Universidade de Aveiro Departamento de Biologia
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PP1 INTERACTOMES AS A MEANS OF CHARACTERIZING PROTEIN FUNCTIONS

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica da Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar Convidada da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro e co-orientação científica da Doutora Odete Abreu da Cruz e Silva, Professora Auxiliar com Agregação da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro.

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palavras-chave Interactoma, Proteína Fosfatase 1, fosforilação, transducção de sinais, cérebro

resumo

A maioria das funções celulares, incluindo expressão de genes, crescimento e proliferação celulares, metabolismo, morfologia, motilidade, comunicação intercelular e apoptose, é regulada por interações proteína-proteína (IPP). A célula responde a uma variedade de estímulos, como tal a expressão de proteínas é um processo dinâmico e os complexos formados são constituídos transitoriamente mudando de acordo com o seu ciclo funcional, adicionalmente, muitas proteínas são expressas de uma forma dependente do tipo de célula.

Em qualquer instante a célula pode conter cerca de centenas de milhares de IPPs binárias, e encontrar os companheiros de interação de uma proteína é um meio de inferir a sua função. Alterações em redes de IPP podem também fornecer informações acerca de mecanismos de doença. O método de identificação binário mais frequentemente usado é o sistema Dois Hibrido de Levedura, adaptado para rastreio em larga escala. Esta metodologia foi aqui usada para identificar os interactomas específicos de isoforma da Proteína Fosfatase 1 (PP1), em cérebro humano. A PP1 é uma proteína fosfatase de Ser/Thr envolvida numa grande variedade de vias e eventos celulares. É uma proteína conservada codificada por três genes, que originam as isoformas α, β, e γ, com a última a originar γ1 e γ2 por *splicing* alternativo. As diferentes isoformas da PP1 são reguladas pelos companheiros de interação – proteínas que interagem com a PP1 (PIPs). A natureza modular dos complexos da PP1, bem como a sua associação combinacional, gera um largo reportório de complexos reguladores e papéis em circuitos de sinalização celular.

Os interactomas da PP1 específicos de isofoma, em cérebro, foram aqui descritos, com um total de 263 interações identificadas e integradas com os dados recolhidos de várias bases de dados de IPPs. Adicionalmente, duas PIPs foram selecionadas para uma caracterização mais aprofundada da interação: Taperina e Sinfilina-1A.

A Taperina é uma proteína ainda pouco descrita, descoberta recentemente como sendo uma PIP. A sua interação com as diferentes isoformas da PP1 e localização celulares foram analisadas. Foi descoberto que a Taperina é clivada e que está presente no citoplasma, membrana e núcleo e que aumenta os níveis de PP1, em células HeLa. Na membrana ela co-localiza com a PP1 e a actina e uma forma mutada da Taperina, no motivo de ligação à PP1, está enriquecida no núcleo, juntamente com a actina. Mais, foi descoberto que a Taperina é expressa em testículo e localiza-se na região acrossómica da cabeça do espermatozoide, uma estrutura onde a PP1 e a actina estão também presentes.

A Sinfilina-1A, uma isoforma da Sinfilina-1, é uma proteína com tendência para agregar e tóxica, envolvida na doença de Parkinson. Foi mostrado que a Sinfilina-1A liga às isoformas da PP1, por co-transformação em levedura, e que mutação do seu motivo de ligação à PP1 diminuiu significativamente a interação, num ensaio de *overlay*. Quando sobre-expressa em células Cos-7, a Sinfilina-1A formou corpos de inclusão onde a PP1 estava presente, no entanto a forma mutada da Sinfilina-1A também foi capaz de agregar, indicando que a formação de inclusões não foi dependente de ligação à PP1.

Este trabalho dá uma nova perspetiva dos interactomas da PP1, incluindo a identificação de dezenas de companheiros de ligação específicos de isoforma, e enfatiza a importância das PIPs, não apenas na compreensão das funções celulares da PP1 mas também, como alvos de intervenção terapêutica.

keywords

Interactome, Protein Phosphatase 1, phosphorylation, signal transduction, brain

abstract

Most of the crucial functions in the cell, including gene expression, cell growth and proliferation, metabolism, morphology, motility, intercellular communication and apoptosis, are regulated by protein-protein interactions (PPIs). Cells respond to a variety of stimuli, thus protein expression is a dynamic process and the complexes formed are transiently assembled and change during their functional cycle; additionally, many proteins are expressed in a cell type-dependent manner.

At any time, cell may contain about hundreds of thousands of binary PPIs, and finding interaction partners of a certain protein it's a mean of discovering its function. Changes in PPIs networks may also provide information about disease mechanisms. The most frequently used binary identification method is the Yeast Two Hybrid system, adapted to high-throughput screening. This approach was here used in order to identify the Protein Phosphatase 1 (PP1) isoform specific interactomes, in the human brain. PP1 is a Ser/Thr protein phosphatase involved in a large variety of cellular pathways and events. It is a conserved protein codified by three genes giving rise to the α, β and γ isoforms, with the last originating γ1 and γ2 by alternative splicing. PP1 isoforms are regulated by the binding partners – PP1 interacting proteins (PIPs). The modular nature of the PP1 complexes, as well as their combinational assembly, generates a large repertoire of regulatory complexes and roles in signaling circuits.

The human brain isoform specific interactomes of PP1 were here described, with a total of 263 interactions identified and integrated with the data collected from several PPIs databases. Also, two PIPs were selected for further characterization of the interaction: Taperin and Synphilin-1A.

Taperin is a poorly described protein, recently found to be a PIP. Its interaction with PP1 different isoforms and localization in the cell was analyzed. Taperin was found to be cleaved and to be present in the cytoplasm, membrane and nucleus and to increase the levels of PP1, in HeLa cells. In the membrane it co-localizes with PP1 and actin and a mutant form of Taperin, in the PP1 binding motif, is enrich in the nucleus together with actin. Moreover, Taperin was found to be expressed in testis and to localize in the acrossome region of the sperm head, a structure where PP1 and actin are also present.

Synphilin-1A, an isoform of Synphilin-1, is an aggregation prone and toxic protein involved in Parkinson`s Disease. Synphilin-1A was shown to bind PP1 isoforms, by yeast co-transformation, and mutation of its PP1 binding motif decrease significantly the interaction in an overlay assay. When overexpressed in Cos-7 cells, synphilin-1A formed inclusion bodies where PP1 was present, but the mutated form of Synphilin-1A was also able to aggregate indicating that inclusions formation was not dependent on PP1 binding.

This work gives a new perspective of PP1 interactomes, including the identification of dozens of isoform specific binding partners, and emphasizes the importance of PIPs, not only in the understanding of PP1 physiological functions but also, as targets for therapeutic interventions.

I have never know whether you should say one with your foot on the sidewalk, two with the following foot on the first step, and so on, or whether the sidewalk shouldn`t count. At the top of the steps I fell foul of the same dilemma.
(...) After all it is not the number of steps that matters.

Samuel Beckett, The Expelled.

INDEX

PUBLICATIONS

This thesis contains experimental results included in the publications indicated below. The author of this thesis declares that she participated in the planning and execution of the experimental work, as well as, in data interpretation and in the preparation of work for publication.

Manuscript 1 | Sara L. C. Esteves, Sara C. Domingues, Odete A. B. da Cruz e Silva, Margarida Fardilha and Edgar F. da Cruz e Silva. 2011. Protein Phosphatase 1α Interacting Proteins in Brain. *OMICS* 16(1-2):3-17.

Manuscript 2 | Sara L. C. Esteves, Luís Korrodi-Gregório, Zita Cotrim, Paula J. M. van Kleeff, Sara C. Domingues, Odete A. B. da Cruz e Silva, Margarida Fardilha and Edgar F. da Cruz e Silva. 2012. Protein Phosphatase 1γ Isoforms Linked Interactions in Brain. *J Mol Neurosci* (Epub ahead of print).

Manuscript 3 | Sara L. C. Esteves, Sandra I. Vieira, Luís Korrodi-Gregório, Joana V. Silva, Georg Luers, Edgar F. da Cruz e Silva, Odete A. B. da Cruz e Silva and Margarida Fardilha. Taperin, a Protein Phosphatase 1 Interacting Protein in Brain and Testis. (In preparation).

Manuscript 4 | Sara L. C. Esteves, Luís Korrodi-Gregório, Georg Luers, Edgar F. da Cruz e Silva, Odete A. B. da Cruz e Silva and Margarida Fardilha. Characterization of the interaction between Synphilin-1A and Protein Phosphatase 1. (In preparation).

Manuscript 5 | Margarida Fardilha, Sara L. C. Esteves, Luís Korrodi-Gregório, Odete A.B. da Cruz e Silva and da Edgar Cruz e Silva. 2010. The physiological relevance of Protein Phosphatase 1 and its interacting proteins to health and disease. *Current Medicinal Chemistry* 17 (33):22.

ABBREVIATIONS

α-Syn	Alpha-Synuclein
AATK	Apoptosis-associated tyrosine kinase
ABPs	Actin binding proteins
Acta2	Actin alpha 2
AD	Activation domain
AI	Autoinhibitory
AKAPs	A-kinase anchoring proteins
AMPA	Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
APP	Amyloid precursor protein
AR	Androgen receptor
ASON	Antisense oligonucleotides
ATM	Ataxia telangiectasia mutated
ATP	Adenosine-5'-triphosphate
AXIN	Axis inhibitor
AXUD1	AXIN1 up-regulated protein
BD	Binding domain
BLAST	Basic local alignment search tool
BMPs	Bone morphogenetic proteins
bp	Base pair
BRCA1	Breast cancer type 1 susceptibility protein
BRCT	BRCA1 C-terminal domain like
BSA	Bovine serum albumin
Ca^{2+}	Calcium

$CaCl_2.2H_2O$	Calcium chloride
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CBD	Ca^{2+} calmodulin-binding domain
CDK	Cyclin-dependent kinases
cDNA	Complementary DNA
CDS	Protein coding sequence
cGMP	Cyclic guanosine monophosphate
CHAPS	3-((3-Cholamidopropyl)dimethylammonium)-1-propanesulfonate
CO_2	Carbon dioxide
COPS	COP9 constitutive photomorphogenic homolog subunit, also known as Jab1/CSN5
CPI-17	Protein kinase C-potentiated inhibitor protein of 17 kDa, also known as PPP1R14A
CSRNP	Cysteine/serine-rich nuclear protein
CTD	C-terminal domain
DAPI	4,6-diamidino-2-phenylindole
DARPP-32	Dopamine- and cAMP-regulated neuronal phosphoprotein, also known as PPP1R1B
ddH_2O	Double distilled water
DIGE	Differential in gel electrophoresis
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPSS laser	Diode-pumped solid-state laser
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid

ER	Endoplasmatic reticulum
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FCP/SCP	TFIIF-associating component of RNA polymerase II CTD phosphatase/small CTD phosphatase
FCPH	FCP homology domain
GADD34	Growth arrest and DNA damage-inducible protein
GFP	Green fluorescent protein
GM	Glycogen binding subunit (muscle)
GSK-3	Glycogen synthase kinase-3
GTP	Guanosine-5'-triphosphate
HCl	Chloridric acid
HDACs	Histone deacetylases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidne
HPRD	Human protein reference database
Hsd17b3	Hydroxysteroid 17 beta dehydrogenase
hsp90	Heat shock protein 90
I1	Inhibitor-1
I2	Inhibitor-2
I3	Inhibitor-3
IB	Immunobloted
IP3R1s	Type 1 inositol (1,4,5)-trisphosphate receptors
IPs	Immunoprecipitates
KIF	Kinesin superfamily proteins
LB	Loading buffer

LB medium	Luria-Bertani medium (Miller)
LBs	Lewy bodies
Leu	Leucine
LGB	Lower gel buffer
LRP	Leucine-responsive regulatory protein
Lrrk2	Leucine-rich repeat kinase 2
MEM	Medium with Earle's salts
Met	Methionine
Mg^{2+}	Magnesium
Mn^{2+}	Manganese
mRNA	Messenger RNA
MYPT1	Myosin phosphatase target subunit 1
NaCl	Sodium chloride
$NaH_2PO_4.2H_2O$	Sodium phosphate monobasic dihydrate
NCBI	National Center for Biotechnology Information
NEDD8	neural precursor cell expressed, developmentally down-regulated 8
NEK2	NIMA related expressed kinase 2
NES	Nuclear export signal
NF-L	Neurofilament-L
NIPP1	Nuclear inhibitor of protein phosphatase 1
NLS	Nuclear localization signal
NMDA	N-Methyl-D-aspartic acid
NMDAR	NMDA receptor
NOM1	Nucleolar protein with MIF4G domain 1
NUB1	NEDD8 ultimate buster 1

PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson`s Disease
PDGFR	Platelet-derived growth factor receptor
PINK1	PTEN-induced putative kinase
PIP	PP1 interacting proteins
PK	Protein kinases
PKA	Cyclic AMP -dependent PK
PMSF	Phenylmethyl-sulfonyl fluoride
PNUTS	Phosphatase 1 nuclear targeting subunit
PP	Protein phosphatase
PP1 BM	PP1 binding motif
PP1c	PP1 catalytic subunit
PPM	Metal-dependent protein phosphatase
PPP	Phosphoprotein phosphatase
Prmn2	Protamine 2
PSD	Post-synaptic density
PSK	Ser/Thr-kinase
PSM	Prostate-specific membrane antigene
PSP	Ser/Thr phosphatase
PTK	Tyr-kinase
PTP	Tyr phosphatase
qRT-PCR	Quantitative RT-PCR
R subunit	Regulatory subunit

RANBP	RAN binding protein
RNA	Ribonucleic acid
ROI	Region of interest
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Real time PCR
SARP	Several ankyrin repeat protein
SD/QDO	Quadruple drop-out synthetic medium
SD/TDO	Triple drop-out synthetic medium
SDS	Sodium dodecyl sulfate
sds22	PP1 regulatory subunit 22
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Ser	Serine
SH2D3C	SH2 domain containing 3C
SIAH	Seven in absentia homolog
SIRT2	Sirtuin 2
SOB	Super optimal broth
Sph1	Synphilin-1
Stmn1	Stathmin 1
Sycp3	Synaptonemal complex protein 3
Tacstd1	Tumor-associated calcium signal transducer 1
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline solution
TBST	Tris-buffered saline solution - Tween
Tctex5	t-complex expressed protein 5

TE	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetramethyl-1,2-diaminomethane
Thr	Threonine
TOPOI	Topoisomerase I
TP53BP2	Tumor suppressor p53-binding protein 2, also known as ASPP2/PPP1R13A
Tpn1	Transition protein 1
TPR	Tetratricopeptide repeat
TPRN	Taperin gene
TSA	Trichostatin A
Tyr	Tyrosine
UGB	Upper gel buffer
UniProt	Universal protein resource
Ura	Uracil
URI	Unconventional prefoldin RPB5 interactor
X-α-Gal	5-Bromo-4-chloro-3-indoxyl-α-D-Galactopyranoside
YLPM1	YLP motif-containing protein 1
YTH	Yeast Two Hybrid

| Chapter I

I | INTRODUCTION

Protein Phosphorylation

Post-translational modifications of proteins, which are not gene-template based, regulate protein function, through changes within its activity, cellular location and interactions with other proteins. The dynamic arrangement, by various combinations, of modular domains in different signaling proteins allows the creation of complex signaling networks and pathways. In addition to performing catalytic functions, signaling proteins modified by phosphorylation, ubiquitination, acetylation, methylation, nitrosylation, etc, serve as scaffolds for the assembly of multiprotein signaling complexes, as adaptors, transcription factors and signal pathway regulators (Figure 1).

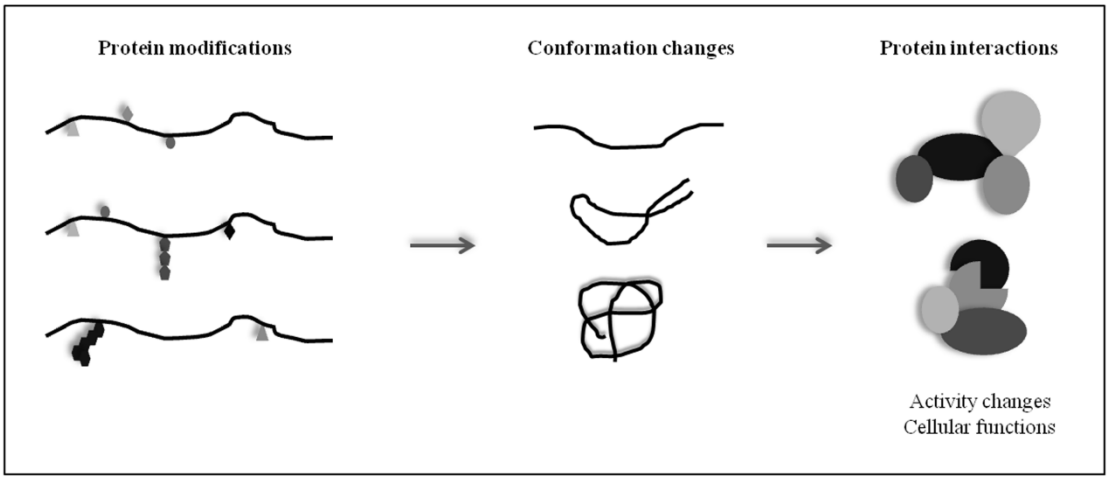

Figure 1 | Schematic representation of protein post-translational modifications related to the regulation of biological processes.

Protein (de)phosphorylation is the best known modification involved in activation and inactivation of enzymes and modulation of molecular interactions in signaling pathways, having the highest number of reported publications in PubMed (Seo, 2004).

Decades of research have shown that reversible phosphorylation of proteins, carried out by protein kinases (PK) and phosphatases (PP), constitutes an essential form of cell

signaling and an important mechanism of regulation in all living organisms. In eukaryotic cells, phosphorylation mainly occurs on three hydroxyl-containing amino acids, serine (Ser), threonine (Thr) and tyrosine (Tyr), of which Ser is the predominant target. A proteomic analysis of 6600 phosphorylation sites on 2244 human proteins revealed that Ser, Thr and Tyr account for 86%, 12%, and 2% of the phosphorylated residues, respectively (Olsen, 2006).

The fully sequenced human genome is thought to contain 518 putative protein kinases (Lander, 2001; Venter, 2001; Johnson, 2005) from two families: 90 Tyr-kinases (PTKs) and 428 Ser/Thr-kinases (PSKs). The specificity of this signaling event and its reversible nature would suggest that there should be similar numbers of protein phosphatases in the human genome. However, there are only 107 putative protein Tyr phosphatases (PTPs) and far fewer (~40) protein Ser/Thr phosphatases (PSPs) (Alonso, 2004; Moorhead, 2007). Whereas the numbers of PTKs and PTPs roughly match each other, the number of catalytic subunits of PSPs is, by far, lower than that of PSKs. This dissimilarity is explained by the combinatorial formation of PSP holoenzymes from a shared catalytic subunit and a large number of target and regulatory subunits. There could be as many distinct PSPs complexes as there are protein PSKs, suggesting that both types of enzymes have a similarly restricted substrate specificity at the holoenzyme level.

Protein Ser/Thr Phosphatases (PSPs)

The initial classification of PSPs was made based on biochemical assays, as either type 1 (PP1) or type 2 (PP2), the last was further subdivided based on metal ion requirement: PP2A, no metal ion; PP2B, Ca^{2+} stimulated; PP2C, Mg^{2+} dependent (Ingebritsen, 1983; Cohen, 1989). The elucidation of complete cDNA and amino acid sequences allowed the separation of PSPs on three major families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and the aspartate-based phosphatases represented by FCP/SCP (TFIIF-associating component of RNA polymerase II CTD phosphatase/small CTD phosphatase) (Chambers, 1994; Cohen, 2002; Yeo, 2003; Moorhead, 2009) (Figure 2).

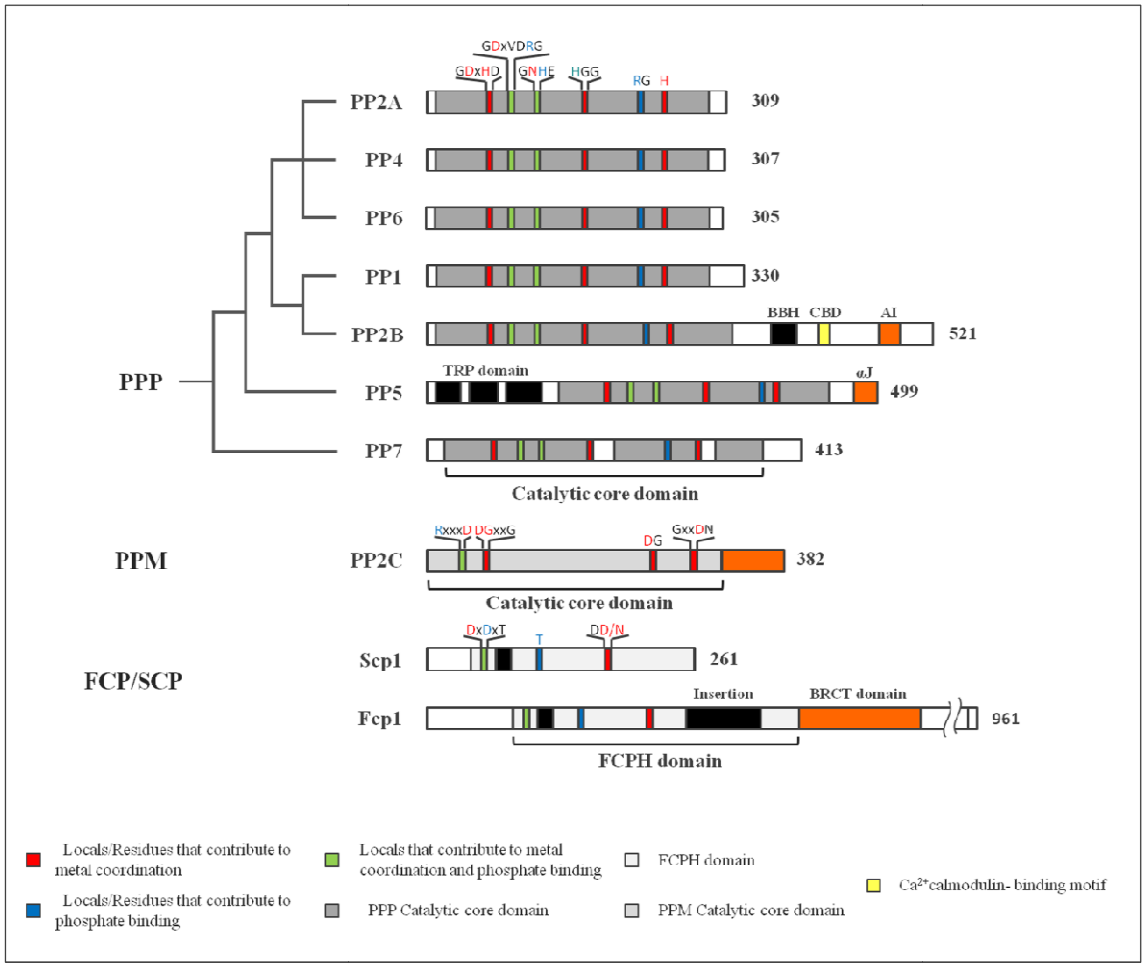

Figure 2 | The Three Families of PSPs: PPP, PPM and FCP/SCP. Representative members of each family are presented here. The catalytic core domains of each protein are indicated below the diagram. Signature sequence motifs are labeled above the diagram. Residues that contribute to metal coordination and phosphate binding are colored in red and blue, respectively. The PPP family contains three characteristic sequence motifs within the conserved 30 kD catalytic domain: GDxHG, GDxVDRG, and GNHE**.** BBH, CNB-binding helix; CTD, carboxy-terminal domain; CBD, Ca^{2+}-calmodulin-binding motif; AI, autoinhibitory sequence; TPR, tetratricopeptide repeat; FCPH, FCP-homology domain. All proteins here are from Homo sapiens except for PP7 (from the model plant Arabidopsis thaliana). Adapted from Shi, 2009.

For several members of the PPP family, the catalytic subunit associates with a great variety of regulatory subunits. Representative members of the PPP family include protein phosphatase 1 (PP1), PP2A, PP2B (commonly known as calcineurin), PP4, PP5, PP6, and PP7 (Figure 2). The PPM family includes protein phosphatases dependent on Mn^{2+}/Mg^{2+}, such as PP2C and pyruvate dehydrogenase phosphatase. In contrast to the

PPP family, members of the PPM family do not have regulatory subunits but contain instead additional domains and conserved sequence motifs that may help determine substrate specificity (Moorhead, 2009). For both PPP and PPM, metal ions play a catalytic and central role through the activation of a water molecule for the dephosphorylation reaction (Egloff, 1995; Goldberg, 1995; Barford, 1996; Moorhead, 2009). In contrast, FCP/SCP uses an aspartate-based catalysis mechanism. The only known substrate for FCP/SCP is the C-terminal domain (CTD) of RNA polymerase II, which contains tandem repeats of a serine-rich heptapeptide (Chambers, 1994; Yeo, 2003; Kamenski, 2004; Zhang, 2010). The conserved structural core of FCP/SCP is the FCP homology (FCPH) domain. FCPs, but not SCPs, contain a BRCT (BRCA1 C-terminal domain like) domain that is C-terminal to the FCPH domain (Yeo, 2003; Qadota, 2008; Zhang, 2010) (Figure 2).

For more than two decades of investigation, cellular functions of PSPs have been documented with increasing detail. Functional characterization has been complemented by biochemical, structural and proteomic research of all three major families of PSPs, giving rise to major advances in mechanistic understanding (Cohen, 2002; Ceulemans, 2004; Cohen, 2004; Kamenski, 2004; Gallego, 2005; Flores-Delgado, 2007; Moorhead, 2008; Fardilha, 2010; Zhang, 2010; Fardilha, 2011b; Esteves, 2012)

Protein Phosphatase 1 (PP1)

PP1 is a major PSP and is ubiquitously expressed in all eukaryotic cells. PP1 regulates a variety of cellular processes, including cell cycle, meiosis, apoptosis, protein synthesis, metabolism, cytoskeletal reorganization, regulation of membrane receptors and channels and muscle contraction (Cohen, 2002; Ceulemans, 2004; Fardilha, 2010).

PP1 exhibits broad substrate specificity *in vitro*, however each assembled and functional PP1 complex is thought to display stringent substrate specificity and elicits specific biological responses. The PP1 holoenzyme consists of a catalytic subunit (PP1c) and a regulatory (R) subunit. PP1c is a 35–38 kDa protein that exists as three isoforms: α (PPP1CA), β/δ (PPP1CB) and γ (PPP1CC) sharing ~90% amino acid sequence

similarity (Figure 3). Two splice variants of PP1γ (PP1γ1/PPP1CC1 and PP1γ2/PPP1CC2) have also been identified.

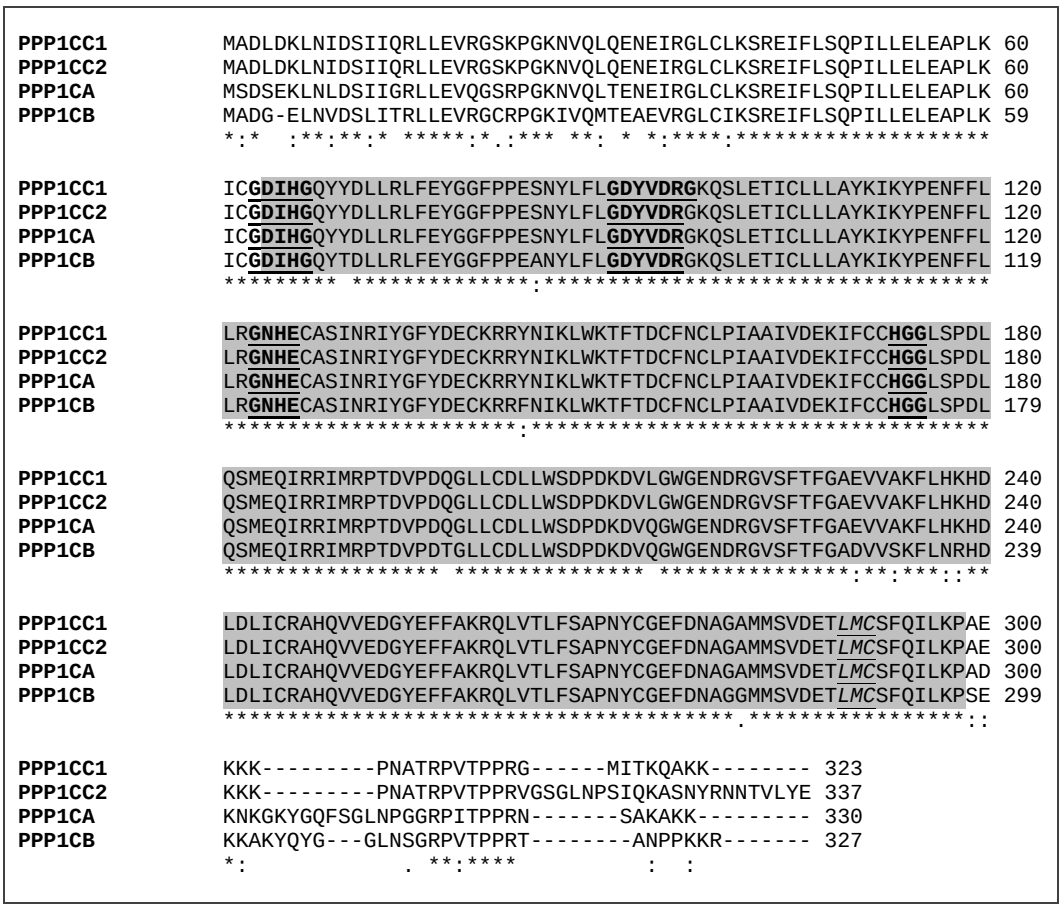

Figure 3 | PP1 isoforms sequence alignment. ClustalW2 alignment shows a great homology between all isoforms: PP1α (PPP1CA), PP1β/δ (PPP1CB), PP1γ1 (PPP1CC1) and PP1γ2 (PPP1CC2). The PPP family is defined by three signature motifs (-GDXHG-, -GDXVDRG-, -GNHE- and -HGG-; in bold underlined; also in figure 2) within the conserved catalytic domain (in grey shadow). The -LMC- hydrophobic pocket (in italics underlined) is important for the recognition of RVxF PP1 binding motif-containing proteins.

All PP1c isoforms are ubiquitously expressed, except for PP1γ2 that is testis and sperm enriched. PP1 cellular activity can be regulated by: reversible phosphorylation of the R subunits, dissociation of the R and the PP1c subunits, allosteric regulation of the R subunits and inducible expression of the R subunits (Cohen, 2002).

PP1c is also highly conserved among all eukaryotes, with ~70% amino acids sequence identity. These sequences support a conserved fold and a similarly positioned active site for all members of the PPP family (Figure 2, 3 and 4).

	Conserved active site residues in PPPs					
PPP1	D64	H66	D92	N124	H173	H248
PPP2	D57	H59	D85	N117	H167	H241
PPP3	D90	H92	D118	N150	H199	H261
PPP4	D54	H56	D82	N114	H164	H238
PPP5	D242	H244	D271	N303	H352	H427
PPP6	D53	H55	D81	N113	H163	H237
PPP7	D84	H86	D113	N149	H197	H303

Figure 4 | Conserved active site metal-binding residues in PPP family members. Position of the residues is indicated, D – Aspartic acid; H – Histidine and N – Asparagine. Adapted from Shi, 2009.

About 200 putative R subunits have been identified, with many more expected to be found (Moorhead, 2008; Hendrickx, 2009; Fardilha, 2010). These R subunits, PP1 interacting proteins – PIPs, may target the PP1 catalytic subunit to specific subcellular compartment, modulate substrate specificity, or serve as substrates themselves. Thus, the interactions between the catalytic subunit and specific R subunits are central to the physiological functions of PP1. PP1c adopts a compact α/β fold, with a β sandwich wedged between two α-helical domains (Egloff, 1995; Goldberg, 1995). Two metal ions, Mn^{2+} and Fe^{2+}, are located in the active site at the three-way joint of the β sandwich and the two helical domains. Coordination of these two metal ions is provided by three histidines, two aspartic acids and one asparagine. These residues are highly conserved in all members of the PPP family (Figure 4), suggesting a common mechanism of metal-catalyzed reaction in the protein family.

The two metal ions are thought to bind and activate a water molecule, which initiates a nucleophilic attack on the phosphorous atom (Egloff, 1995; Goldberg, 1995). Three shallow surface grooves roughly follow the domain boundaries and converge at the catalytic center, forming a Y-shaped surface feature.

PP1 Binding Motifs

Early studies suggested that most PIPs contain the sequence motif RVxF. This notion was supported by the crystal structure of PP1 bound to a peptide containing the sequence RRVSFA (Egloff, 1997). The valine and phenylalanine residues of the peptide stack against hydrophobic amino acids on the surface of PP1, whereas the side chain of the first, but not the second, arginine residue makes hydrogen bonds to PP1. Subsequent studies focused on the identification and improvement of the consensus peptide sequence. Most of the PIPs were thought to contain a surface motif of [RK][X]0-1[VI]{P}[FW] (Wakula, 2003). It seems that RVxF-consensus sequences function as PP1 interaction sites only when they are present in a flexible and exposed loop that can be modeled into a β-strand (Wakula, 2003). A systematic analysis of the docking peptides that combined biochemistry with molecular dynamics gave rise to the refined consensus sequence [HKR][ACHKMNQRSTV][V][CHKNQRST][FW] (Meiselbach, 2006). This consensus sequence allowed the accurate prediction and experimental confirmation of several previously unknown PIPs. Within this consensus sequence, the most conserved valine and phenylalanine/tryptophan residues appear to anchor the binding of the PIP to PP1, whereas the other residues provide the needed specificity for recognizing different PIPs. More recently, a new redefinition of the RVxF motif and its flanking residues was made, based on the sequences of 143 PIPs: [KRL][KRSTAMVHNQ][VI]{FIMYDP}[FW] (Hendrickx, 2009).

Other PP1 binding motif (BM) the MyPhoNE motif, was found in the myosin phosphtase targeting subunit (Mypt) family RXXQ[VIL][KR]X[YW] (Terrak, 2004; Hendrickx, 2009).

Another consensus site for PP1 binding has been described, the so called apoptotic signature, F-X-X-R-X-R, that also appears to exist in several PP1 interactors, and first identified in the apoptotic Bcl family (Ayllon, 2002). Recently, the generic PP1 BM was identified, the SILK-motif: [GS]-IL-[KR]. It was first described for I2, a specific PP1 inhibitor (Huang, 1999; Wakula, 2003; Lin, 2005; Hurley, 2007; Hendrickx, 2009). This motif is present in nearly 10% of proteins containing the RVxF-motif and is usually N-terminal to it. The SILK and RVxF-motifs are functionally interchangeable and can both be essential for PP1 anchoring.

Comprehensive mechanistic understanding of PP1 will likely require the structural elucidation of PP1 bound to different classes of PIPs whose functions have been biochemically characterized.

Protein Phosphatase 1 Interactome

The broad *in vitro* substrate specificity of PP1 leads to the idea that its enzymatic specificity is mainly dictated by the PIPs. PP1 is a highly specific and regulated PP, owing to the unusual diversity and structural design of its regulatory toolkit. A variety of approaches has identified more than 200 mammalian proteins known to interact with PP1 but relatively little is known about isoform specific PP1 regulators.

PIPs were originally identified using classical biochemical approaches as well as the Yeast Two Hybrid (YTH) technique. Recently, *in silico* screenings based on stringent definitions of the RVxF PP1 BM, combined with a biochemical validation procedure, have led to a near doubling of the PP1 interactome (Meiselbach, 2006; Hendrickx, 2009). Novel PP1 complexes also have been identified by affinity chromatography with covalently bound microcystin-LR, a potent small-molecule inhibitor of PP, in combination with the selective elution of PP1-bound proteins, by competition with a synthetic RVxF-type docking peptide (Moorhead, 2008). Yet another set of PP1 complexes has been identified using antibody arrays (Flores-Delgado, 2007). More recently, high throughput YTH screens were used to characterize the human testis PP1γ interactome and was shown that there are isoform tissue-specific PIPs (Fardilha, 2011b). Some PIPs were identified when PP1γ1 was used as bait while others were only obtained when the bait was PP1γ2. Even more interesting was the fact that the majority of PIPs obtained with a single bait were with the unique C-terminal of PP1γ2.

Given the number of PPs and phosphoprotein substrates encoded in the human genome, a large number of PIPs surely remain to be discovered. In fact, a bioinformatics-assisted screen recovered only about one-third of the previously known mammalian PIPs with an RVxF motif (Hendrickx, 2009), indicating that about 450 genes, instead of the currently validated 150, are likely to encode this type of PIP. Clearly, much more work is required to uncover the true diversity of the PP1 interactome.

Although only a minority of PP1 complexes has been functionally analyzed it is known that PIPs function as inhibitors, substrate specifiers, and substrate targeting proteins, or a combination thereof.

More than a dozen vertebrate PIPs have been identified as PP1 substrates. They are often activated by dephosphorylation, as is the case for BRCA1, an E3 ubiquitin ligase,

focal adhesion kinase (FAK), the PP CDC25C and caspase 2 (Zhao, 1991; Allen, 2000; Ban, 2000; Bottini, 2002). By contrast, PP1 maintains the associated PKs NEK2 and Aurora-A in an inactive state (Lopez, 1996). Dephosphorylation by PP1 stabilizes the transcription factor Ikaros (Orengo, 1976) and regulates the binding of ligands to various PIPs (Barford, 1996; Draznin, 2006; Fry, 2008; Qadota, 2008). Some substrate-PIPs also regulate PP1 function. Inhibitor-2 (I2) and the PKC potentiated inhibitor (CPI-17) are both substrates and potent inhibitors of PP1 (Ohnishi, 2005; Hurley, 2007).

Many PIPs contain specific domains that mediate the binding of PP1 to specific cellular compartments or macro-molecular complexes. Indeed, PIPs can target PP1 to such diverse structures as the plasma membrane (e.g. integrin aIIB), mitochondria (e.g. URI), endoplasmic reticulum (e.g. the stress-induced protein GADD34), glycogen particles (e.g. G-subunits), the actin cytoskeleton (e.g. Neurabin II), chromatin (e.g. Repo-man) and nucleoli (e.g. NOM1). The targeting by PIPs brings PP1 into close proximity to specific subsets of substrates; the associated increased local substrate concentration is sufficient to increase the dephosphorylation rate by up to several orders of magnitude (Bertorello, 1991).

More than half of all PIPs inhibit PP1 when glycogen phosphorylase is used as a substrate (Hendrickx, 2009). Most of these PIPs are poor inhibitors, but some substrate and targeting PIPs, including GADD34 (Walaas, 1991b), the Neurabins (Walaas, 1991a), PNUTS (Auerbach, 2002) and NIPP1 (Suter, 2008), are inhibitory at the low nanomolar range.

Nonetheless, some PIPs are true PP1 inhibitors because they block access to the active site and inhibit the dephosphorylation of all substrates. Some PIPs, including Inhibitor-1 (I1), CPI-17 and DARPP-32 (Walaas, 1991b) are inhibitory only when phosphorylated, functioning as pseudosubstrates (Desdouits, 1995; Ceulemans, 2004; Ohnishi, 2005) .

Some PIPs, including MYPT1 (Terrak, 2004) and the Neurabins (Colbran, 1997; Terry-Lorenzo, 2002a), interact with PP1 in an isoform-dependent manner, suggesting that they possess isoform-specific docking sites. Because the PP1 isoforms differ mainly at the N- and C-termini, these represent obvious binding places for specific docking sequences.

A complete understanding of PP1 function requires the identification of the associated subunits that direct PP1 specific functions, as well as functional analysis of PP1 holoenzymes.

PP1 and its regulators in Brain

Protein phosphorylation is a fundamental process associated with memory, learning and brain function, with prominent roles in the processing of neuronal signals and in short-term and long-term modulation of synaptic transmission (Winder, 2001; Koshibu, 2009; Graff, 2010). Of all mammalian tissues, the brain expresses the highest levels of PKs and PPs. A wide range of specific inhibitors and targeting partners such as scaffolding, anchoring, and adaptor proteins also contribute to the control of PKs and PPs and recruit them into signaling complexes in neuronal cells (Sim, 1999; Winder, 2001). Phosphorylation events, therefore, are controlled not only by the balanced activity of PKs and PPs but also by their restricted localization.

The importance of PP1 in neural systems became apparent after the discovery that its catalytic subunits α, β, γ1 and γ2, are expressed in the brain (Takizawa, 1994; da Cruz e Silva, 1995; Ouimet, 1995; Strack, 1999). PP1 is highly expressed in brain both in neurons and glia (da Cruz e Silva, 1995; Ouimet, 1995). In the brain the mRNAs for PP1α, PP1β and PP1γ1 were found to be particularly abundant in hippocampus and cerebellum (da Cruz e Silva, 1995). At the protein level PP1α and PP1γ1 were found to be more highly expressed in brain than in peripheral tissues (Figure 5), with the highest levels being measured in the striatum, where they were shown to be relatively enriched in the medium-sized spiny neurons (da Cruz e Silva, 1995). Mammalian PP1α, PP1β and PP1γ1 localize to distinct subcellular locations in mammalian cells (Andreassen, 1998; Trinkle-Mulcahy, 2001; Lesage, 2005). At the electron microscopic level, PP1 immunoreactivity was demonstrated in dendritic spine heads and spine necks, and possibly also in the postsynaptic density (Ouimet, 1995). PP1 immunoreactivity has also been reported in human hippocampal neuronal cytoplasm (Pei, 1994). In addition, most neuronal nuclei were not immmunoreactive for PP1γ1 but were usually strongly immunoreactive for PP1α (Ouimet, 1995). The detection of the PP1γ2 isoform in brain extracts was unexpected (Takizawa, 1994; Strack, 1999), because its expression was

previously reported to be testis specific (Kitagawa, 1990, Shima, 1993). PP1γ2 protein is uniformly expressed in most forebrain regions, but is especially enriched in the striatum. Lower levels were detected in hindbrain and cerebellum, similarly to PP1γ1 and PP1α (Strack, 1999).

