin solution (Gibco BRL, Invitrogen)

MEM + GLUTAMAX Components

Amino Acids:	**Concentration (mg/L)**
L-Alanyl-Glutamine	406
L-Arginine hydrochloride	126
L-Cystine	24
L-Histidine hydrochloride	42
L-Isoleucine	52
L-Leucine	52
L-Lysine hydrochloride	73
L-Methionine	15
L-Phenylalanine	32
L-Threonine	48
L-Tyrosine	10
L-Valine	46
Vitamins:	

Choline chloride	1
D-Calcium pantothenate	1
Folic Acid	1
Niacinamide	1
Riboflavin	0.1
Thiamine hydrochloride	1
i-Inositol	2
Inorganic Salts:	
Calcium Chloride ($CaCl_2.2H_2O$)	264
Magnesium Sulfate ($MgSO_4.7H_2O$)	200
Potassium Chloride	400
Sodium Bicarbonate	2200
Sodium Chloride	6800
Sodium Phosphate monobasic ($NaH_2PO_4.2H_2O$)	158
Other components:	
D-Glucose	1000
Phenol Red	10

- **Immunoprecipitation**

Lysis Buffer

50 mM Tris-HCl pH 8.0

120 mM NaCl

4% CHAPS

Lysis Buffer + Protease inhibitors

Add to 4 mL of Lysis buffer the following quantities for a final volume of 5 mL:

23,8 µl Pepstatin A (1 mg/mL stock solution in DMSO)

0,72 µl Leupeptin (5 mg/mL stock solution)

180 µl Benzamidine (200 mM stock solution)

43,2 µl Aprotinin (2.1 mg/mL stock solution)
176 µl PMSF 100X

Washing solution

50 mM Tris-HCl
120 mM NaCl

Protein A Sepharose (for one sample)

Incubate 20 mg in 1ml of lyses buffer for 30 min. Wash 2 times with 500 µl of lyses buffer and resuspend in 50 µl of lyses buffer

Table 18: List of suppliers and reagents.

Supplier	Reagents
Applied Biosystems	Ready Reaction Mix (dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA polymerase, FS, rTth pyrophosphatase, magnesium chloride and buffer), formamide.
	SOC medium (SOB, $MgCl_2$, $MgCO_4$)
QIAGEN	QIAquick
Invitrogen Live Technologies	GAL4-AD primer (sequence T ACC ACT ACA ATG GAT G) and the 1 Kb ladder DNA molecular weight markers.
New England Biolabs	Hind III restriction endonuclease
UBS	ethidium bromide
Sigma	Ampicillin, EDTA, glucose, MgSO4, MnCl2.4H2O, RbCl, RNAse, sodium acetate, Triton x-100, glass beads.
Merck	Ethanol, agar, LB broth, KCl,

APPENDIX II- BACTERIA AND YEAST STRAINS

- *E. coli* XL1- blue: *recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacZΔM15 Tn10(Tet^r)]*

- S. cerevisiae AH109: MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal 80Δ, LYS2:: $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ, MEL1

- S. cerevisiae Y187: MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met-, gal 80Δ, URA3:: $GAL1_{UAS}$-$GAL1_{TATA}$-lacZ, MEL1

APPENDIX III-PRIMERS

PRIMER	**SEQUENCE (5':::: 3')**	Nt. No.
GAL4 AD	TAC CAC TAC AAT GGA TG	17
pBri3-Eco RI-ATG	CCT TGA ATT CGA ATG GTG AAG ATT AGC TTC	30
pBri3-STOP-Xho I	GAA ACT CGA GTC ACA CCA CCC CGC AG	26
pBri2-Eco RI- ATG	CCT TGA ATT CGA ATG GTG AAG GTG ACG	27
pBRI2-STOP-XhoI	GAAA CTC GAG TCA AGA AGA AAT TAA AG	27
pPINK1-Eco RI- ATG	CCT TGA ATT CGA ATG GCG GTG CGA CAG	27
pPINK1-STOP-XhoI	GAAA CTCGAG TCA CAG GGC TGC CCT C	26
M13/pUC	AGC GGA TAA CAA TTT CAC ACA GG	23
pBri3-seq1-496	ACG AGC GCA TCA ACG TGC	18
pBri3-seq2-731	GCT CCG GCG CCG GGC AAC	18
pPINK1-seq2-187	GGG CTC GGG CTC CCT AAC	18
pPINK1-seq3-542	TGC CCC AGA ACC TGG AGG	18
pPINK1-seq4-1034	TGC TGC TGC AGC TGC TGG	18
pPINK1-seq5-1588	TGG TTG GCT GGC TCC TCC	18
pBRI2-seq1-240	GAG GAG GCG CTC ATC ATC	18
pBRI2-seq2-636	TAT TTA GAT CTT AAC CTG	18
PINK1FWXho1	G CTC GAG GA ATG GCG GTG CGA CAG GCG	28
PINK1RVEcoR1	C GAA TTC TA CAG GGC TGC CCT CCA TG	27