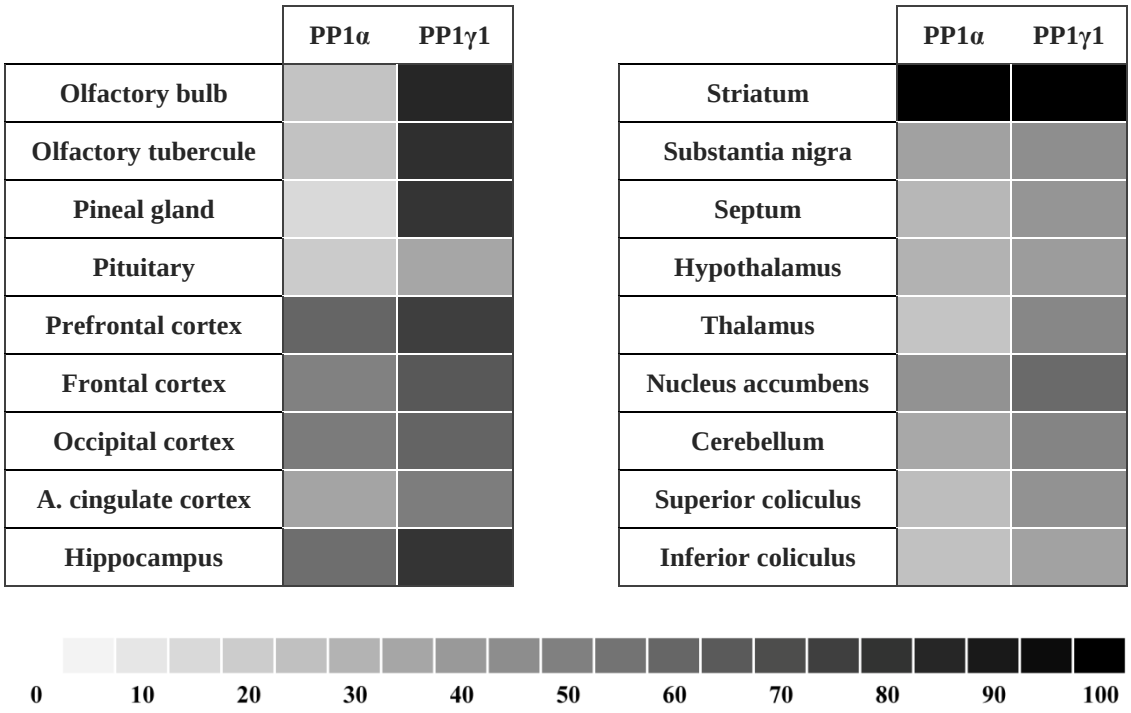

Figure 5 | Brain distribution of PP1α and PP1γ1. Diagram of the comparison of the relative abundance of PP1α and PP1γ1 isoforms in different rat brain tissues. Levels of abundance indicated in grey color intensity, expressed in relation to the striatum (black). Adapted from da Cruz e Silva, 1995.

In the nervous system PP1 regulates short term signaling such as the phosphorylation status of receptors, ion channels, and signaling proteins, as well as long term signalling, requiring changes in protein translation, gene expression and neuronal morphology that together modify neuronal plasticity (Lisman, 1989; Nairn, 1992; Mulkey, 1994; Terry-Lorenzo, 2000; Winder, 2001; Tweedie-Cullen, 2011). PP1 activity controls both long term potentiation (Blitzer, 1995; Blitzer, 1998) and long term depression (Mulkey, 1994; Morishita, 2001) in the hippocampal synapse. High concentrations of PP1, specifically PP1γ1 and PP1α, in the actin-rich structure known as the Post Synaptic Density (Ouimet, 1995; Strack, 1997a; Terry-Lorenzo, 2000), correlate with the regulation of substrates such as NMDA (Snyder, 1998; Westphal, 1999) and AMPA

subtypes of glutamate receptors (Wang, 1991; Yan, 1999), calcium (Surmeier, 1995) and potassium (Endo, 1995) channels, and CaMKII (Strack, 1997a; Blitzer, 1998). In summary, PP1 is an essential regulator of protein function and neuronal activity, with multiple PIPs regulating their activity and substrate specificity, some of which will be discussed below.

DARPP-32 (PPP1R1B) is one of several PKA-regulated inhibitors of PP1. It is expressed only in some specific neuronal populations in the brain and at low levels in a few non-neuronal cell types in the brain (e.g., tanycytes, choroid plexus) and other organs (e.g., adrenal medulla, parathyroid cells, kidney) (Ouimet, 1984; Hemmings, 1986; Meister, 1989). DARPP-32 phosphorylation status has been thoroughly investigated and it is the core of a rich network of regulation events. It controls a wide variety of neuronal properties, from ion channel permeability and synaptic plasticity, to nuclear chromatin response. The combination of experimental and modeling studies suggests that in medium-size spiny neurons DARPP-32 is a robust integrator of signaling whose main role may be to increase the reliability in decoding the information mediated by glutamate and dopamine, as well as other inputs (Yger, 2011).

DARPP-32 is phosphorylated at various sites, the most extensively studied is at Thr34 which turns DARPP-32 into a potent inhibitor of PP1 (Hemmings, 1984a). Thr34 in DARPP-32, like Thr35 of I1 are excellent substrates for both PKA and cGMP-dependent protein kinases (Hemmings, 1984b). They are dephosphorylated by PP2B (calcineurin/PPP3) and by the catalytic subunit of PP2A (PPP2) *in vitro* (King, 1984; Hemmings, 1984a) and in striatal neurons (Halpain, 1990; Nishi, 1999). DARPP-32 has been implicated in the regulation by dopamine of several ion channels in striatal neurons, including the AMPA an dNMDA glutamatereceptors (NMDAR) and voltage-gated Na^+ and Ca^{2+} channels (Svenningsson, 2004). Studies in neurons in culture with the inhibitor of nuclear export leptomycin B revealed that DARPP-32 undergoes a continuous shuttling between the cytoplasm and the nucleus (Stipanovich, 2008). Since PP1 has many substrates in the nucleus (Moorhead, 2007), it is likely that the consequences of the accumulation of phosphorylated Thr34-DARPP-32 has multiple consequences on chromatin and other nuclear targets. For example, a recent report shows that DARPP-32 interacts with tra2-beta1, a factor involved in the regulation of alternative splicing (Benderska, 2010).

Neurabin I (PPP1R9A) and spinophilin (Neurabin II/PPP1R9B) are brain actin binding protein specifically enriched in dendritic spines (Allen, 1997; Satoh, 1998; Grossman, 2002) as is PP1 (Ouimet, 1995) and are modulators of PP1 activity. In hippocampal slices, inhibition of PP1 activity caused a rapid increase in dendritic spines and filopodia (Jourdain, 2003), suggesting that the ongoing synaptic activity associated with the Neurabin I/PP1 complex maintains normal spine number and morphology. Neurabin II has the properties expected of a scaffold protein localized to the cell membrane (Allen, 1997) it plays an important role in the regulation of glutamate receptors by anchoring PP1 in the proximity of AMPA and NMDA receptors, thus regulating the efficacy of postsynaptic glutamatergic neurotransmission. As phosphorylation of neurofilament proteins is associated with depolymerization, neurofilament-associated PP1 may regulate neurofilament stability. Neurabin I and Neurabin II were identified to bind PP1γ1 and PP1α, but not PP1β (MacMillan, 1999; Terry-Lorenzo, 2002a). Because the PP1 isoform-binding selectivity of Neurabin II and Neurabin I *in vitro* matches the selective enrichment of PP1 isoforms in isolated postsynaptic densities (Strack, 1997a; Strack, 1999; Terry-Lorenzo, 2002b), it is likely that spinophilin and/or Neurabin selectively target PP1α and PP1γ1 activity to actin-rich dendritic spines and postsynaptic densities (Colbran, 2004). Neurabins target the PP1-I2 complex to actin cytoskeleton and regulate cell morphology (Terry-Lorenzo, 2002b). In fact Neurabins bind PP1 and I2 and mutation of a conserved PP1 BM abolished Neurabins binding to both proteins. PP1 association to brain microtubules also occurs through the microtubule-associated protein tau. Tau acts as targeting protein that bridges PP1 to microtubules and can also be a substrate for the PP (Liao, 1998).

Myr8 myosins comprise a new class of myosins that has been designated class XVI. The head domain contains a large N-terminal extension composed of multiple ankyrin repeats implicated in mediating an association with PP1α and γ1 (Patel, 2001). The structural features and restricted expression patterns suggest that members of this novel class of unconventional myosins comprise a mechanism to target selectively the PP1 in developing brain (Patel, 2001).

Neurofilament-L (NF-L), a membrane-bound protein, was identified as a PP1 binding protein in bovine brain cortex plasma membranes. Bovine NF-L, at nanomolar concentration, inhibited the activity of rabbit skeletal muscle PP1c but not of PP2A. NF-

L may target the functions of PP1 in membranes and cytoskeleton of mammalian neurons (Terry-Lorenzo, 2000).

Type 1 inositol (1,4,5)-trisphosphate receptors (IP3R1s) play a major role in neuronal calcium (Ca^{2+}) signaling. The IP3R1s are phosphorylated by PKA. PP1α was isolated in a YTH screen of rat brain cDNA library using InsP3R1 as bait (Tang, 2003). The specificity of the IP3R1-PP1α association was confirmed and the IP3R1-PP1 complex was immunoprecipitated from rat brain synaptosomes and neostriatal lysate. PP1 facilitates the dephosphorylation of PKA-phosphorylated IP3R1.

PP1 complexes in response to ischemia-reperfusion stress and ischemic tolerance were studied in brain (Cid, 2007). PP1α and PP1γ were immunoprecipitated and resolved by 2-D electrophoresis. DIGE analysis detected 14 different PIPs that exhibited significant changes in their association with PP1α or PP1γ. Ischemia-reperfusion altered the interaction of heat shock cognate 71 kDa-protein, creatine kinase B and DARPP-32 with both PP1α and PP1γ, and the interaction of phosphodiesterase-6B, transitional endoplasmic reticulum ATPase, lamin-A, glucose-regulated 78 kDa-protein, dihydropyrimidinase-related protein-2, gamma-enolase, neurofilament-L, and ubiquitin ligase SIAH2 with PP1γ.

PP1 and its regulators in Testis and Sperm

Splicing of the PP1γ gene originates two isoforms, PP1γ and PP1γ2, PP1γ2 is enriched in testis and virtually exclusive to sperm (Kitagawa, 1990; Shima, 1993; da Cruz e Silva, 1995; Smith, 1996; Fardilha, 2008). In testis, PP1γ2 localizes in the cytoplasm of secondary spermatocytes and round spermatids, as well as elongating spermatids and testicular and epididymal spermatozoa, while PP1γ1 expression is observed in interstitial cells and PP1α in spermatogonia, pachytene spermatocytes and interstitial cells (Chakrabarti, 2007). PP1γ gene null male mice are infertile due to impaired spermatogenesis, leading to the absence of epididymal spermatozoa (Varmuza, 1999). Although PP1α expression was increased and its localization altered, it could not substitute for PP1γ, further suggesting a specific role for PP1γ2 in sperm differentiation and morphogenesis (Chakrabarti, 2007). Several studies have demonstrated that PP1γ2

is important in the regulation of sperm motility (Smith, 1996; Smith, 1999; Huang, 2002; Mishra, 2003). In sperm, PP1γ2 is present along the entire flagellum including the middle-piece, consistent with a role in sperm motility, but it is also found in the posterior and equatorial regions of the head, suggesting a role in the acrosome reaction (Huang, 2002). Furthermore, Visconti and co-workers have recently shown that inhibition of PPs induces capacitation-associated signaling (Krapf, 2010). Immotile spermatozoa possess higher activity levels of PP1γ2 compared with motile spermatozoa. Inhibition of protein phosphatase activity by okadaic acid and calyculin A initiates motility in caput epididymal sperm without requirement for a change in cAMP levels (Smith, 1996; Vijayaraghavan, 1996).

Significantly, some testis/sperm-specific PIPs have been identified. For example, the spermatogenic zip protein 1 (Szp1), a member of the basic helix-loop-helix family of transcription factors, which binds to PP1γ2 in mouse testis (Hrabchak, 2004). Overexpression of Szp1 and loss of PP1γ in the testis show similar phenotypes, such as spermatogenic arrest and germ cell apoptosis (Hsu, 2004). Another example is endophilin B1t. This testis enriched isoform of endophilin B1a was shown to bind PP1γ2 but did not interact with a mutant form of PP1γ2, lacking the specific C-terminus, nor with PP1α (Hrabchak, 2007). Moreover, the somatic isoform did not interact with any of the PP1 isoforms and the characteristic punctuate expression pattern of endophilin, in testis, was absent in PP1γ null mice. Finally, endophilin B1t was able to inhibit recombinant PP1γ2 activity (Hrabchak, 2007).

Inhibitor-2 (I2) is capable of inhibiting the catalytic subunit of PP1 leading to the production of a stable PP1-I2 complex. GSK-3 phosphorylates I2 in the PP1-I2 complex, relieving the inhibition and producing active PP1. The complex PP1γ2-I2 is inactive in motile caudal sperm and the PP activity is re-established in immotile sperm by the higher GSK-3 activity (Smith, 1996; Vijayaraghavan, 1996).

Inhibitor-3 (I3) is a potent PP1 inhibitor (Giffon, 1996; Zhang, 1998) and a human homologue of the mouse t-complex expressed protein 5 (Tctex5), being genetically linked to the male sterility phenotypes of impaired sperm tail development and poor sperm motility in t complex mice (Cebra-Thomas, 1991; Pilder, 1991; Pilder, 1993). In epididymal mouse spermatozoa Tctex5 is present in the head and principal piece of the tail (Pilder, 2007). These are also the locations where PP1γ2 is expressed (Huang,

2005). I3 is a substrate for caspase-3 and is degraded *in vivo* during apoptosis (Huang, 2008) and PP1γ2 has an anti-apoptotic effect in testis that might result from its ability to increase I3 stability (Cheng, 2009).

In male germ cells PP1γ2, I3, sds22 and actin form a multimeric complex in which PP1γ2 seems to be inactive (Cheng, 2009). The stability of the complex depends on functional PP1 interaction sites in sds22 and I3, indicating that PP1 mediates the interaction between sds22 and I3, forming a catalytically inactive complex in the cell (Lesage, 2007). Sds22 (PPP1R7) was identified in sperm (Mishra, 2003) and rat testis (Chun, 2000), and inhibits the PP1 catalytic subunit in rat liver nuclei (Dinischiotu, 1997). The expression pattern of rat sds22 matches that of PP1γ2, suggesting that its involvement in spermatogenesis relates to the control of PP1γ2 activity. Sds22 was identified in motile caudal spermatozoa as a regulator of PP1γ2 catalytic activity (Huang, 2002). In caput sperm sds22 is bound to a 17 kDa protein, suggesting that binding to PP1γ2 requires sds22 dissociation from p17.

The cyclic AMP-dependent PK (PKA) and PP1 are broad specificity signaling enzymes with opposing actions that catalyze changes in the phosphorylation state of cellular proteins. PKA is a ubiquitous, multifunctional enzyme involved in the regulation of several cellular events. PKA holoenzyme consists of four subunits, two catalytic and two regulatory (RI and RII). compartmentalization of PKA is mediated through association of its regulatory subunits with A-kinase anchoring proteins (AKAPs) (Faux, 1996). Several anchoring proteins have been identified that can simultaneously associate with PKs and PPs (Faux, 1996). In testis and/or sperm there are three AKAPs that have been shown to be related to PP1γ2, AKAP220, AKAP4 and AKAP3. AKAP220 binds PKA and PP1, being a competitive inhibitor of PP1 (Schillace, 2001). In spermatozoa, *Akap4* gene knockout mice that lack flagellar movement, exhibit a significant change in the activity and phosphorylation of PP1γ2 (Huang, 2005). This suggests the involvement of AKAP4 in the regulation of PP1γ2 activity in the principal piece of mouse spermatozoa.

Given that many AKAPs have been shown to be present in germ cells and localized to compartments related to motility where PP1γ2 is also present (for instance, mitochondrial sheath or axoneme, Table 1), they might be putatively involved in motility acquisition (Fardilha, 2011a).

A study showed that inhibition of PPs, with calyculin A, resulted in an enhancement of the phosphorylated state at the activation loop of the PKA catalytic subunit in the mouse sperm principal and middle-pieces (Goto, 2009). Also, PKA and PP1γ2 co-localized in the principal piece, but PP1γ2 was not present in the middle piece. It was suggested that PP1 and PP2A suppress full activation of PKA, as well as enhancement of the phosphorylated states of other flagellar proteins, in order to prevent precocious changes of flagellar movement from the progressive type to hyperactivation (Goto, 2009). Nevertheless, a study from Fardilha and co-workers has shown that PP1γ2 is also present in the middle-piece of human sperm attributing to this PP the dephosphorylation of PKA in the entire tail (Fardilha, 2008). Together, these findings suggest that the AKAP/PKA/PP1 complex is important for regulation of sperm motility.

Table 1 | Functions and sub-cellular localization of the AKAPs present in male germinative tissues. Adapted from Fardilha, 2011a.

Name	Tissue and cell expression	Subcellular localization in sperm	Sperm associated function	References
AKAP220/ AKAP11	*Testis Spermatid Spermatocyte Spermatozoa*	*Cytoplasm, centrosome, middle piece and peroxisomes*	*Sperm maturation and motility*	(Lester, 1996) (Vijayaraghavan, 1997) (Reinton, 2000)
AKAP3/ AKAP110/ FSP95/ SOB1	*Testis Spermatozoa Spermatid*	*Fibrous sheath, principal piece and acrosome*	*Acrosome reaction and sperm motility*	(Horowitz, 1984) (Vijayaraghavan, 1997) (Lin, 1995) (Mandal, 1999) (Lefevre, 1999)
AKAP4/ AKAP82/ FSC1	*Testis Spermatid Spermatozoa*	*Fibrous sheath and principal piece*	*Sperm motility*	(Carrera, 1994) (Fulcher, 1995) (Johnson, 1997) (Visconti, 1997) (Turner, 1998) (Turner, 2001)
D-AKAP1/ S-AKAP84/ AKAP121/	*Testis Spermatid*	*Middle piece*	*Sperm maturation and motility ?*	(Lin, 1995) (Huang, 1999) (Feliciello, 1998)
TAKAP80	*Testis*	*Fibrous sheath*	*Sperm motility ?*	(Mei, 1997)
AKAP14/ AKAP28	*Testis*	*Axoneme*	*Sperm motility ?*	(Kultgen, 2002)
WAVE1	*Testis Spermatid Spermatocyte Spermatozoa*	*Mitochondrial sheath /middle piece*	*Sperm motility ?*	(Rawe, 2004)

Other proteins have also been implicated in the regulation of PP1γ2 in testis/sperm either by inducing (14-3-3) or inhibiting (hsp90) PP1γ2 activity during sperm

maturation, which correlates with increased spermatozoa motility (Shima, 1993b; Jurisicova, 1999; Huang, 2002; Mishra, 2003; Hrabchak, 2004; Huang, 2004; Huang, 2005).

PP1 regulation

The existence of common binding sites for PIPs explains why a relatively small protein such as PP1 can interact with numerous different regulatory proteins and why the binding of most regulatory subunits is mutually exclusive. The relative abundance of each PP1 isoform may be an important factor in determining the composition of numerous PP1 holoenzymes and the relative contribution of each PP1 isoform to different biological functions.

The role played by PPs in health and disease, and particularly the involvement of PP1, makes it and the proteins that regulate its function (PIPs) excellent targets for pharmacological intervention. PP1/PIPs complexes seem to be the future targets for several diseases since PP1 has been associated to several disorders. Nevertheless, the specificity of PP1 targeting should be achieved by two means: PP1 isoform specificity and differential PIP association. These meaning that each PP1 isoform has its tissue and event-specific expression pattern and the same happen with the PIPs, leading to the formation of a specific PP1/PIP complex in a certain place and time and event. This highly specific complex formation can then be target by an inhibiting or stimulating molecule.

The exquisite specificity of PP1 *in vivo* is explained by the structural design and diversity of its toolkit, and by various regulatory mechanisms that impose on PIPs. Some PIPs are expressed in a cell type-dependent manner, accounting for cell type-specific PP1 activity (Ceulemans, 2004; Moorhead, 2007; Virshup, 2009). Recent data show that the concentration of several PIPs is controlled by regulated proteolysis (Kloeker, 1997; Strack, 1997a; Strack, 1997b; Huang, 2008). Moreover, many signaling pathways interfere with the affinity of specific PIPs for PP1. For example, phosphorylation of Ser/Thr residues in or near RVxF-type docking sequences is often associated with a reduced binding affinity for the RVxF-binding channel (Bollen, 2001). Signaling through phosphorylation can also result in the recruitment or release of inhibitory PIPs (Tavalin, 1999; Ceulemans, 2004; Ohnishi, 2005).

Another PIP control mechanism involves positive or negative allosteric regulation by metabolites or other proteins (Chen, 1999; Westphal, 1999). The PP1-mediated dephosphorylation of some substrate - PIPs - is restrained through regulated masking of

the phosphorylated residues by 14-3-3 proteins (Ree, 1991; Schillace, 1999; Allen, 2000; Trinkle-Mulcahy, 2001; Bottini, 2002). Finally, PP1–PIP complexes are highly dynamic and different PIPs compete for the same PP1 binding sites (Schillace, 1999; Trinkle-Mulcahy, 2001). Ultimately, the concentration and PP1-binding affinities of PIPs determines which PP1 holoenzymes are formed.

In recent years, PKs have become highly successful drug targets, mainly for the treatment of cancer (Scott, 1999). As most phosphorylations are reversible, PPs are equally powerful drug targets to interfere with protein phosphorylation. This is impressively illustrated by the PP2B inhibitors cyclosporin A and FK506, which are clinically used as potent immunosuppressants.

Clearly, PP1 inhibitors hold great promise for the treatment of various human pathologies, including cancer, neurodegenerative diseases, type 2 diabetes, heart failure and viral diseases (Strack, 1997c; Chen, 1999; Schillace, 1999; Tavalin, 1999; Koshibu, 2009). However, highly specific cell-permeating inhibitors for the PP1 catalytic subunit are not yet available and it is questionable whether such agents could ever be used therapeutically, as they would be likely to inhibit all PP1 holoenzymes (Trinkle-Mulcahy, 2001). A more selective approach, could involve the functional disruption of subsets of PP1 holoenzymes with small molecule compounds that bind to PIP interaction sites on PP1, such as the hydrophobic binding grooves for the RVxF, SILK and MyPhoNE sequences (Bollen, 2010; Fardilha, 2010). Blocking the less prevalent SILK and MyPhoNE motifs will affect smaller subsets of PP1 holoenzymes; however, even compounds that interfere with the docking of RVxF sequences can provide greater selectivity than predicted from the abundance of this motif, as its importance is holoenzyme-dependent (Bollen, 2010). Moreover, RVxF competing agents can be used at concentrations that disrupt only the binding of low-affinity RVxF variants. Another PP1 targeting strategy aims to interfere with substrate recruitment at extended docking sites of specific holoenzymes (Bollen, 2010). At the very least, inhibitors of subsets of PP1 holoenzymes could be employed for functional studies (Bollen, 2010; Fardilha, 2010).

An increasing number of proteins have been identified in diverse cell types that regulate the catalytic activity of PP1. Indeed, the diversity of such PP1 regulatory subunits makes them attractive pharmacological targets. Besides, PP1 isoforms are highly mobile in cells and can dynamically re-localize through the direct interaction with targeting

subunits (Trinkle-Mulcahy, 2001). Clearly, a full understanding of the regulation of different cellular processes by PP1 requires the identification and characterization not only of the various PP1 regulatory proteins and holoenzyme, but also the isoform and tissue specific PP1-PIPs complexes.

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AIMS

Protein Phosphatase 1 (PP1) is a major Ser/Thr phosphatase whose function is highly dependent on the proteins it binds to - referred as PP1 Interacting Proteins - PIPs. Today, more than 200 PIPs have been identified that specify PP1 subcellular localization, substrates and activity. The identification of novel PIPs is a mean to unravel novel PP1 functions. A novel approach was followed, in which the aim was to determine PP1 isoform-specific functions, using a high throughput strategy - the YTH system.

Thus, the main aims of this PhD thesis were:

1 | To characterize PP1α, PP1γ1 and PP1γ2 isoform-specific interactomes in human brain;

2 | To confirm and describe two novel PP1/PIP complexes - PP1/Taperin and PP1/Synphilin-1A - and to address their physiological relevance.

| Chapter II

II | PP1 INTERACTION NETWORKS IN BRAIN

A variety of approaches has identified more than 200 mammalian proteins known to interact with PP1, known as PIPs (Moorhead, 2008; Hendrickx, 2009; Fardilha, 2010; Fardilha, 2011b). PIPs function as inhibitors, substrate specifiers, and substrate targeting proteins, or a combination thereof and sometimes PP1 interactors are themselves substrates for the associated PP1 (Bollen, 2001; Ceulemans, 2004; Fardilha, 2010; Fardilha, 2011a). Given the number of PPs and phosphoprotein substrates encoded in the human genome, a large number of PIPs surely remain to be discovered. Moreover, relatively little is known about PP1 isoform-specific regulators.

The majority of the putative PP1 interactions proposed derived primarily from biochemical approaches, high-throughput YTH screens, mass spectrometry and *in silico* screenings (Hrabchak, 2004; Bennett, 2006; Trinkle-Mulcahy, 2006; Flores-Delgado, 2007; Moorhead, 2008; Hendrickx, 2009; Fardilha, 2011b). Recently, the human testis PP1γ interactome was characterized, by YTH, and it was shown that there are isoform tissue-specific PIPs (Fardilha, 2011b). Some PIPs were identified when PP1γ1 was used as bait while others were only obtained when the bait was PP1γ2. Even more interesting was the fact that the majority of PIPs obtained with a single bait were with the unique C-terminal of PP1γ2 (Fardilha, 2011b). Thus, clearly, there exists a PP1 isoform specificity in what concerns PIPs binding, which is highly relevant for PP1 isoform particular functions.

The YTH technique, as originally developed by Fields and Song, relies on the modular properties of eukaryotic transcription factors, which typically comprise at least two structurally and functionally separate domains: a DNA-binding domain (BD) and an activator domain (AD) (Fields, 1989; Chien, 1991). A bait gene is expressed as a fusion to the BD, of a specific transcription factor, while another gene or cDNA is expressed as a fusion to the AD. It exploits the fact that the BD cannot function as a transcription activator unless physically near the AD, through an interaction that does not need to be covalent, thus activating transcription of reporter genes. The YTH assays are performed by expressing the two fusion proteins in yeast. When using a large scale screen, a plasmid library, expressing cDNA-encoded AD-fusion proteins, can be screened by being

introduced into adequate yeast strains. These larger scale YTH approaches typically rely on interaction by yeast mating (Finley, 1994; Serebriiskii, 2001). When the two transformant cultures, from compatible yeast strains, are matted, diploid cells containing the reporter genes are originated. If the protein interaction occurs, transcription of the reporter genes is activated allowing growth on selective media.

The YTH system provides a sensitive method for detecting relatively weak and transient protein interactions. High-throughput YTH screens, which generated most of the binary protein interaction data currently available, are providing samples of complete interactomes. Some online databases function as interaction repositories with data from literature compiled through comprehensive curation efforts, enabling data sharing of protein-protein interactions (PPIs). The databases include direct and predicted PPIs and integrate interaction of a large number of organisms.

Even though PPIs mapping lacks sufficient coverage and dynamic information for a complete interactome, they greatly increased our knowledge, although understanding the global organization of proteomes is still far from complete.

Using the YTH system we made high throughput screens into human brain libraries using the different PP1 isoforms (α, γ1, γ2 and the specific C-terminal of the γ2 isoform) as baits, in order to identify the isoform-specific PP1 interactors. After, an in depth database search for PPIs was made, to build PP1 isoform-specific networks, so that key functional proteins could be identified.

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Original Articles

Protein Phosphatase 1α Interacting Proteins in the Human Brain

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Abstract

Protein Phosphatase 1 (PP1) is a major serine/threonine-phosphatase whose activity is dependent on its binding to regulatory subunits known as PP1 interacting proteins (PIPs), responsible for targeting PP1 to a specific cellular location, specifying its substrate or regulating its action. Today, more than 200 PIPs have been described involving PP1 in panoply of cellular mechanisms. Moreover, several PIPs have been identified that are tissue and event specific. In addition, the diversity of PP1/PIP complexes can further be achieved by the existence of several PP1 isoforms that can bind preferentially to a certain PIP. Thus, PP1/PIP complexes are highly specific for a particular function in the cell, and as such, they are excellent pharmacological targets. Hence, an in-depth survey was taken to identify specific PP1α PIPs in human brain by a high-throughput Yeast Two-Hybrid approach. Sixty-six proteins were recognized to bind PP1α, 39 being novel PIPs. A large protein interaction databases search was also performed to integrate with the results of the PP1α Human Brain Yeast Two-Hybrid and a total of 246 interactions were retrieved.

Introduction

The correct targeting and localization of proteins to specific subcellular compartments represent an important biological mechanism for regulating cellular function. These processes are of pivotal importance to correct cell development and differentiation, apoptosis, intercellular communication, proteostasis, and metabolism. Therefore, elucidating the constituent molecular parts of these signaling events, where functionally related proteins are arranged in close proximity, represents a fundamental step toward understanding the function of biological systems.

Among posttranslational modifications, reversible protein phosphorylation mediates most of signal transduction pathways in living cells, through the action of protein kinases and phosphatases (Cohen, 2001). These events occur under tight and transient regulation and abnormal phosphorylation mechanisms lead to disorders such as cancer, diabetes, heart failure,and neurological degeneration (Cohen, 2001; da Cruz e Silva et al., 1995a; Fardilha et al., 2010; Gandy and Greengard, 1994; Neumann, 2002; Sridhar et al., 2000).

Of all Ser/Thr protein phosphatases, Protein Phosphatase 1 (PP1) forms a major class and is highly conserved among all eukaryotes (Lin et al., 1999). Three genes are known to encode PP1 catalytic subunits, termed PP1α, PP1β, and PP1γ, with diversity increased by alternative splicing (da Cruz e Silva et al., 1995b). PP1 regulates a variety of cellular events through the dephosphorylation of multiple substrates and its multifunctionality is due to its association with different regulators and/or targeting subunits (Bollen, 2001; Ceulemans and Bollen, 2004; Fardilha et al., 2010, 2011a) known as PP1 Interacting Proteins (PIPs). The PP1 isoforms are highly conserved across their large catalytic domain, but are divergent at the N and C termini. Thus, PIPs bind to the unique C terminus to direct their isoform specific activities. To exert their dephosphorylation reactions that are important in time and space, the diverse functions of PP1 must be independently regulated. For this reason, PIPs are believed to be much more specific for individual functions and are therefore better targets for specific pathways (Ceulemans and Bollen, 2004; Cohen, 2002; Fardilha et al., 2010, 2011a; Virshup and Shenolikar, 2009).

The large majority of PIPs contain a degenerate, so-called RVxF-motif that conforms to the consensus sequence [R/K]-X0-1-[V/I]-[F/W], where X denotes any residue except proline (Bollen, 2001; Wakula et al., 2003). This motif binds with

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high affinity to a hydrophobic channel that is remote from the catalytic site of PP1 (L288-M290-C291) (Gibbons et al., 2005). The binding of the RVxF-motif by itself has no major effects on the conformation or activity of PP1 (Egloff et al., 1997). However, RVxF-mediated anchoring of PP1 promotes the occupation of secondary, lower affinity binding sites, and this often does affect the activity and/or substrate specificity of PP1 (Bollen, 2001; Wakula et al., 2003). The RVxF-motif is present in about one-third of all eukaryotic proteins but only a small fraction are PIPs. It seems that RVxF-consensus sequences function as PP1 interaction sites only when they are present in a flexible and exposed loop that can be modeled into a β-strand (Wakula et al., 2003).

Other PP1-binding motifs (PP1-BMs) have been described, F-X-X-R-X-R, present in several PP1 interactors (Ayllon et al., 2002), and the MyPhoNE motif, RXXQ[VIL][KR]X[YW], present in MYPT-1 (Terrak et al., 2004). An additional generic PP1-binding motif was identified, the SILK-motif: [GS]-IL-[KR]. It was first described for I2, a specific PP1 inhibitor (Hurley et al., 2007; Lin et al., 2005). This motif is present in nearly 10% of proteins containing the RVxF-motif and is normally N-terminal to it. The SILK and RVxF-motifs are functionally interchangeable and can both be essential for PP1 anchoring. More recently, work from Bollen and coworkers allowed the redefinition of the RVxF motif and its flanking residues based on the sequences of 143 PIPs: [KRL]-[KRSTAMVHNQ]-[VI]-{FIMYDP}-[FW] (Hendrickx et al., 2009).

The existence of common binding sites for PIPs explains why a relatively small protein such as PP1 can interact with numerous different regulatory proteins and why the binding of most regulatory subunits is mutually exclusive. The relative abundance of each PP1 isoform may be an important factor in determining the composition of numerous PP1 holoenzymes and the relative contribution of each PP1 isoform to different biological functions.

The broad *in vitro* substrate specificity of PP1 leads to the idea that the enzymatic specificity is mainly dictated by the PIPs. Thus, a complete understanding of PP1 function requires the identification of the associated subunits that direct PP1 specific functions, as well as functional analysis of PP1 holoenzymes. A variety of approaches has identified more than 100 mammalian proteins known to interact with PP1 (Fardilha et al., 2010). These PIPs function as inhibitors, substrate specifiers, and substrate targeting proteins, or a combination thereof. Sometimes PP1 interactors are themselves substrates for associated PP1 (Bollen, 2001; Ceulemans and Bollen, 2004; Fardilha et al., 2010, 2011a). Given the number of protein phosphatases and phosphoprotein substrates encoded in the human genome, a large number of PIPs surely remain to be discovered. Moreover, relatively little is known about isoform specific PP1 regulators. Recently, we have characterized the human testis PP1γ interactome and have shown that there are isoform tissue-specific PIPs (Fardilha et al., 2011b). Some PIPs were identified when PP1γ1 was used as bait while others were only obtained when the bait was PP1γ2. Even more interesting was the fact that the majority of PIPs obtained with a single bait were with the unique C-terminal of PP1γ2 (Fardilha et al., 2011b). Thus, clearly, there exists a PP1 isoform specificity in what concerns PIPs binding that is highly relevant for PP1 isoform particular function.

The majority of the putative PP1 interactions proposed derived primarily from biochemical approaches, high-throughput Yeast Two-Hybrid (YTH) screens, mass spectrometry and *in silico* screenings (Bennett et al., 2006; Fardilha et al., 2011b; Flores-Delgado et al., 2007; Hendrickx et al., 2009; Hrabchak and Varmuza, 2004; Moorhead et al., 2008; Trinkle-Mulcahy et al., 2006). The YTH system provides a sensitive method for detecting relatively weak and transient protein interactions (Fields and Song, 1989). High-throughput YTH screens, which generated most of the binary protein interaction data currently available, are providing samples of complete interactomes. Even though the resulting interaction mapping lacks sufficient coverage and dynamic information for a complete interactome, they greatly increased our knowledge, although understanding the global organization of proteomes is still far from complete.

Of all mammalian tissues, the brain expresses the highest levels of protein kinases and phosphatases, and PP1 is highly expressed both in neurons and glia (da Cruz e Silva et al., 1995b; Ouimet et al., 1995). It is increasingly evident that protein phosphorylation is a fundamental process associated with memory, learning, and brain function, with prominent roles in the processing of neuronal signals and in short-term and long-term modulation of synaptic transmission (Graff et al., 2010; Koshibu et al., 2009). Because PP1α is known to be highly enriched in the brain (da Cruz e Silva et al., 1995b) the main goal of this work was to identify the proteins expressed in human brain that interact with PP1α by the YTH method. Indeed, we identified 66 PIPs of which 39 represent novel interactions. Also, we integrated the YTH results with protein–protein interactions data from several sources (previously PIPs described in the literature and public Web repositories) and developed physical maps to validate *in silico* the novel interactions obtained in our YTH. The PP1α interactome thus obtained allowed the identification of novel key proteins in signaling pathways that were not previously taught as such, addressing novel functions to PP1α in the brain.

Materials and Methods

Human brain library screening by Yeast Two-Hybrid

The PP1α cDNA was directionally subcloned into *EcoRI/BamHI* digested pAS2-1 (GAL4 binding domain expression vector) to produce pAS-PP1α. This expression vector was first used to confirm the expression of the resulting fusion proteins (GAL4-PP1α) in yeast strain AH109. For library screening, the yeast strain AH109 transformed with pAS-PP1α, was mated with yeast strain Y187 expressing the human brain cDNA library (from an adult male brain, Clontech, HL4004AH) in the pACT-2 vector (Gal4 activation domain expression vector). Half the mating mixture was plated onto high stringency medium (quadruple dropout medium (QDO): SD/-Ade/-His/-Leu/-Trp) and the other half onto low stringency medium (triple dropout medium (TDO): SD/-His/-Leu/-Trp), and the plates were incubated at 30°C. Colonies obtained in the low stringency plates were replica plated onto high stringency medium. Finally, all high stringency surviving colonies were plated onto selective medium containing X-α-Gal and incubated at 30°C to check for MEL-1 expression (indicated by the appearance of a blue color). All the YTH reagents were purchased from Clontech, Saint-Germain-en-Laye, France. All other nonspecified reagents were purchased from Sigma-Aldrich, Portugal.

Recovery of plasmids from yeast and sequence analysis

Yeast plasmid DNA was recovered and used to transform *Escherichia coli* XL1-Blue. Plasmid DNA was obtained from each resulting bacterial colony and digested with the restriction enzyme *Hind*III (NEB, Ipswich, MA, USA) to identify the corresponding library plasmids. DNA sequence analysis was performed using an Automated DNA Sequencer (Applied Biosystems, Carlsbad, CA, USA) using the GAL4-AD primer—TACCACTACAATGGATG (Clontech). The DNA sequences obtained were compared to the GenBank database, using the BLAST algorithm, to identify the corresponding encoded proteins.

Databases search of PIPs

The human specific PP1α interactors available in eight online databases: BioGRID, BIND, STRING, HPRD, IntAct, MINT, Reactome-FLS, and InnateDB, were retrieved. Afterward, an exhaustive analysis to the PIPs was made and the proteins were grouped. The PP1α interaction map was made using Cytoscape (Shannon et al., 2003).

Results

Identification of 66 proteins by Yeast Two-Hybrid Screening of a human brain cDNA library

In order to identify PP1α Interacting Proteins expressed in the human brain, an YTH screen of a human brain cDNA library was carried out using full-length human PP1α. The screen yielding 298 positive clones from a total of 2×10^7 clones screened, corresponding to 66 different protein–protein interactions. After partial or complete sequence analysis (depending on the length of the positive clone's cDNAs), *in silico* searches of the GenBank database allowed their identification and classification into four separate groups. Two groups are listed on Tables 1 and 2. The third group corresponds to clones putatively encoding novel PIPs with homology to genomic sequences and lists positives where the Genbank sequence similarity, although apparent, did not correspond to an annotated gene (Appendix 1). These cDNA clones may correspond to transcripts derived from novel, previously unidentified genes. The fourth group corresponds to possible false positive hits. Table 1 (known PP1 interacting proteins) lists positives encoding previously

Table 1. Known PP1 Interacting Proteins

		PP1-BMs				
Clone ID	*No. Clones*	*RVxF*	*SILK*	*Chr*	*Uniprot accession number*	*Reference of interaction discovery*
AATK	1	KAVSF		17	Q6ZMQ8	(Gagnon et al., 2007)
AXIN1	4	RVAF/RVEF	SILK	3	Q96S65	(Luo et al., 2007)
C1QA	16	RSLGF/KGLF		1	P02745	(Fardilha et al., 2011b)
CNST	25	RRVRF	SILK	1	Q6PJW8	(Fardilha et al., 2011b)
C9orf75	45	KISF / RAIRW		9	Q4KMQ1	(Esteves, 2008; Trinkle-Mulcahy et al., 2006)
KIAA1949	4	KISF		6	Q6NYC8	(Kao et al., 2007; Trinkle-Mulcahy et al., 2006)
LAP1B	14	REVRF/ KVNF/ KVKF	SILK	1	Q5JTV8	(Santos, 2009)
NEK2	2	KVHF		1	P51955	(Helps et al., 2000a)
PHACTR3	2	RNIF		20	Q96KR7	(Sagara et al., 2003)
PPP1R2	1	KLHY	GILK	3	P41236	(Huang and Glinsmann, 1976)
PPP1R3C	5	KRVVF / KNVSF /RITF / KIEF		10	Q9UQK1	(Doherty et al., 1996)
PPP1R3D	2	RVQF / LRVRF		6	O95685	(Armstrong et al., 1997)
PPP1R3E	1	RVRF		14	Q9H7J1	(Ceulemans et al., 2002; Munro et al., 2005)
PPP1R9B	14	RKIHF		17	Q96SB3	(Allen et al., 1997a)
PPP1R13A	17	RVKF		1	Q13625	(Helps et al., 1995)
PPP1R13B	5	LRVRF		14	Q96KQ4	(Helps et al., 1995)
PPP1R3G	1	KRVQF		6	B7ZBB8	(Ceulemans et al., 2002)
PPP1R13L	2			19	Q8WUF5	(Colland et al., 2004)
PPP1R15B	3	KKVTF		1	Q5SWA1	(Jousse et al., 2003)
PPP1R16A	1	KQVLF		8	Q96I34	(Skinner and Saltiel, 2001)
RANBP9	20	RMIHF		6	Q96S59	(Fardilha et al., 2011b)
RIF1	9	KKIAF/ RRVSF	SILK	2	Q5UIP0	(Moorhead et al., 2008; Trinkle-Mulcahy et al., 2006)
SH3RF2	4	KTVRF		5	Q8TEC5	(Chen et al., 2009)
STAU1	16	RKVTF		20	O95793	(Monshausen et al., 2002)
WBP11	1	RKVGF / LSVRF	SILK	12	Q9Y2W2	(Llorian et al., 2004)
YLPM1	8	RVGF / KRVRW / RAIGF		14	P49750	(Tran et al., 2004; Ulke-Lemee et al., 2007)
ZFYVE9	3	RRVWF / KVIRW		1	O95405	(Bennett and Alphey, 2002; Colland et al., 2004)

Number of clones indicate the count of isolated cDNA clones for the respective protein.
PP1-BMs, PP1 binding motifs; Chr, chromosome.

Table 2. Novel PP1 Interacting Proteins

Clone ID	No. Clones	PP1BMs RVxF	PP1BMs SILK	Chr	Uniprot accession number
ANKRD15	2			9	Q14678
BTBD10	1	RHVDF		11	Q9BSF8
CLCN2	1			3	P51788
CEP170	1	RILF		1	Q5SW79
CLTC	1	RAIQF	GILR	17	Q00610
CKB	1			14	P12277
CNP1	1	KIFF		17	P09543
CNTN1	2	LTITW		12	Q12860
CRK	1			17	P46108
CXXC1	1			18	Q9P0U4
DCTN1	2	KIKF / KVTF	SILK	2	Q14203
DEAF1	1			11	O75398
FRMPD4	1	KVRF / KVSF		X	Q14CM0
GLIPR1L2	1			12	Q4G1C9
GLTSCR2	1			19	Q9NZM5
IBTK	1	KKVSF		6	Q9P2D0
IIP45	1	RVTF		1	Q5JXC2
JPH3	1			16	Q8WXH2
KCTD20	3	RHVDF		6	Q7Z5Y7
KIAA0460	1	RVGW	SILK	1	Q5VT52
KIAA1377	8	KLRW	SILK	11	Q9P2H0
LPIN2	1			18	Q92539
MAFG	1			17	O15525
MAL2	1			8	Q969L2
MAP4K4	2			2	O95819
NDP	2			X	Q00604
PHC1	2			12	P78364
PIAS1	1			15	O75925
PIAS3	1			1	Q9Y6X2
PREX1	1	KKVCF / KVIF		20	Q8TCU6
PRR16	2	RVRF		5	Q569H4
SLC45A1	1	RNVTF	GILK	1	Q9Y2W3
SNCAIP	6	LRVTF		5	Q9Y6H5
SorLA-1	1			11	Q92673
SPRED1	1	RHVSF		15	Q7Z699
UBE2Z	1			17	Q9H832
ULK1	1			12	O75385
ZBTB11	1		GILK	3	O95625
ZNF827	1	LNVQF		4	Q17R98

Number of clones indicate the count of isolated cDNA clones for the respective protein.
PP1-BMs, PP1 binding motifs; Chr, chromosome.

identified PIPs, such as Nek2 (Helps et al., 2000b) and PPP1R9B (Allen et al., 1997a). Table 2 (novel PIPs) lists positives encoding known proteins that were not previously associated with PP1 and uncharacterized proteins (present in the database) that are novel PP1 interactors.

The YTH screen yielded 298 positive clones (Tables 1 and 2 and Appendix 1) that correspond to 66 different proteins (Tables 1 and 2), not considering PIPs with homology to genomic sequences, some with more than one hit.

Careful analysis of the YTH screen revealed that the most abundant interaction was detected with C9orf75 (Table 1). Thus, 45 positives out of the 298 detected encoded C9orf75, which corresponds to 15% of the positive clones obtained. This protein, also known as Taperin, was associated with autosomal-recessive nonsyndromic hearing loss by target genome capture combined with next-generation capture (Rehman et al., 2010) and by homozygosity mapping (Auluck et al., 2010). Immunolocalization studies of mouse cochlea by Rehman et al. (2010) demonstrated the presence of C9orf75/Taperin at the taper regions of hair cell stereocilia. Nevertheless, the function of C9orf75/Taperin still needs to be elucidated. Interestingly, together, seven well- known PIPs from the 27 correspond to 122 positive clones, 41% of the total interactions. Among them is C1QA (16 positive clones/5%), a protein related to the innate immune response and associated to oxidative stress responses in the brain (Luo et al., 2003; Ten et al., 2010) and PPP1R9B (14 positive clones/5%), also known as Spinophilin, highly abundant in neuronal spines where it is involved in synaptic transmission (Allen et al., 1997b; Feng et al., 2000).

Table 2 presents the list of known proteins or uncharacterized proteins (present only in the database) that have not been previously associated with PP1 and thus represent potential novel PP1 interacting proteins. This group of 39 different PIPs was encoded by 60 cDNAs. Twenty-nine proteins (74%) corresponded to single hits. Two independent positive clones codify to each of seven proteins. KIAA1377, SNCAIP (synuclein, α interacting protein), and KCTD20 (potassium channel tetramerisation domain containing 20) were encoded by eight, six, and three independent positive cDNA clones, respectively. KIAA1377, although of unknown function, is codified by several human brain ESTs (UniGene Hs.156352). SNCAIP, Synphilin-1, predominantly expressed in neurons is located in the cytoplasm and presynaptic nerve terminals and associated with synaptic vesicles, was initially identified as an α-synuclein-interacting protein (Engelender et al., 1999; Ribeiro et al., 2002). However, in several neurodegenerative disorders called α-synucleinopathies, such as Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy, synphilin-1, is mainly localized in neuronal and glial cytoplasmic inclusions (Wakabayashi et al., 2002, 2000). KCTD20 may have a voltage-gated potassium channel activity being involved in potassium ion transport (Gene Ontology GO:0005249) and has also been shown to be expressed in human brain (UniGene Hs.188757).

For all the already known PIPs and for the newly identified (Tables 1 and 2, respectively) a search for the PP1BMs RVxF and SILK was performed. All the proteins already known to be PIPs have the RVxF-motif, except PPP1R13L, and six contain the SILK-motif (Table 1). For the group of proteins that are novel PIPs (Table 2) there are 18 proteins that possess the RVxF motif but 54% does not, and also six have the SILK-motif. Only one PIP, ZBTB11, has a SILK-motif without an RVxF-BM.

Positives whose nucleotide sequence did not align with mRNA or cDNA sequences in the Genbank database are listed in Appendix 1. Thus, because the positives identified were derived from the brain cDNA library, the seven genomic sequences listed probably contain hitherto unidentified genes encoding putative novel PIPs, although the possibility of genomic contamination cannot be excluded.

Only three different possible false positive interactions were detected in our screen corresponding to proteins present in the mitochondria. One clone codifies for the 16s ribosomal

RNA, one independent clone codifies for IDH2 (isocitrate dehydrogenase 2), and the other for CYCS (Cytocrome *c*). These clones might be an artefact from the cDNA library. The last two clones, although being mitochondrial proteins, are codified by nuclear chromosomes, so they may interact with PP1α nonetheless.

Retrieval of PP1α interacting proteins from free access online databases for protein interactions

Eight free access online databases for protein interactions were searched in order to retrieve PP1α interacting proteins (Appendix 2). The specificity of each database is different and with variations in the type and depth of their annotations. STRING is a database of known and predicted protein interactions. Reactome-Fls functions as a data mining resource. MINT focuses on experimentally verified protein–protein interactions mined from the scientific literature by curators. IntAct provides an open source database system and analysis tools for protein interaction data, interactions are derived from literature curation or direct user submissions. BioGRID is an online interaction repository with data compiled through curation efforts. InnateDB database has experimentally verified interactions involved in the innate immune response by integrating known interactions public databases together with curated data. HPRD is a platform to visually depict and integrate information, which is extracted from the literature. Finally, BIND documents molecular interactions by including high-throughput data submissions and hand-curated information gathered from the scientific literature.

After having collected all PP1α interactors from the above databases, the nomenclature for each protein was normalized for the Uniprot accession number in order for the interactions in each database to be compared. In total, we obtained 246 PP1α interactions from the databases and from our YTH screen (Appendix 2). The corresponding number of interactions found in each database are the following: Biogrid, 28; STRING, 113; HPRD, 70; Reactome, 52; InnateDB, 10; IntAct, 18; MINT, 33; and BIND, 30.

Discussion

Protein phosphorylation is critical to the health and vitality of eukaryotic cells and probably their major metabolic control mechanism. Consequently, of note, many disease processes are associated with abnormal phosphorylation of key proteins, thus suggesting a possible common molecular basis for some apparently unrelated and diverse diseases processes. The role played by protein phosphatases in health and disease, and particularly the involvement of PP1, makes it and the proteins that regulate its function (PIPs) excellent targets for pharmacological intervention. Indeed, there are different compounds that act by disrupting PP1/PIPs complex. For example salubrinal, a small molecule that protects cells from endoplasmic reticulum stress, inhibits the formation of PP1-PPP1R15A (Boyce et al., 2005). Treatment of cells with trichostatin A, a deacetylase inhibitor, disrupted HDAC6-PP1 complexes (Brush et al., 2004). In another study, a GADD34-derived peptide competitively disrupted the PP1/GADD34 complex, when added to cells (Kepp et al., 2009). PP1/PIPs complexes seem to be the future targets for several diseases, because PP1 has been associated to several disorders. Nevertheless, the specificity of PP1 targeting should be achieved by two means: PP1 isoform specificity and differential PIP association. These meaning that each PP1 isoform has its tissue and event-specific expression pattern and the same happens to the PIPs, leading to the formation of a specific PP1/PIP complex in a certain place and time event/mechanism. This highly specific complex can then be target by an inhibiting or stimulating molecule. In summary, determining PP1 isoform and tissue specific PIPs is crucial to identify particular complexes. We have previously identified PP1γ1 and PP1γ2 human testis interactome, identifying common and specific interactors in a total of 72 novel interactions (Fardilha et al., 2011b).

In order to identify potential PP1α interacting and regulating proteins, and to characterize the PP1α human brain interactome a large scale screen for PP1α binding proteins was performed, using the YTH system (Fardilha et al., 2004). A total of 66 proteins were identified from the 298 positive clones obtained that are expressed in human brain and bind PP1α. Furthermore, seven extra proteins codified by cDNA, but present in the database as genomic clones, were also obtained. Moreover, three proteins were considered to be false positive hits because they were mitochondrial proteins. One is, in fact, codified by mitochondrial DNA but the other two are codified by nuclear DNA, and afterward, translocated to the mitochondria. Thus, they could interact with PP1 during this process. These 10 proteins (Appendix 1) were left out of the functional analysis (Fig. 1). For the remainder of the clones (286 clones/96%), corresponding to 66 proteins, the most abundant group are proteins involved in signaling (20%), followed by proteins involved in splicing and transcription (17%) and metabolism (14%). The remaining proteins identified include components of the cytoskeleton, proteins involved in apoptosis, as well as cell cycle and transport (Fig. 1) and 18% still have unknown cellular function. This analyzes reflects the functional diversity of PP1, and all the proteins here mentioned and described illustrate the great multiplicity of cellular pathways and events in which PP1 is involved, and controls targets, and is regulated by its PIPs.

This YTH screen, as already verified in other YTH screens (Fardilha et al., 2011b), shows the inconsistency between the frequency of the cDNAs isolated and the total number of protein interactors identified. For example, the already known PIPs (Table 1) are encoded by 76% (226) of the total cDNAs isolated and account for 41% (27) of the PIPs identified. Neverthless, 20% (60) of the positive clones isolated code for 39 proteins (59%), all novel PIPs (Table 2). In the first case the ratio cDNA/protein is 8.4 and in the second case is 1.6. There are, in the case of the novel PIPs, more proteins identified from single hits (29 proteins). As stated by Fardilha et al. (2011b) this may be the explanation why these novel PIPs were not yet identified, or alternatively, they may have been discarded given that they are less abundant clones in the YTH screens, or they could have just been missed because usually the screens are not that exhaustive. However, as we took an in-depth exhaustive strategy, and decided to sequence all the YTH positive clones, we did not miss the rare interactions or low abundant proteins, difficult to pick up by proteomic methodologies. The identification of both abundant and rare known PIPs in our screens confirms the specificity and reliability of the YTH approach.

All, except one of the previously known PIPs possess an RVxF PP1BM. In contrast, only 46% of the novel PIPs

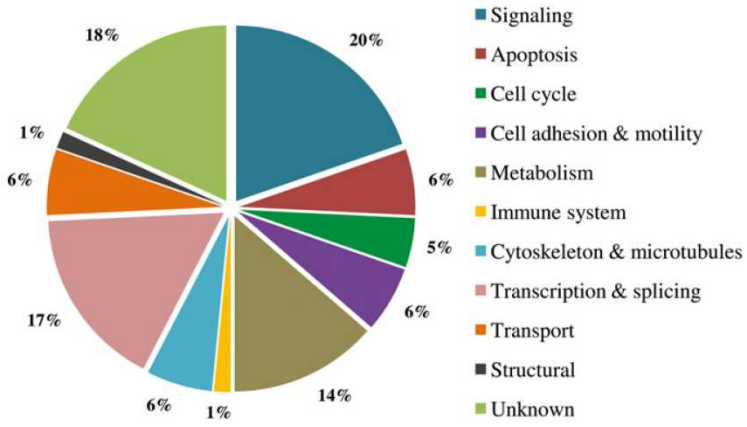

FIG. 1. Distribution of the PP1α regulators according to function. Biological functions attributed to PIPs identified; according to databases by search based on functional motifs present, interacting proteins, cellular localization, and molecular function.

(18 proteins) appear to have the consensus PP1BM. In fact, the number of proteins carrying PP1-BMs may be much higher if one considers the fact that some of the positive clones were not fully sequenced, and thus the analysis had to rely on the amino acid sequences available in the Genbank database, which may not reflect the frequent occurrence of alternatively spliced variants in the brain. The identification of previously identified PIPs and the presence of the PP1BM in the novel PIPs strengthens and validates the results of the YTH screen. In fact, the latter was present in 44 (67%) out of the 66 proteins identified.

Eight protein–protein interaction databases were searched for PP1α interactions; only human proteins were considered in this study (Appendix 2). After a careful analysis of the interactions and organization of the retrieved results they were compared to the interactions obtained in the human brain PP1α YTH screen. From the PIPs identified in the YTH some were present in the databases searched (Fig. 2A) but other were not, the 39 novel PIPs (Table 2). Yet, the first bar in Figure 2A, corresponding to novel interactions, includes 46 proteins instead of 39. This is because some already characterized interactions are not yet present in the free online databases (e.g., PPP1R3E and PPP1R3G). The highest number of times an interaction appears in a database the stronger is the credibility of the interaction, considering the different criteria used for inclusion of the interactions in the different databases. Of course, this is also dependent on the detection methods of the interaction, some being more reliable than others (Appendix 2). Among the 246 interactions 156 interactions appeared in a single database (Fig. 2B). Forty-one interactions were common to two databases and 25 to three. Fifteen interactions were present in four databases, six interactions in five databases, and finally three interactions were present in six databases. These three PIPs are well-known PIPs: PPP1R15A (or GADD34), PPP1R8 (or NIPP1), and ZFYVE9 (or SARA), which strengths the above idea. The first is involved, together with PP1, in protein synthesis, regulation of calreticulin exposure, and TGF-β signaling (Brush et al., 2003; Kepp et al., 2009; Shi et al., 2004). The second, also with PP1,

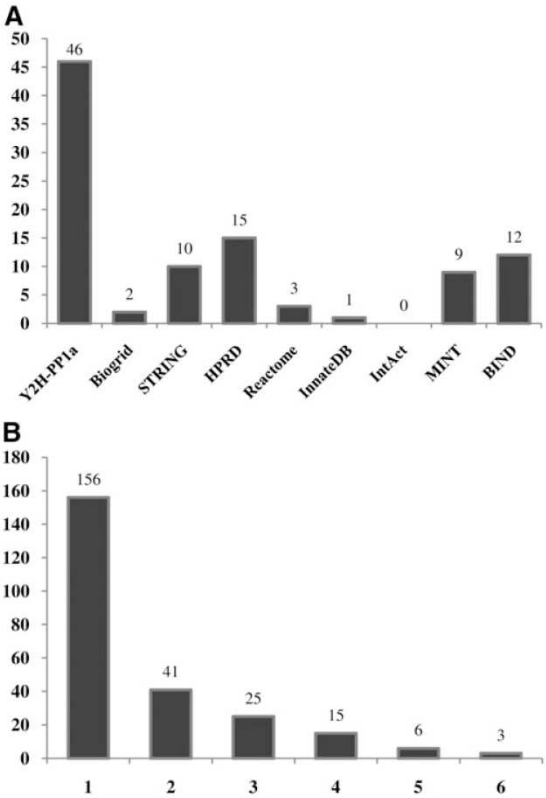

FIG. 2. Analysis of the PP1α interacting proteins in the databases searched. (**A**) Number of PIPs identified in the Yeast Two-Hybrid Screen (Y2H-PP1a) present in the different databases. (**B**) Number of PIPs identified in all the databases (including the Yeast Two-Hybrid Screen) that are present in only one database; two or more databases.

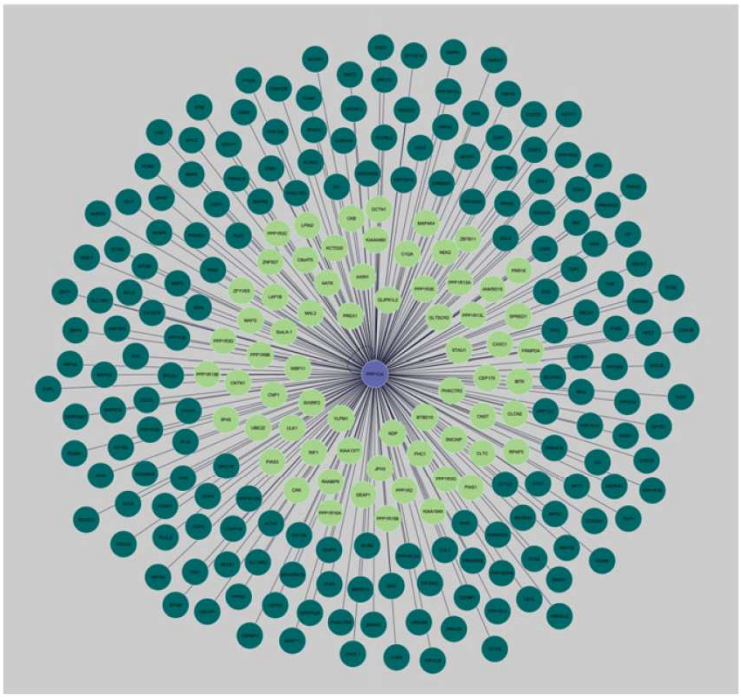

FIG. 3. PP1α interactions mapping. PP1α Interacting proteins retrieved from online databases (dark green) and identified in the Human Brain Yeast Two- Hybrid Screen using PP1α as bait (light green). The total number of interactors is 247 and the proteins obtained in the Yeast Two-Hybrid Screen is 66. Of the 66 proteins 39 are novel PP1α interactors while the remainder are also common to one or more databases (Appendix 2).

regulates RNA splicing (Tanuma et al., 2008; Trinkle-Mulcahy et al., 1999). ZFYVE9 is involved in signal transduction enhancing the recruitment of PP1 to the TGF-β receptor 1 (Bennett and Alphey, 2002; Shi et al., 2004).

Finally, we used cytoscape (Shannon et al., 2003) to assemble a diagram of the interactome of PP1α (Fig. 3) using the databases interactions (Fig. 3, in dark green) plus our YTH interactions (Fig. 3, in light green). This interaction map clearly shows that our YTH screen contributes in many interactions to the overall picture. Considering the above discussion, we have added 39 new PIPs to the PP1α interactome. Also, we have identified at least three key molecules in the PP1α interactome, which are present in six databases. Indeed, PP1/PPP1R15A is a target for salubrinal in the treatment of Herpes simplex virus infection (Boyce et al., 2005). Thus, we propose that the complexes PP1/PPP1R8 and PP1/ZFYVE9 could also be relevant targets.

Conclusions

In this study we report the identification of 66 proteins expressed in the human brain that bind PP1α. The majority of the detected interactions were novel (39/60%) and the functions of the new PIPs still need to be fully characterized. Only then will the precise roles of PP1α/brain-specific PIPs be fully elucidated. We have tried to do the complete picture of the PP1α interactome taking advantage of databases interaction information. We concluded that some of the PIPs identified in our YTH screen were present in other databases but some were novel. Thus, the present work added 39 novel interactions to PP1α interaction network. Together, our results and drawn conclusions allowed us to identify key PIPs in human brain that bind preferentially PP1α. This study also points to the importance of addressing PP1 isoforms as independent entities and consider PP1α/brain-specific PIP (or other PP1 isoform/PIP tissue specific complex) as an excellent target for specific pharmacologic therapy.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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(Appendix follows →)

Appendix Table A1. Clones Putatively Encoding Novel PIPs With Homology to Genomic Sequences and Mitochondrial Proteins

Clone ID	*No. clones*	*Chromosome*	*NCBI reference sequence*
Genomic clones			
C-2190G12	1	14	AL_139194
C2genomic	1	2	NW_001838769
C2genomic	1	2	NW_001838818
C2genomic	1	2	NW_001838863
C3genomic	1	3	NW_001838877
C4genomic	1	4	NW_001838915
C17genomic	3	17	NW_001838448
Mitochondrial proteins			
16S ribosomal RNA	1	Mitochondria	AM_263191.1
CYCS	1	7	NM_018947
IDH2	1	15	NM_002168

Number of clones indicate the count of isolated cDNA clones for the respective protein.

Appendix Table A2. Complete List of PP1α Interacting Proteins Identified in the YTH Screen and Present in the Searched Databases

Protein ID	*Uniprot accession number*	*Interaction detection*	*Databases*
AATK	Q6ZMQ8	Y2H	Y2H-PP1a
ACTA1	P68133	AC	BIOGRID
ADORA1	P30542	P	STRING
ADORA2A	P29274	P	STRING
ADORA2B	P29275	P	STRING
ADORA3	P33765	P	STRING
AKAP1	Q92667	E, P	HPRD, STRING
AKAP11	Q9UK44	AC+B, P, PP	BIOGRID, STRING, HPRD
AKAP9	Q99996	E+IC, P	HPRD, STRING
AKT1	P31749	P	STRING
ANKRD15	Q14678	Y2H, PP	Y2H-PP1a, BIND, HPRD
AP2S1	P53680	E	Reactome
APAF	O14727	CO-IP, AA+CO-IP, PP	InnateDB, IntAct, HPRD
ATF5	Q9Y2D1	P	STRING
ATF6	P18850	P	STRING
ATM	Q13315	AA	IntAct
AURKA	O14965	E	HPRD
AURKAB	Q96GD4	AA, E	IntAct, Reactome
AXIN1	Q96S65	Y2H, CO-IP, P	Y2H-PP1a, MINT, STRING
BAD	Q92934	CO-IP	IntAct, HPRD, STRING
BAX	Q07812	CO-IP, PP	InnateDB, HPRD
BCL2	P10415	AA, E, P, PP	IntAct, Reactome, STRING, HPRD
BCL2L1	Q07817	AC+B, AA, P	BIOGRID, IntAct, STRING, HPRD,
BCL2L2	Q92843	AC+B, P, PP	BIOGRID, STRING, HPRD
BMP2	P12643	E, P	Reactome, STRING
BMP4	P12644	E, P	Reactome, STRING
BMP6	P22004	P	STRING
BMPR1A	P36894	E	Reactome, STRING
BMPR1B	O00238	E	Reactome
BMPR2	Q13873	E, P	Reactome, STRING
BRCA1	P38398	AC+B, CO-IP, P, PP	BIOGRID, IntAct, STRING, HPRD
BTBD10	Q9BSF8	Y2H, PD	Y2H-PP1a, InnateDB
C1orf114	Q5TID7	Y2H, PP	MINT, HPRD
C1QA	P02745	Y2H	Y2H-PP1a
C9orf75	Q4KMQ1	Y2H, PP	Y2H-PP1a, MINT, HPRD
CAD	P27708	PP	HPRD
CAMK2B	Q13554	P	STRING
CAMK2G	Q13555	P	STRING
CASC5	Q8NG31	PP, Y2H	HPRD, MINT

(*continued*)

Appendix Table A2. (Continued)

Protein ID	*Uniprot accession number*	*Interaction detection*	*Databases*
CCDND1	P24385	P	STRING
CDC5L	Q99459	AC+B, CO-IP, E, P, PP	BIOGRID, MINT, Reactome, STRING, HPRD
CDH1	P12830	CO-IP, AA, PP	InnateDB, IntAct, HPRD
CDK1	P06493	AA, E, P	IntAct, Reactome, STRING
CDK2	P24941	AA, E	IntAct, Reactome
CDK3	Q00526	E	Reactome
CDK4	P11802	AA, E	IntAct, Reactome
CDK5	Q00535	E	Reactome
CDK6	Q00534	E	Reactome
CDK7	P50613	E	Reactome
CEP170	Q5SW79	Y2H	Y2H-PP1a
CHD1	O14646	P	STRING
CHEK	O96017	E	Reactome
CHI3L1	P36222	P	STRING
CKB	P12277	Y2H	Y2H-PP1a
CLCN2	P51788	Y2H	Y2H-PP1a
CLTC	Q00610	Y2H	Y2H-PP1a
CNP1	P09543	Y2H	Y2H-PP1a
CNST	Q6PJW8	Y2H	Y2H-PP1a
CNTN1	Q12860	Y2H	Y2H-PP1a
CRK	P46108	Y2H	Y2H-PP1a
CSRNP2	Q9H175	Y2H, E, PP	BIND, MINT, Reactome, HPRD
CTRL	P40313	P	STRING
CUED2	Q9H467	CO-IP	InnateDB
CUL1	Q13616	CO-IP, PP	IntAct, HPRD
CXXC1	Q9P0U4	Y2H	Y2H-PP1a
DCTN1	Q14203	Y2H	Y2H-PP1a
DCX	O43602	E, P+PP	HPRD, STRING
DDX17	Q92841	P	STRING
DEAF1	O75398	Y2H	Y2H-PP1a
EED	O75530	B	BIOGRID, STRING
EIF2AK2	P19525	AC+B, P, PP	BIOGRID, STRING, HPRD
EIF2S1	P05198	P+IM	STRING
EP300	Q09472	E	Reactome
ERBB2IP	Q96RT1	E	Reactome
ESR1	P03372	AC	IntAct
EVPL	Q92817	P	STRING
EXOSC8	Q96B26	P	STRING
FRMPD4	Q14CM0	Y2H	Y2H-PP1a
FXYD1	O00168	E, P	HPRD, STRING
GIYD2	P50224	P	STRING
GLIPR1L2	Q4G1C9	Y2H	Y2H-PP1a
GLTSCR2	Q9NZM5	Y2H	Y2H-PP1a
GPX1	P07203	E	Reactome
GSK3B	P49841	AC, E, P	BIOGRID, Reactome,STRING
GSTP1	P09211	P	STRING
GYS1	P13807	P+IM	STRING
GYS2	P54840	P+IM	STRING
H2AFX	P16104	E, P	HPRD, Reactome, STRING
HCFC1	P51610	AC+B, P	BIOGRID, STRING
HEYL	Q9NQ87	Y2H, PP	MINT,BIND, HPRD
HFE2	Q6ZVN8	E	Reactome
HNF4A	P41235	CROSSLINK	BIND
HOXA10	P31260	P	STRING
HSPA4	P34932	PP	HPRD
HSPA8	P11142	AC, P	BIOGRID, STRING
IBTK	Q9P2D0	Y2H	Y2H-PP1a
ID2	Q02363	PP	HPRD
IDI1	Q13907	P	STRING
IIP45	Q5JXC2	Y2H	Y2H-PP1a
IKKA	O15111	CO-IP	InnateDB

(*continued*)

Appendix Table A2. (Continued)

Protein ID	*Uniprot accession number*	*Interaction detection*	*Databases*
IKKB	O14920	CO-IP	InnateDB
IQGAP1	P46940	P	STRING
IRS4	O14654	P	STRING
JPH3	Q8WXH2	Y2H	Y2H-PP1a
KCNQ1	P51787	AC + B, PP	BIOGRID, STRING
KCTD20	Q7Z5Y7	Y2H	Y2H-PP1a
KIAA0460	Q5VT52	Y2H	Y2H-PP1a
KIAA0649	Q5T8A7	Y2H	MINT, BIND
KIAA1377	Q9P2H0	Y2H	Y2H-PP1a
KIAA1949	Q6NYC8	Y2H, P, E	Y2H-PP1a, BIND, STRING, HPRD
KIF13A	Q9H1H9	E	HPRD
KIF18A	Q8NI77	Y2H, PP	MINT, HPRD
LAP1B	Q5JTV8	Y2H	Y2H-PP1a, BIND
LMTK2	Q8IWU2	AC + B + Y2H, P	BIOGRID, STRING
LPIN2	Q92539	Y2H	Y2H-PP1a
LRRC68	O75864	Y2H	MINT
MAFG	O15525	Y2H	Y2H-PP1a
MAL2	Q969L2	Y2H	Y2H-PP1a
MAP3K3	Q99759	AP, CO-IP	IntAct, MINT
MAP4K4	O95819	Y2H	Y2H-PP1a
MAPK1	P28482	E, P	HPRD, Reactome, STRING
MAPK3	P27361	E, P	HPRD, Reactome, STRING
MAX	P61244	AA	IntAct
MEN1	O00255	P	STRING
MPHOPH10	O00566	Y2H	MINT, BIND
MRLC3	P19105	P	STRING
MYC	P01106	AA	IntAct
MYL2	P10916	P	STRING
MYL9	P24844	P	STRING
MYO16	Q9Y6X6	E, P	HPRD,STRING
N33	Q13454	Y2H, PP	BIND, MINT, HPRD
NCOR1	O75376	AC	BIOGRID
NDP	Q00604	Y2H	Y2H-PP1a
NEK2	P51955	E, P, Y2H	Y2H-PP1a, HPRD, Reactome,STRING
NOC2L	Q9Y3T9	B	BIOGRID
NOM1	Q5C9Z4	Y2H, PP	MINT, HPRD
OAZ1	P54368	E	Reactome
PCDH7	O60245	E	HPRD
PCNA	P12004	PP	HPRD
PHACTR1	Q9C0D0	Y2H + AC, P, PP	BIOGRID, STRING, HPRD
PHACTR3	Q96KR7	Y2H + AC + FWB, E	Y2H-PP1a, STRING, BIND, HPRD, BIOGRID
PHACTR4	Q8IZ21	E, P	HPRD, STRING
PHC1	P78364	Y2H	Y2H-PP1a
PHKA2	P46019	P	STRING
PIAS1	O75925	Y2H	Y2H-PP1a
PIAS3	Q9Y6X2	Y2H	Y2H-PP1a
PKN1	Q16512	P	STRING
PLCL2	Q9UPR0	Y2H, PP	BIND, MINT, HPRD
PLIN	O60240	E, P	Reactome, STRING
PLK1	P53350	E	Reactome
PLP1	P60201	P	STRING
PLP2	Q04941	P	STRING
PPP1CA	P62136	PP	HPRD
PPP1CB	P62140	E	Reactome
PPP1CC	P36873	E, P + IM + PP	Reactome,STRING
PPP1R10	Q96QC0	Y2H, B, E	BIND, BIOGRID, MINT,STRING, HPRD
PPP1R11	O60927	P, E	STRING, HPRD
PPP1R12A	O14974	P	STRING
PPP1R12B	O60237	P	STRING
PPP1R13A	Q13625	Y2H, PP	Y2H-PP1a, MINT, HPRD, BIND
PPP1R13B	Q96KQ4	Y2H, PP	Y2H-PP1a, MINT, HPRD, BIND

(*continued*)

Appendix Table A2. (Continued)

Protein ID	*Uniprot accession number*	*Interaction detection*	*Databases*
PPP1R13L	Q8WUF5	Y2H, PP	Y2H-PP1a, HPRD, BIND
PPP1R15A	O75807	Y2H, AC, E, P, PP	BIND, BIOGRID, MINT, Reactome, STRING, HPRD
PPP1R15B	Q5SWA1	Y2H, PP	BIND, Y2H-PP1a, MINT, HPRD
PPP1R16A	Q96I34	Y2H	Y2H-PP1a
PPP1R1A	Q13522	P	STRING
PPP1R1B	Q9UD71	E+IC, E, P	HPRD,Reactome, STRING
PPP1R2	P41236	E, Y2H, E, P	Y2H-PP1a, Reactome, STRING, HPRD
PPP1R2P9	O14990	E, P	HPRD,STRING
PPP1R3A	Q16821	P	STRING
PPP1R3B	Q86XI6	Y2H, P, PP	BIND, MINT, STRING, HPRD
PPP1R3C	Q9UQK1	Y2H, P	Y2H-PP1a, STRING
PPP1R3D	O95685	Y2H, P	Y2H-PP1a, STRING
PPP1R3E	Q9H7J1	Y2H	Y2H-PP1a
PPP1R3G	B7ZBB8	Y2H	Y2H-PP1a
PPP1R8	Q12972	Y2H, E, P+IM, PP, B	MINT, Reactome, STRING, HPRD, BIND, BIOGRID
PPP1R9A	Q9ULJ8	E,P+IM	HPRD, STRING
PPP1R9B	Q96B17	Y2H, AC+B, P+IM, PP	Y2H-PP1a, BIOGRID, STRING, HPRD, MINT
PPP2CA	P67775	E,P+IM	HPRD, Reactome,STRING
PPP2CB	P62714	E	Reactome
PPP2R4	Q15257	P+IM	STRING
PPP2R5D	Q14738	P+IM	STRING
PPP4C	P60510	E	Reactome
PPP5C	P53041	E	Reactome
PPP6C	O00743	E	Reactome
PREX1	Q8TCU6	Y2H	Y2H-PP1a
PRKACA	P17612	P	STRING
PRKACB	P22694	P	STRING
PRKACG	P22612	P	STRING
PRKAG1	P54619	IM	STRING
PRKAR2A	P13861	P	STRING
PRKAR2B	P31323	P	STRING
PRKCD	Q05655	E	HPRD
PRKCE	Q02156	P	STRING
PRR16	Q569H4	Y2H	Y2H-PP1a
PTEN	P60484	CO-IP, AA+CO-IP, E, PP	InnateDB, IntActReactome, HPRD
PYGM	P11217	P	STRING
RANBP9	Q96S59	Y2H	Y2H-PP1a
RB1	P06400	AA+CO-IP, E, P, PP	IntAct, Reactome, STRING,HPRD
RGMA	Q96B86	E	Reactome
RGMB	Q6NW40	E	Reactome
RIF1	Q5UIP0	Y2H	Y2H-PP1a
ROCK1	Q13464	P	STRING
RON	Q04912	CO-IP	InnateDB
RPAP2	Q8IXW5	AC	BIOGRID
RPAP3	Q9H6T3	AC	BIOGRID
RRP1B	Q14684	AC+B	BIOGRID
RUVBL2	Q9Y230	AC	BIOGRID
RYR2	Q92736	B, P	BIOGRID,STRING
SERPING1	P05155	P	STRING
SF3A2	Q15428	AC	MINT
SH2D3A	Q9BRG2	P	STRING
SH2D3C	Q8N5H7	P	STRING
SH3RF2	Q8TEC5	Y2H	Y2H-PP1a
SIRT2	Q8IXJ6	E	Reactome
SKP1	P63208	AA+CO-IP, PP	IntAct, HPRD
SLC18A1	P54219	P	STRING
SLC18A2	Q05940	P	STRING
SLC45A1	Q9Y2W3	Y2H	Y2H-PP1a
SMAD1	Q15797	E	Reactome
SMAD7	O15105	E, P	Reactome, STRING
SMARCB1	Q12824	AC+B, P	BIOGRID, STRING

(*continued*)

Appendix Table A2. (Continued)

Protein ID	*Uniprot accession number*	*Interaction detection*	*Databases*
SNCAIP	Q9Y6H5	Y2H	Y2H-PP1a
SNW1	Q13573	CO-IP	MINT
SorLA-1	Q92673	Y2H	Y2H-PP1a
SPRED1	Q7Z699	Y2H, PP	Y2H-PP1a, MINT, HPRD, BIND
STAM	Q92783	Y2H, PP	BIND, MINT, HPRD
STAU1	O95793	Y2H, E, P	Y2H-PP1a, MINT, HPRD, STRING, BIND
SYTL2	Q9HCH5	Y2H, PP	BIND, MINT, HPRD
TEP1	Q99973	P	STRING
TGFBR1	P36897	AC, E, P, CO-IP	BIOGRID, Reactome, STRING, BIND
TGFBR2	P37173	E, Y2H	Reactome, BIND
TIAM1	Q13009	P	STRING
TNF A	P01375	AC	InnateDB
TP53	P04637	Y2H, PP	BIND, MINT, HPRD
UBE2Z	Q9H832	Y2H	Y2H-PP1a
ULK1	O75385	Y2H	Y2H-PP1a
VDR	P11473	E	HPRD
VIP	P01282	P	STRING
WBP11	Q9Y2W2	Y2H	Y2H-PP1a
YLPM1	P49750	PP, Y2H	Y2H-PP1a, HPRD, BIND
ZBTB11	O95625	Y2H	Y2H-PP1a
ZDBF2	Q9HCK1	Y2H	BIND
ZFYVE16	Q7Z3T8	Y2H, E, P, PP	MINT, Reactome, STRING, HPRD, BIND
ZFYVE9	O95405	Y2H, E, IM, PP	BIND, Y2H-PP1a, MINT, Reactome, STRING, HPRD
ZNF827	Q17R98	Y2H	Y2H-PP1a

Y2H-PP1a, human brain PP1α YTH screen; AA, antibody array; AP, affinity purification; B, biochemical; CO-IP, coimmunoprecipitation; E, experimental knowledge based; FWB, Far-Western Blot; IC, inferred by curator; IM, interlogs mapping; P, predicted text mining; PD, pull down; PP, philogenetic profile; Y2H, Yeast Two-Hybrid.

Manuscript 2 - Protein Phosphatase 1γ Isoforms Linked Interactions in the Brain

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Abstract Posttranslational protein modifications, in particular reversible protein phosphorylation, are important regulatory mechanisms involved in cellular signaling transduction pathways. Thousands of human proteins are phosphorylatable and the tight regulation of phosphorylation states is crucial for cell maintenance and development. Protein phosphorylation occurs primarily on serine, threonine, and tyrosine residues, through the antagonistic actions of protein kinases and phosphatases. The catalytic subunit of protein phosphatase 1 (PP1), a major Ser/Thr-phosphatase, associates with a large variety of regulatory subunits that define substrate specificity and determine specific cellular pathway responses. PP1 has been shown to bind to different proteins in the brain in order to execute key and differential functions. This work reports the identification of proteins expressed in the human brain that interact with PP1γ1 and PP1γ2 isoforms by the yeast two-hybrid method. An extensive search of PP1-binding motifs was performed for the proteins identified, revealing already known PP1 regulators but also novel interactors. Moreover, our results were integrated with the data of PP1γ interacting proteins from several public web databases, permitting the development of physical maps of the novel interactions. The PP1γ interactome thus obtained allowed for the identification of novel PP1 interacting proteins, supporting novel functions of PP1γ isoforms in the human brain.

Keywords Protein phosphorylation · Yeast two-hybrid system · Protein interaction · Interactome

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Edgar F. da Cruz e Silva passed away on March 2, 2010.

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Introduction

Cellular signal transduction mechanisms typically transmit information via posttranslational protein modifications, among the most important is reversible protein phosphorylation. Several thousands of human proteins have been found to be phosphorylated in vivo, and only a small subset of the total phosphorylation sites are regulated in response to any given stimulus (Olsen et al. 2006). These are tightly regulated events of crucial importance for the correct cell development and differentiation, apoptosis, intercellular communication, proteostasis, and metabolism (Cohen 2001). Therefore, abnormal phosphorylation mechanisms lead to disorders such as cancer, diabetes, heart failure, and neurological degeneration (Gandy and Greengard 1994; da Cruz e Silva et al. 1995a; Sridhar et al. 2000; Cohen 2001; Neumann 2002; Fardilha et al. 2010).

In eukaryotic cells, phosphorylation occurs mainly on serine (Ser), threonine (Thr), and tyrosine (Tyr) residues, through the reversible action of protein kinases (PKs) and protein phosphatases (PPs). The fully sequenced human genome contains 518 putative PKs from two families: Tyr-

kinases with 90 members and Ser/Thr-kinases with 428 members (Lander et al. 2001; Venter et al. 2001; Manning et al. 2002; Johnson and Hunter 2005). The specificity of phosphorylation events and their reversible nature would suggest that there should be similar numbers of PPs in the human genome. However, there are 107 putative protein Tyr-phosphatases (PTPs) but far fewer (~40) protein Ser/Thr-phosphatases (PSPs) (Bollen 2001; Alonso et al. 2004; Ceulemans and Bollen 2004). This contrast in the number of family members is diminished when one considers the combinatorial formation of PSP holoenzymes from a common catalytic subunit and a large number of target and regulatory subunits (Bollen 2001).