APPENDIX IV- VECTORS

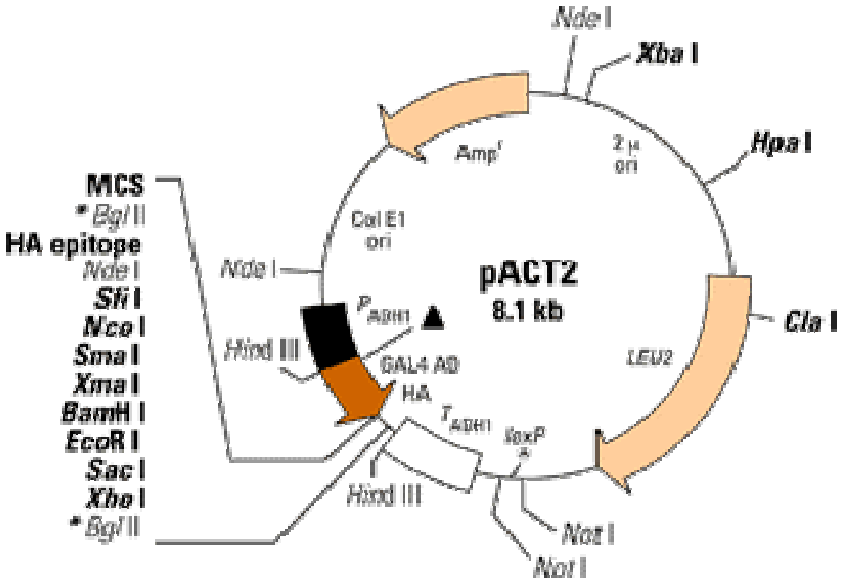
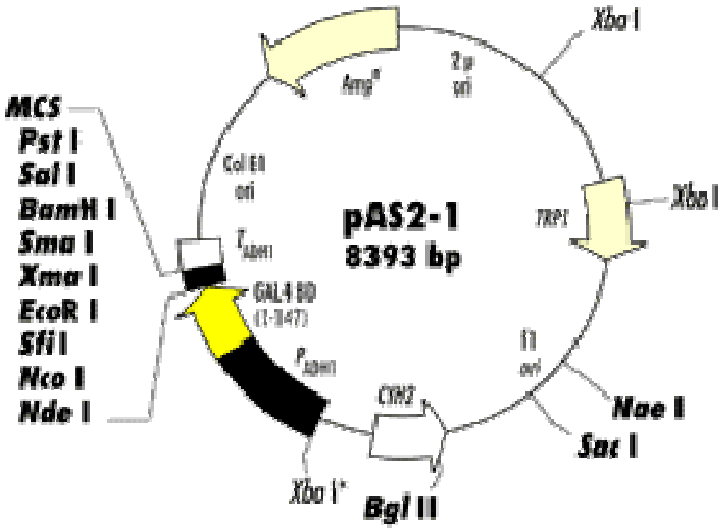


Figure 43: pACT2 map and MCS. Unique sites are coloured blue. pACT2 is used to generate a hybrid containing the GAL4 AD, an epitope tag and a protein encoded by a cDNA in a fusion library. The hybrid protein is expressed at medium levels in yeast host cells from an enhanced, truncated ADH1 promoter and is target to the nucleus by the SV40 T-antigen nuclear localization sequence. pACT2 contains the LEU2 gene for selection in Leu$^-$ auxotrophic yeast strains.