The elucidation of the complete cDNA and amino acid sequences allowed for the separation of PSPs into three major families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases, and the aspartate-based phosphatases represented by FCP/SCP (TFIIF-associating component of RNA polymerase II CTD phosphatase/small CTD phosphatase) (Gallego and Virshup 2005; Moorhead et al. 2007; Shi 2009).

The catalytic subunit of several members of the PPP family associates with a large variety of regulatory subunits. In fact, protein phosphatase 1 (PP1), a major PSP, exhibits broad substrate specificity in vitro. However, each functional PP1 complex is thought to have a stringent substrate specificity, each triggering a specific cellular pathway response (Bollen 2001; Ceulemans and Bollen 2004; Fardilha et al. 2010, 2011a). The PP1 holoenzyme consists of a catalytic subunit (PP1c) and regulatory subunits (PP1 interacting proteins—PIPs). PP1c exists as three isoforms: α (PPP1CA), β/δ (PPP1CB), and γ (PPP1CC), and PP1γ is known to undergo alternative splicing to yield two variants PP1γ1 and PP1γ2 (da Cruz e Silva et al. 1995b). All forms of the PP1c share ~90 % amino acid sequence similarity which supports a conserved fold and a similarly positioned active site, but diverge at the N- and C-termini (Zhang et al. 1993a; Lin et al. 1999; Schillace and Scott 1999; Shi 2009). The remarkable degree of evolutionary conservation of these enzymes is undoubtedly related to their essential role in the regulation of fundamental cellular processes (Barford and Neel 1998; Barford et al. 1998; Cohen 2002; Fardilha et al. 2010).

PIPs can function as modulators of PP1 activity, determining targets and substrate specificity, as well as subcellular localization of the holoenzyme or may even serve as substrate themselves. Hence, PP1 activity can be regulated by reversible phosphorylation of the regulatory subunits, dissociation of the regulatory and the catalytic subunits, allosteric regulation of the regulatory subunits, and inducible expression of PP1 and the regulatory subunits (Cohen 2002). All PP1c isoforms are ubiquitously expressed, but PP1γ2 is significantly enriched in the sperm and testis (Takizawa et al. 1994; Strack et al. 1999).

More than 200 putative PIPs have been identified, with many more expected to be found (Moorhead et al. 2008; Hendrickx et al. 2009; Fardilha et al. 2010; 2011b; Heroes et al. 2012). Thus, the interactions between PP1 and the specific PIPs are central to the physiological functions of PP1, and PIPs are superior regulators at targeting specific pathways, when compared to PP1 alone (Cohen 2002; Ceulemans and Bollen 2004; Terrak et al. 2004; Virshup and Shenolikar 2009; Fardilha et al. 2010, 2011a).

Most PIPs contain the RVxF motif that binds, with high affinity, to hydrophobic amino acids on the surface of PP1, typically remote from the catalytic site (Egloff et al. 1997; Gibbons et al. 2005). Several studies permitted further characterization of the consensus sequence, and the RVxF motif was redefined: [RK][X]0-1[VI]{P}[FW] (Wakula et al. 2003), [HKR][ACHKMNQRSTV][V][CHKNQRST][FW] (Meiselbach et al. 2006), and [KRL][KRSTAMVHNQ][VI]{FIMYDP}[FW] (Hendrickx et al. 2009). Other PP1-binding motifs (PP1 BMs) have been described, and these strengthen the interaction of the PIPs with PP1, the apoptotic signature F-X-X-[KR]-X-[KR] (Ayllon et al. 2002; Godet et al. 2010), the MyPhoNE motif RXXQ[VIL][KR]X[YW] (Terrak et al. 2004; Hendrickx et al. 2009), and the SILK-motif [GS]IL[KR] (Huang et al. 1999; Connor et al. 2000; Wakula et al. 2003; Lin et al. 2005; Hurley et al. 2007; Hendrickx et al. 2009).

It is increasingly evident that protein phosphorylation is a fundamental process associated with memory, learning, and brain function, with prominent roles in the processing of neuronal signals and in short-term and long-term modulation of synaptic transmission (Koshibu et al. 2009; Graff et al. 2010). PP1 itself has already been implicated in this process, as a suppressor of learning and memory and as a potential mediator of cognitive decline during ageing (Genoux et al. 2002). Of all the mammalian tissues, the brain expresses the highest levels of PKs and PPs, and PP1 is also expressed at higher levels in several brain regions (Takizawa et al. 1994; da Cruz e Silva et al. 1995b; Ouimet et al. 1995; Strack et al. 1999; Bordelon et al. 2005). PP1 has been shown to bind to different PIPs in the brain in order to execute key and differential functions depending on the PIP it binds to. In essence, PP1 dephosphorylation specificity towards such a large array of targets is not only highly dependent on the expression of differentially spliced isoforms, but also on the diversity and structural design of the assembled PIPs.

Recently, we have characterized the human testis PP1γ and the human brain PP1α interactomes and have shown that there are isoform tissue-specific PIPs (Fardilha et al. 2011b; Esteves et al. 2012), supporting isoform and tissue PP1-specific functions. In the testis, some PIPs were identified solely when PP1γ1 was used as the bait, while others were only obtained when the bait was PP1γ2. In the brain, some PIPs were also PP1α specific. Thus, PP1 isoform

specificity clearly exists with respect to specific PIPs, which is highly relevant for PP1 isoform-dependent functions. Likewise, this is also relevant if one considers tissue- and cell-specific functions. At least, in vivo, a specific PP1 isoform is predominantly present in a particular tissue or in a precise intracellular location, together with a particular PIP. This does not exclude the fact that in vitro PP1c is promiscuous and may bind to a protein to which in fact never would do so in vivo.

The main goal of the work here presented was to identify the proteins expressed in the human brain that interact with PP1γ1 and PP1γ2 isoforms by the yeast two-hybrid (YTH) method. Also, we integrated the YTH results obtained, with PP1γ interaction data from several public web databases, and developed physical maps for the interactions. The PP1γ interactome thus obtained allowed for the identification of novel PIPs, addressing novel functions to PP1γ isoforms in the brain.

Materials and Methods

Human Brain Library Screening by the Yeast Two-Hybrid Method

The PP1γ1 cDNA was directionally subcloned into *Sal*I/*Sma*I digested pAS2-1 (GAL4-binding domain expression vector, Clontech) to produce pAS-PP1γ1. The 200-bp PP1γ2-specific C-terminal containing the *Pst*I fragment was transferred from pTacTacγ2 (Zhang et al. 1993b) into *Pst*I digested pAS-PP1γ1 to produce pAS-PP1γ2 or into *Pst*I digested pAS2-1 to produce pAS-PP1γ2C (Fardilha et al. 2011b). These expression vectors were first used to confirm the expression of the resulting fusion proteins (GAL4-PP1γ1, GAL4-PP1γ2, and GAL4-PP1γ2C) in the yeast strain AH109. For library screening, the yeast strain 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) transformed either with pAS-PP1γ1, pAS-PP1γ2, or pAS-PP1γ2C was mated with the yeast strain Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met$^-$, gal 80Δ, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ, MEL1) expressing a human brain cDNA library (from an adult male brain, Clontech, HL4004AH) in the pACT-2 vector (Gal4 activation domain expression vector, Clontech). Half the mating mixture was plated onto high stringency medium (quadruple dropout: SD/-Ade/-His/-Leu/-Trp) and the other half onto low stringency medium (triple dropout: SD/-His/-Leu/-Trp), and the plates were incubated at 30 °C. Colonies obtained in the low stringency plates were replica-plated onto the high stringency medium. Finally, all high stringency surviving colonies were plated onto selective medium containing X-α-Gal and incubated at 30 °C to check for MEL-1 expression (indicated by the appearance of a blue color). All the YTH reagents were purchased from Clontech, Saint-Germain-en-Laye, France. All other nonspecified reagents were purchased from Sigma-Aldrich, Portugal.

Recovery of Plasmids from Yeast and Sequence Analysis

Yeast plasmid DNA was recovered and used to transform *Escherichia coli* XL1-Blue (recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F′ proAB lacZΔM15 Tn10 (Tet^r)]). Plasmid DNA was obtained from each resulting bacterial colony and digested with the restriction enzyme *Hin*dIII (NEB, Ipswich, USA) to identify the corresponding library plasmids. DNA sequence analysis was performed using an Automated DNA Sequencer (Applied Biosystems, Carlsbad, USA) using the GAL4-AD primer—TACCAC TACAATGGATG (Clontech, Saint-Germain-en-Laye, France). The cDNA sequences obtained were compared to the GenBank database, using the BLAST algorithm, to identify the corresponding encoded proteins.

The PP1 BMs search was made based on the consensus sequences previously described, RVxF (Wakula et al. 2003; Meiselbach et al. 2006; Hendrickx et al. 2009), SILK (Huang et al. 1999; Wakula et al. 2003; Lin et al. 2005; Hurley et al. 2007), MyPhoNE (Terrak et al. 2004; Hendrickx et al. 2009), and the apoptotic signature (Ayllon et al. 2002; Godet et al. 2010). This search was executed using the ScanProsite program (EXPASy Proteomics Server) for all the PIPs obtained from the YTH clones. Context of the motifs was also further studied in order to assign the significance of the motifs.

Database Search of PIPs

The human-specific PP1γ interactors were retrieved from 11 available online databases: APID, BIND, BioGRID, HPRD, InnateDB, IntAct, iRefIndex, MINT, PINA, Reactome-FLS, and STRING, searched for the human PPP1CC gene. The UniProt accession numbers were used to normalize all proteins found to interact with PP1γ, in order to avoid protein duplications of alias from different databases. The cellular function and localization for each PIP was obtained from UniProt, Human Protein Reference (HPRD), and the Gene Ontology databases. The PP1 interaction maps were produced using Cytoscape (Shannon et al. 2003) and Cerebral plugin to create a layered view of subcellular localizations (Barsky et al. 2007).

Results

Identification of PP1γ Isoform-Specific Binding Proteins in the Human Brain by YTH Screening

With the purpose of identifying PP1γ isoform-specific binding proteins in the human brain, three separate YTH screens

were performed, using full-length PP1γ1 (YTH1), full-length PP1γ2 (YTH2), and the specific C-terminal 39 amino acids of PP1γ2 (PP1γ2C, YTH3) as bait, on a human brain cDNA library. PP1γ isoforms are originated by alternative splicing, PP1γ1 is ubiquitously expressed, and PP1γ2 is particularly enriched in the testis and sperm; nonetheless, the latter is also present in the brain (Takizawa et al. 1994; Strack et al. 1999). PP1γ2C lacks the surface hydrophobic pocket (Leu288-Met290-Cys291) (Gibbons et al. 2005), which works as a docking site for the PP1 BM present in the vast majority of PIPs. This bait was included in the YTH screens with the intention of unravelling unknown PIPs specific for PP1γ2.

A total of 1.0×10^6, 6.6×10^5, and 2.4×10^5 clones were screened yielding 241 positive clones in YTH1, 228 positive clones in YTH2, and 18 positives in YTH3, respectively. After partial or complete sequence analysis (depending on the length of the positive clone's cDNAs), in silico searches of the GenBank database allowed for the identification of the clones, corresponding to a total of 52 proteins in YTH1 (Table 1), 141 proteins in YTH2 (Table 2), and 4 proteins in YTH3 (Table 3) that bind to PP1γ1, PP1γ2, and PP1γ2C, respectively. Also, an exhaustive search was carried out for the PP1 BMs: RVxF, SILK, MyPhoNE, and apoptotic signature, present in the proteins identified (Tables 1, 2, and 3).

From the 52 proteins identified that bind to PP1γ1, in YTH1, 22 are already known PP1 regulators (data in italics in Table 1). In fact, all the most abundant clones correspond to previously described PIPs, like AATK (apoptosis-associated tyrosine kinase) with 31 clones isolated, TP53BP2 (tumor suppressor p53-binding protein 2) with 29 clones, TPRN (Taperin) with 23, and YLPM1 (YLP motif-containing protein 1) with 20 clones, all having PP1 BMs (Table 1).

AATK binds to PP1 and the interaction is required for inhibition of NKCC1 activity in *Xenopus laevis* oocytes (Gagnon et al. 2007). This protein is an active cytosolic kinase expressed in different brain regions, induces neuronal differentiation, and also promotes differentiation upon stimulation by other agents in SH-SY5Y cells (Raghunath et al. 2000). This protein was found to be up-regulated during apoptosis in myeloid precursor cells, induced by IL-3 deprivation (Gaozza et al. 1997), and in CGC apoptosis, induced by low K^+ concentrations (Tomomura et al. 2001). Moreover, the phosphorylation states of AATK are closely related to the high potassium-induced survival and low potassium-induced apoptosis of cerebellar granule cells (Tomomura and Furuichi 2005).

TP53BP2 (also known as ASPP2/PPP1R13A) is a member of the apoptosis stimulating proteins of p53 (ASPP) family, is a p53-binding protein and was identified as a PIP and inhibitor of PP1, suggesting that PP1 may be involved in dephosphorylation and regulation of p53 through interaction with TP53BP2 (Helps et al. 1995). TP53BP2 enhances the ability of p53 to stimulate specifically the expression of pro-apoptotic target genes but not genes involved in the cell cycle arrest (Samuels-Lev et al. 2001; Bergamaschi et al. 2004).

Taperin is associated with autosomal recessive nonsyndromic hearing loss by target genome capture combined with next generation capture and by homozygosity mapping (Li et al. 2010; Rehman et al. 2010). Immunolocalization places taperin predominantly at the taper regions of the inner ear hair cells. The interaction of PP1 with taperin was detected in several proteomics studies (Trinkle-Mulcahy et al. 2006; Moorhead et al. 2008; Fardilha et al. 2011b; Esteves et al. 2012), but was only recently characterized (Ferrar et al. 2012). Taperin binds to PP1 through a RVxF BM and can inhibit its activity. It shuttles between the nucleus and the cytoplasm but remains almost exclusively nuclear, where it also interacts with DNA damage response proteins (Ferrar et al. 2012). Even so taperin cellular function is still unclear.

The putative RNA-binding protein YLPM1 was established as a nuclear PIP and has a putative PP1-binding site (KRVRW). These results further support a key role for PPs in several nuclear functions, including the regulation of pre-mRNA splicing (Tran et al. 2004; Ulke-Lemee et al. 2007).

Six clones were found corresponding to neurabin II (spinophilin/PPP1R9B), an actin-binding protein that is specifically enriched in dendritic spines (Allen et al. 1997; Satoh et al. 1998; Grossman et al. 2002) as is PP1 (Ouimet et al. 1995). Neurabin II plays an important role in the regulation of glutamate receptors by anchoring PP1 in the proximity of AMPA and NMDA receptors, thus regulating the efficacy of postsynaptic glutamatergic neurotransmission. Blocking the interaction between neurabin II and PP1 disrupts targeting of PP1 and the dephosphorylation of AMPA receptors and abolishes the rundown of AMPA currents (Yan et al. 1999). In what concerns neuronal cytoskeletal organization, neurabin II facilitates the dephosphorylation of doublecortin by PP1 to mediate microtubule bundling at the axonal wrist (Bielas et al. 2007). Furthermore, neurabin II has been shown to play an important role in the actions of drugs of abuse (Allen et al. 2006; Charlton et al. 2008), and changes in PP1 function have been associated with Parkinson's disease (Brown et al. 2008).

CSRNP1 and KIAA1377 were the most abundant clones (five clones) from the novel PIPs. CSRNP1 (cysteine/serine-rich nuclear protein 1) is a nuclear protein and a member of the CSRNP family (Gingras et al. 2007). The CSRNP proteins have the hallmark of transcription factors, and the CSRNP1 gene was strongly induced in response to IL-2 in mouse T cells (Gingras et al. 2007). CSRNP1 was first identified as AXIN1 up-regulated protein (AXUD1) in a AXIN1 cDNA microarray and was found to be down-

Table 1 Complete list of proteins found in YTH1

Protein ID	UniProt ID	No. of clones	PP1 BMs			
			RVxF	SILK	MyphoNE	Apoptotic signature
AATK	*Q6ZMQ8*	*31*	*KAVSF*			*FERRWR*
ABT1	Q9ULW3	1	RVFF			FVRRKK
B3GNT1	O43505	1				FDERFR
BTBD10	Q9BSF8	3	RHVDF			
C15orf59	Q2T9L4	1	RVRF			
C1QA	*P02745*	*10*				
CLMN	Q96JQ2	1	KTVHF			
CLTC	Q00610	3	KEVCF, RAIQF			
CNST	*Q6PJW8*	*7*	*RRVRF*	*SILK*		FLGKDK
COPS5	Q92905	1				
CSRNP1	Q96S65	5	RVAF, RVEF	SILK		
DEAF1	O75398	1				
ELP4	Q96EB1	1	KIAW			FKLKRK
FTL	P02792	3				
FXYD6	Q9H0Q3	1				FNQKPK
HMGN1	P05114	1				
KCTD20	Q7Z5Y7	1	RHVDF			
KIAA1377	Q9P2H0	5		KSILK		FEEKRK
LMTK2	Q8IWU2	10	KAVTF			FDERSR
NAPEPLD	Q6IQ20	1	LRVTW, KVTF, KVLW			
NEK2A	*P51955*	*2*	*KKVHF*			
NUAK1	O60285	1	RIIDW	KGILK, KGILK, KGILK		FENKDK
PHACTR4	*Q8IZ21*	*1*				
PHC1	P78364	1				FRLKRK
PIAS1	O75925	1				
PPP1R9B	*Q96SB3*	*6*	*RKIHF, RVRF, KEIEF*			
PPP1R13B	*Q96KQ4*	*7*	*LRVRF*			
PPP1R15B	*Q5SWA1*	*4*	*KKVTF*			*FEHRER*
PPP1R16A	*Q96I34*	*4*	*KQVLF*		*RAQQVKMW*	
PPP1R18	*Q6NYC8*	*2*	*LKISF*			
PPP1R3C	*Q9UQK1*	*4*	*KRVVF, KNVSF, RITF, KIEF*			FVNKLK
PPP1R8	*Q12972*	*1*	*RVTF*			
PRR16	Q569H4	2	RVRF			
RANBP9	*Q96S59*	*7*	*RMIHF*			FTLKVR
RIF1	*Q5UIP0*	*9*	*KKIAF, RRVSF, HSVNF*	*SILK*		FLHKNK, FLPKAK
RPL7	P18124	2				FVIRIR
RPRD2	Q5VT52	1	RVGW	SILK		
RRP1B	Q14684	1	KKVTF, RVAF			
SH3RF2	*Q8TEC5*	*1*	*KTVRF*			FDLRGK
SHANK3	F2Z3L0	1	KEVRF			FRYKRK, FQRRSK
STAM1	Q92783	2	LMVEW, KTVQF			
STAU	*O95793*	*7*	*RKVTF*			FNGKGK
TACC2	O95359	1				
TEFM	Q96QE5	1	RRIAW, RVFF	SILK		FCCRKK
TOR1AIP1	*Q5JTV8*	*5*	*REVRF, KVNF, LKVKF*	*SILK*		*FVKRNR*
TP53BP2	*Q13625*	*29*	*RVKF*			*FQQKQR*
TPRN	*Q4KMQ1*	*23*	*RAIRW, KISF*			

Table 1 (continued)

Protein ID	UniProt ID	No. of clones	PP1 BMs			
			RVxF	SILK	MyphoNE	Apoptotic signature
WBP11	*Q9Y2W2*	*3*	*RKVGF, LSVRF*	*SILK*		
YLPM1	*P49750*	*20*	*RVGF, KEVEF, KRVRW, RAIGF*			
ZDBF2	Q9HCK1	1	KLVLW, KITF, RINF, KKVTW, KIHF			FICKYK
ZNF318	Q5VUA4	1		SILK		
ZNF667	Q5HYK9	2				

The number of clones indicates the count of isolated cDNA clones for the respective protein. Data in italics correspond to already known PP1 regulators

PP1-BMs PP1-binding motifs

regulated in lung, kidney, liver, and colon cancers when compared to normal tissues, suggesting that AXUD1 may have a tumor-suppressor function (Ishiguro et al. 2001).

KIAA1377 was found to be expressed in the brain, lung, skeletal muscle, kidney, pancreas, testis, and ovary in a large cDNA sequencing and expression profile study of the coding sequences of unidentified human genes (Nagase et al. 2000). KIAA1377 cellular function is still uncharacterized.

In YTH2, in contrast to YTH1, the most abundant clones corresponded to novel PIPs: KIF1A with a total of nine clones, COPS5 (seven clones), and PINK (six clones) (Table 2). KIF1A was identified as member of the kinesin superfamily proteins (KIFs) and proposed as a neuron-specific microtubule plus end-directed motor transporter of synaptic vesicle precursors (Okada et al. 1995). Marked neuronal degeneration and death occurred both in KIF1A mutant mice and in cultures of mutant neurons, suggesting that KIF1A-mediated axonal transport plays a critical role in the viability, maintenance, and function of neurons, particularly mature neurons (Yonekawa et al. 1998). Recently, mutations on the KIF1A gene have been implicated in hereditary sensory and autonomic neuropathy type 2 (Riviere et al. 2011), nonsyndromic intellectual disability (Hamdan et al. 2011), and hereditary spastic paraparesis (Erlich et al. 2011). Several members of this family are present in the databases as PP1 interactors: KIF18A, KIF2A, KIF2B, and KIF2C (Behrends et al. 2010; Wu et al. 2010) (Supplementary data); also, KIF13A was already described as binding to PP1 (Santra et al. 2009).

COPS5 was first identified as a co-activator of the transcription factor c-Jun (Claret et al. 1996). COPS5 (also known as Jab1/CSN5) is also a subunit of the COP9 signalosome complex (Kwok et al. 1998; Seeger et al. 1998), a multimeric complex, essential for the regulation of ubiquitin conjugation and other signal transduction pathways (Deng et al. 2000; Wolf et al. 2003; Kim et al. 2004; Shackleford and Claret 2010).

PTEN-induced putative kinase (PINK1) localizes predominantly within mitochondria, specifically at the inner mitochondrial membrane. Mutations in its gene were identified as a cause of autosomal recessive parkinsonism and associated with mitochondrial dysfunction and kinase pathways in neurodegeneration (Silvestri et al. 2005; Gandhi et al. 2006; Narendra and Youle 2011). PINK1 directly phosphorylates parkin, which induces its translocation to the mitochondria (Kim et al. 2008).

From the 141 proteins identified in YTH2, only 12 were already known PIPs (data in italics in Table 2); among them are also AATK and TP53BP2 and the well known PIP neurabin I (PPP1R9A). Neurabin I binds to actin filaments and may be involved in neurite formation. Abolishing its binding to PP1 delays the onset of dendritic spines, in cultured hippocampal neurons (Hsieh-Wilson et al. 1999; Terry-Lorenzo et al. 2002). Also, in hippocampal slices, inhibition of PP1 activity caused a rapid increase in dendritic spines and filopodia, suggesting that the ongoing synaptic PP1 activity associated with the neurabin/PP1 complex maintains normal spine number and morphology (Jourdain et al. 2003; Oliver et al. 2002). Finally, in contrast to the neurabin II/PP1 complex, which down-regulates AMPA receptors (Feng et al. 2000), the neurabin I/PP1 complex leads to an increased targeting of AMPA receptors mediating synaptic transmission (Terry-Lorenzo et al. 2005). Neurabins associate with PP1 through the canonical PP1 BMs. Also, neurabins heterodimerize in neurons (MacMillan et al. 1999), which suggests a complex interplay between neurabin I/PP1 and neurabin II/PP1 to regulate protein composition and function of excitatory synapses (Terry-Lorenzo et al. 2005).

In YTH3, a total of 18 clones were obtained, corresponding to four proteins that interact with the C-terminus of PP1γ2 (Table 3). From those proteins, RANBP9 (15 clones) is the only already described as a binding partner of PP1 and will be

Table 2 Complete list of proteins found in YTH2

Protein ID	UniProt ID	No. of clones	PP1 BMs			
			RVxF	SILK	MyphoNE	Apoptotic signature
AATK	*Q6ZMQ8*	*5*	*KAVSF*			*FERRWR*
ACAD8	Q9UKU7	1	KVAF, KKVGW, RAVIF			
ADAR	P55265	1	KQIEF, KTVSF			
ADCY1	Q08828	1	LAIGF, RRILF			
ADRA1A	P35348	1	KIVFW			
ALDOC	P09972	5	RQVLF			
ANKRD42	*Q8N9B4*	*1*	*KKVHF*			
APBB1	O00213	1	RVRF, RRVGW			
ARF3	P61204	1	KNISF			
ARHGEF17	Q96PE2	2	RRSVSF, KVSF			
ARIH2	O95376	1				
ARPP21	Q9UBL0	1				
ATG2B	Q96BY7	1	KVTF, LSVRF, RRVMF, KVKF, LVISF			FRPRPR
ATP2A2	P16615	2	KIVEF, RVSF			
ATP6V0A1	Q93050	1	KVQF, RVWF			
ATP6V1H	Q9UI12	2	KVNW			
B3GNT1	O43505	1				FDERFR
BAP1	Q92560	1	KVLF			
BCR	P11274	1	LSVKF			
C5orf45	Q6NTE8	1	KSVKW			
C6orf136	Q5SQH8	1				
CDC23	Q9UJX2	3				
CDK5R1	*Q15078*	*1*				
CHD5	Q8TDI0	1				FNARQR
CNST	*Q6PJW8*	*2*	*RRVRF*	*SILK*		
COL4A2	P08572	2				
COPS5	Q92905	7				
COX11	Q9Y6N1	6	KISF			
CREB5	Q02930	3				FLERNR
CTSH	P09668	1	KAIGF			
CYB561D2	O14569	1	LAVLW			
DAZAP2	Q15038	1				
DBNDD2	Q9BQY9	2				
DCAF8	Q5TAQ9	1	KVVVW, KGVNF			FVQRFR
DDO	Q99489	2				
DDX5	P17844	1	RLIDF, KTIVF			
DENND4B	O75064	1	RVVW, REILF			
DGKE	P52429	1				
DGKZ	Q13574	1	KINF			FNSRFR
DTX3	Q8N9I9	1				
ENO2	P09104	1				
ENTPD6	O75354	3	KHVDF			
EPB49	Q08495	1				
EXD2	Q9NVH0	1				
FAIM2	Q9BWQ8	1				
FAM127A	A6ZKI3	1	LKVTF, KVTF			
FAM168B	A1KXE4	1				
FGFR3	P22607	1	HNVTF			
FTL	P02792	2				

Table 2 (continued)

Protein ID	UniProt ID	No. of clones	PP1 BMs			
			RVxF	SILK	MyphoNE	Apoptotic signature
FXYD6	Q9H0Q3	1				
GAA	P10253	1				
GABBR1	Q9UBS5	2	KAINF, RVDF, KYVWF, KIWW, LAIVF, LVVLF			
GBAS	O75323	1	RAIRF			
GIPC1	O14908	1				
GLUL	P15104	1				
GPM6B	Q13491	2				
GPR56	Q9Y653	3				
GPX1	P07203	1	KLITW			
HIPK2	Q9H2X6	3	KVIDF			
ITM2C	Q9NQX7	4	KISF			
ITSN1	Q15811	2	RQIGW			
KCNQ2	O43526	1	LKVGF			FGDRSR
KIAA0513	O60268	1				
KIAA0895L	Q68EN5	1				
KIF1A	Q12756	9	RILF, KKVQF, KISF			
KLHL21	Q9UJP4	4				
LMNB2	Q03252	1				
LPXN	O60711	2				
LRP3	O75074	5				
MBP	P02686	1				FLPRHR
MED13L	Q71F56	1				
MEGF8	Q7Z7M0	1	REVFW, RTVLF			
MPPED1	O15442	1				
MPRIP	Q6WCQ1	1				
MTMR6	Q9Y217	1	RTVHF			
NCAM1	*P13591*	*1*	*KDISW, KDVRF*			
NDUFB7	P17568	1				
NEUROD2	Q15784	1				
NFAT5	O94916	2	KVIF	GILK		
NICN1	Q9BSH3	1				
NKX2-2	O95096	1	RRVLF, KIWF			
NQO2	P16083	2				
OLFM1	Q99784	1				
OPRL1	P41146	1				
PEX16	Q9Y5Y5	1	RILF, KIYF			
PGD	P52209	1	KGILF			
PIDD	Q9HB75	1	LQVEF, KVIF, RICF			
PINK1	Q9BXM7	6	KMVGW			
PKIA	P61925	1				
PKM2	P14618	1	LRVNF			
PKNOX2	Q96KN3	1				
POLDIP3	Q9BY77	1				
POM121C	A8CG34	1				
PPP1R9A	*Q9ULJ8*	*1*	*RKIKF, KVRW*			
PRKCB	P05771	1	KSVDW, RYIDW			
PSMD8	P48556	1	KILF, RILF			
PTPN5	P54829	1				

Table 2 (continued)

Protein ID	UniProt ID	No. of clones	PP1 BMs			
			RVxF	SILK	MyphoNE	Apoptotic signature
PTPRN	Q16849	1				
PURA	Q00577	1				
PWP2	Q15269	1	HSVSF, LAVTF, RVLF			
RAB11B	Q15907	1				
RAB1B	Q9H0U4	1				
RAB5C	P51148	1	RAVEF			
RAB6A	P20340	1				
RANBP9	*Q96S59*	*2*	*RMIHF*			
RASGRF2	O14827	1				
RBFOX3	A6NFN3	1				
RHBDD2	Q6NTF9	1				
RIF1	*Q5UIP0*	*2*	*KKIAF, HSVNF, RRVSF*	*SILK*		
RNF187	Q5TA31	1				
RTN4R	Q9BZR6	1	LTILW			
SCARB2	Q14108	1	KIVEW			
SCD5	Q86SK9	1	LNISW			
SDHB	P21912	2				
SEC61A1	P61619	1	RKIQF, KVLW			
SEMA4D	Q92854	1	RITW, RVYF, RVFW			
SLC36A1	Q7Z2H8	4	RVVDF			
SMPD1	P17405	3	RVLW, RILF, LAVAF			
SMTN	P53814	1				
SPTBN1	Q01082	1				
SRPR	P08240	1	KISF, KPIVF			
STAU1	*O95793*	*1*	*RKVTF*			
STMN3	Q9NZ72	3				
STX1B	P61266	1				
SUMO2	P61956	1	RQIRF			
SYNGAP1	Q96PV0	1	RTVAW			
TCFL5	Q9UL49	1				
TECPR1	Q7Z6L1	4	KVWF, RAVYF, RGVVW			
THY1	P04216	1	KHVLF			
TMBIM6	P55061	1	RKINF			
TMEM229B	Q8NBD8	1				
TMEM8B	A6NDV4	1				
TNS1	*Q9HBL0*	*2*	*RDVIF, RVQF, KVEF, KVKF*			
TOR1AIP1	*Q5JTV8*	*3*	*REVRF, KVNF, LKVKF*	*SILK*		*FVKRNR*
TP53BP2	*Q13625*	*2*	*RVKF*			
TSPAN7	P41732	1				
TUBB2A	Q13885	2				
TYRO3	Q06418	3				
UBA7	P41226	1				
VRK3	Q8IV63	1	KKVKW			
WSB1	Q9Y6I7	2	RSVSF, KMVRF			

The number of clones (*No.*) indicates the count of isolated cDNA clones for the respective protein. Data in italics correspond to already known PP1 regulators

PP1-BMs PP1-binding motifs

Table 3 Complete list of proteins found in YTH3

The number of clones indicates the count of isolated cDNA clones for the respective protein. Data in italics correspond to already known PP1 regulators

PP1-BMs PP1-binding motifs

Protein ID	UniProt ID	No. of clones	PP1 BMs			
			RVxF	SILK	MyphoNE	Apoptotic signature
BMPR2	Q13873	1				FGSKHK
GOLGA7	Q7Z5G4	1				
IL3RA	P26951	1	KFVVF, KLVVW			FNRKFR
RANBP9	*Q96S59*	*15*	*RMIHF*			*FTLKVR*

described below. A single clone was obtained for the other three proteins: BMPR2, GOLGA7, and IL3RA.

Bone morphogenetic proteins (BMPs) comprise the largest subfamily of TGF-beta-related ligands and are known to bind to type I and type II receptor serine/threonine kinases. BMPR2, bone morphogenetic protein receptor type-2, binds to BMP-7, BMP-2 and, less efficiently, BMP-4 (Nohno et al. 1995; Rosenzweig et al. 1995). Defects in BMPR2 have been reported to be the cause of several pulmonary pathologies (Lane et al. 2000; Runo et al. 2003; Machado et al. 2006; Talati et al. 2010).

GOLGA7 also known as GCP16 is the Golgi acylated membrane protein, associated with GCP170 (GOLGA3), and possibly involved in vesicular transport from the Golgi to the cell surface (Ohta et al. 2003). GCP16 associates with DHHC9 and forms a protein complex. DHHC9 requires GCP16 for protein stability and fatty acyltransferase activity with specificity for H- and N-Ras (Swarthout et al. 2005).

Finally, IL3RA is a receptor of interleukin-3. Cytokines interact with cell surface receptors initiating signaling cascades that promote cell growth and survival, and hence, several pathways are activated by a variety of cytokines, including interleukin-3 (McCubrey et al. 2001; Lee and McCubrey 2002; Hantschel and Superti-Furga 2004).

Retrieval of PP1γ Interacting Proteins from Free Access Online Databases

A total of 348 unique interactions were obtained for PP1γ from the 11 databases searched (Supplementary data). Only human proteins were considered in this analysis. A number of interactions found in each database are the following: APID—40, BIND—2, BioGRID—8, HPRD—17, InnateDB—2, IntAct—31, iRefIndex—85, MINT—6, PINA—52, Reactome-FLS—137, and STRING—248. After gathering all PP1γ interacting partners from the above databases, the UniProt accession number was used to identify alias duplication, so that the interactions found in each database and in the YTHs could be compared, and an interaction network map was elaborated (Fig. 1).

Only seven interactions present in the databases are also identified in the YTH screens: four (PPP1R9B, PPP1R8, PPP1R3C, WBP11) with YTH1, one (GBAS) with YTH2, and two interactions (COPS5, TP53BP2) are common to the databases and both YTH1 and YTH2. None of the four interactions found in YTH3 were found in the databases. Eight proteins were found to bind to both PP1γ1 and PP1γ2: AATK, CNST, RIF1, STAU, and TOR1AIP1—all are previously described PIPs—and B3GNT1, FTL, and FXYD6, which are novel PIPs (Fig. 1). The only PIP found in all the three YTH screens was RANBP9, also known as RANBPM.

RANBP9 interacts with several signaling molecules, including cell surface receptors, nuclear receptors, transcription factors, cytosolic kinases, and microtubules (Nakamura et al. 1998; Murrin and Talbot 2007). Its interaction with PP1 was previously described in our laboratory (Fardilha et al. 2011b). RANBP9 may play a key role in the etiology of Alzheimer's disease given that it binds the low-density lipoprotein receptor-related protein (LRP), the amyloid precursor protein (APP), and BACE1 and robustly increases Aβ generation in a variety of cell lines, primary neuronal cultures and in transgenic mice (Lakshmana et al. 2009, 2012). In fact, the increased Aβ levels were translated in an increased deposition of amyloid plaques (Lakshmana et al. 2012). Also, it seems that RANBP9 simultaneously inhibits cell-adhesive processes and enhances Aβ generation by accelerating APP, LRP, and β1-integrin endocytosis (Woo et al. 2012).

Discussion

Deregulated protein phosphorylation is associated with different disease processes, for instance, cancer, metabolic, and neurological disorders, suggesting a common molecular basis for apparently unrelated and diverse pathologies. In addition to reversible phosphorylation and other posttranslational modifications, the correct targeting and localization of proteins to specific subcellular compartments represents an important biological mechanism for regulating cellular functions. Thus, the role of PP1 and its regulatory PIPs are fundamental areas of research, not only for the understanding of their different physiological functions but also as

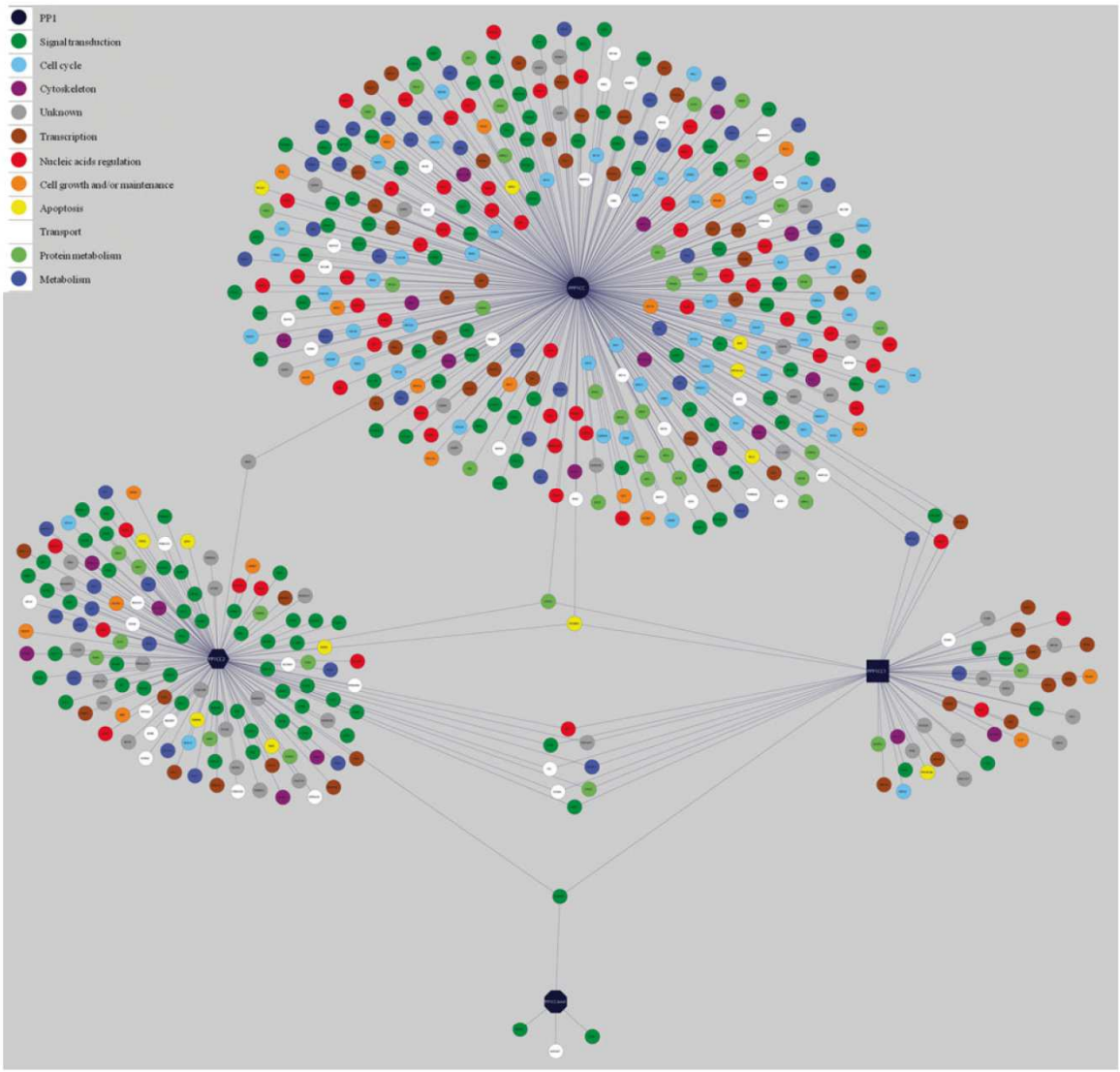

Fig. 1 PP1γ interactions mapping. PP1γ interacting proteins retrieved from online databases searches (PPP1CC, *circle*) and identified in the human brain yeast two-hybrid screens using PP1γ1 as bait (PPP1CC1, *rectangle*), PP1γ2 (PPP1CC2, *hexagon*), and PP1γ2 C-terminal (PPP1CC2end, *octagon*). *Different colors* correspond to the functional characterization of all the PIPs (also in Fig. 2)

possible targets for therapeutic intervention. Thus, it is important to unravel PIP diversity and how each PP1 isoform interacts with a given PIP profile. In fact, each PP1 isoform exhibits its own physiological roles, cellular localizations, and tissue-specific expression pattern. One would expect the same to be true for PIPs, leading to the formation of PP1/PIP complexes in a highly regulated manner with respect to localization, timing, and event-dependent mechanisms.

We have previously identified the PP1γ1 and PP1γ2 human testis interactome and PP1α human brain interactome, including 72 and 39 novel PIPs, respectively (Fardilha et al. 2011b; Esteves et al. 2012). Here, we present a complete map of PP1γ isoform interactors by integrating YTH screening results with the information retrieved from 11 databases (Fig. 1). Also, a functional characterization of all the PIPs identified in the YTHs and databases was extrapolated (different colors in Figs. 1 and 2). From the 348 interactions retrieved from the online database searches, for the PPP1CC gene, 21.6 % are involved in signal transduction events, 15.6 % in cell cycle, and 13. 5 % in nucleic acid regulation,

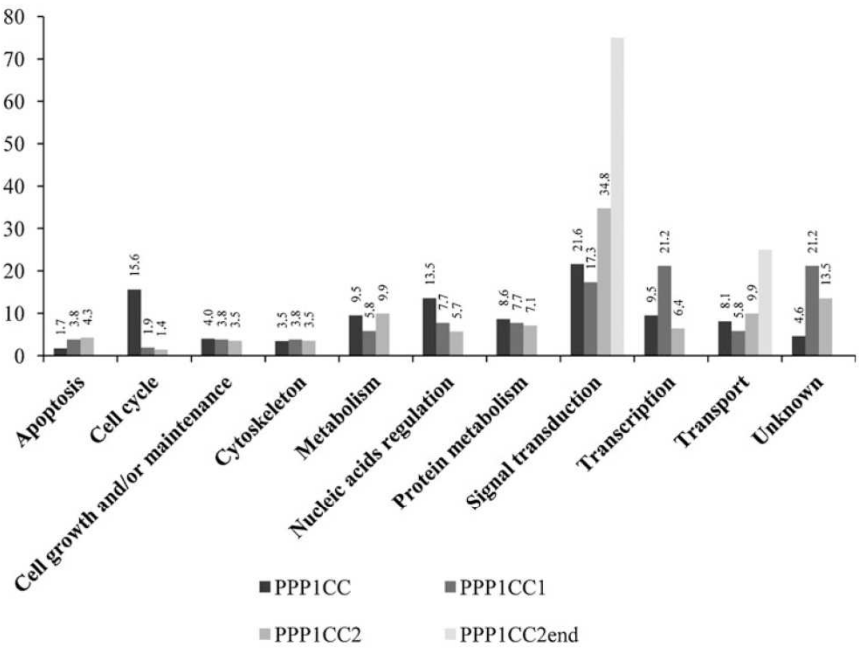

Fig. 2 Distribution of the PP1γ regulators according to function. Biological functions attributed to PIPs identified on YTH1 (PPP1CC1), YTH2 (PPP1CC2), YTH3 (PPP1C2end), and protein interaction databases (PPP1CC). Database searches were based on functional motifs presented, interacting proteins, cellular localization, and molecular function

and 4.6 % still have unknown functions. From the 52 PIPs identified that bind to PP1γ1 in YTH1 (PPP1CC1), 21.2 % have functions related to transcription, 17.3 % are also involved in signal transduction, and 21.2 % have unknown functions. In the YTH2 (PPP1CC2), the majority (34.8 %) of PP1γ2-binding proteins are related with signal transduction, 9.9 % are associated with metabolism and transport, and for 13.5 %, functions have not yet been described. Finally, from the four proteins that interact with the specific C-terminal of PP1γ2 (PPP1CC2end), three are involved in signal transduction and one in transport.

Some of the already characterized PP1 interactions, also identified in the YTHs, are not yet present in the free online databases, like PPP1R8 (NIPP1), PPP1R13B (ASPP1), and PPP1R9A (neurabin I), which justifies the fact that only seven proteins found in the YTHs also came up in the database searches.

In the YTH2, from a total of 228 positive clones isolated, 141 proteins were identified, a much higher number than the 52 proteins identified in YTH1 from 241 cDNA clones. This may be attributed to nonspecific interactions since it is known that PP1γ2 is less expressed in the brain, probably having a less functional significance. Further, when PP1γ2-specific C-terminal was used in the YTH3 as bait only 18 clones were recovered corresponding to four proteins. Compared to the previous YTH screens performed in the laboratory, namely PP1γ in the testis, a tissue were PP1γ2 is largely expressed (Fardilha et al. 2011b), PP1γ2 had 156 positive clones and only 27 interacting proteins and the PP1γ2 C-terminal resulted in 83 clones corresponding to 40 interacting proteins identified. PP1γ2 is an isoform particularly enriched in the testis and sperm, where it distributes along the tail and in the equatorial region of the head (Fardilha et al. 2011b). With this tissue and cell expression pattern, it is expected that its interactions are highly regulated by the testis/sperm-specific PIPs. A complete sequence search for PP1 BMs, RVxF, SILK, MyphoNE, and apoptotic signature for all the proteins from the YTHs was performed (Tables 1, 2, and 3). From the proteins in YTH1, nine do not have any PP1 BM, including PHACTR4, a known PIP (Kim et al. 2007; Zhang et al. 2012). The SILK motif was described as being present in seven different PIPs (Hendrickx et al. 2009) and always N-terminal to a RVxF motif, being 7 to 55 aa distant. In the YTH1, all the proteins that hold a SILK motive (10 in total) also have a RVxF motif, with the exception of KIAA1377 and ZNF318. NUAK1, despite having three SILK motifs, they are all C-terminal to the RVxF. WBPII and CNST are known to have a SILK motif and CSRNP1 is a novel PIP but has family members (CSRNP2 and CSRNP3) with studied SILK motifs (Hendrickx et al. 2009). RIF1 and TEFM have SILK motifs found to be at the expected distance to the RVxF (18 and 66 aa, respectively), and finally, RPRD2 and TOR1AIP with SILK motifs more distant (265 and 227 aas, respectively) than the already described examples; thus, the functional relevance will have to be experimentally addressed.

The apoptotic signature, F-X-X-[KR]-X-[KR], is present in 22 proteins, 16 also bear the RVxF motif. The apoptotic signature is present in the Bcl2 family of proteins (e.g., Bcl2, Bcl-W, Bcl-WS) and is related to cell death. This

motif is usually close to the RVxF motif (~30 aa distance). A new apoptotic signature was also found by studying the peptides from apoptosis inducing-factor and apoptosis protease-activating factor 1, composed of a new combinational PP1 docking motif: [RK]-$X_{(0,1)}$-[VI]-X-F-X-X-[KR]-X-[KR] (Godet et al. 2010). PPP1R15B, ABT1, PPP1R3C, and ZDBF2 have a pattern similar to the Bcl2 family one.

From the 141 proteins identified in the YTH2, 66 proteins do not have any PP1 BM, including CDK5R1, which was found to bind the complex PP1/I2 in the brain (Agarwal-Mawal and Paudel 2001). Four proteins that were found to bind PP1γ2 carry a SILK motif in their amino acid sequence and also a RVxF motif; of these, three also interacted with PP1γ1 (CSNT, RIF1, and TORAIP1), and the fourth, NFAT5, is a novel PIP with the SILK motif more distant from the RVxF than the other known SILK containing PIPs. Thirty-seven proteins have the apoptotic signature motif, of which 23 also have the RVxF and three (CSNT, RIF1, and TORAIP1) the RVxF and the SILK motif. With respect to the apoptotic signature, ATG2B, DCAF8, and DENND4B have a pattern similar to the Bcl2 family, and GABBR1, ACAD8, and UBA7 have the new combinational apoptotic signature.

In YTH3, four proteins were identified, but only two, RANBP9 and IL3RA, have the PP1 BM, RVxF. RANBP9, IL3RA, and BMPR2 also have the apoptotic signature, although only IL3RA has a pattern similar to the Bcl2 family.

Also interesting is the fact that from all of the novel PIPs identified, in the YTH screens, none presented the MyPhoNE motif. Comparing PIPs from YTH1 and YTH2 that have the SILK motif, in the YTH2, there are about three times more proteins but two times less PIPs with a SILK motif. This may indicate that for the PP1γ2 isoform, which is less expressed in the brain, most of its interactions were less specific and, therefore, form less tight interactions with fewer contact points.

In order to have a complete picture of the PP1 interactome in the human brain and isoform-specific PIPs, the results obtained for PP1γ were compared with the previous PP1α YTH (Esteves et al. 2012). The map in Fig. 3 shows that five proteins were found to bind PP1α, PP1γ1, and PP1γ2 (AATK, CNST, RIF1, STAU, and TOR1AIP1), and RANBP9 was the only protein found in all the four YTH screens. Consortin (CNST) is a *trans*-Golgi network cargo receptor for the targeting of transmembrane proteins to the plasma membrane, identified as a binding partner of connexins (del Castillo et al. 2010) and of PP1 (Hendrickx et al. 2009).

RIF1 is a nuclear protein found to co-immunoprecipitate with PP1 (Moorhead et al. 2008). It is required for checkpoint-mediated arrest of cell cycle progression, in response to DNA damage during S-phase (Silverman et al. 2004; Buonomo et al. 2009; Wang et al. 2009). Yeast RIF1 associates with telomeres and regulates their length, but human RIF1 does not accumulate at functional telomeres, instead it localizes to dysfunctional telomeres and to telomeric DNA clusters, in accordance to DNA damage response factors, dependent on ATM (ataxia telangiectasia mutated) and TP53BP1 (Silverman et al. 2004; Xu and Blackburn 2004).

STAU (Staufen) interaction with PP1 was first described in the brain (Monshausen et al. 2002), and it is a double-stranded RNA-binding protein and emerges as a relatively ubiquitous RNA granule-forming factor (Ferrandon et al. 1994; Duchaine et al. 2000). This protein was initially described in *Drosophila* oocytes, where it is found in granules involved in microtubule-dependent localization of maternal mRNAs to define the anterior–posterior axis of the embryo (Lasko 1999; Kloc et al. 2002). Staufen also participates in actin-dependent segregation of mRNA during asymmetric division of embryonic nervous system cells (Kiebler and DesGroseillers 2000; Kloc et al. 2002). Moreover, *Drosophila* Staufen is essential for long-term memory acquisition, a phenomenon known to require mRNA targeting followed by local translation at the synapse (Dubnau et al. 2003; Barbee et al. 2006).

TOR1AIP1 (LAP1B) is a type-2 transmembrane protein that interacts with torsinA (Goodchild and Dauer 2005) and appears to participate in the relative distribution of torsinA between the nuclear envelope and the main endoplasmic reticulum (Vander Heyden et al. 2009; Jungwirth et al. 2010). The biological function of torsinA is not well understood but a deletion in its gene is associated with dystonia (Ozelius et al. 1997, 1999). TOR1AIP1 interaction with PP1 was described and both co-localize near the nuclear envelope (Santos 2009). Figure 4 corresponds to a network that provides a visualization of the PIP subcellular localization, in a layered view of the cell, placing PIPs in the region(s) corresponding to the appropriate subcellular localization.

PP1 has a variety of cellular functions in the cell and is present in the nucleus and cytoplasm (nucleus + cytoplasm in Fig. 4), as are a considerable number of PIPs. In fact, the plasma membrane, cytoplasm, and nucleus are the main cellular locations of the PIPs found. This broad cellular distribution is in accordance with a wide diversity of PP1 signaling functions in many cellular pathways.

The formation and breakdown of protein complexes in cellular pathways are regulated by a variety of mechanisms, but phosphorylation is implicated as the regulatory mechanism in many of these cases. PP1 is highly specific at dephosphorylating its numerous targets. This specificity and multiplicity is achieved through the diversity, structural design, and localization of the assembled regulatory subunits. Thus, the interactions between the different catalytic subunits and specific PIPs are central to the physiological functions of PP1. Different approaches have identified

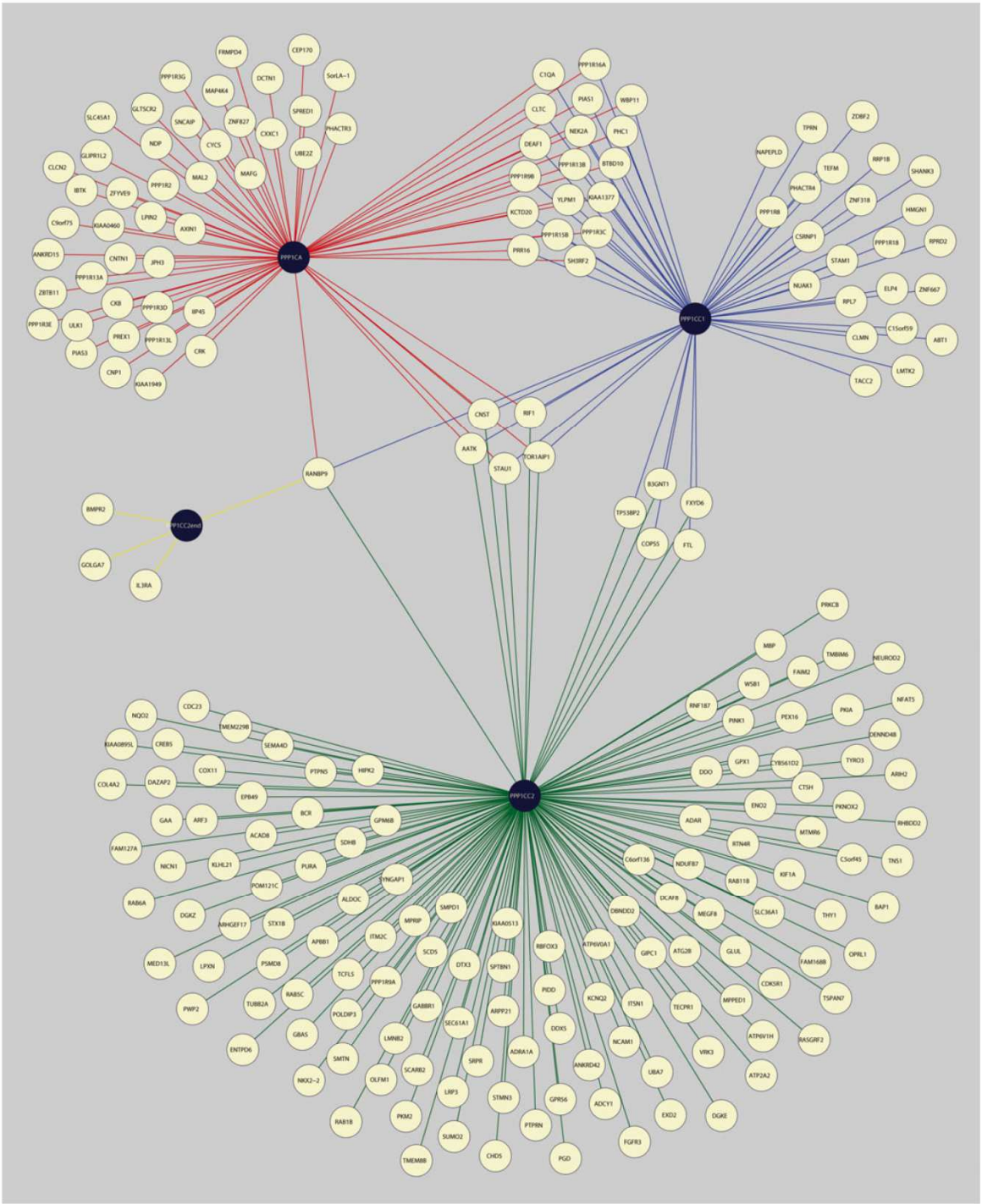

Fig. 3 Mapping of PP1 interactions from human brain. PP1α (PPP1CA) (Esteves et al. 2012) in *red*, PP1γ1 (PPP1CC1/YTH1) in *blue*, PP1γ2 (PPP1CC2/YTH2) in *green*, and PP1γ2 C-terminal (PPP1CC2end/YTH3) in *yellow*

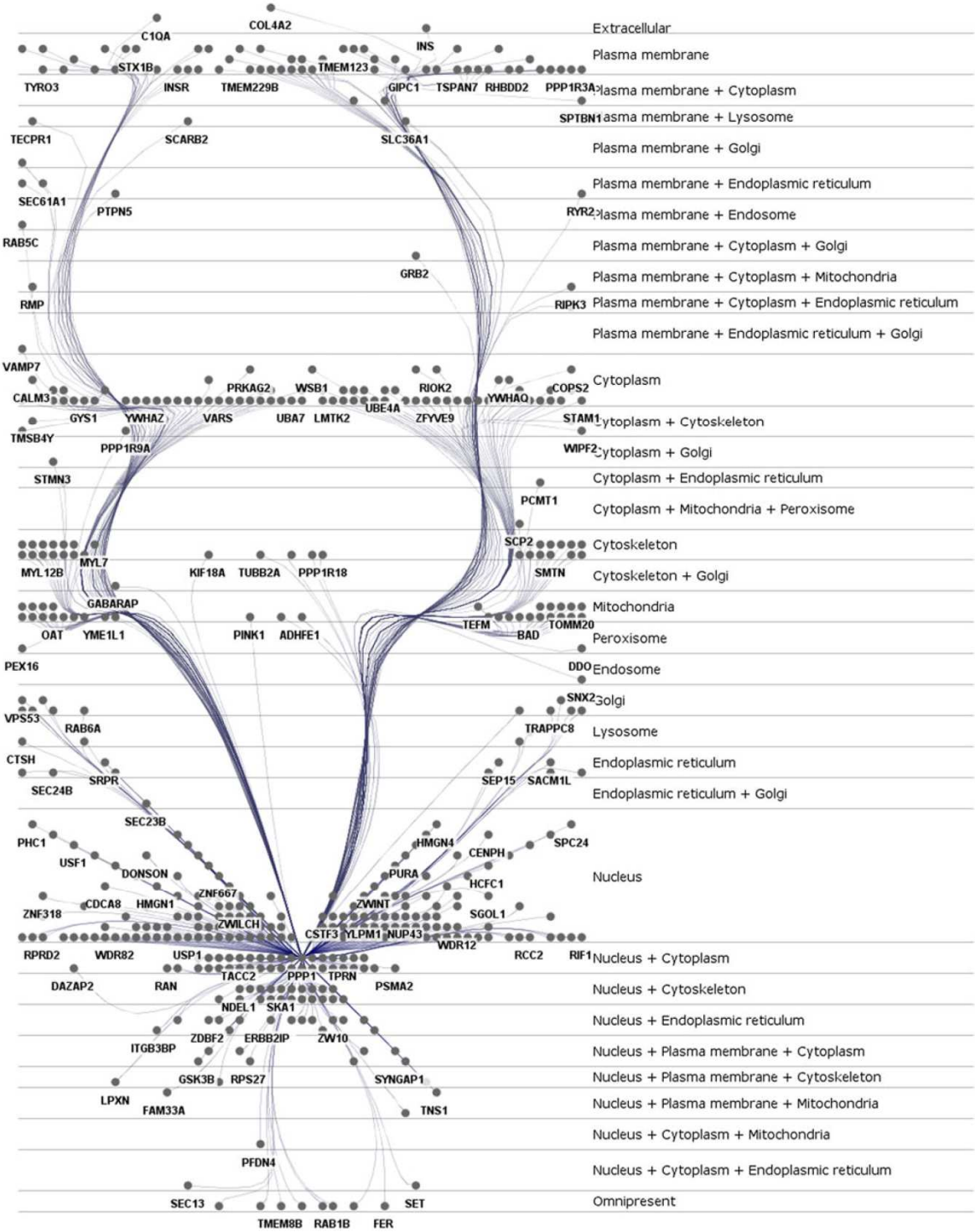

Fig. 4 Cellular distribution of the PP1γ regulators. PIPs identified in the YTHs and retrieved from the databases were distributed according to their subcellular localization, in a layered view of the cell. The cellular function and localization for each PIP was obtained from UniProt, Human Protein Reference (HPRD), and the Gene Ontology databases

approximately 200 mammalian PIPs (Meiselbach et al. 2006; Moorhead et al. 2008; Hendrickx et al. 2009; Fardilha et al. 2010, 2011b; Heroes et al. 2012), but the real number could be much higher. Understanding PP1 interactions is critical for a full understanding of the processes in which it is involved and the mechanisms by which it is regulated.

A complete picture of the PP1 interactome in the brain was built taking advantage of database interaction information and several human brain YTH screens. Some of the PIPs identified in the YTH screens were present in the databases, but the majority were novel PIPs. Thus, the identification of 197 proteins expressed in the human brain that bind PP1γ isoforms is here reported. Most of the detected interactions (162) are novel and the functions of the new PIPs still need to be fully characterized. Only then, the precise roles of PP1 isoform-specific PIPs will be fully elucidated. Thus, the present work added to the number of novel interactions in the PP1 interaction network. Together, our results allowed for the identification of key PIPs in the human brain that bind differently to the PP1γ isoforms. These findings help to explain how such a restricted number of highly conserved proteins can have such diverse roles in a panoply of cellular pathways. Additionally, the data here presented highlight the need to address PP1 isoforms as independent entities and consider PP1 isoform/PIP tissue-specific complexes as an excellent target for a more specific pharmacological based therapy.

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Conflict of interest No competing financial interests exist.

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Supplementary data

Table 1 | Complete list of PPP1CC interacting proteins present in the searched databases.

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
ACTB	P60709	ICU TM	STRING	20562859 omim:00602021 omim:00602468 omim:00608724
ADHFE1	Q8IWW8	PRED	iRefIndex	15657099
AGL	P35573	PRED	iRefIndex	15657099
AHCTF1	Q8WYP5	ICU EX	STRING Reactome	20482850
AK2	P54819	PRED	iRefIndex	15657099
AKAP1	Q92667	TM	STRING	omim:00602449 09238861 09880537
AKAP11	Q9UKA4	VT AC WB EX TM	iRefIndex APID STRING BioGrid HPRD PINA	10209101 12147701 11152471 19008911
AKAP4	Q5JQC9	TM	STRING	15385410
AKT1	P31749	ICU TM EX	STRING Reactome	omim:00601728 omim:00605035 08798763 20482850
AKT2	P31751	ICU EX	STRING Reactome	20482850
AKT3	Q9Y243	ICU EX	STRING Reactome	20482850
ANAPC7	Q9UJX3	EX	Reactome	20482850
APITD1	Q8N2Z9	EX	Reactome	20482850
ARFGEF1	Q9Y6D6	TM	STRING	10212259 14724321 16737766
ARFGEF2	Q9Y6D5	TM	STRING	10212259 16737766 17360629
ARHGAP9	Q9BRR9	TM	STRING	omim:00176914 08914631
AT5F1	P24539	PRED	iRefIndex	15657099
ATM	Q13315	AA	APID IntAct PINA	17274640
ATP5C1	Q8TAS0	PRED	iRefIndex	15657099
ATP6V1E1	P36543	PRED	iRefIndex	15657099
ATPO	P48047	PRED	iRefIndex	15657099
ATXN2	Q24JQ7	TM	STRING	9529352

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
AURKA	O14965	YTH VV VT EX TM	APID STRING iRefIndex HPRD PINA	11551964 11039908 11551964 12490715
AURKB	Q96GD4	ICU TM EX	STRING Reactome	10385519 11350965 11801737 20482850
B9D2	Q9BPU9	ICU EX	STRING Reactome	20482850
BAD	Q92934	TM IM	STRING	omim:00138079 omim:00176875 omim:00176911
BCL2	P10415	AA EX	APID IntAct PINA Reactome	17274640 20482850
BCL2L1	Q07817	AA	APID IntAct PINA	17274640
BIRC5	O15392	AA ICU EX	APID STRING IntAct PINA Reactome	17274640 20482850
BMI1	P35226	COEX	STRING	
BRCA1	P38398	COIP EX TM	APID STRING IntAct iRefIndex PINA	17511879 omim:00601728 omim:00601772 12438214
BUB1	O43683	ICU EX	STRING Reactome	20482850
BUB1B	O60566	ICU EX	STRING Reactome	20482850
BUB3	O43684	COEX ICU EX	STRING Reactome	20562859 20482850
BYSL	Q13895	PRED	iRefIndex	15657099
C11orf58	O00193	COEX TM	STRING	02987020 16467385
CALM1	P62158	ICU TM EX	STRING Reactome	omim:00176915 01328240 01331060 20482850
CALM3	Q9BRL5	EX	Reactome	20482850
CALML3	P27482	ICU EX	STRING Reactome	20482850
CALML5	Q9NZT1	ICU EX	STRING Reactome	20482850
CALML6	Q8TD86	ICU EX	STRING Reactome	20482850
CAMK2A	Q9UQM7	ICU EX	STRING Reactome	20562859 20482850

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
CAMK2B	Q13554	ICU TM EX	STRING Reactome	omim:00176875 01660400 01719969 20482850
CAMK2D	Q13557	ICU EX	STRING Reactome	20482850
CAMK2G	Q13555	ICU TM EX	STRING Reactome	20562859 omim:00176960 01660400 01719969 20482850
CASC5	Q8NG31	ICU TM EX	STRING Reactome	20231380 20482850
CBS	P35520	PRED	iRefIndex	15657099
CBX1	P83916	COEX TM	STRING	omim:00601772
CCDC99	Q96EA4	ICU EX	STRING Reactome	20482850
CCND1	P24385	AA EX	APID IntAct PINA Reactome	17274640 20482850
CCND3	P30281	AA EX	APID IntAct PINA Reactome	17274640 20482850
CCNG1	P51959	COEX	STRING	
CDC14A	Q9UNH5	PRED	iRefIndex	15657099
CDC20	Q12834	ICU TM EX	STRING Reactome	12490715 20482850
CDC25C	P30307	ICU TM	STRING	20562859 omim:00300415 omim:00601728 omim:00607566
CDC34	P49427	AA	APID IntAct PINA	17274640
CDCA2	Q69YH5	TM	STRING	16492807 16998479
CDCA8	Q53HL2	ICU EX	STRING Reactome	20482850
CDK1	P06493	AA EX	APID IntAct PINA Reactome	17274640 20482850
CDK2	P24941	AA EX	APID IntAct PINA Reactome	17274640 20482850
CDK3	Q00526	EX	Reactome	20482850
CDK4	P11802	AA EX	APID IntAct PINA Reactome	17274640 20482850

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
CDK5	Q00535	EX	Reactome	20482850
CDK6	Q00534	EX	Reactome	20482850
CDK7	P50613	EX	Reactome	20482850
CDKN1B	P46527	VT	HPRD PINA	
CDKN2A (isoform 1/2/3)	P42771	AA	IntAct	17274640
CDKN2A (isoform 4)	Q8N726	AA	APID PINA	17274640
CENPA	P49450	ICU TM EX	STRING Reactome	omim:00117141 20482850
CENPC1	Q03188	ICU TM EX	STRING Reactome	omim:00117141 20482850
CENPE	Q02224	ICU TM EX	STRING Reactome	omim:00602259 20482850
CENPF	P49454	ICU EX	STRING Reactome	20482850
CENPH	Q9H3R5	ICU EX	STRING Reactome	20482850
CENPI	Q92674	ICU EX	STRING Reactome	20482850
CENPK	Q9BS16	ICU TM EX	STRING Reactome	1312079 20482850
CENPL	Q8N0S6	ICU EX	STRING Reactome	20482850
CENPM	Q9NSP4	ICU EX	STRING Reactome	20482850
CENPN	Q96H22	ICU EX	STRING Reactome	20482850
CENPO	Q9BU64	ICU EX	STRING Reactome	20482850
CENPP	Q6IPU0	ICU EX	STRING Reactome	20482850
CENPQ	Q7L2Z9	EX	Reactome	20482850
CENPT	Q96BT3	ICU EX	STRING Reactome	20482850
CEP250	Q9BV73	TM	STRING	17283141
CHEK2	O96017	EX	Reactome	20482850
CKAP5	Q14008	ICU EX	STRING Reactome	20562859 20482850
CLASP1	Q7Z460	ICU EX	STRING Reactome	20482850
CLASP2	O75122	ICU EX	STRING Reactome	20482850
CLIP1	P30622	ICU EX	STRING Reactome	20482850
CNBP	P62633	COEX	STRING	
CNIH	Q8TBE1	COEX	STRING	
COPB1	P53618	COEX	STRING	
COPS2	P61201	COEX	STRING	
COPS5	Q92905	COEX	STRING	

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
CPSF3	Q9UKF6	PRED	iRefIndex	15657099
CPSF4	O95639	PRED	iRefIndex	15657099
CROCC	Q5TZA2	TM	STRING	17283141
CSNK1E	P49674	PRED	iRefIndex	15657099
CSTF3	Q12996	PRED	iRefIndex	15657099
CTCF	P49711	COEX	STRING	
DCP2	Q8IU60	PRED	iRefIndex	15657099
DDX1	Q92499	COEX TM	STRING	omim:00400010 omim:00605281 17661632
DDX10	Q86VR6	PRED	iRefIndex	15657099
DDX18	Q9NVP1	PRED	iRefIndex	15657099
DDX3X	O00571	TM	STRING	omim:00400010 omim:00605281 17661632
DDX3Y	O15523	TM	STRING	omim:00117141 omim:00176914 omim:00182465
DDX4	Q9NQI0	TM	STRING	omim:00117141 omim:00126420 omim:00176914
DEK	P35659	COEX TM	STRING	19103207
DHX15	O43143	COEX TM	STRING	omim:00400010 omim:00605281 17661632
DKC1	O60832	PRED	iRefIndex	15657099
DLD	P09622	COEX	STRING	
DNM1L	O00429	PRED	iRefIndex	15657099
DONSON	Q9NYP3	TM	STRING	omim:00182465
DPM1	O60762	COEX PRED	STRING iRefIndex	15657099
DPYS	Q14117	TM	STRING	17683050 20701987
DSN1	Q9H410	ICU EX	STRING Reactome	20482850
EFHA1	Q8IYU8	COEX	STRING	
EIF2AK2	P19525	YTH VV VT EX TM	APID STRING iRefIndex HPRD PINA	12138106 20562859 01556141 10696424 11070019
EIF2B1	Q14232	PRED	iRefIndex	15657099
EIF2S1	P05198	AA EX TM PRED IM	APID STRING IntAct iRefIndex PINA Reactome	17274640 20562859 omim:00611048 omim:00613257 02246237 15657099 20482850
EIF2S2	P20042	PRED	iRefIndex	15657099
EIF3E	P60228	COEX TM	STRING	02246237 03021534 06301469

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
EIF3H	O15372	COEX TM	STRING	02246237 03021534 06301469
EP300	Q09472	EX	Reactome	20482850
ERBB2IP	Q96RT1	EX	Reactome	20482850
ERCC6L	Q2NKX8	ICU EX	STRING Reactome	20482850
ESR1	P03372	AA	APID IntAct PINA	17274640
FAM33A	Q8WVK7	EX	Reactome	20482850
FBL	Q96BS4	PRED	iRefIndex	15657099
FER	P16591	EX	IntAct	20711500 IM-13779
FGR	P09769	TM	STRING	omim:00601728 01637843 02854198
FTSJ3	Q8IY81	PRED	iRefIndex	15657099
GABARAP	O95166	COIP	IntAct	20562859 IM-15184
GABARAPL2	P60520	COIP	IntAct PINA	20562859 IM-15184
GBAS	O75323	COEX	STRING	
GLO1	Q04760	COEX TM	STRING	16555297
GRB2	P62993	COIP	IntAct	IM-15417 21706016
GRM7	Q14831	TM	STRING	02153935 07517497 09326275
GRPEL1	Q9HAV7	PRED	iRefIndex	15657099
GSK3A	P49840	TM	STRING	omim:00601792 01312697 02822414
GSK3B	P49841	ICU TM EX	STRING Reactome	omim:00601792 omim:00607566 omim:00613275 20482850
GYS1	P13807	ICU TM IM PRED EX	STRING iRefIndex Reactome	20562859 omim:00601792 omim:00602999 omim:00607566 15657099 20482850
GYS2	P54840	ICU TM IM EX	STRING Reactome	20562859 omim:00601792 omim:00602999 omim:00607566 20482850
H2AFV	Q71UI9	COEX TM	STRING	omim:00117141 omim:00600849 omim:00601772
H2AFX	P16104	TM	STRING	omim:00117141 omim:00126420 omim:00176914

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
H2AFZ	P0C0S5	COEX TM	STRING	omim:00117141 omim:00600849 omim:00601772
HAT1	O14929	COEX	STRING	
HCFC1	P51610	VV VT EX ICU TM	APID STRING iRefIndex HPRD PINA	10637318 12788939
HCK	P08631	TM	STRING	15090593 16283202
HDAC1	Q13547	COEX EX TM AC	STRING iRefIndex PINA	20562859 omim:00176883 omim:00613275 12567184 19070599
HDAC10	Q969S8	EX TM	STRING PINA	14670976 omim:00600849 omim:00613275 10207071
HDAC6	Q9UBN7	EX TM	STRING PINA	14670976 omim:00600849 omim:00613275 10207071
HDAC8	Q9BY41	EX AC	STRING iRefIndex PINA	19070599
HMGN4	O00479	COEX	STRING	
HNRNPA2B1	P22626	COEX	STRING	
IARS2	Q9NSE4	COEX	STRING	
IF5A1	P63241	PRED	iRefIndex	15657099
IKBKG	Q9Y6K9	PRED COIP AP	APID MINT IntAct PINA iRefIndex	15657099 14743216
INCENP	Q9NQS7	ICU EX	STRING Reactome	20482850
INS	P01308	TM EX	STRING Reactome	omim:00138079 omim:00176883 omim:00600849 20482850
INSR	P06213	ICU TM EX	STRING Reactome	omim:00176883 omim:00601728 01637843 20482850
ITGB3BP	Q13352	EX	Reactome	20482850
KIF18A	Q8NI77	ICU EX	STRING Reactome	20482850
KIF2A	O00139	ICU EX	STRING Reactome	20562859 20482850
KIF2B	Q8N4N8	ICU EX	STRING Reactome	20482850
KIF2C	Q99661	ICU EX	STRING Reactome	20562859 20482850
KM-PA-2	Q96Q25	PRED	iRefIndex	15657099

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
KNTC1	P50748	ICU EX	STRING Reactome	20482850
LIPA	P38571	PRED	iRefIndex	15657099
LYPLA1	Q6IAQ1	COEX	STRING	
MAD1L1	Q9Y6D9	ICU EX	STRING Reactome	20482850
MAD2L1	Q13257	EX	Reactome	20482850
MAP1LC3A	Q9H492	COIP	IntAct PINA	20562859 IM-15184
MAP1LC3B	Q9GZQ8	COIP	IntAct PINA	20562859 IM-15184
MAP3K3	Q99759	AP COIP PRED	APID MINT IntAct iRefIndex PINA	15657099 14743216
MAPRE1	Q15691	COEX ICU EX	STRING Reactome	20482850
MAX	P61244	AA	APID IntAct PINA	17274640
MDH2	P40926	PRED	iRefIndex	15657099
MIS12	Q9H081	ICU EX	STRING Reactome	20482850
MLF1IP	Q71F23	ICU EX	STRING Reactome	20482850
MLL5	Q8IZD2	ICU COIP	STRING IntAct	19377461
MRPL3	P09001	COEX	STRING	
MST1R	Q04912	COIP	InnateDB	14505491
MYL10	Q9BUA6	ICU EX	STRING Reactome	20482850
MYL12A	P19105	ICU EX	STRING Reactome	20482850
MYL12B	O14950	ICU EX	STRING Reactome	20482850
MYL2	P10916	ICU TM EX	STRING Reactome	omim:00603768 omim:00609172 01657915 20482850
MYL5	Q02045	ICU EX	STRING Reactome	20482850
MYL6	P60660	ICU	STRING	20562859
MYL6B	P14649	ICU	STRING	
MYL7	Q01449	ICU EX	STRING Reactome	20482850
MYL9	P24844	ICU TM EX	STRING Reactome	omim:00603768 omim:00609172 omim:00613245 20482850
MYLPF	Q96A32	ICU EX	STRING Reactome	20562859 20482850

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
MYO16	Q9Y6X6	EX TM VT	STRING iRefIndex HPRD PINA	11588169 09332696 09332704 14989261
NACA	B2R4P8	PRED	iRefIndex	15657099
NAE1	Q13564	COEX IM	STRING	
NAP1L1	P55209	COEX	STRING	
NARS	O43776	COEX	STRING	
NCOR1	O75376	EX TM	STRING PINA	12410313 omim:00600849 12410313 15773917
NDC80	O14777	ICU TM EX	STRING Reactome	20921135 20482850
NDE1	Q9NXR1	ICU EX	STRING Reactome	20482850
NDEL1	Q9GZM8	ICU EX	STRING Reactome	20482850
NDUFB5	O43674	COEX	STRING	
NEK2	P51955	TM	STRING	10880350 12221103 15659832
NOM1	Q5C9Z4	TM	STRING	17965019
NOP56	O00567	PRED	iRefIndex	15657099
NOP58	Q9Y2X3	PRED	iRefIndex	15657099
NSL1	Q96IY1	ICU EX	STRING Reactome	20482850
NUDC	Q9Y266	ICU EX	STRING Reactome	20562859 20482850
NUF2	Q9BZD4	ICU TM EX	STRING Reactome	10385519 20482850
NUP107	P57740	COEX ICU EX	STRING Reactome	20562859 20482850
NUP133	Q8WUM0	ICU EX	STRING Reactome	20562859 20482850
NUP160	Q12769	ICU EX	STRING Reactome	20562859 20482850
NUP37	Q8NFH4	COEX ICU EX	STRING Reactome	20562859 20482850
NUP43	Q8NFH3	ICU EX	STRING Reactome	20562859 20482850
NUP85	Q9BW27	ICU EX	STRING Reactome	20562859 20482850
NUP98	P52948	EX	Reactome	20482850
OAT	P04181	PRED	iRefIndex	15657099
OGT	O15294	ICU TM	STRING	20562859 12510058 15014073 15247246

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
P2RY1	P47900	TM	STRING	10919872 19286657 20570683
PAFAH1B1	P43034	ICU EX	STRING Reactome	20562859 20482850
PAPOLA	P51003	PRED	iRefIndex	15657099
PCMT1	P22061	COEX	STRING	
PCNA	P12004	AA EX	APID IntAct PINA Reactome	17274640 20482850
PES1	B2RDF2	PRED	iRefIndex	15657099
PFDN4	Q9NQP4	TM	STRING	omim:00117141
PHKA1	P46020	ICU EX	STRING Reactome	20482850
PHKA2	P46019	ICU EX	STRING Reactome	20482850
PHKB	Q93100	COEX ICU EX	STRING Reactome	20482850
PHKG1	Q16816	ICU EX	STRING Reactome	20482850
PHKG2	P15735	ICU EX	STRING Reactome	20482850
PLA2G6	O60733	TM	STRING	08978503 09882488 16099093
PLIN1	O60240	ICU TM EX	STRING Reactome	01313435 01321155 01324971 20482850
PLK1	P53350	ICU TM EX IM	STRING Reactome	11572854 15090593 17028581 20482850
PMF1	Q6P1K2	EX	Reactome	20482850
POLR1D	Q9Y2S0	PRED	iRefIndex	15657099
POLR2A	P24928	MI EX	APID BIND	12036313
POLR2B	P30876	COEX TM	STRING	01313364 01429657 02537149
PPA1	Q15181	PRED	iRefIndex	15657099
PPP1R11	O60927	TM	STRING	omim:00109091 omim:00126449 omim:00138079
PPP1R14A	Q96A00	ICU TM	STRING	omim:00109091 omim:00126449 omim:00138079
PPP1R14B	Q96C90	COIP EX TM	APID STRING MINT iRefIndex PINA	17022978 omim:00109091 omim:00126449 omim:00138079

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
PPP1R15A	O75807	WB EX TM AC GI	APID STRING BioGrid iRefIndex PINA	12016208 11564868 14670976 omim:00109091 omim:00126449 omim:00138079
PPP1R1A	Q13522	ICU TM EX	STRING Reactome	omim:00109091 omim:00126449 omim:00138079 20482850
PPP1R1B	Q9UD71	TM	STRING	omim:00176915 omim:00604399 01313435
PPP1R2	P41236	YTH VT EX TM IM PRED	APID STRING iRefIndex HPRD PINA Reactome	15657099 10807923 8119416 20482850 omim:00109091 omim:00126449 omim:00138079
PPP1R3A	Q16821	ICU TM EX	STRING Reactome	omim:00109091 omim:00126449 omim:00138079 20482850
PPP1R3C	Q9UQK1	TM	STRING	omim:00109091 omim:00126449 omim:00138079
PPP1R3D	O95685	RC EX TM BC VV PD	APID STRING BioGrid iRefIndex HPRD PINA Reactome	20562859 omim:00109091 omim:00126449 omim:00138079 9414128
PPP1R7	Q15435	TM IM PRED	STRING iRefIndex	omim:00109091 omim:00126449 omim:00138079 15657099
PPP1R8	Q12972	VV RC EX COEX TM IM BC PRED PD VT	APID STRING BioGrid iRefIndex HPRD PINA Reactome	11104670 omim:00109091 omim:00126449 omim:00138079 20482850 15657099 7499293
PPP1R9B	Q96SB3	WB RC EX TM IM AC BC PD	APID STRING BioGrid iRefIndex PINA	10194355 omim:00602021 omim:00602468 omim:00603325 10391935

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
PRKACA	P17612	TM	STRING	omim:00613256 15385410
PRKAG2	Q9UGJ0	PRED	iRefIndex	15657099
PRKCH	P24723	TM	STRING	omim:00176960 01310215 01312697
PRKG1	Q13976	ICU TM	STRING	06330098 06501303 07592887
PRKRIR	O43422	COEX	STRING	
PSMA2	P25787	COEX	STRING	
PSMC6	P62333	COEX	STRING	
PSMG2	Q969U7	COEX	STRING	
PYGL	P06737	PRED	iRefIndex	15657099
RAF1	P04049	YTH EX TM VV VT COIP	APID STRING iRefIndex HPRD PINA InnateDB	12374792 omim:00176960 02562181 02690080
RAN	P62826	COEX	STRING	
RANBP2	P49792	EX	Reactome	20482850
RANGAP1	P46060	ICU EX	STRING Reactome	20562859 20482850
RB1	P06400	VV VT	HPRD PINA	
RBM34	P42696	PRED	iRefIndex	15657099
RCC2	Q9P258	ICU EX	STRING Reactome	20562859 20482850
RIOK2	Q9BVS4	PRED	iRefIndex	15657099
RIPK3	Q9Y572	AP COIP PRED	APID MINT IntAct iRefIndex PINA	15657099 14743216
RMP	O94763	COIP COEX TM	IntAct PINA STRING	17936702 18285608 IM-15772 21397856
ROCK1	Q13464	EX	Reactome	20482850
ROCK2	O75116	ICU EX	STRING Reactome	20482850
RPL6	Q02878	COEX TM	STRING	9529352
RPS27	P42677	EX	Reactome	20482850
RUVBL1	Q9Y265	PRED	iRefIndex	15657099
RYR2	Q92736	CoFR EX TM BC	APID STRING BioGrid iRefIndex PINA	10830164 omim:00180902 omim:00600620 07836435
SACM1L	Q9NTJ5	COEX TM	STRING	17605038
SARS	P49591	PRED	iRefIndex	15657099
SCP2	P22307	COEX	STRING	