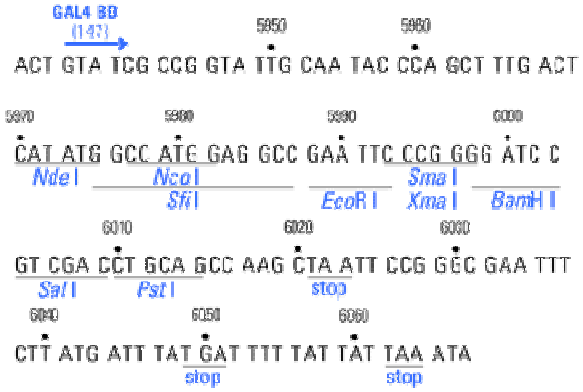


Figure 44: pAS2-1 map and MCS. Unique sites are coloured blue. pAS2-1 is a cloning vector used to generate fusions of a bait protein with the GAL4 DNA-BD. The hybrid protein is expressed at high levels in yeast host cells from the full-length ADH1 promoter. The hybrid protein is target to the yeast nucleus by nuclear localization sequences. pAS2-1 contains the TRP1 gene for selection in Trp$^-$ auxotrophic yeast strains.

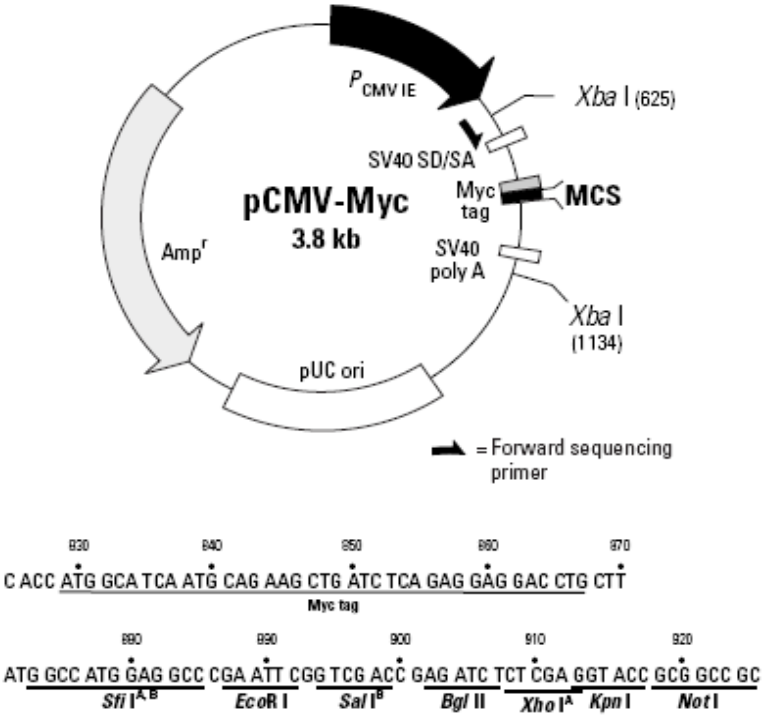
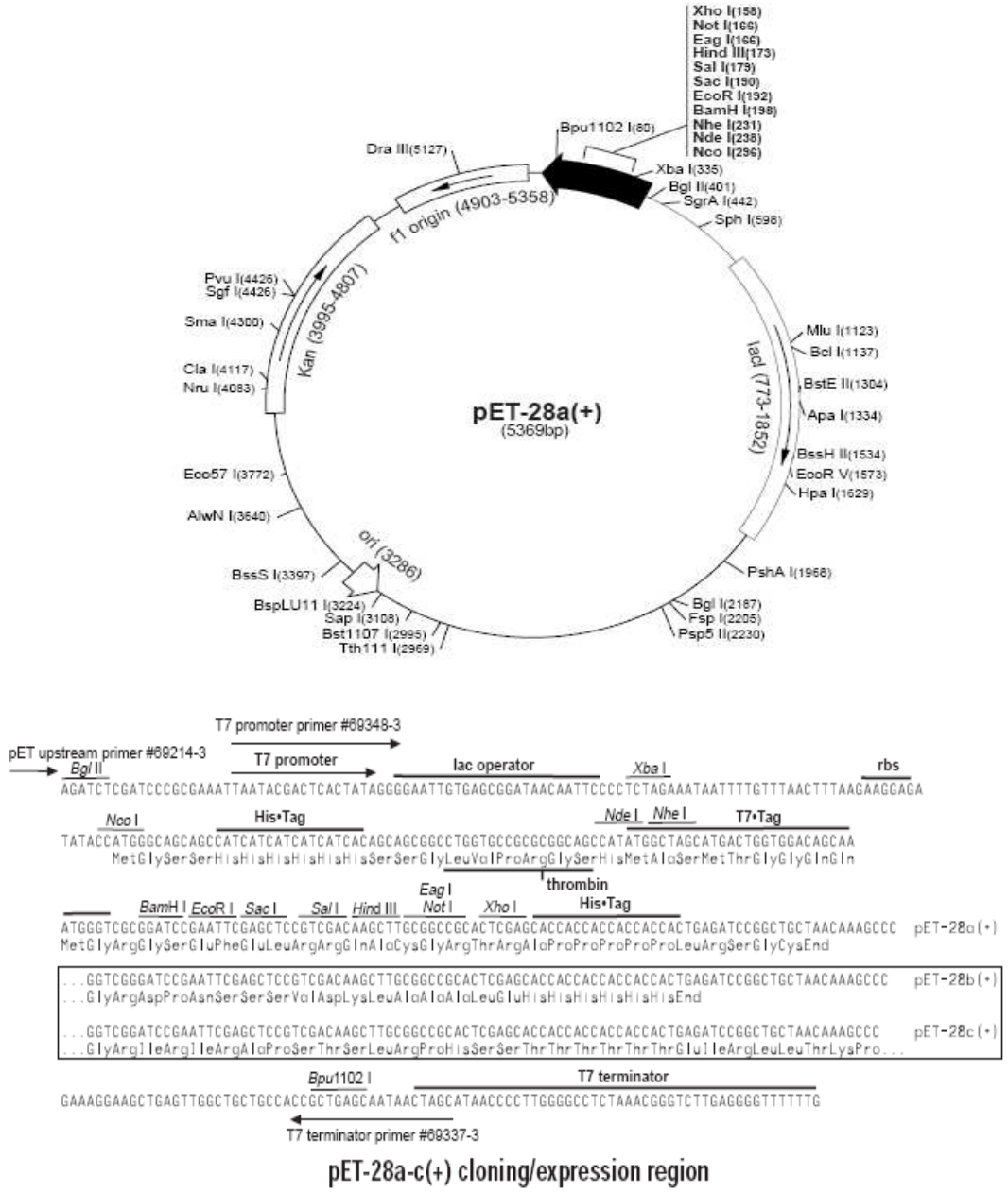