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
SDHD	O14521	COEX	STRING	
SEC13	P55735	ICU PRED EX	STRING iRefIndex Reactome	20562859 15657099 20482850
SEC23B	Q15437	PRED	iRefIndex	15657099
SEC24B	O95487	COEX	STRING	
SEH1L	Q96EE3	EX	Reactome	20482850
SEP15	O60613	COEX	STRING	
SEPT7	Q16181	EX	Reactome	20482850
SERBP1	Q8NC51	COEX	STRING	
SET	Q01105	COEX TM	STRING	07531497 08626647 18214640
SF3B3	Q15393	PRED	iRefIndex	15657099
SGOL1	Q5FBB7	ICU TM EX	STRING Reactome	20562859 omim:00176915 20482850
SGOL2	Q562F6	ICU TM EX	STRING Reactome	omim:00176915 20482850
SIAH2	O43255	TM	STRING	17683050
SIRT2	Q8IXJ6	EX	Reactome	20482850
SKA1	Q96BD8	ICU EX	STRING Reactome	20482850
SKP1	P63208	AA COEX EX TM	APID STRING STRING IntAct PINA Reactome	omim:00607566 17274640 20482850
SLITRK1	Q96PX8	TM	STRING	18061458 20013999
SLTM	Q9NWH9	COEX	STRING	
SMARCB1	Q12824	WB RC EX TM BC AC GI PD VV	APID STRING BioGrid iRefIndex HPRD PINA	20562859 omim:00601607 omim:00611048 12016208
SMC1A	Q14683	EX	Reactome	20482850
SMNDC1	O75940	COEX IM	STRING	
SNX2	O60749	COEX	STRING	
SON	P18583	COEX TM	STRING	omim:00182465
SPA17	Q15506	TM	STRING	15385410
SPC24	Q8NBT2	ICU EX	STRING Reactome	20482850
SPC25	Q9HBM1	ICU EX	STRING Reactome	20562859 20482850
SRM	P19623	PRED	iRefIndex	15657099
SSU72	Q9NP77	PRED	iRefIndex	15657099

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
ST13	P50502	COEX	STRING	
STK38	Q15208	ICU	STRING	
SUMO1	P63165	COEX TM	STRING	11861864
TAF7	Q15545	COEX	STRING	
TAOK1	Q7L7X3	ICU EX	STRING Reactome	20482850
TCEA1	P23193	COEX	STRING	
THOC7	Q6I9Y2	COEX	STRING	
TLX1	P31314	WB RC BC AC PD	APID BioGrid iRefIndex PINA	9009195
TLX1NB	P0CAT3	EX TM	STRING	9009195 09009195 09673847 15090593
TMEM123	Q8N131	COEX	STRING	
TMSB4Y	O14604	TM	STRING	omim:00400010 omim:00602021 omim:00602468
TOMM20	Q15388	COEX	STRING	
TOP1	P11387	TM	STRING	omim:00117141 omim:00126420 omim:00176914
TOP2B	Q02880	COEX TM	STRING	omim:00126420 07954457 08297104
TOX4	O94842	ICU TM	STRING	20516061
TP53	P04637	COIP AA VT VV	APID IntAct IntAct iRefIndex HPRD PINA	17274640
TP53BP2	Q13625	YTH COIP PD EX TM PA VV VT PRED	APID STRING MINT iRefIndex HPRD PINA	8549741 20562859 omim:00606455 omim:00607463 doi:10.1074/jbc.M708717200 15657099
TPT1	P13693	PRED	iRefIndex	15657099
TRAPPC8	Q9Y2L5	COEX	STRING	
TSR1	Q2NL82	PRED	iRefIndex	15657099
UBA2	Q9UBT2	COEX IM	STRING	
UBE4A	Q14139	COEX	STRING	
USF1	P22415	EX TM COIP	STRING IntAct iRefIndex PINA	19303849 omim:00601728 18572192 IM-12076

Protein	*Interactor ID*	*Interaction Detection Method*	*Database*	*Pubblication Identifier*
USP1	O94782	COEX	STRING	
USP9Y	O00507	TM	STRING	omim:00400010
VAMP7	P51809	COEX	STRING	
VARS	P26640	PRED	iRefIndex	15657099
VBP1	P61758	COEX IM	STRING	
VPS53	Q5VIR6	PRED	iRefIndex	15657099
WBP11	Q9Y2W2	YTH VT EX TM VV	APID STRING iRefIndex HPRD PINA	14640981
WDR12	Q53T99	PRED	iRefIndex	15657099
WDR82	Q6UXN9	ICU TM IM	STRING	18342605 20516061 20562859
WIPF2	Q8TF74	TM	STRING	omim:00601772
XPO1	O14980	COEX ICU TM IM EX	STRING Reactome	20562859 16251346 17661632 20482850
YME1L1	Q96TA2	COEX	STRING	
YWHAQ	P27348	COEX TM	STRING	9705329
YWHAZ	P63104	VV VT COIP PD EX TM	HPRD APID STRING iRefIndex PINA Reactome	20562859 15469938 18753613 15028637 20482850
ZFYVE9	O95405	YTH PRED EX	APID BIND MINT iRefIndex HPRD PINA Reactome	15231748 15657099 20482850
ZW10	O43264	ICU EX	STRING Reactome	20482850
ZWILCH	Q9H900	ICU EX	STRING Reactome	20482850
ZWINT	O95229	ICU EX	STRING Reactome	20562859 20482850

Legend:

Interaction Detection Method	Acronym
Inferred by curator (STRING)	ICU
In vivo (APID, HPRD) *vv (iRefIndex)*	VV
In vitro (APID, HPRD) *vt (iRefIndex)*	VT

Interaction Detection Method	Acronym
Two hybrid (APID, BIND, MINT, iRefIndex) *Two hybrid pooling approach (IntAct)* *Yeast 2 Hybrid (HPRD)*	YTH
Western blot (APID)	WB
Antibody array (APID, IntAct)	AA
OPHID Predictive protein interaction (iRefIndex)	PRED
Predictive text mining (STRING)	TM
Tandem-affinity purification (APID, IntAct) *Tap (iRefIndex)*	AP
Experimental knowledge based (Reactome) *Experimental interaction detection (STRING)* *Experimental (BIND)*	EX
Unspecified method - coexpression (STRING) *Co-expression (STRING)*	CoEX
Co-immunoprecipitation *Anti bait coimmunoprecipitation (APID, IntAct)* *Anti tag coimmunoprecipitation (MINT, InnateDB, IntAct)* *Anti bait coip (iRefIndex)* *Anti tab coip (iRefIndex)*	CoIP
Affinity chromatography (BioGrid) *Affinity chromatography technology (iRefIndex)*	AC
Peptide array (MINT)	PA
Biochemical (BioGrid, iRefIndex)	BC
Interologs Mapping (STRING)	IM
Molecular interaction (APID)	MI
Co-fractionation (APID)	CoFR
Reconstituted complex (APID)	RC
Pull down (APID, iRefIndex)	PD
Genetic interference (BioGrid, iRefIndex)	GI

| Chapter III

III | NOVEL PIPS IN BRAIN AND TESTIS

Protein-Protein interactions (PPIs) are a common aspect in virtually all biological processes, including formation of macromolecular complexes, signaling pathways, regulation and metabolism. PPIs are also a clue of functional relationships between the interacting proteins.

The YTH system allowed the identification of a large number of putative PP1 interaction partners. Nevertheless, particular PP1-PIP interactions need to be validated by other methods, to exclude the possibility of a false positive interaction. From the four screens performed (Chapter II) 263 interactors were retrieved for PP1 different isoforms. From the PP1 interactions identified, two proteins were chosen for further studies: Taperin and Synphilin-1A.

Taperin was the most abundant clone in the screen with PP1α in brain, with a total of 45 hits, and was also identified in a screen with PP1γ1 in brain and testis (Fardilha, 2011; Esteves, 2012a,b).

Synphilin-1A was chosen based on its relevance to PD, besides being a less characterized isoform of Synphilin-1. Furthermor, phosphorylation is an important regulatory method in the formation of LBs and the phosphatases involved in the pathways are far from being fully described.

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Manuscript 3 - Taperin, a Protein Phosphatase 1 Interacting Protein in Brain and Testis

In preparation

Taperin, a Protein Phosphatase 1 Interacting Protein in Brain and Testis

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Abstract

C9ORF75, also known as taperin, was associated with autosomal-recessive non-syndromic hearing loss by target genome capture combined with next-generation capture and by homozygosity mapping. Immunolocalization studies of mouse cochlea demonstrated the presence of taperin at the taper regions of hair cell stereocilia. Taperin was identified as a Protein Phosphatase 1 (PP1) α and γ binding protein, by SILAC-based quantitative proteomics, displacement affinity chromatography and Yeast Two Hybrid. Recently, taperin was found to shuttle between the nucleus and cytoplasm, to accumulate at sites of DNA damage in the nucleus and to interact with DNA damage response proteins.

In our study, taperin was not only found in nucleus and cytoplasm but also in the membrane, where it was observed to co-localize with PP1 and actin. When mutated in the PP1 binding motif, the taperin mutant accumulates in the nucleus with actin. In agreement with a role in actin cytoskeleton, taperin overexpression in HeLa cells altered the cellular morphology and actin dynamics. Interestingly, taperin has homology with phostensin, a known PP1 binding protein and an actin filament pointed-end-capping protein. Confirming a role for taperin and PP1 in actin depolymerisation, mutant taperin leads to a high F-actin depolymerisation and altered surface homeostasis.–We also detected taperin presence in testis and found that it is expressed and is primarily present in the acrosome of elongating spermatids, a structure where PP1 and actin have important functions in capacitation and acrosome reaction. This study first reveals a functional relation between taperin, PP1, and actin that, together with taperin high expression in sperm cells, has potential interest in male reproductive physiology

Introduction

The C9orf75 protein is also known as taperin due to its localization in the taper regions of hair cells stereocilia (Rehman, 2010). Targeted genome capture, applied to the genomic DNA of an affected individual from a DFNB79 (nonsyndromic recessive deafness locus) linked family, identified a nonsense mutation in the taperin gene, and three additional frameshift mutations in the same gene were also found in other three DFNB79-linked families (Rehman, 2010). Immunolocalization in mouse cochlea demonstrated that taperin is located prominently at the taper regions of hair cell stereocilia. Sequencing of the four exons of taperin gene (TPRN), revealed a homozygous 11 bp deletion in exon 1, which cosegregated with the disease in all affected members from a Morocco family; and a 1 bp deletion, also in exon 1, predicted to cause a premature protein truncation, in a Dutch family (Li, 2010). Taken together, both studies suggest that mutations in TPRN can be a frequent cause of autosomal-recessive nonsyndromic hearing loss, by taperin loss-of-function, although its actual cellular function(s) is still unknown.

In various Protein Phosphatase 1 (PP1) interactome studies, taperin was found to be a PP1 Interacting Protein (PIP). This was shown by SILAC-based quantitative proteomics (Trinkle-Mulcahy, 2006), displacement affinity chromatography (Moorhead, 2008) and Yeast Two Hybrid (YTH) screens with cDNA libraries of human testis (Fardilha, 2011) and human brain (Esteves, 2012a,b). PP1 is a major protein Ser/Thr phosphatase involved in a broad diversity of cellular functions. The holoenzyme consists of a catalytic subunit (PP1c) and a regulatory subunit, the PIPs. PP1c is a 35–38 kDa protein that exists as three isoforms: PP1α (PPP1CA), PP1β/δ (PPP1CB) and PP1γ (PPP1CC) with two splice variants PP1γ1 (PPP1CC1) and PP1γ2 (PPP1CC2). All PP1c isoforms are ubiquitously expressed, except for PP1γ2 that is testis and sperm enriched. About 200 putative PIPs

have been identified, with many more expected to be found (Moorhead, 2008; Hendrickx, 2009; Fardilha, 2010; Heroes, 2012). PIPs may target PP1c to specific subcellular compartments, modulate substrate specificity, inhibit its activity or serve as substrates themselves. Thus, the interactions between the different PP1 isoforms and specific PIPs are central to the understanding of PP1 physiological functions. The binding of PIPs to PP1 is made through binding motifs (BMs) present on the PIPs, the most common being the RVxF motif (Egloff, 1997; Wakula, 2003; Meiselbach, 2006; Hendrickx, 2009).

A recent study characterized the PP1 and taperin interaction (Ferrar, 2012) and described that taperin binds preferentially PP1α over PP1γ (but does not bind PP1β), and can inhibit PP1 activity. Taperin was also found to shuttle between the nucleus and cytoplasm, but accumulates in the nucleus at sites of DNA damage. Of note, this study also identified other nuclear interaction partners of taperin, by SILAC-based quantitative immunoprecipitation, and among them were DNA damage proteins like PARP1 and TOPOI (Ferrar, 2012).

Taperin is a vertebrate protein with the highest level of sequence conservation on the C-terminal, where the RVxF (KISF) PP1 BM is present (Ferrar, 2012). Database search with taperin sequence showed that it has some homology with phostensin (Rehman, 2010), with the region with more similarity being the one containing the KISF PP1 BM, present in both proteins (Ferrar, 2012). Phostensin is also a known PIP (Kao, 2007) and, most importantly, an actin filament pointed-end-capping protein, which modulates actin dynamics by targeting PP1 to the F-actin cytoskeleton (Kao, 2007; Lai, 2009).

This study aimed to characterize in more detail the interaction between PP1 and taperin, and this protein isoforms in brain, testis and sperm. Our results show that taperin, *in vivo* and *ex vivo,* binds to PP1α and γ isoforms. Taperin and PP1α co-localize in the nucleus,

cytoplasm and membrane of HeLa cells, with the PP1 membranar localization appearing to be depend on its binding to taperin, once it is altered by the absence of the PP1 BM in taperin. Further, binding to PP1 was found to be of major importance in regulating taperin effects on the actin cytoskeleton. Also, the expression of taperin in male germ cells and its localization in testis were analysed, as it was detected as a human testis PIP (Fardilha, 2011). Taperin was found to have a high expression level in spermatocytes and shows an acrosome pattern staining in elongating spermatids. Taperin, actin and PP1 share the same localization in the acrosome, which suggests that in sperm they can form a complex, with a possible function in F-actin dynamics necessary for capacitation and acrosome reaction.

Material and Methods

Interaction of PP1 and taperin by yeast co-transformation

Small-scale LiAc yeast transformation procedures were performed combining the bait plasmid pAS2-PP1α (Gal4 binding domain expression vector) with specific taperin positive clones isolated from an human brain cDNA library (Esteves, 2012a) in pACT2 vector (Gal4 activation domain expression vector) (Fardilha, 2004). In brief, salmon testes carrier DNA was added to the plasmidic DNA, and then freshly prepared yeast strain AH109 competent cells were further added, followed by 600 µL of sterile PEG/LiAc (40% PEG 4000/ 1X TE/ 1X LiAc). The mixture was incubated at 30 ºC for 30 min with shaking (200 rpm). After adding DMSO, the solution was mixed gently and then heat-shocked at 42 ºC for 15 min. The cells were chilled on ice and pelleted by centrifugation for 5 sec at 14,000 rpm and resuspended in 0.5 mL of 1X TE buffer. In parallel, co-transformation with the empty vectors pAS2-1 and pACT-2 was performed, as a negative control. The association of murine p53 (encoded by plasmid pVA3) and SV40 large T antigen (plasmid pTD1) served as a positive control. To confirm protein-protein interactions, the fresh diploid colonies were assayed for growth on SD/QDO (high stringency medium) plates with X-α-Gal to check for MEL-1 expression (indicated by the appearance of blue color). All reagents and media were purchased from Clontech (Saint-Germain-en-Laye, France).

Taperin-GFP construct

Taperin cDNA was subcloned in pEGFP-N1 vector (Clontech) to produce taperin-GFP. Taperin cDNA, recovered from an YTH screen (Esteves, 2012a), with the incomplete

coding sequence of isoform 1 was PCR amplified to originate the CDS of isoform 2; specific primers with *Eco*RI and *Sal*I restriction sites were used. PCR product and pEGFP vector were digested with *Eco*RI and *Sal*I (New England Biolabs), following the manufacturer's instructions. Ligation was performed according to ligase manufacturer's instructions (New England BioLabs).

Direct Mutagenesis

Taperin-RVTA-GFP - Mutagenesis of the PP1 BM RVTF in taperin cDNA was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, now Agilent Technologies) by mutating the last amino acid (phenylalanine) to alanine in order to disrupt the PP1 BM.

Cell culture and transfection

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% Non-Essential aminoacids (Gibco, Invitrogen) and 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco). Cultures were maintained at 37º C and 5% CO_2.

For transfection, the culture medium was replaced with complete medium (antibiotic/antimycotic-free) and the DNA diluted in Opti-MEM (serum- and antibiotic/antymicotic-free). The Lipofectamine 2000 reagent (Invitrogen) was diluted appropriately in the same medium, and the DNA solution was added to the Lipofectamine solution drop by drop, mixed by gentle bubbling with the pipette and allowed to rest for 25 min at room temperature. The complexes 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 24 hrs.

Antibodies

Anti-PP1α antibody (CBC2C) – antiserum was raised in rabbits against the PP1α C-terminal peptide NKGKYGQFSGLNPGG. Anti-PP1γ antibody (CBC3C) – antiserum was raised in rabbits against the PP1γ C-terminal peptide, KKPNATRPVTPPRGMITKQAKK, which detects the two γ isoforms (da Cruz e Silva, 1995). Both were affinity purified, at the Centre for Cell Biology, University of Aveiro.

Anti-GFP antibody – Monoclonal JL8 antibody, obtained from Clontech.

Anti-taperin antibodies – Rabbit polyclonal SAB2103870 (Ab70) and SAB2103485 (Ab85) antibodies were obtained from Sigma-Aldrich.

Anti β-tubulin – Monoclonal antibody 32-2600, from Invitrogen.

Anti pan-Cadherin antibody – Rabbit polyclonal Ab6529, from Abcam.

Anti actin antibody – Monoclonal anti-α-actin 2G2 antibody, kindly provided by Brigitte M. Jockusch, Cell Biology, Zoological Institute, Technical University of Braunschweig, D-38092 Braunschweig, FRG.

Secondary antibodies used were Texas Red-conjugated anti rabbit IgGs, Alexa Fluor 350/488-conjugated anti-mouse IgGs (Molecular Probes), for immunocytochemistry analyses; and horseradish peroxidase-linked IgGs antibodies (GE Healthcare), for enhanced chemiluminescence detection (ECL kit; Pierce).

Immunoprecipitation

After 24 hrs of transfection, cells were washed once with PBS 1X and then collected with lysis buffer (50 mM Tris HCl, pH 8; 120 mM NaCl; 4% CHAPS) containing a protease inhibitor cocktail. The samples were sonicated for 10 sec and mass normalized lysates were precleared with Protein A Sepharose beads (Pharmacia) for 1 hr at 4 ºC with agitation. After centrifuging for 5 min at 10000 g at 4 ºC, the supernatant was transferred to a new Sepharose beads and the primary antibody was added and incubated overnight with shaking at 4º C. The mixture was then centrifuged for 1 min at 4 ºC at 10000 g and the pellet washed four times with washing solution (50 mM Tris HCl, pH 8; 120 mM NaCl), for 15 min with agitation at 4ºC. After the last wash, the tubes were centrifuged for 10 min at 18000 g and 4 ºC, and the supernatant was fully discarded. The beads were then resuspended in fresh Loading Buffer/1% SDS; boiled for 10 min. Lysates were also collected, 10% SDS solution was added in order to obtain a final concentration of 1% SDS, and lysates boiled for 10 min and frozen at - 80 ºC.

Immunoprecipitates and lysates were electrophoreted through in a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then incubated with anti-GFP, anti-PP1α or anti PP1γ1 antibodies and developed by enhanced chemiluminescence (ECL, Amersham). Between the different antibodies membrane stripping was performed. Briefly, the membrane was incubated for 30 min with stripping solution (62,5 mM Tris-HCl, pH 6,7; 2%SDS; 100mM β-mercaptoethanol) at 50º C and 75 rpm, washed three times with TBST, for 15 min with agitation, and two times with water and left to air dry; membrane exposure to film was also performed, in order to confirm the loss of signal from the previous antiboby.

Overlay blot assay

Transfect cells lysates were separated on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with TBST/5% non-fat milk for 1 hr and then overlaid with purified PP1γ1 protein (1 μg/mL) (Browne, 2007) in TBST/3% non-fat milk for 1 hr. After washing three times with TBST, to remove excess protein, the bound PP1γ1 was detected by incubating the membrane with anti-PP1γ antibody in TBST/3% non-fat milk, for 1 hr. Immunoreactive bands appeared after incubating with horseradish peroxidase conjugated secondary antibody, in 3% non-fat milk in TBST for 1 hr, and developing with ECL (Pierce).

Immunocytochemistry

Cells were cultured in glass coverslips until 70-80% confluence and transfected as described above. Each well was washed three times with 1X PBS and then a 4% paraformaldehyde fixative solution was gently added and left to stand for 30 min. Finally, cells were washed three times with 1X PBS. For permeabilization, methanol was added for 2 min followed by 5 washes with 1X PBS. Blocking was carried out for 1 hour with PBS/3%BSA, and then primary antibodies (and phalloidin) diluted in PBS/3% BSA were added and incubated at room temperature for 2 hrs. After three washes with 1X PBS, the secondary antibody was added using the same methodology and incubated for 2 hrs. Finally, three washes were performed and coverslips were mounted on microscope glass slides with anti-fading reagent containing DAPI for nucleic acid staining (Vectashield, Vector Laboratories). For confocal microscopy, images were acquired in a LSM 510 META confocal microscope (Zeiss) using an Argon laser line of 488 nm (GFP channel),

a 561 nm DPSS laser (Texas red labels channel), and a Diode 405-430 laser (Alexa Fluor 350/DAPI labels channel).

Isolation of testicular germ cells

C57/Bl6 mice were used for the isolation of testicular germ cells. Mouse housing was approved by the governmental commission and all animals were kept under standard conditions at the animal facility of the Philipps University of Marburg, with light/dark cycles of 12 hours. Isolation of cell populations from mouse testis was performed according to (Bellve, 1993). Animals were killed by cervical dislocation and testes were removed and decapsulated. Decapsulated testes of six mice were incubated in PBS without Ca/Mg (PAA, Colbe, Germany), supplemented with collagenase, dispase and DNase (20 µg/mL each) at 37°C for 30 min in a Falcon tube under slow rotating motion to dissociate the seminiferous tubules and to liberate interstitial cells. The suspension was then filtered through a Nylon sieve (mesh with 70-µm pore size) and the retained seminiferous tubules were resuspended in the same buffer and triturated with a Pasteur pipette until they were dissociated to a single cell suspension. Cells were sedimented by centrifugation for 10 min at 500g at 4°C to remove the enzymes and resuspended in DMEM (PAA, Colbe, Germany) containing 0.5% BSA (w/v). Cells were further subjected to velocity sedimentation at unit gravity for 2 hours in a BSA gradient (1–5% of BSA (w/v) in DMEM), using a Celsep sedimentation chamber (ECET Celsep-System 5440, Eppendorf, Hamburg, Germany) with a cushion of DMEM supplemented with 10% (w/v) BSA at the bottom of the sedimentation chamber (total volume 1 L). Gradient fractions of 50 mL each were collected and cells were sedimented by centrifugation at 500g for 10 min at 4°C. Isolated cells were analyzed by phase contrast microscopy and

DAPI staining, and homogeneous cell populations were used for further analysis. Isolated cells were processed for RNA isolation (RNeasy mini kit, Qiagen, Hilden, Germany).

Expression analysis in isolated testicular germ cells

Total RNA from the isolated cells was prepared using the RNeasy Plus Universal Midi Kit (Quiagen, Germany). RNA concentration was determined spectrophotometrically (NanoDrop 1000 Spectrophotometer, Thermo Scientific, Germany).

For synthesis of cDNA, a reverse transcription reaction was carried out using 1 µg of RNA and the Transcriptor First Strand cDNA Synthesis Kit for RT-PCR (Roche, Germany). Expression levels of distinct mRNAs were determined by qRT-PCR using the LightCycler 480 SYBR Green I Master (Roche, Germany) and the following cycle conditions. An initial denaturation at 95°C for 15 min was followed by 45 cycles of denaturation (94°C, 15 sec), annealing (58°C, 30 sec) and polymerization (72°C, 30 sec). Thereafter, a melting curve was generated over temperatures of 55–95°C with 30 sec/1°C.

PCR analysis was performed on 96-well plates with the LightCycler® 480 Real-Time PCR System (Roche, Germany). A combination of *Gapdh* and *Hprt* was identified as optimal reference genes for the testis and all expression levels were calculated as relative values using the mean of both reference genes. All samples were run in triplicate and the averages were used for the calculation of the relative expression levels of the genes. The expression quantification of the target gene was calculated using the difference of the C_T-values (ΔC_T) from the mean of the target gene and the mean of the C_T-values from both housekeeping genes (C_T reference gene). The expression levels were further related ($\Delta\Delta C_T$) to control samples using the difference of the ΔC_T-value from the sample (ΔC_T

sample) and the ΔC_T-value from a control (ΔC_T control) and the relative values were calculated as the $2_T^{-\Delta\Delta C}$ (Livak and Schmittgen, 2001). A cDNA obtained from the cell suspension of whole testis was used as control sample for qRT-PCR reactions. All primers used for qRT-PCR are listed in a supplementary Table 1.

Human testicular biopsies

Testicular biopsies for immunofluorescence microscopy were obtained from patients presenting at the Department of Andrology, University Hospital Hamburg-Eppendorf, Germany. Informed consent was obtained and the study conducted in accordance with the guidelines of the 'Helsinki Declaration'. Only samples presenting normal spermatogenesis on the histological analysis were used.

Immunohistochemistry

For immunofluorescence microscopy of paraffin-embedded sections, human testes biopsies were fixed with 4% paraformaldehyde, 0.1 M HEPES pH 7.4, and embedded in paraffin. Paraffin embedded testis were cut on a microtome into 5 µm thick sections. Sections were deparaffined and rehydrated as follows: Xylol 3 x 10 min, absolute ethanol 2 x 5 min 96% ethanol, 80% ethanol, 70% ethanol, and ddH2O, each step for 1 x 5 min at RT.

Non-specific binding sites were blocked with 4% BSA for 2 hrs at RT and washed 3 x with TBST. After, sections were incubated with Ab70 primary antibody in 1% BSA in TBST overnight at 4°C. On the following day, sections were washed 3 x 5 min in TBST, incubated with fluorochrome-conjugated rabbit secondary antibody (diluted in 1% BSA in TBST) for 2 hrs and lastly washed 2 x 5 min with TBST. Negative control was

processed in parallel by adding TBST instead of the first antibody. Nuclei were visualized with DAPI (1:200) for 5 min at RT and washed 2 x with TBST for 5 min. Finally, slides were inspected with LEICA fluorescence microscope (Leica, Germany).

Results

Interaction of Taperin with PP1

Previous studies from our laboratory, aiming to identify the PP1 interactomes in human brain and testis by the YTH method, screened human cDNA libraries using PP1α, γ1 and γ2 as baits, and yielded many PP1 isoform specific binding partners (Fardilha, 2011; Esteves, 2012a,b). One of the most abundant positive clones obtained in the screens was taperin, found to interact with PP1γ1 in testis and brain, and with PP1α in brain.

Taperin has four isoforms present on Uniprot (entry: Q4KMQ1) and only one on NCBI (acession number: NM_001128228.2), corresponding to isoform 1 of Uniprot. The clones recovered from the YTHs some corresponded to isoform 1 and others could correspond to isoform 1, 2 or 4, as they were incomplete in the N-terminus. Isoform 1 is the longest one, with 711 aminoacids, while the other 3 isoforms have between 405 and 433 aminoacids, with isoform 3 having a longer C-terminal (Figure 1). Taperin from rat and mouse (with only one isoform each) is very similar to human isoform 1 (Figure 1), in fact, a recent analysis (Ferrar, 2012) showed that taperin is a vertebrate specific protein with the highest conservation found in the C-terminal, were the PP1 BM (KISF) is located. Actually, human taperin has two potential PP1 BMs (RAIRW and KISF; red underlined in Figure 1). However, while the predicted KISF motif is a conserved PP1 BM, the other one (RAIRW) is quite different from the common PP1 BMs and is less likely to be physiologically functional; indeed the motif RAIRW is not present in the other species.

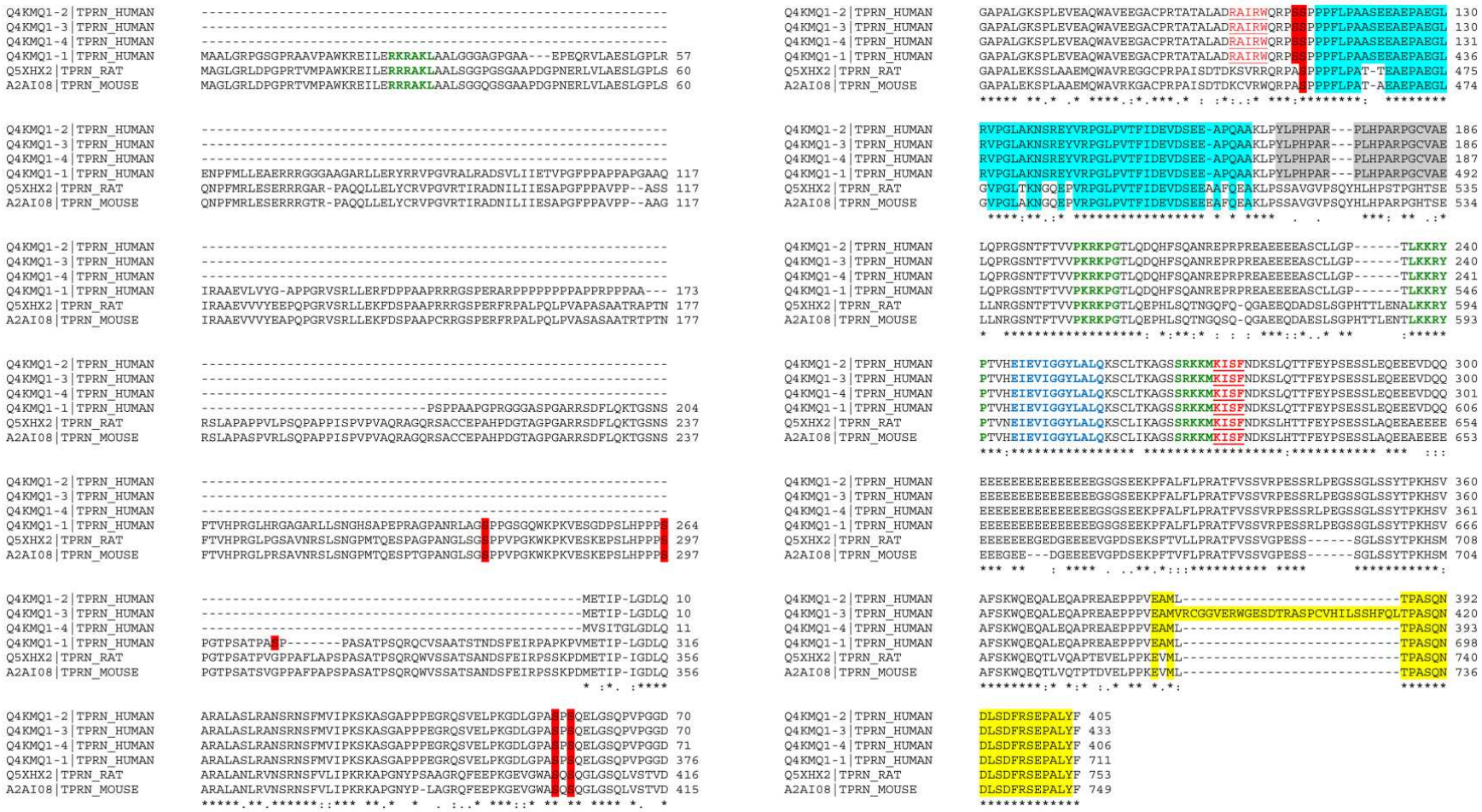

Figure 1 | Sequence alignment of human taperin isoforms and taperin from rat and mouse. ClustalW2 alignment shows a great homology between human, rat and mouse taperin, and conserved PP1 BM, NLSs and NES motifs in all the isoforms. PP1 BMs in red underlined; NLSs in green; NES in blue; antibodies anti-taperin epitopes: Ab70, yellow shadow and Ab85, ligh blue shadow; putative cleavage site in grey shadow and putative phosphoserines (Olsen, 2010) in red shadow.

Several taperin clones from the human brain library, found in the YTH screen (Esteves, 2012a), were selected for analysis of the interaction with PP1 isoforms, by yeast co-transformation (Figure 2A). Clones C1 and C3 have the complete coding sequence of taperin isoform 2 (corresponding to incomplete isoform 1) in the correct frame. Clones C2 and C6 are also complete clones, but were out of frame. Clone C5 does not has the RAIRW PP1 BM and is out of frame, and clone C4 misses part of the same PP1 BM and is also out of frame. All the clones have a second PP1 BM, corresponding to the aminoacids KISF. All clones tested were found to interact with PP1α and PP1γ1, as expected, but also revealed a positive interaction with PP1γ2. Light blue colonies also represent positive interactions that took longer to turn blue in the presence of X-α-GAL.

Cleavage of Taperin in cells

The expression of taperin-GFP fusion protein in human HeLa cells revealed the existence of two bands, when the anti-GFP antibody was used (Figure 2B). The immmunoreactive proteins have apparent molecular mass of 86,6 kDa and 66,4 kDa (Figure 3). As the theoretical molecular mass of taperin isoform 2 is 44.2 kDa and the GFP-tag has approximately 30 kDa, the expected molecular mass for the fusion protein would be around 74 kDa. The observed higher molecular mass protein (86,6 kDa) probably represents the full length fusion protein, and its abnormal higher migration is most probably due to post-translational modifications on the protein. The smaller band has a corresponding molecular mass of approximately 66 kDa and may result from proteolytic cleavage of the full length protein. This fragment contains the PP1 BM. Interestingly, an apparent increase of PP1α protein cellular levels could be observed with increasing amounts of transfected taperin-GFP (Figure 2B).

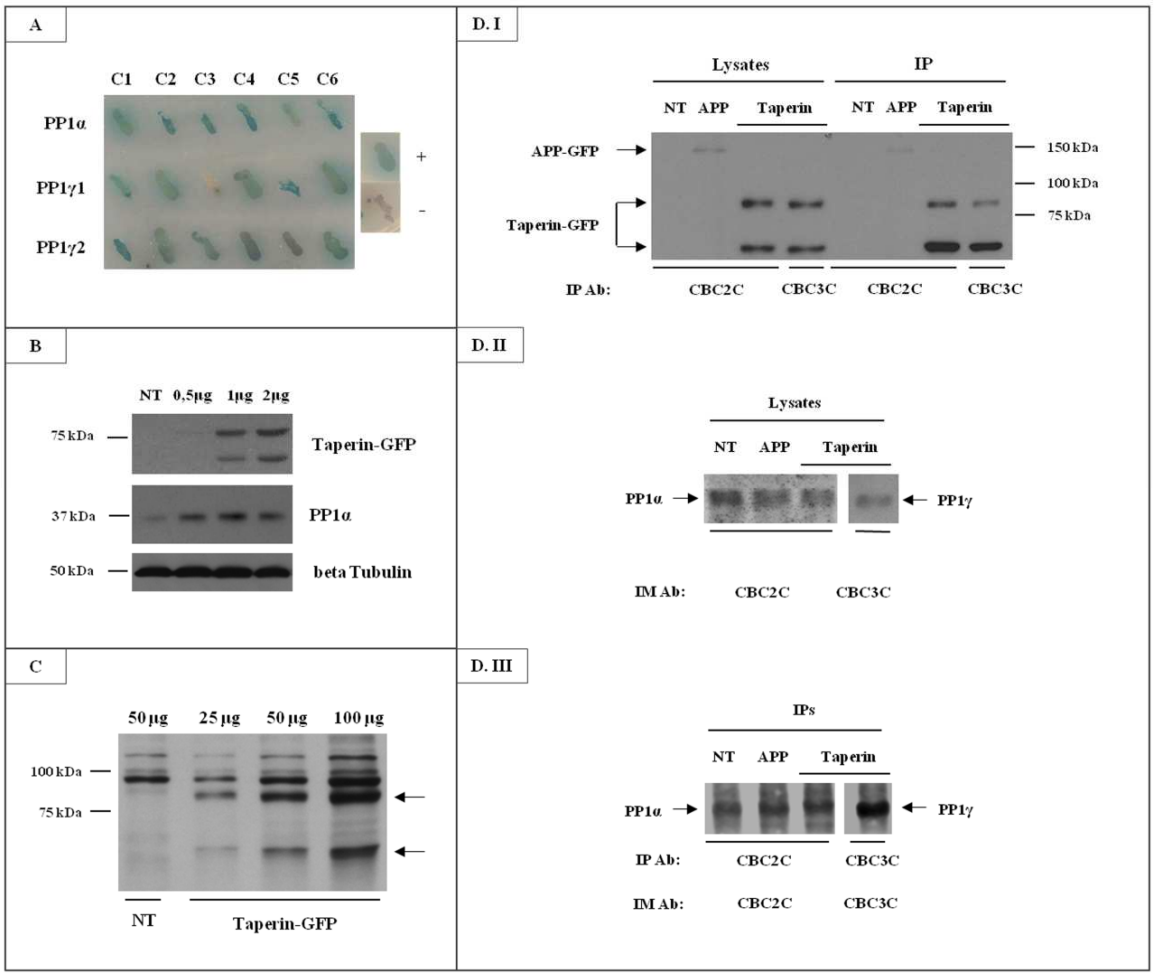

Figure 2 | Taperin binds PP1 and is cleaved in the cell. **A.** Analysis of interaction of taperin YTH clones and PP1 isoforms by yeast co-transformation. Blue color indicates positive interaction. Plus and minus represent positive and negative controls, respectively. **B.** Taperin-GFP expression in HeLa cells. The lanes correspond to non transfected (NT) cells and cells transfected with 0,5 µg, 1 µg and 2 µg of taperin-GFP DNA. Immunoblot analysis of taperin-GFP in lysates of transfected HeLa cells, using an antibody against the GFP tag. Immunoblot analysis of endogenous PP1 levels with increasing amounts of transfected taperin, detected with anti-PP1α antibody. Beta-tubulin was used as a loading control. **C.** Overlay assay of taperin with PP1γ1. The total protein amounts of HeLa cell lysates loaded on each well are indicated on top. NT, lysate from non transfected cells; taperin-GFP, lysate from cells transfected with the taperin-GFP construct. **D.** Co-immunoprecipitation of taperin with PP1. Immunoblot analysis of HeLa cells tranfected with taperin-GFP (or APP-GFP, Amyloid Precursor Protein, as a control) and immunoprecipitated with anti-PP1α (CBC2C) and anti-PP1γ1 (CBC3C) antibodies. **D.I.** Membrane immunoblotted with anti-GFP antibody. **D.II:** Membrane immunoblotted with anti-PP1α and anti-PP1γ antibodies. **D.III.** Membrane immunoblotted with anti-PP1α and anti-PP1γ antibodies. NT, non-tranfected cells; APP, cells transfected with APP-GFP; Taperin, cells transfected with taperin-GFP; IP Ab, immunoprecipitation antibody; and IM Ab, immunoblot antibody.

In order to further confirm the interaction of taperin with PP1, a blot overlay analysis was performed (Figure 2C). The results obtained from this experiment confirm the interaction. Once more, two bands were observed for taperin and both fragments showed an interaction with PP1, which is in agreement with the presence of the PP1 BM (Figure 3). Also with the purpose of confirming and provide an *ex vivo* evidence for the interaction of taperin with PP1, immunoprecipitation of protein extracts obtained from HeLa cells transfected with taperin-GFP was performed, using highly specific anti-PP1α (CBC2C) and anti-PP1γ (CBC3C) antibodies (Figure 2D.I). Proteins from cells lysates and immunoprecipitates (IPs), immunobloted (IB) with anti-GFP (Figure 2D.I), anti-PP1γ and PP1α antibodies (Figure 2D.II and III) were used.

From the analysis of Figure 2D.I it is obvious that taperin co-immunoprecipitates with PP1α and PP1γ. Appropriate controls were included in parallel: immunoprecipitation from non-transfected cells and from cells transfected with APP (Alzheimer's amyloid precursor protein). In agreement with the observed in Figures 2B and C, two bands were again observed following taperin-GFP transfection, with the previously referred molecular mass. Since protein degradation is an unlikely explanation, given the use of protease inhibitors during the process of immunoprecipitation, this observation suggests that the complete protein may have been cleaved *in vivo*. This cleavage must occur on the N-terminus side of the protein, since the GFP tag is on the C-terminus (Figure 3) and detection was achieved with an anti-GFP antibody. This experiment clearly shows that taperin interacts with PP1 isoforms α and γ, in agreement with the yeast co-transformation assays (Figure 2A) and previous data from Ferrar and co-workers (Ferrar, 2012). This interaction is likely to be direct in accordance with the presence of a canonical PP1 binding motif (KISF) in taperin, and with the result of the overlay.

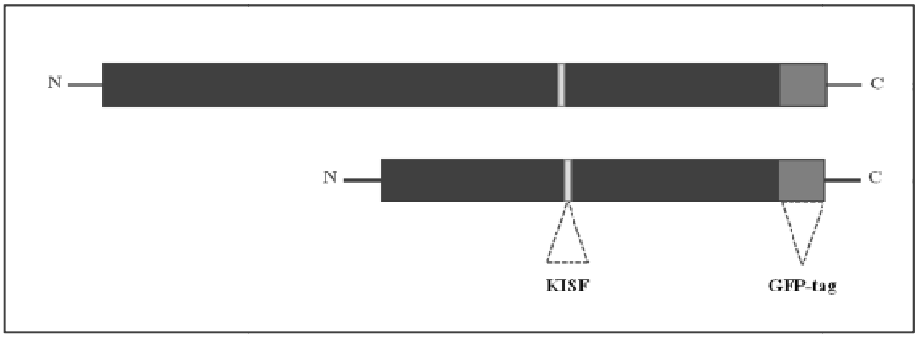

Figure 3 | Schematic representation of the observed taperin protein forms. The two forms of taperin detected in cellular lysates are here represented: the full-length protein (~86,6 kDa) on the top and the cleaved shorter fragment below (~66,4 kDa). Cleavage occurs N-terminally and could lead to the loss of approximately 178 aminoacids but keeps the KISF PP1 BMs (indicated in light grey). GFP, C-terminal tag.

Taperin and PP1 colocalize in the nucleus and plasma membrane of HeLa cells

The expression of taperin-GFP fusion protein in HeLa cells allowed the analysis of its subcellular localization and co-localization with PP1α, revealing a highly enriched nuclear taperin distribution (Figure 4). The majority of taperin-GFP population has an exclusively nuclear distribution, but some was also found distributed throughout the cytoplasm and plasma membrane (Figure 4). Of note, none of these two distribution patterns was apparently dependent on the levels of taperin-GFP expression. Previous results from (Ferrar, 2012) also showed that taperin could associate with PP1 in both nucleoplasm and cytoplasm, although a clear enrichment of taperin in the nucleoplasm was detected. These observations are in accordance with the putative Nuclear Localization Signals (NLS, green in Figure 1) and Nuclear Export Signal (NES, blue in Figure 1) that are conserved between the species, found by taperin sequence search.

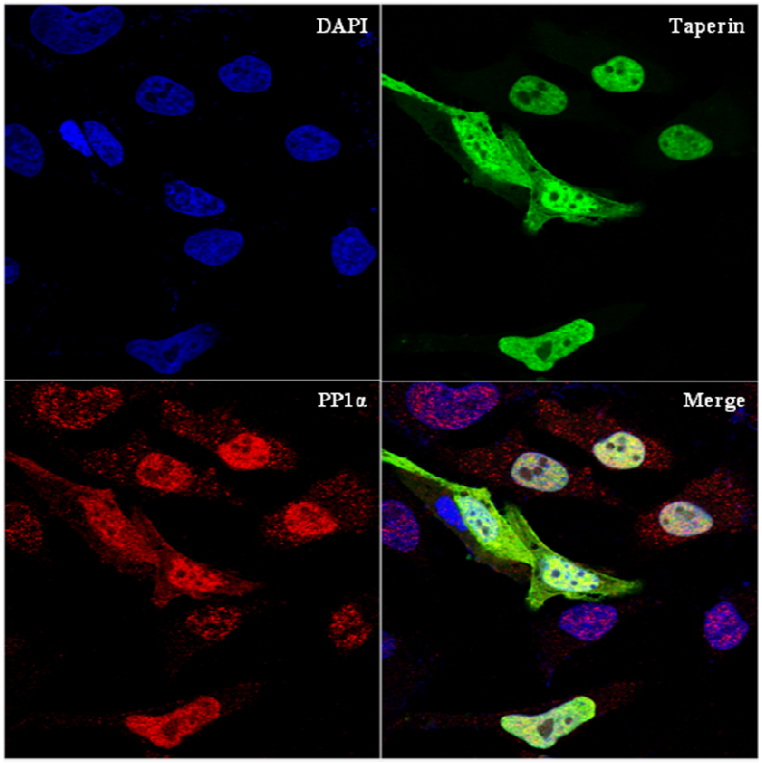

Figure 4 | Subcellular localization of taperin-GFP and co-localization with PP1α. Taperin has nuclear and cytoplasmic distribution, being enriched in the nucleus, and co-localizes with PP1α in the plasma membrane and nucleus, where PP1α is also enriched, in HeLa cells. Nucleic acids were stained using DAPI (blue); taperin-GFP green fluorescence; endogenous PP1α (red) detected with Texas Red-conjugated anti-PP1α antibody; co-localization observed in the merged image (yellow/orange).

Interestingly, cells with high taperin cytoplasmic distribution exhibited a less rounded morphology (around 90% of this population), and taperin could also be observed at the plasma membrane and in fillopodia-like structures (ROIs in Figure 5A). Remarkably, while in non-transfected cells cytoplasmic PP1α is not observed in the plasma membrane (under the conditions used), taperin cytoplasmic presence relocates PP1α to the referred structures (Figures 4 and 5A), where they co-localize.

Co-localization analysis using an anti-PP1α antibody revealed a high degree of nuclear taperin/PP1α co-localization. Very interestingly, PP1α nuclear staining increases in taperin transfected cells, in comparison to non-transfected cells, in a taperin dose-

dependent manner. Further, in the most highly transfected cells not only the nuclear abundance of PP1α increases but its cytoplasmic amounts also appear to increase slightly, suggesting that PP1α protein levels are up-regulated by taperin, in accordance with Figure 2B.

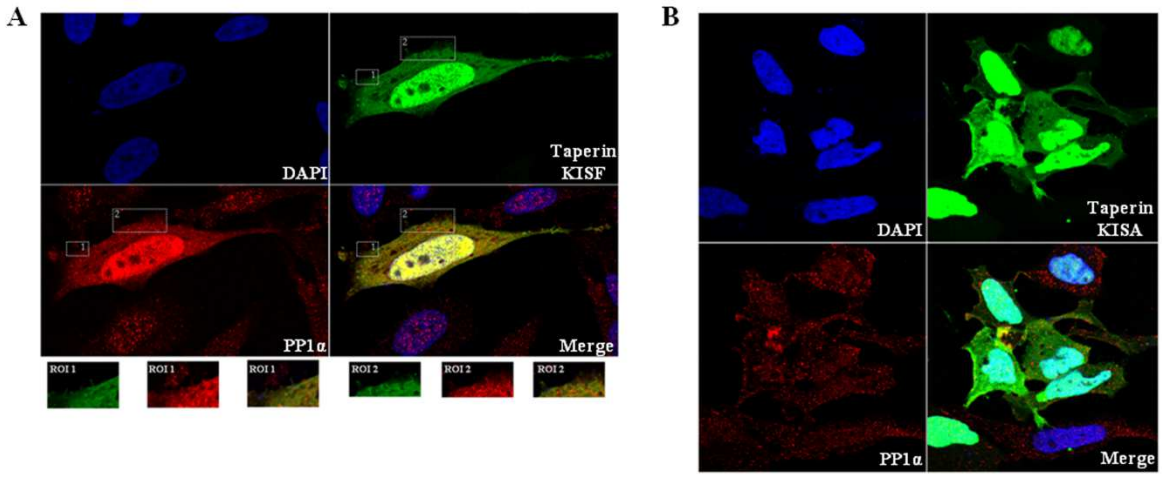

Figure 5 | Subcellular localization of wildtype taperin and taperin mutated in the PP1 BM, in HeLa cells. A. wildtype KISF taperin and PP1α co-localize in the nucleus and in the membrane. **B.** the PP1 BM mutant form of taperin (KISA) does not localizes in the cell membrane and does not increases the levels of PP1α in the nucleus. Nucleic acids were stained using DAPI (blue); Taperin-KISF/KISA-GFP green fluorescence; endogenous PP1α (red) detected with Texas Red-conjugated anti-PP1α antibody; co-localization observed in the merged image (yellow/orange). ROI, Region of interest.

When a mutant form of taperin in the PP1 BM (KISF to KISA) was used (Figure 5B) the same localization of taperin was observed, but the enrichment of PP1 levels on the transfected cells was highly decreased and the distribution of PP1 was different. Some of the transfected cells also showed an enrichment of PP1α in the nucleus, but to a much lesser extent, in comparison with the KISF form of taperin. Also much less PP1 localization in the cell membrane and cytosolic co-localization with taperin was observed with the mutant taperin. Of note, we were still be able to see some co-localization of PP1

with mutant taperin, despite the mutated PP1 BM, but probably as a result of the great overexpression of this form of taperin.

The morphology of the mutant taperin transfected cells was also highly irregular and seemed deregulated. This, together with the homology of this protein to the PIP phostensin (Rehman, 2010), led us to analyze actin distribution on these cells (Figure 6A). Interestingly, on the cells expressing taperin-KISF, actin co-localized with PP1 and taperin on the membrane (ROIs on Figure 6A). But more significant was the fact that, on the cells expressing mutant taperin-KISA, this localization was no longer observed and actin was clearly enriched in the nucleus, especially on the cells where taperin had an high nuclear localization (Figure 6A). Only in about 20% of the cells expressing taperin-KISF actin was enriched in the nucleus (in comparison to non-transfected neighbour cells) while, in contrast, 78% of the cells expressing the taperin-KISA had an obvious enrichment of actin nuclear levels. This was accompanied with a decrease in actin localization at the plasma membrane. We went further and look for pan-cadherin, as a membrane marker, to analyse if mutant taperin was altering its homeostasis. As it can be seen in Figure 6B, when the mutant form of taperin was expressed, the levels of cadherin in the membrane were greatly decreased, particularly when higher amounts of this form of taperin were in the cytoplasm and membrane. When cells were stained with phalloidin (Figure 6C), which binds to filamentous actin (F-actin), a clear function of taperin in actin polimerization could be observed. In cells where wildtype taperin-GFP was expressed, less F-actin stress fibers were observed (70% of the cells), and when taperin was at the cytoplasm stress fibers were abolished and F-actin was only cortical. This was strongly enhanced in cells expressing taperin-KISA, where F-actin staining was greatly decreased (in 94% of the cells), and no stress fibers could be observed, indicating highly depolymerised F-actin, (32% out of the 94%).

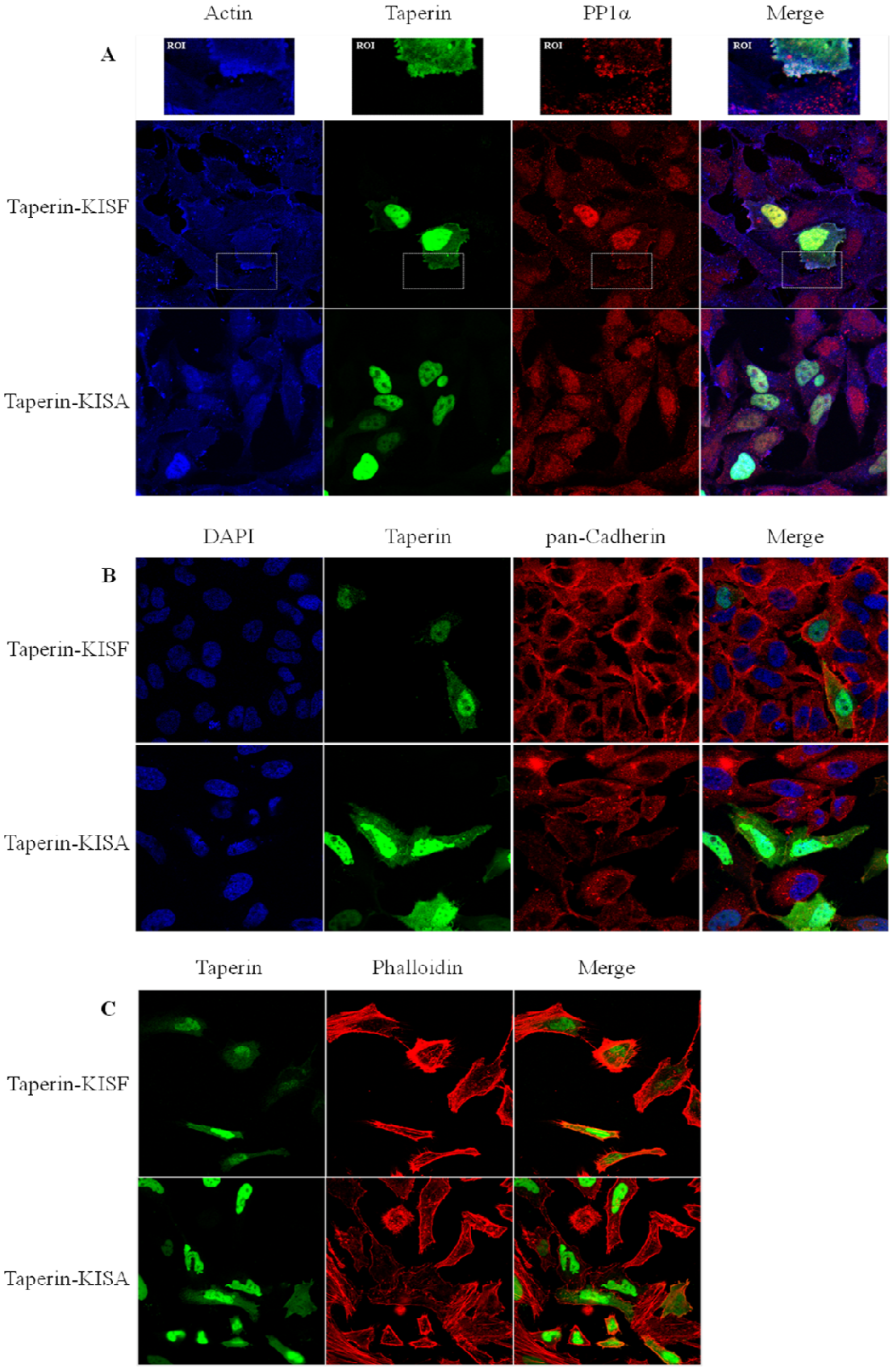
Actin
Taperin
PP1α
Merge
A
ROI
ROI
ROI
ROI
Taperin-KISF
Taperin-KISA
DAPI
Taperin
pan-Cadherin
Merge
B
Taperin-KISF
Taperin-KISA
Taperin
Phalloidin
Merge
C
Taperin-KISF
Taperin-KISA

Figure 6 | Taperin, PP1α and actin co-localize in the cell membrane of HeLa cells. A. Wildtype taperin/Taperin-KISF co-localizes with PP1α and actin in the cell membrane and mutant taperin/Taperin-KISA accumulates with actin in the nucleus. Endogenous actin (blue) detected with Alexa Fluor 350-conjugated anti-actin antibody; Taperin-KISF/KISA-GFP green fluorescence; endogenous PP1α (red) detected with Texas Red-conjugated anti-PP1α antibody; co-localization observed in the merged image (yellow/orange). ROI, Region of interest. **B.** Cells overexpressing mutant taperin (Taperin-KISA) have lower level of cadherin in the membrane than cells expressing wildtype taperin (Taperin-KISF). Nucleic acids were stained using DAPI (blue); Taperin-KISF/KISA-GFP green fluorescence; endogenous cadherin (red) detected with Texas Red-conjugated anti-cadherin; co-localization observed in the merged image. **C.** Wildtype (Taperin-KISF) and mutant (Taperin-KISA) taperin overexpression affect actin dynamics in cells. Taperin-KISF/KISA-GFP green fluorescence; endogenous F-actin detected with plalloidin red fluorescence; co-localization observed in the merged image.

Taperin differential expression in male germ cells

Taperin was shown to be expressed in rat testis by western blot (Ferrar, 2012) and was identified in a human testis cDNA library in a screen for PIPs by the YTH technique (Fardilha, 2011). Thus, we decided to look for taperin expression and localization during spermatogenesis.

We made use of an already established protocol based on unit gravity sedimentation to isolate germ cell populations from a heterogeneous suspension of mouse whole testis (Dastig, 2011). Three major homogenous fractions were separated (fractions 7, 13 and 17) and microscopically evaluated. Moreover, genes already established as markers for each step of the spermatogenesis were analyzed. The fraction 7 showed high relative expression levels for the spermatocytes markers, synaptonemal complex protein 3 (*Sycp3*) and stathmin 1 (*Stmn1*), as well as, the tumor-associated calcium signal transducer 1 (*Tacstd1*), a marker for spermatogonia. Fraction 13 showed the highest expression for the spermatid marker, transition protein 1 (*Tpn1*), and fraction 17 for other spermatid marker, protamine 2 (*Prmn2*). These results are in accordance with previous data that showed the use of these germ cell markers for the isolated fractions validation

(Dastig, 2011). Additionally, other expression markers were also used to check for fraction contamination with somatic cells. We found low relative expression levels in all the fractions analyzed for actin alpha 2 *(Acta2)* for peritubular myoid cells, hydroxysteroid 17 beta dehydrogenase (*Hsd17b3*) and *c-kit* for Leydig cells and the androgen receptor (AR) for Sertoli cells. The exception was for *Acta2* in fraction 13. The expression profiling of the somatic and germ cells specific markers showed that cell populations isolated were enriched in spermatocytes (fraction 7), round spermatids (fraction 13) and elongated spermatids (fraction 17).

Subsequently, we went to check for the relative expression levels of taperin isoform 1 and taperin isoform 3, two alternatively spliced isoforms of the gene *TPRN* in humans. Mouse taperin 3 is not present in any database when searching for mouse ESTs. Our approach to overcome this was to mimic, in the mouse Tprn gene, the alternative splicing that occurs in humans. So, we design the forward primer to match the corresponding exon-exon junction that exists in humans for taperin 3 and the reverse primer for an exon-exon junction present in both, mouse and humans. We were able to detect both transcripts in mouse testis showing for the first time that taperin 3 message is also made. Moreover, expression levels of these isoforms in each germ cell pool were compared with the original testicular cell suspension to retrieve the relative expression values (Table 2, Supplementary data). Both isoforms show a high relative expression level in spermatocytes with 1.9-fold and 2.5-fold, for taperin 1 and 3, respectively. However, whereas taperin 1 maintains its levels in round spermatids (1.5-fold) and lowers in elongating spermatids (0.3-fold), taperin 3 expression decreases in round spermatids (0.6-fold) and recovers to the same levels seen in the whole testis lysates.

Furthermore, since taperin was recovered from a human brain library we also went to check for the expression in hippocampus, by comparison with the whole testis relative

expression levels. Surprisingly, both isoforms showed very low levels of expression (0.002-fold and 0.027-fold for taperin1 and 2, respectively) in hippocampus, which could unveil an important role for these proteins in the male reproductive physiology.

Taperin localization in human testis

Formaldehyde-fixed human testis sections were incubated with taperin antibodies followed by incubation with fluorochrome-labeled secondary antibody (Figure 7).

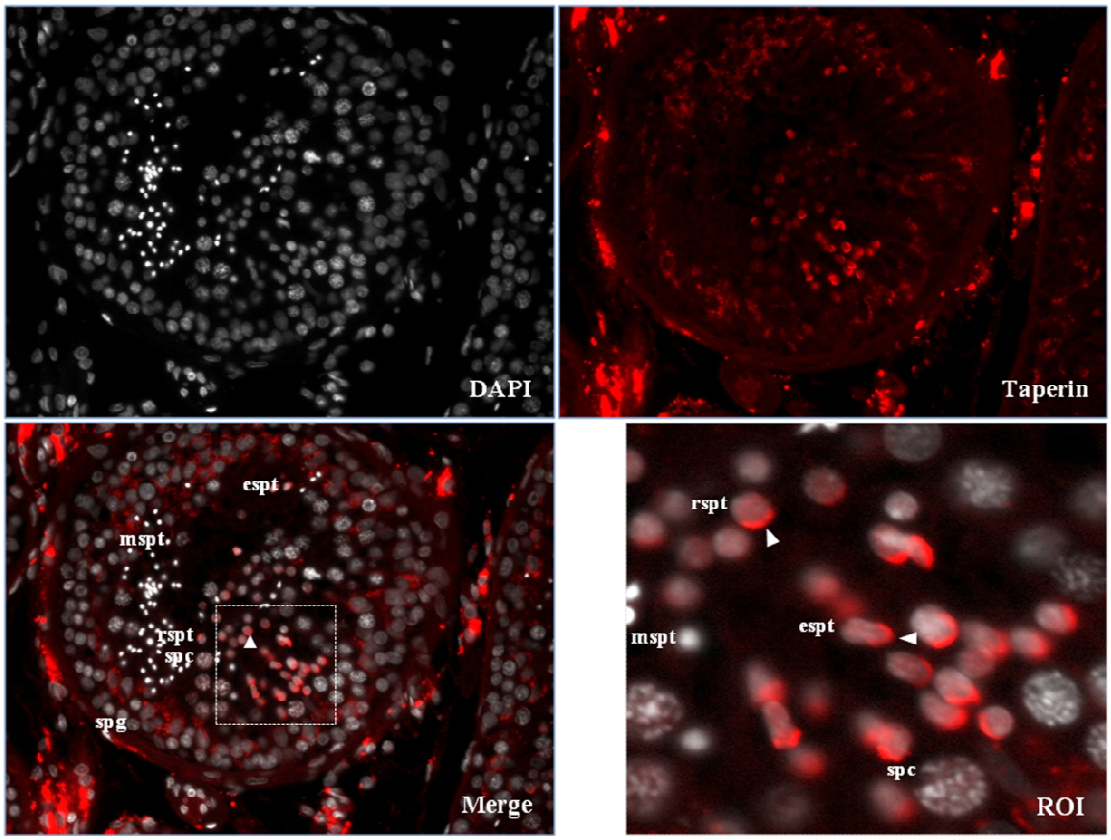

Figure 7 | Taperin cellular localization in human testis. Human testis paraffin sections were stained with DAPI (nucleus, grey) and an anti-taperin antibody Ab70, and visualized with Alexa Fluor 488-labelled secondary antibody (red). Image obtained at 40x magnification. Arrowheads indicate the acrosome. spg, spermatogonia; spc, spermatocyte; rspd, round spermatid; espd, elongating spermatid; mspt, mature spermatid; D. ROI (region of interest), showing round and elongating spermatids.

The primary antibodies recognize all taperin isoforms, however higher specificity of Ab70 should be assigned to taperin 3, given the fact that the epitope corresponds to this isoform in a larger extent (Figure 1). Fluorescence patterns showed staining of elongating spermatids with “cap-shaped” concentration of the immunoreactivity at one pole of the nucleus (acrosome pattern). Moreover, fluorescence was weak in the nucleus of spermatocytes and round spermatids. Sertoli cells, spermatogonia and almost mature spermatozoa cells showed no immunoreactive taperin staining. Non-specific fluorescence was observed in interstitial cells. Human testis cryosections showed the same results and for Ab85 no apparent specific staining was obtained (data not shown).

Discussion and Conclusion

The regulation of the subcellular localization of signaling pathway components is a key determinant in the effective initiation and maintenance of signaling cascades. Regulation of signal transduction pathways through protein-protein interactions can thus make possible the activation of a particular pathway by co-localizing protein kinases and phosphatases with their downstream substrates. Therefore, the identification and characterization of the proteins implicated in cellular pathways are imperative as a mean to understand these cellular events and associated pathologies.

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. The plasticity of PP1 is due to interaction with a diverse set of PIPs. Using the YTH system, new PP1 interactors from human brain and testis were identified in order to gain insight into the various roles of PP1 in several cellular processes, including brain function and neurodegeneration and male infertility. One of the PIPs found on those screens was taperin (Fardilha, 2011; Esteves, 2012a,b). The various techniques used revealed that taperin may occur as a precursor protein, given that it suffers cleavage near its N-terminus. This apparent cleavage originates a fragment reduced in about 20 kDa, which corresponds to approximately 178 aminoacids (Figure 3). This being the case, the smaller protein fragment lacks the most N-terminal putative PP1 BM, but conserves the canonical motif, thus being still able to bind PP1, and in fact both proteins forms bind strongly to PP1. This was confirmed by all the binding assays used: yeast co-transformation, immunoprecipitation and overlay. As a result, the simplest interpretation of this results indicates that the binding likely occurs directly and not through a third bridging protein.

Although both these fragments can bind PP1, the higher abundance of the full-length protein in transfected cells (Figures 2B and C), contrast the smaller fragment enriched in the co-immunoprecipitates (Figure 2D.I). This suggests that the taperin C-terminally truncated form could be the favoured PP1 binding partner.

Another important validation of the putative interactions, discovered with the YTH system, is the confirmation of co-expression of the two binding partners either in the same cell-type or in 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 determine the subcellular localization of these proteins, the particular structures to which they associate and the processes where they are involved. With this intent, the subcellular distribution and co-localization with PP1α was evaluated for taperin. The expression of taperin fused to GFP in HeLa cells revealed that it is mainly nuclear, and co-localizes with PP1α in this subcellular compartment. Increasing expression of taperin enhances the amount of PP1α in the cell, although it remains to verify if this is due to a gene transactivation effect or to an alteration on mRNA/protein half-life. Furthermore, cytoplasmic/plasma membrane localization of taperin recruits PP1α to the same subcellular compartments (Figure 4). In another study, overexpression of taperin recruited most of the nuclear PP1γ, including the nucleolar pool, to the nucleoplasm, and mutation of the PP1 BM abolished relocalization of PP1γ (Ferrar, 2012). It has been shown that over-expression of PP1 regulatory subunits can cause a relocation of PP1 itself, which functions as a strong indicator of their *in vivo* interaction (Trinkle-Mulcahy, 2001).

In our co-localization studies it became clear that not only PP1 and taperin but also actin co-localize in the membrane (Figure 6A). In fact, when taperin was mutated in the PP1

BM, this re-localization of PP1 and its co-localization with actin was not seen, suggesting that taperin interaction with actin might be dependent on or regulated by PP1 binding.

Already known PIPs also bind to actin

Actin is a eukaryotic protein implicated in a number of cellular activities. It comprises a highly conserved family of proteins that fall into three broad classes: α, β and γ isoforms. It is mainly located in the cytoplasm, but it is also present in the nucleus (dos Remedios, 2003). Cellular actin plays a variety of roles including myosin-independent changes of cell shape, motor-based organelle transport, regulation of ion transport, and receptor-mediated responses of the cell to external signals (dos Remedios, 2003). Many of these processes require the dynamic behavior of the actin cytoskeleton which involves the polymerization and depolymerization of actin filaments (Welch, 1997). Monomeric actin (globular/G-actin) polymerizes in a head to tail fashion to form helical actin filaments (filamentous/F-actin) (Wegner, 1976; Pollard, 1981; Holmes, 1990). Several types of actin binding proteins (ABPs) facilitate the disassembly and assembly of actin. ABPs can be classified as: monomer-binding proteins, filament-depolymerizing proteins, filament end-binding (capping) proteins, filament severing proteins, cross-linking proteins, stabilizing proteins and, finally, motor proteins (dos Remedios, 2003). Some ABPs are not limited to one class and many bind to the same loci on the surface of actin and, therefore, can bind with positive cooperativity, and tend to form ternary complexes, but rather more bind with negative cooperativity (dos Remedios, 2003).

PP1α and PP1γ1, but not PP1β, are enriched in dendritic spines (da Cruz e Silva, 1995; Ouimet, 1995; Strack, 1999) where they associated with the actin-rich structure known as the post-synaptic density (PSD) (Terry-Lorenzo, 2000). Neurabin I (PPP1R9A) and

Neurabin II (Spinophilin/PPP1R9B) also localize in PSD, bind PP1 and are actin cross-linking proteins (Allen, 1997; Nakanishi, 1997; Satoh, 1998; MacMillan, 1999; Oliver, 2002; Terry-Lorenzo, 2002a). In parallel with its localization, Neurabins also display a significant preference for PP1α and PP1γ1 over PP1β (Colbran, 1997), in fact there is a selective targeting of PP1α and PP1γ1 to the neuronal actin cytoskeleton (Terry-Lorenzo, 2002a). Taperin was also found to preferentially bind PP1α over PP1γ, and not to bind PP1β (Ferrar, 2012).

Neurabin I is specifically expressed in neural tissue whereas Neurabin II is ubiquitously expressed (Nakanishi, 1997; Satoh, 1998). The later was found to be also enriched in adherens junction fractions in rat liver and at the cadherin-based cell-cell adhesion sites in Madin-Darby canine kidney cells, playing an important role in linking the actin cytoskeleton to the plasma membrane (Satoh, 1998). Also a multiprotein complex containing Neurabin, PP1, and I2 (PPP1R2) that potentially regulates dephosphorylation events at the actin cytoskeleton, has been described (Terry-Lorenzo, 2002b). Binding of Neurabin II to F-actin is inhibited by phosphorylation by PKA (Hsieh-Wilson, 2003) or CaMKII (Grossman, 2004). The Neurabin I/PP1 complex controls actin rearrangement to promote spine development in mammalian neurons (Oliver, 2002). Disrupting the functions of cytoskeletal Neurabin/PP1 complex enhances filopodia and impaired surface AMPA-type glutamate receptors expression in hippocampal neurons, hindering the morphological and functional maturation of dendritic spines (Terry-Lorenzo, 2005). Postsynaptic actin-bound Neurabin I-PP1 complex regulates synaptic transmission and bidirectional changes in hippocampal plasticity (Hu, 2006). Neurabin II also facilitates the dephosphorylation of doublecortin by PP1 to mediate microtubule bundling at the axonal wrist (Bielas, 2007).

Phostensin was identified as a PP1 F-actin cytoskeleton targeting subunit (Kao, 2007). Localization studies revealed that the phostensin/PP1 complex was localized with the actin cytoskeleton at the cell periphery in Madin–Darby canine kidney epithelial cells. Phostensin targets PP1 to F-actin cytoskeleton and the complex may play a vital role in modulating actin rearrangements (Kao, 2007). Phostensin was later found to decrease the elongation and depolymerization rates of actin filament pointed ends, suggesting that phostensin is an actin filament pointed end-capping protein that is capable of modulating actin dynamics (Lai, 2009).

Analyzis of taperin sequence showed that it has some homology with phostensin (Rehman, 2010), the region with more similarity containing the KISF PP1 BM, present in both (Ferrar, 2012). The presence of the same PP1 BM in both proteins could indicate that they can even compete for binding to PP1, once they also have the same localization in the membrane.

Actin, PP1 and taperin nuclear function

In the nucleus, actin is part of the chromatin remodeling complex, it is associated with the transcription machineries, it associates with newly synthesized ribonucleoproteins, and it influences long-range chromatin organization [for review see (Visa, 2010)]. PP1 is also involved in transcription, mRNA processing and translation [for review see (Ceulemans, 2004 and Moorhead, 2007)]. We found, by sequence analysis, three NLSs and one NES in taperin (Figure 1), and this protein was previously shown to translocate between cytoplasm and nucleus, besides having two RGG motifs that may facilitate nucleic acid binding (Ferrar, 2012). Hence, most likely, taperin also has important roles in the nucleus and possibly related to nucleotides regulation, once it is recruited to sites of DNA damage

and associates with several proteins known to play a role in the DNA damage response (Ferrar, 2012). In our study in HeLa cells, when taperin mutated in the PP1 BM was used, taperin and actin were accumulated in the nucleus, and taperin-actin nuclear export could therefore be dependent on PP1 binding.

Taperin alters cell morphology and actin dynamics

According to our results, transfected HeLa cells with high levels of cytoplasmic taperin presented a more elongated morphology and relocalized PP1 to the membrane. On the other hand, when a PP1 BM mutant form of taperin was expressed, the levels of cadherin and F-actin in the membrane were decreased and PP1 no longer localized to this structure (Figure 6B).

Cadherins interact homo and heterophilic via their extracellular domain with cadherins on adjacent cells (Yamada, 2005). The cytoplasmic region of cadherin binds β-catenin; and this catenin, in turn, associates with α-catenin (Abe, 2008). Binding between cadherin extracellular domains is relatively weak, but cell-cell adhesion may be strengthened by lateral clustering of cadherins mediated by protein linkages between the cadherin cytoplasmic domain and the actin cytoskeleton (Jamora, 2002). Biochemical studies showed that α-catenin can interact with actin filaments (Rimm, 1995; Pokutta, 2002), but, more recently, it was shown that α-catenin binding to β-catenin and α-catenin binding to actin filaments are mutually exclusive (Yamada, 2005). Several ABPs interact with α-catenin and could mediate linkage of the cadherin-catenin complex to actin filaments (Jamora, 2002).

In HeLa cells where wildtype taperin-KISF was present, and especially when taperin was at the cytpoplasm/plasma membrane, F-actin had mainly a cortical distribution and, in

cells expressing the taperin-KISA, F-actin staining was highly decreased, the remaining F-actin was also cortical, indicating massive F-actin depolymerisation (Figure 6C). In none of the conditions actin presented a normal distribution and polimerization dynamics. We can conclude that overexpression of taperin induces actin depolimerization, a process highly regulated by taperin binding to PP1, which appears to be a negative regulator of taperin-induced actin depolymerisation. We thus hypothesize that taperin-PP1 complexes can reach the plasma membrane, where they bind to F-actin and regulate its rate of (de)polymerization.

Actin and taperin in sperm

In human sperm the regions reported to contain actin include the acrosomal space, the equatorial and post acrosomal regions, and the tail (Clarke, 1982; Virtanen, 1984; Ochs, 1985; Fouquet, 1992). The presence of actin in the tail might be important for the regulation of sperm motility, and its presence in the head suggests a possible involvement in the acrosome reaction. It was reported that actin polymerization is important for initiation of sperm motility during post-testicular maturation (Lin, 2002).

Only capacitated sperm can undergo the acrosome reaction after binding to the egg zona pellucida, a process which enables sperm to penetrate into the egg and fertilize it (Wassarman, 1988; Breitbart, 2003). Polymerization of G-actin to F-actin occurs during capacitation, depending on protein kinase A activation, protein tyrosine phosphorylation, and phospholipase D activation (Brener, 2003; Cohen, 2004). Activation of protein kinase C during bovine sperm capacitation causes a rapid increase in actin polymerization which is followed by fast depolymerisation (Cohen, 2004). F-actin formation is important for the translocation of phospholipase C from the cytosol to the sperm plasma membrane

during capacitation (Breitbart, 2005; Lai, 2009). Prior to the occurrence of the acrosome reaction, F-actin should undergo depolymerization, a necessary process which enables the outer acrosomal membrane and the overlying plasma membrane to come into close proximity and fuse. The binding of the capacitated sperm to the zona pellucida induces a fast increase in sperm intracellular calcium, and the activation of actin severing proteins that break down the actin fibers, and allows the acrosome reaction to take place (Tomes, 1996; O'Toole, 2000).

Taperin was found to also bind the PP1γ2 isoform (Figure 2A), an isoform very enriched in testis and sperm. In sperm, PP1γ2 is present along the entire flagellum including the middle-piece, consistent with a role in sperm motility, but it is also found in the posterior and equatorial regions of the head, suggesting a role in the acrosome reaction (Huang, 2002). In testis, PP1γ2 forms an inactive complex with actin, sds22 (PPP1R7), I3 (PPP1R11), the latter being a potent PP1 inhibitor (Cheng, 2009). The formation of the complex was described in both differentiating and terminally differentiated male germ cells, in which PP1γ2 appears to be held in a catalytically inactive state. Of note, PP1γ2 was also shown to have an anti-apoptotic effect in the testis, possibly mediated by its ability to enhance the stability of I3 (Cheng, 2009).

By immunohistochemistry, taperin staining was found in elongating spermatids with acrosome pattern (Figure 7). Fluorescence was weak in the nucleus of spermatocytes and round spermatids, while spermatogonia and almost mature spermatozoa cells showed no immunoreactive taperin staining. This suggests that taperin is expressed in an advanced phase of sperm maturation, but not in mature sperm. Isoforms 1 and 3 of taperin showed a high relative expression level in spermatocytes. However, whereas taperin 1 maintains its levels in round spermatids and lowers in elongating spermatids, taperin 3 expression lowers in round spermatids and recovers in elongating spermatids to the same levels seen

in the whole testis lysates, which is in accordance to the immunohistochemistry results (Figure 7).

Taperin, actin and PP1 share the same localization in the acrosome, which suggests that in sperm they could form a complex, as was seen in HeLa cells, with a possible function in capacitation and acrosome reaction through the regulation of F-actin dynamics.

Taperin shuttles between the nucleus and cytoplasm, where it can bind PP1 and actin, and it would be interesting to perform functional assays for both, the nuclear and the cytoplasmic taperin pools. Since it was also found to be cleaved, it remains necessary to analyze if the two protein forms are targeted to different subcellular compartments. Taperin and actin direct binding steel needs to be confirmed, but the complex PP1-taperin appears to have a role in regulating actin dynamics. In sperm, taperin may have a function in the acrosome, since it localizes in this structure, where PP1γ2 and actin may have important roles in capacitation and acrosome reaction.

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Author Disclosure Statement

No competing financial interests exist.

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Supplementary data

Table 1 - List of primers used in the qRT-PCR.

Primer	*Gene*	Sequence	Positions	Acession number
Mus musculus, Taperin 1- FW	*Tprn*	ACCGAAGGAGGTCATGCTCACAC	2262 – 2284	NM_175286.4
Mus musculus, Taperin 1 - RV		TTGACTGGCTTCCAGGACCACC	2404 – 2383	
Mus musculus, Taperin 3- FW		GAAGGAGGTCATGGTAAGCCAGGTG	2265 – 2289	
Mus musculus, Taperin 3- RV		GCAGGTGTGAGCTTATCCAGGCTTC	2373 – 2349	

Table 2 - **mRNA expression levels of Taperin isoforms**. Isolated cell fractions were spermatocytes (Spc), round spermatids (rSpt) and elongated spermatids (eSpd). Relative expression values were calculated by the ΔΔ Ct-method. Increased mRNA expression is indicated by orange shading and decreased expression levels by blue shading. Light shading indicates alterations above 1.5-fold or below 0.75-fold and intense shading indicates changes in expression levels of more than 2-fold or below 0.5-fold

Gene	Isoforms	Spc	rSpd	elSpd	Hippocampus
Tprn	Taperin 1	1,863	1,521	0,27	0,002
	Taperin 3	2,488	0,624	1,139	0,027

Manuscript 4 - Characterization of the interaction between Synphilin-1A and Protein Phosphatase 1

In preparation

Characterization of the interaction between Synphilin-1A and the Protein Phosphatase 1

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Abstract

Aggregation of proteins that lead to the formation of inclusion bodies in neurons is a common feature of many neurodegenerative diseases. In Parkinson`s Disease (PD) this inclusion bodies are referred as Lewy Bodies (LB) and result from the aggregation of several proteins, being α-Synuclein the most studied. Synphilin-1 is another component of LBs and was first identified as a binding partner of α-Synuclein. Synphilin-1A, an alternative spliced isoform of Synphilin-1, was described as aggregation-prone and toxic to neurons. It forms inclusions and recruits α-Synuclein and Synphilin-1 to the inclusion bodies. Diverse factors lead to the development of LBs in the affected cells, but deregulation in phosphorylation is probably one of the most relevant. Many kinases involved in those deregulated events are known but the counterpart protein phosphatases are far from being elucidated. Here we report the interaction of Synphilin-1A and Protein Phosphatase 1 (PP1), a Ser/Thr phosphatase, highly expressed in brain tissues and enrolled in many signaling pathways. The interaction was identified by the Yeast Two Hybrid system and validated by biochemical methods and by co-localization studies. Mutation of the PP1 binding motif, present in the vast majority of the PP1 binding proteins and also in Synphilin-1A, strongly decreases the interaction. Finally, an interaction mapping of α-Synuclein, Synphilin-1 and PP1 was made by a large database search of protein-protein interactions and five proteins were found to be common interactors, suggesting functional relationship among them in the process of LBs formation.

Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized by the degeneration and progressive loss of dopaminergic neurons in the substantia nigra pars compacta, being one of the most common neurodegenerative diseases.

An hallmark of PD is the presence of cytoplasmic inclusions known as Lewy bodies (LBs) in surviving neurons. A large number of proteins have been identified in LBs (Shults, 2006; Beyer, 2007; Wakabayashi, 2007), but the process of LB formation remains unclear. Alpha-Synuclein (α-Syn), a presynaptic protein of 140 amino acid residues, is the major component of protein inclusion found *post-mortem* in affected subjects with synucleinopathies like PD (Spillantini, 1997), dementia with LBs (Spillantini, 1998b), and multiple system atrophy (Spillantini, 1998a; Tu, 1998), in which it is assumed to be in a fibrillar β-sheet conformation (Yoshimoto, 1995; El-Agnaf, 1998). α-Syn (non-A4 component of amyloid precursor) is one of three synuclein family members (α, β, γ) encoded by the gene SNCA identified in humans (Jakes, 1994; Polymeropoulos, 1997; Lavedan, 1998). Three mutations in the SNCA gene have been found in familial PD: A53T, A30P and E46K, (Polymeropoulos, 1997; Kruger, 1998; Zarranz, 2004), besides duplications and triplications of the gene (Nuytemans, 2010). In fact, overexpression of these mutant forms in neuroblastoma cells and in mice was found to stimulate aggregate formation (Ostrerova-Golts, 2000; Sung, 2001; Kang, 2011).