Figure 45: The pCMV-Myc Mammalian Expression Vector c-Myc epitope tag is well-characterized and highly immunoreactive. High-level expression in mammalian cells is driven from the human cytomegalovirus immediate early promoter/enhancer (*P*CMV IE). The vector contains an intron (splice donor/splice acceptor); the epitope tag; an MCS; and a polyadenylation signal from SV40. This vector also possesses the ampicillin resistance gene for selection in *E. coli.*



pET-28a-c(+) cloning/expression region

Figure 46: The pET-28a-c(+) vectors carry an N-terminal His•Tag®/thrombin/T7•Tag® configuration plus an optional C-terminal His•Tag sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand.

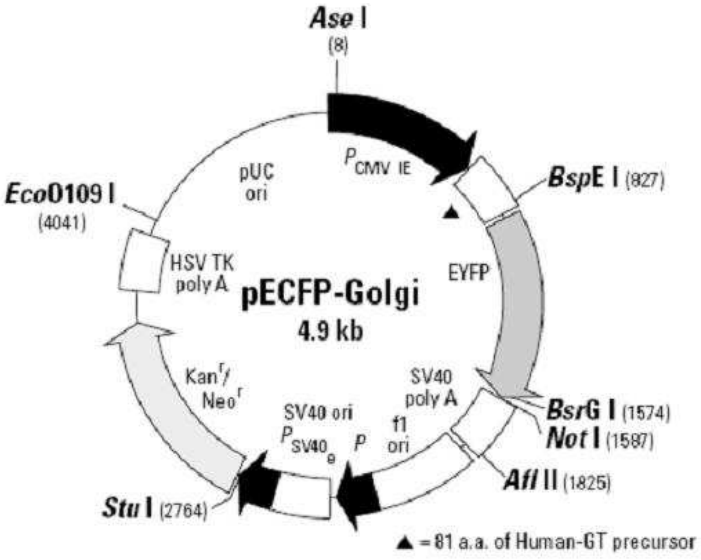


Figure 47: pECFP-Golgi encodes a fusion protein consisting of enhanced cyan fluorescent protein (ECFP) and a sequence encoding the N-terminal 81 amino acids of human beta 1,4galactosyltransferase. This region of human beta 1, 4-GT contains the membrane-anchoring signal peptide that targets the fusion protein to the trans-medial region of the Golgi apparatus.