Another important component of LBs is synphilin-1 (Sph1), which was shown to interact with α-Syn (Engelender, 1999; Wakabayashi, 2000; Kawamata, 2001; Xie, 2009). Sph1 is a protein with 919 amino acid residues and, although its physiological

functions still needs to be clarified, it is known that it gradually accumulates from neuronal cell bodies to nerve terminals during development, and that it is enriched in the presynaptic terminals, where it associates with synaptic vesicles (Ribeiro, 2002).

Overexpression of Sph1 in cultured cells induces large juxtanuclear ubiquitin-positive inclusions (O'Farrell, 2001; Lee, 2002) and its co-transfection with the non-A component of α-Syn leads to the formation of inclusion bodies (Ribeiro, 2002). Sph1 has also been implicated in PD as a protein ubiquinated by Parkin for which loss of function results in juvenile or early-onset disease (Chung, 2001; Ribeiro, 2002). More recently, it was reported that expression of Sph1 in mouse brain by adenoviral vectors induces the formation of aggregates and death of dopaminergic neurons (Krenz, 2009). These observations, all together, suggest that the interaction of α-Syn and Sph1 may contribute to the formation of cellular inclusions and the neural degeneration in PD.

Sph1 has several protein-protein interacting motifs, including Ankyrin repeats, a coiled coil domain and an ATP and GTP binding domain (Engelender, 2000). By comparing the human and mouse Sph1 cDNA sequence it was confirmed that the regions containing these protein interacting motifs were among the most conserved together with a relatively long internal conserved region (O'Farrell, 2002). Amino acid substitutions in any of these domains impair protein function (Marx, 2003).

Of note, Sph1 phosphorylation by GSK3β blocks its ubiquitylation and decreases inclusion body formation and, phosphorylation by CKII decreases its interaction with α-Syn and, consequently, inclusions formation but not its ubiquitylation (Lee, 2003; Avraham, 2005). Thus the interaction between Sph1 and α-Syn is regulated by phosphorylation and it seems that the phosphorylation of Sph1 is more critical to this interaction (Lee, 2003). Sph1 also interacts with other proteins involved in PD such as

Parkin, Lrrk2 and SIAH (Chung, 2001; Liani, 2004; Smith, 2006). An alternative spliced isoform of Sph1, synphilin-1A (Sph1A), was also found in PD LBs and shown to interact with α-Syn and Sph1 (Eyal, 2006a; Eyal, 2006b; Szargel, 2009). Sph1A is an aggregation-prone protein that can cause neuronal toxicity, and has a higher tendency to aggregate than Sph1 (Eyal, 2006b).

Although many efforts have been made in the identification of the kinases involved in the phosphorylation of α-Syn and Sph1 (Lee, 2003; Avraham, 2005; Ishii, 2007; Szargel, 2008; Inglis, 2009; Qing, 2009) much less data is available on the phosphatases responsible for the dephosphorylation of these proteins. So far, evidence exist supporting *in vitro* dephosphorylation of α-Syn by PP2A and/or PP1, and PP2C (Ishii, 2007; McFarland, 2008; Waxman, 2008). These results need further clarification and there are no specific data regarding Sph1 dephosphorylation.

Protein Phosphatase 1 (PP1) is a major protein Ser/Thr phosphatase involved in a broad diversity of cellular functions. The holoenzyme consists of a catalytic subunit (PP1c) and a regulatory subunit (PP1 Interacting Proteins - PIPs). PP1c exists as three isoforms: PP1α (PPP1CA), PP1β/δ (PPP1CB) and PP1γ (PPP1CC) with two splice variants PP1γ1 (PPP1CC1) and PP1γ2 (PPP1CC2). Of all mammalian tissues, the brain expresses the highest levels of protein kinases and phosphatases. All PP1 isoforms are ubiquitously expressed but PP1γ1 and PP1α are expressed at higher levels in several brain regions (Takizawa, 1994; da Cruz e Silva, 1995; Ouimet, 1995; Strack, 1999; Bordelon, 2005).

Sph1A was shown to interact with PP1 in human brain using the Yeast Two Hybrid (YTH) system (Esteves, 2012a). Here, we validated the interaction by overlay and demonstrated that the conserved PP1 binding motif “RVTF” (Wakula, 2003; Meiselbach, 2006; Hendrickx, 2009), which is present in Sph1A, was crucial for the

interaction. Additionally, Sph1A and PP1 co-localized in aggregates present in Cos-7 cells over-expressing Sph1A-GFP. Sph1A mRNA was also found in late stages of sperm maturation, a process in which PP1 is believed to have an important role.

Material and Methods

PP1-Synphilin-1A binding by yeast co-transformation

Small-scale LiAc yeast transformation procedures were performed combining the bait plasmid pAS2-PP1α (Gal4 binding domain expression vector) with specific Sph1A positive clones isolated from an human brain cDNA library in vector pACT2 (Gal4 activation domain expression vector), as previously described (Esteves, 2012a).

In brief, salmon testes carrier DNA was added to the plasmidic DNA and then freshly prepared yeast strain AH109 competent cells, followed by 600 µL of sterile PEG/LiAc (40% PEG 4000/ 1X TE/ 1X LiAc). The mixture was incubated at 30 ºC for 30 minutes (min) with shaking (200 rpm). After adding DMSO the solution was mixed gently and then heat-shocked at 42 ºC for 15 min. The cells were chilled on ice and pelleted by centrifugation for 5 sec at 14,000 rpm and resuspended in 0.5 mL of 1X TE buffer. In parallel, co-transformation with the empty vectors pAS2-1 and pACT-2 was performed, as a negative control. The association of murine p53 (encoded by plasmid pVA3) and SV40 large T antigen (plasmid pTD1) served as a positive control. To confirm protein-protein interactions, the fresh diploid colonies were assayed for growth on SD/QDO (high strigency medium) plates with X-α-Gal to check for MEL-1 expression (indicated by the appearance of blue color). All reagents and media were purchased from Clontech.

Sph1A constructs

pET-Sph1A - Sph1A cDNA, IMAGE clone 4838145 (BC094759.1), was subcloned in pET-28a vector (Novagen). The cDNA was PCR amplified using specific primers with

SalI and XhoI restriction sites. PCR product and pET vector were digested with SalI and XhoI (New England Biolabs), the manufacturer's instructions were followed. Ligation was performed according to ligase manufacturer's instructions (New England BioLabs).

Sph1A-GFP - Sph1A cDNA, IMAGE clone 4838145 (BC094759.1), was subcloned in pEGFP-N1 vector (Clontech). Sph1A cDNA was amplified by PCR using specific primers with XhoI and SalI restriction sites. PCR product and pEGFP vector were digested with XhoI and SalI (New England Biolabs), the manufacturer's instructions were followed. Ligation was performed according to ligase manufacturer's instructions (New England BioLabs).

Direct Mutagenesis

pET-Sph1A-RVTA and Sph1A-RVTA-GFP - Mutagenesis of the PP1 BM, RVTF to RVTA, in Sph1A cDNA was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene now Agilent Technologies) by mutating the last amino acid to alanine in order to disrupt the PP1 BM.

Overlay blot assay

A single *Rosetta* (DE3) (Novagen) colony expressing His-tagged Sph1A was selected and grown overnight in 3 ml Luria Bertani medium containing ampicillin (50 μg/mL) at 37ºC. Expression was induced with 0,1mM isopropyl--D-thio-galactopyranoside at 37ºC. Samples were then treated as described elsewhere (Browne, 2007). The same procedure was also performed for pET-Sph1A-RVTA, pET-NEK2A (Wu, 2007) and pET empty. Bacteria cells lysates were separated on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with TBST/5% non-fat milk for

1 hour (hr) and then overlaid with purified PP1γ1 protein (1 μg/mL) (Browne, 2007) in TBST/3% non-fat milk for 1 hr. After washing three times with TBST, to remove excess protein, the bound PP1γ1 was detected by incubating the membrane with anti-PP1γ antibody in TBST/3% non-fat milk, for 1 hr. Immunoreactive bands appeared after incubating with horseradish peroxidase conjugated secondary antibody, in 3% non-fat milk in TBST for 1 hr, and developing with ECL (Pierce).

Antibodies

Anti-PP1α (CBC2C) antibody - antiserum was raised in rabbits against the PP1α C-terminal peptide, NKGKYGQFSGLNPGG. Anti-PP1γ (CBC3C) antibody - antiserum was raised in rabbits against the PP1γ C-terminal peptide, KKPNATRPVTPPRGMITKQAKK, which detects the two γ isoforms (da Cruz e Silva, 1995); both were affinity purified.

Cell culture and transfection

Monkey kidney COS-7 cells were maintained with Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 U/ml penicillin/100 mg/ml streptomycin (p/s) and 3.7 g/l $NaHCO_3$ (complete DMEM) at 37°C and 5% CO_2 For transfection, the culture medium was replaced with complete medium (antibiotic/antimycotic-free) and the DNA diluted in Opti-MEM (serum- and antibiotic/antymicotic-free). The Lipofectamine 2000 reagent (Invitrogen) was diluted appropriately in the same medium, and the DNA solution was added to the Lipofectamine solution drop by drop, mixed by gentle bubbling with the pipette and allowed to rest for 25 min at room temperature. The complexes 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 24 hrs.

Immunocytochemistry

Cells were cultured in glass coverslips until 80-90% confluence and transfected as described above. Each well was washed three times with 1X PBS and 4% paraformaldehyde fixative solution was then gently added and left to stand for 30 min. Finally, cells were washed three times with 1X PBS. For permeabilization, methanol was added for 2 min followed by 5 washes with 1X PBS. Blocking was carried out for 1 hr with PBS/3%BSA, and then primary antibody diluted in PBS/3% BSA was added and incubated at room temperature for 2 hrs. After three washes with 1X PBS, the secondary antibody was added using the same methodology and incubated for 2 hrs. Finally, three washes were performed and coverslips were mounted on microscope glass slides with anti-fading reagent containing DAPI for nucleic acid staining (Vectashield, Vector Laboratories). Epifluorescence microphotographies were acquired with an Olympus IX-81 inverted epifluorescence microscope, equipped with EGFP (Chroma 41020) and Texas Red (Chroma 41004) filter cubes for fluorophore microscopy visualization.

Animals

C57/Bl6 mice were used for the isolation of testicular germ cells. Housing of mice was approved by the responsible governmental commission and all animals were kept under standard conditions at the animal facility of the Philipps University of Marburg, with light and dark cycles of 12 hrs. Male mice of 6–10 weeks of age were used

Isolation of testicular germ cells

Isolation of cell populations from mouse testis was performed as previously described (Bellve, 1993). Animals were killed by cervical dislocation and testes were removed and decapsulated. Decapsulated testes of six C57/Bl6 mice were incubated in PBS without Ca/Mg (PAA, Colbe), supplemented with collagenase, dispase and DNase (20 µg/mL each) at 37°C for 30 min, in a Falcon tube under slow rotating motion to dissociate the seminiferous tubules and to liberate interstitial cells. The suspension was then filtered through a Nylon sieve (mesh with 70-µm pore size) and the retained seminiferous tubules were resuspended in the same buffer and triturated with a Pasteur pipette until they were dissociated to a single cell suspension. Cells were sedimented by centrifugation for 10 min at 500g at 4°C to remove the enzymes and resuspended in DMEM (PAA, Colbe) containing 0.5% BSA (w/v). Cells were further subjected to velocity sedimentation at unit gravity for 2 hrs in a BSA gradient (1–5% of BSA (w/v) in DMEM), using a Celsep sedimentation chamber (ECET Celsep-System 5440, Eppendorf) with a cushion of DMEM supplemented with 10% (w/v) BSA at the bottom of the sedimentation chamber (total volume 1 L). Gradient fractions of 50 mL each were collected and cells were sedimented by centrifugation at 500g for 10 min at 4°C. Isolated cells were analyzed by phase contrast microscopy and DAPI staining, homogeneous cell populations were used for further analysis. Isolated cells were processed for RNA isolation (RNeasy mini kit, Qiagen).

Expression analysis in isolated testicular germ cells

Total RNA from the isolated cells was prepared using the RNeasy Plus Universal Midi Kit (Quiagen). The concentration of RNA was determined spectrophotometrically

(NanoDrop 1000 Spectrophotometer, Thermo Scientific). For synthesis of cDNA, a reverse transcription reaction was carried out using 1 µg of RNA and the Transcriptor First Strand cDNA Synthesis Kit for RT-PCR (Roche). Expression levels of distinct mRNAs were determined by qRT-PCR using the LightCycler 480 SYBR Green I Master (Roche) and the following cycle conditions. An initial denaturation at 95°C for 15 min was followed by 45 cycles of denaturation (94°C, 15 s), annealing (58°C, 30 s) and polymerization (72°C, 30 s). Thereafter, a melting curve was generated over temperatures of 55–95°C with 30 s/1°C.

PCR analysis was performed on 96-well plates with the LightCycler® 480 Real-Time PCR System (Roche). A combination of *Gapdh* and *Hprt* was identified as optimal reference genes for the testis and all expression levels were calculated as relative values using the mean of both reference genes. All samples were run in triplicate and the averages were used for the calculation of the relative expression levels of the genes. The expression quantification of the target gene was calculated using the difference of the C_T-values (ΔC_T) from the mean of the target gene and the mean of the C_T-values from both housekeeping genes (C_T reference gene). The expression levels were further related ($\Delta\Delta C_T$) to control samples using the difference of the ΔC_T-value from the sample (ΔC_T sample) and the ΔC_T-value from a control (ΔC_T control) and the relative values were calculated as the $2_T^{-\Delta\Delta C}$ (Livak, 2001). A cDNA obtained from the cell suspension of whole testis was used as control sample for qRT-PCR reactions. All primers used for qRT-PCR are listed in supplementary Table 1.

Databases search of Protein-Protein Interactions (PPIs)

The human specific α-Syn, Sph1 and PP1 interactors were retrieved from thirteen available online databases: APID, BioGRID, HPRD, InnateDB, IntAct, iRefIndex, KEGG PATHWAY, MINT, Ophid, PINA, Reactome-FLS, Spike and STRING. Also, PPIs from previews work were used (Esteves, 2012a,b). Afterwards, an exhaustive analysis to the PPIs was made and the proteins were grouped; only the interactions common to at least two of the proteins search were considered for this analysis. The UniProt accession numbers were used to normalize all proteins found, in order to avoid protein duplications of alias from different databases. The interactions maps were made using Cytoscape (Shannon, 2003).

Results

Interaction of Synphilin-1A with PP1

PP1, a Ser/Thr phosphatase, whose function is dependent on the proteins it binds to, has more than 200 PIPs described so far (Moorhead, 2008; Hendrickx, 2009; Fardilha, 2010; Heroes, 2012). We have already described the PP1 interactomes from human brain and testis, by carrying out YTH screens using PP1α, γ1 and γ2 as bait, that yielded several PP1 specific-isoform binding partners (Fardilha, 2011b; Esteves, 2012a,b). Sph1A was found to interact with PP1 in an YTH screen of a human brain library using the bait PP1α (Esteves, 2012a). Six clones, from the total of 298 obtained, corresponded to this isoform of Sph1. The PP1-Sph1A interaction was further confirmed by other techniques. None of the clones obtained corresponded to the complete sequence of Sph1A, the positions of the beginning of the YTH clones are indicated in the amino acids sequence in Figure 1A (arrows).

The majority of PIPs have one or more PP1 binding motifs (PP1 BM), being the so called RVxF motif the most common and described, with its consensus sequence being continuously updated in the last decade (Wakula, 2003; Meiselbach, 2006; Hendrickx, 2009). By analysis of Sph1 and 1A amino acid sequences it was found that both contain an RVxF PP1 BM (RVTF, Figure 1) at their C-terminal. As it was previously described, mouse Sph1 shows extensive homology with the human form, with greatest similarities in the regions that contain the Ankyrin-like motifs and the coiled-coil domain (O'Farrell, 2002). In fact, Sph1 is a highly conserved protein, not only in primates and mammals, but also in zebrafish. Sph1 PP1 BM RVTF is also conserved among some species analyzed: Homo sapiens, NP_005451.2 and NP_001229864.1; Pan troglodytes, XP_003310838.1; Macaca mulatta, XP_001090698.1; Canis lupus familiaris,

XP_538600.3; Bos taurus, NP_001192707.1; Mus musculus, NP_080684.2; Rattus norvegicus, NP_001100849.1; Gallus gallus, XP_424409. and Danio rerio, NP_001107108.1 (Figure 1B).

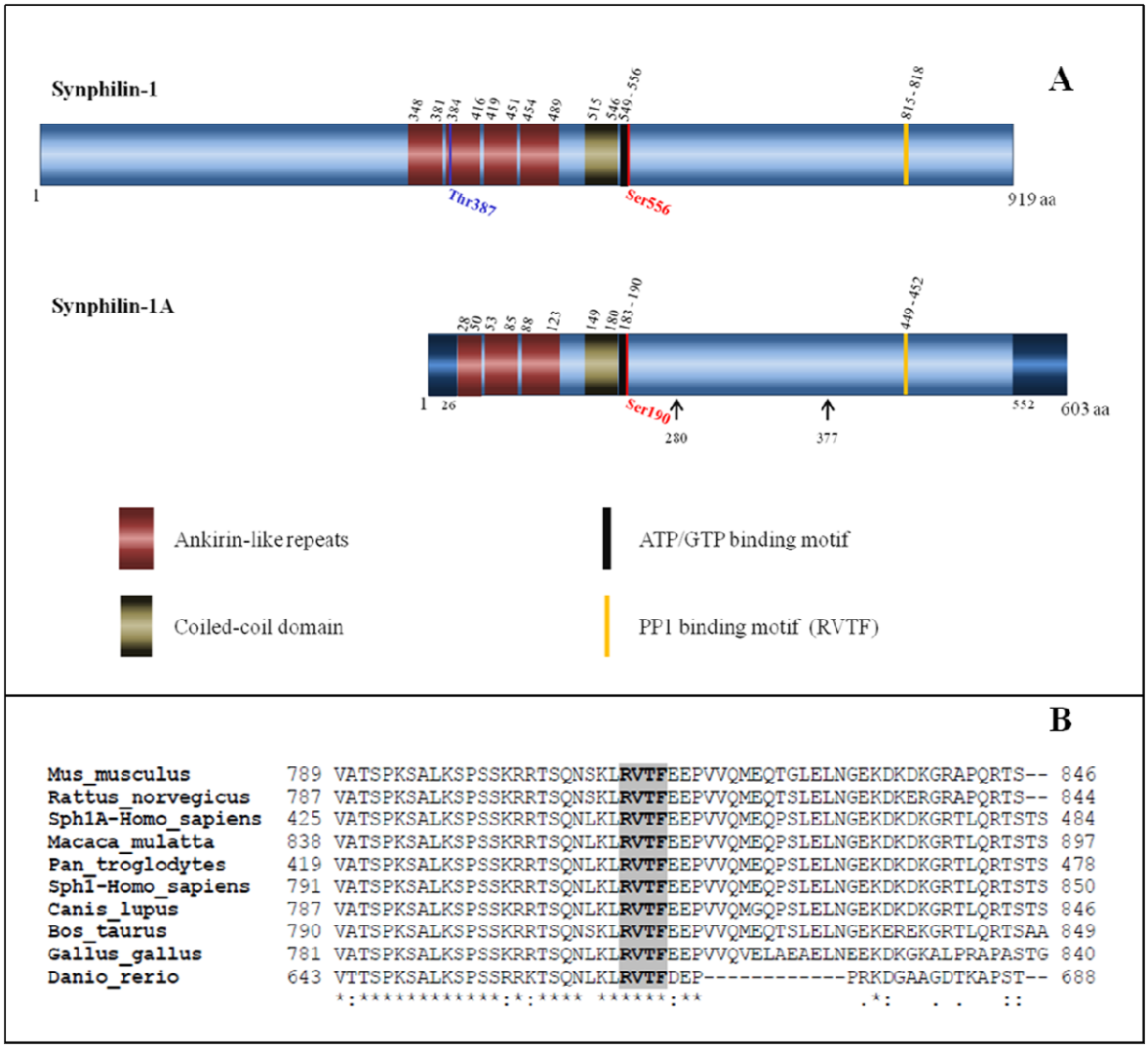

Figure 1 | Synphilin-1 and Synphilin-1A isoforms. **A.** Schematic representation of Synphilin-1 and 1A amino acid sequences, domains and motifs (indicated below the diagram). Due to differential alternative splicing, Synphilin-1A lacks part of the Synphilin-1 N-terminus, including the first and part of the second ankyrin-like domains. In addition, it contains additional amino acid stretches at the N-terminus (28 amino acids) and C-terminus (51 amino acids), shown in dark blue. **B.** ClustalW2 alignment of human Synphilin-1 and 1A isoforms and its homologues. Protein sequences were obtained from GenBank and HomoloGene. Grey shadow indicates conservation of the RVTF PP1 BM motif across the species. Numbers indicate position on the amino acid sequences.

The interaction of all the Sph1A clones, identified in the YTH screen (Esteves, 2012a), and PP1 isoforms was tested by yeast co-transformation (Figure 2A). The interaction of all clones with PP1α was corroborated, and they all also interacted with the others two PP1γ isoforms (Figure 2A). Light blue colonies represent positive interactions also, but took longer to turn blue in the presence of X-α-Gal. This is in accordance with the presence of a PP1 BM (RVTF) predicted by bioinformatic analysis and present in all the Sph1A positives isolated. Clone B in Figure 2A is the only one that stars at the position 280 of Sph1A amino acid residue sequence; all the others start at position 377 (Figure 1A). The fact of the clones not having the complete Sph1A sequence was not sufficient to hamper the interaction with the PP1 isoforms, in accordance to the presence of the PP1 BM (Figure 1)

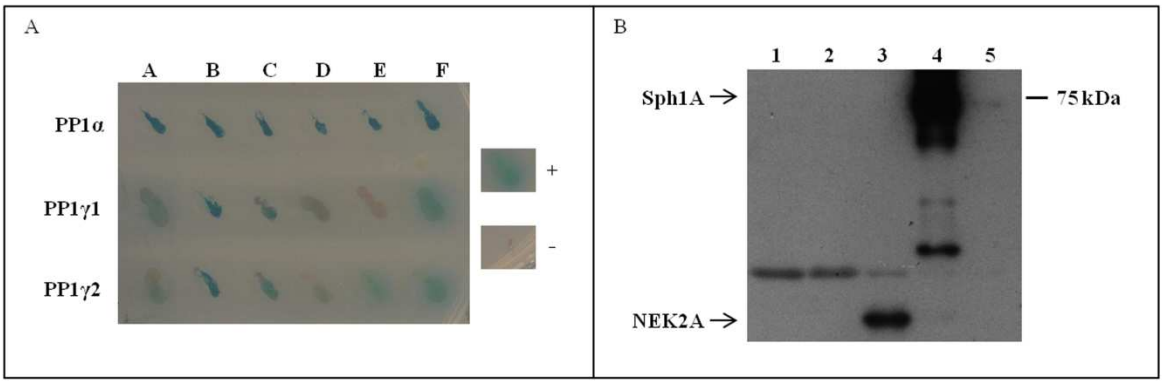

Figure 2 | **Synphilin-1A binds PP1**. **A.** Analysis of interaction of Synphilin-1A YTH clones and PP1 isoforms by yeast co-transformation. Blue color indicates positive interaction. Plus and minus represent positive and negative controls, respectively. **B.** Overlay assay of Synphilin-1A and PP1γ1. Detection with anti-PP1γ antibody(CBC3C). Lanes: **1**- Non transformed Rosetta cells (20µg of total protein loaded); **2** - pET vector (20µg); **3** - pET-NEK2A (1µg); **4** - pET-Sph1A-WT (1µg) and **5** - pET-Syn-1A-MT (1µg). WT, wildtype; MT, mutant.

In order to further characterize the interaction between PP1 and Sph1A, a mutant form of Syph1A, in the RVxF PP1 BM, was created. The mutant Sph1A was obtained by directed mutagenesis of the phenylalanine residue in the RVTF PP1 BM motif to alanine, originating the motif RVTA. To this end, the cDNA from the clone IMAGE 4838145 (BC094759.1) was used, once none of the YTH clones was complete. This cDNA clone corresponds to the complete CDS, but has an aspartic acid in position 346 instead of a glycine, this substitution is not located in any of the Sph1A relevant motifs.

When the mutant form of Sph1A, pET-Sph1A-RVTA expressed in bacteria, was used on an overlay assay with PP1γ1, a significant decrease in the interaction was detected, when compared with the wild type Sph1A, and the well known PIP, Nek2A (Wu, 2007) (Figure 2B).

The expression of Sph1A-GFP fusion protein in Cos-7 cells allowed the analysis of its subcellular localization, aggregates formation and co-localization with PP1α and γ isoforms. Transfected Cos-7 cells with Sph1A-GFP showed numerous aggregates with an exclusively cytoplasmic distribution (Figure 3), both for the wildtype (WT - PP1 BM RVTF) and mutant (MT – PP1 BM RVTA) forms of Sph1A. PP1α (Figure 3A and C) and PP1γ (Figure 3B and D) co-localized with Sph1A on the aggregates; although in a less extend on the aggregates formed by the RVTA form of Sph1A.

The expression of the MT Sph1A did not originate fewer aggregates; in fact, there was no difference in the number and types of the aggregates formed by WT and MT Sph1A (Table 1). Our original hypothesis was that PP1 could be a phosphatase involved in the regulation of Sph1/1A-α-Syn interaction, by controlling the phosphorylation status of at least Sph1/1A. It is not probable that α-Syn and PP1 could bind directly, once α-Syn does not have any PP1 BM. However, the formation of the aggregates on Cos-7 cells

overexpressing the WT and MT forms of Sph1A suggests that it is not dependent on their interaction with any of the PP1 isoforms.

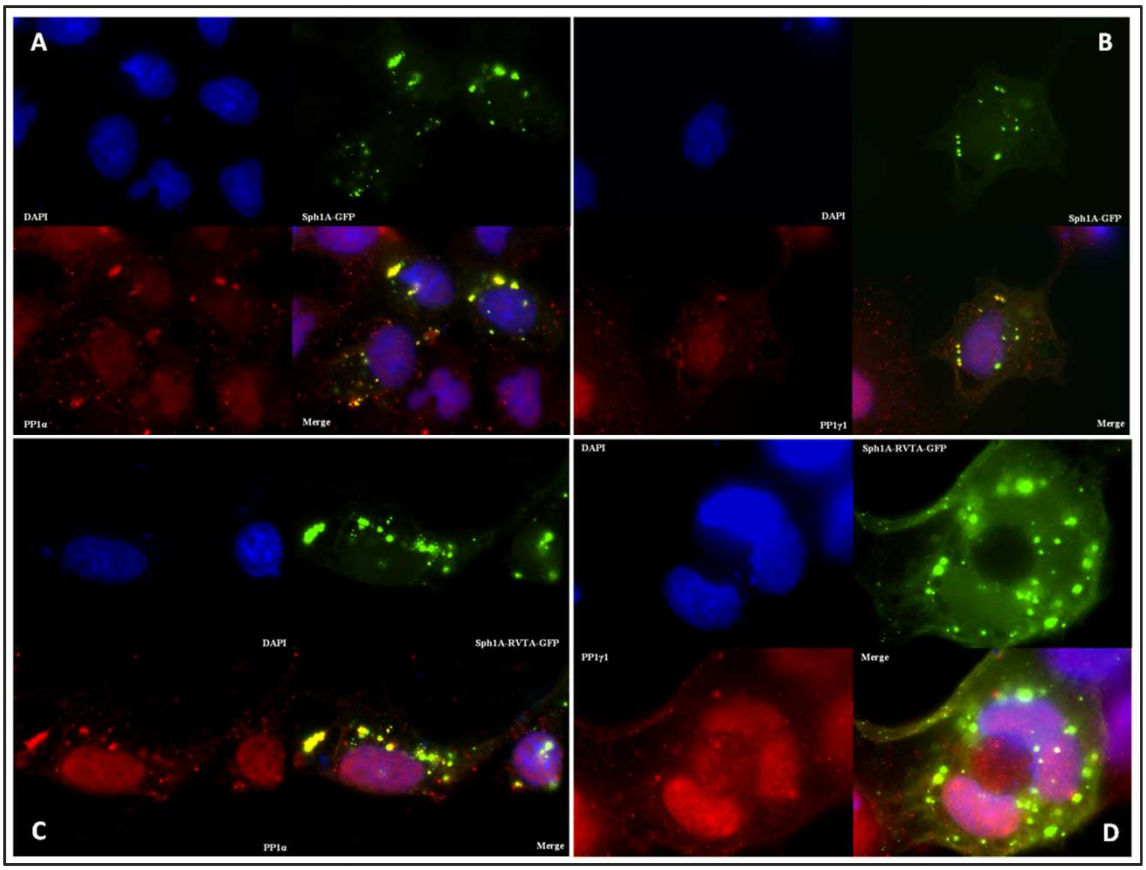

Figure 3 | Synphiln-1A and PP1 co-localization in cytosolic inclusions. A. PP1α (Red) and Sph1A (Green) co-localize in aggregates formed in Cos-7 cells overexpressing Sph1A-RVTF-GFP. **B.** PP1γ (Red) and Sph1A (Green) co-localize in aggregates formed in Cos-7 cells overexpressing Sph1A-RVTA-GFP. **C.** PP1α (Red) and Sph1A (Green) co-localize in aggregates formed in Cos-7 cells overexpressing Sph1A-RVTA-GFP. **D.** PP1γ (Red) and Sph1A (Green) co-localize in aggregates formed in Cos-7 cells overexpressing Sph1A-RVTA-GFP. Nucleic acids were stained using DAPI (blue); Sph1A-GFP green fluorescence; endogenous PP1 (red) detected with Texas Red-conjugated anti-PP1 antibody; co-localization observed in the merged image (yellow/orange).

Table 1 | Aggregates formation in Cos-7 cells overexpressing Synphilin-1A wildtype and mutated in the PP1 BM. A total of 400 cells were counted for each condition.

DNA	% transfection	% cells with aggregates	% small aggregates/cell	% medium aggregates/cell	% big aggregates/cell
pSph1A-WT_GFP	25	68	32	5	0.5
pSyn-1A-MT_GFP	25	79	30	3	1

α-Synuclein, Synphilin-1 and PP1 interaction networks

With the purpose of finding key proteins involved in the process of LBs formation that could be modulating PP1-Sph1/1A interaction and consequently Sph1/1A–α-Syn interaction, a map of Protein-Protein Interactions (PPIs) was drawn (Figure 4). PP1, Sph1 and α-Syn PPIs were retrieved from several available online databases and from previous results of PP1 interactomes in brain (Esteves, 2012a,b).

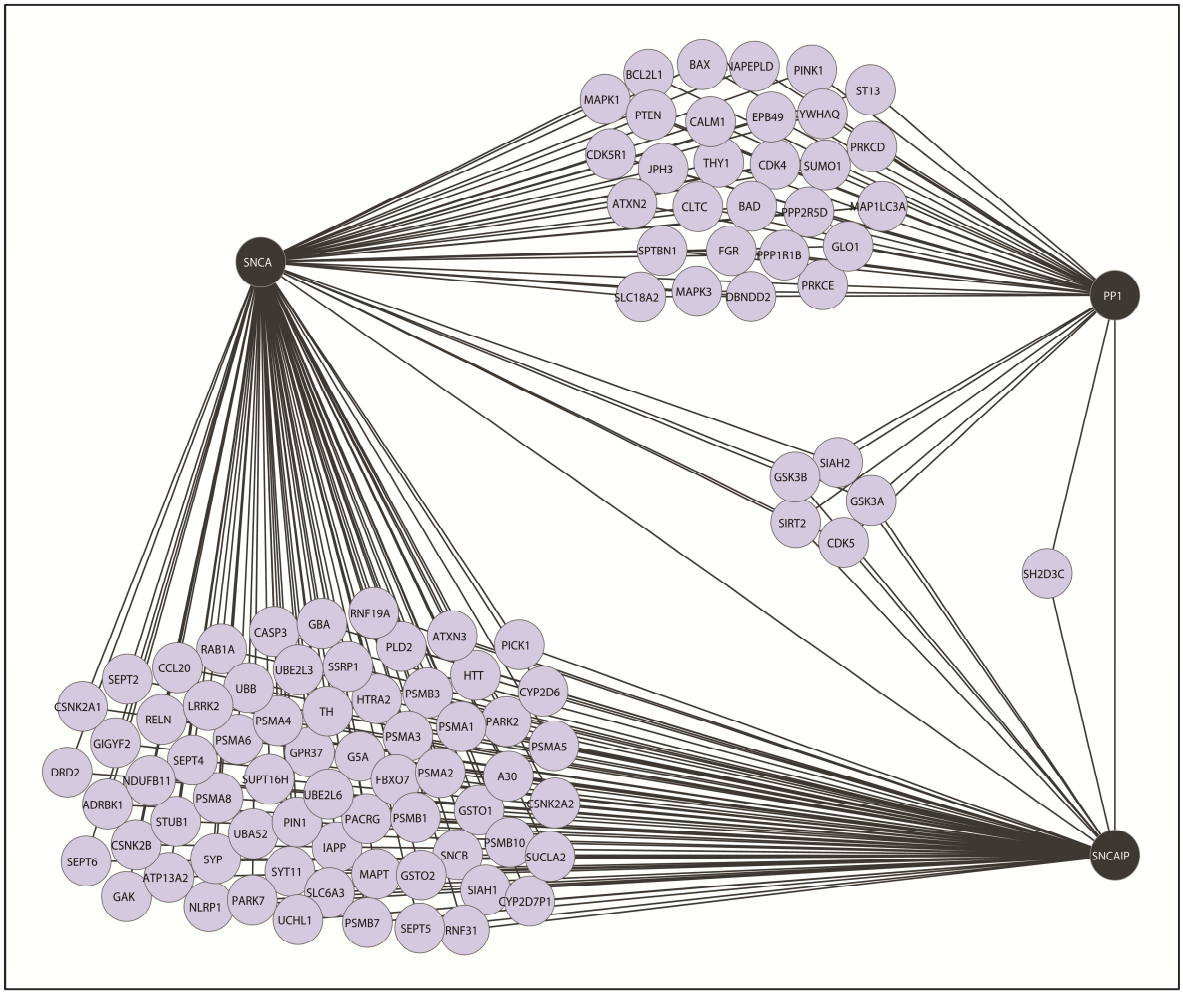

Figure 4 | Synphilin-1, α-Synuclein and PP1 common protein interactions. The human specific Sph1, α-Syn and PP1 interacting proteins were retrieved from available online databases, PPIs from previews work were also added (Esteves, 2012a,b).

From a total of 102 different interactions analyzed (supplementar Table 2), five were found to be common to PP1, Sph1 and α-Syn (GSK3A and B, CDK5, SIAH2 and SIRT2). Those proteins retrieved may be important in the process of inclusion body formation.

Sph1A expression during spermatogenesis

In an YTH screen using NUB1 (NEDD8 ultimate buster 1) as bait the cDNA of Sph1 was isolated from a human testis cDNA library (Tanji, 2006). Once the Sph1A IMAGE clone cDNA used in this work was obtained from testis and also because PP1 has an important role in sperm development and function (Fardilha, 2011a) we decided to analyze the expression of Sph1 isoforms in testis, more specifically during spermatogenesis.

We took advantage of an already established protocol using unit gravity sedimentation for the isolation of germ cell populations from a crude heterogeneous suspension of testicular cells (Dastig, 2011). We were able to separate three major homogenous fractions (fractions 7, 13 and 17) that were microscopically evaluated. Further, genes already established as markers for each step of the spermatogenesis were analysed (supplementary Table 3). The fraction 7 showed high relative expression levels of the synaptonemal complex protein 3 (*Sycp3*) and stathmin 1 (*Stmn1*), markers for spermatocytes, and also of the tumor-associated calcium signal transducer 1 (*Tacstd1*), a marker for spermatogonia. In fraction 13 the highest expression was observed for the spermatid marker, transition protein 1 (*Tpn1*), and in fraction 17 of other spermatid marker protamine 2 (*Prmn2*). These results are in accordance with previous data (Dastig, 2011). Additionally, expression markers for somatic cells were used to check

for contamination in each fraction. The levels of actin alpha 2 (*Acta2*) for peritubular myoid cells, hydroxysteroid 17 beta dehydrogenase (*Hsd17b3*) and *c-kit* for Leydig cells and the androgen receptor (AR) for Sertoli cells were low in the fractions analyzed. The only exception was for *Acta2* in fraction 13. The expression profiling of the somatic and germ cell specific markers showed that cell populations isolated was indeed enriched in spermatocytes (fraction 7), round spermatids (fraction 13) and elongated spermatids (fraction 17).

Next, we went to check for the relative expression levels of Sph1 and Sph1A, two alternatively spliced isoforms of the gene SNCAIP. Expression levels of these isoforms in each germ cell pool were compared with the original testicular cell suspension to retrieve the relative expression values. Both isoforms show a similar pattern of expression with higher levels in round spermatids and lower levels in elongating spermatids. Furthermore, the expression pattern of the SNCAIP isoforms in hippocampus was also assessed. Interestingly, the levels for the relative expression were extremely low when compared with testis, with values of 0.005 and 0.006 for Sph1 and Sph1A, respectively, when compared 1.647 and 1.504 in round spermatids (supplementary Table 3).

Discussion and Conclusion

Sph1A was identified as a PIP first by an YTH screen from a human brain library (Esteves, 2012a) and later confirmed by biochemical methods. Sph1A is an isoform of Sph1 and an aggregation prone and neurotoxic protein, that interacts with α-Syn and Sph1 (Eyal, 2006b) and relevant to LBs formation. Here we suggest that Sph1A may target PP1 to protein complexes involved in the initial steps of inclusion body formation, thus potentiating the dephosphorylation of other proteins and/or being itself a substrate. An emerging concept that is already in clinical trials is the targeting of PP1/PIP complexes for therapy, e.g. by interfering with viral replication and cell division, in order to disrupt cellular events where PP1 containing complexes are crucial players in dephosphorylating key proteins (Fardilha, 2010). Modulating phosphorylation status in order to change the interaction of α-Syn and Sph1/1A, is crucial in the understanding of inclusion bodies formation and, subsequently, interfering with their formation. Additionally, by disturbing the system a step further can be made towards therapeutic strategies in LBs related disorders, including PD.

Here we showed that, at least in Cos-7 cells, the formation of aggregates is not dependent of Sph1A-PP1 interaction, since Sph1A (RVTF) and Sph1A (RVTA) behaved exactly the same way, although we also show, in an overlay assay, that the interaction PP1-Sph1A (RVTF) was much stronger than PP1-Sph1A (RVTA). An explanation may be the fact that in the overlay the protein normal conformation may not be acquired, while *in vivo* it is fully accomplished and other point of contact (besides the PP1 BM) may account for the PP1-Sph1A interaction. Also, other proteins may take part in the complexes formed in the aggregates and may be stabilizing them, and we have to consider the presence of endogenous Sph1 and Spn-1A bound to PP1 in the

aggregates. Furthermore, we cannot forget that Sph1A-RVTA was being overexpressed in the experiment.

PP1, Sph1 and α-Syn interactions network

In order to draw the interaction maps of α-Syn, Sph1 and PP1 and gain some insight in their possible interrelation to PD, several PPIs were retrieved from online databases and our previous PP1 interactomes (Esteves, 2012a,b). A large number of PPIs (67) were found to be common to α-Syn and Sph1, as expected considering the importance of both and their interaction in LBs formation, but also between α-Syn and PP1 (Figure 4). Only a few PPIs were found to be interconnected with the three: GSK3, CDK5, SIAH2 and SIRT2; and SH2D3C is the only interactor common to Sph1A and PP1.

Glycogen syntase kinase 3 (GSK3) is a Ser/Thr kinase encoded by two genes GSK3A GSK3B. The two isoforms are highly homologous in their kinase domains but differ in their N-terminal and C-terminal regions that may have a regulatory role. Also, the levels of both isoforms are different in diverse tissues, being GSK3B particularly abundant in brain (Woodgett, 1990). Phosphorylated tau is another protein also present in Lewy bodies (Ishizawa, 2003) and α-Syn directly stimulates tau phosphorylation through GSK3B (Duka, 2009) by forming a heterotrimeric complex with tau and GSK3B (Kawakami, 2011). Mutation analysis showed that Ser556 (Ser190 on Sph1A, Figure 1) is a major GSK3B phosphorylation site in Sph1 (Avraham, 2005). GSK3B was also shown to co-immunoprecipitate with Sph1, and protein 14-3-3, an activator of GSK3B activity and a PIP. GSK3B decreased the *in vitro* and *in vivo* ubiquitylation of Sph1 as well as its degradation promoted by SIAH. Several kinases have been found to phosphorylate GSK3 isoforms at their N-terminal region (Grimes, 2001). This

phosphorylation was found to be reverted, at least, by the action of two phosphatases PP1 and PP2A (Stambolic, 1994; Grimes, 2001). A recent study determined the *in vitro* and *in vivo* dephosphorylation of phospho GSK3 isoforms, in the absence or the presence of okadaic acid and lithium (PP1 and PP2A inhibitors), and showed the preferential dephosphorylation of phospho GSK3A by PP2A and of phospho GSK3B by PP1 (Hernandez, 2010). It was also demonstrated that GSK3B complexed with AKAP220 is regulated by PKA and PP1 more efficiently than GSK3B free from AKAP220 (Tanji, 2002). Thus PP1 could be a major regulator of GSK3B in brain and therefore of Sph1/1A phosphorylation states.

Cyclin-dependent protein kinase 5 (Cdk5), another Ser/Thr kinase, is an heterodimer of a catalytic Cdk5 and a regulatory p25 subunit (Lew, 1995; Dhavan, 2001; Grant, 2001). Cdk5 is inactive in dividing cells but becomes progressively more active in differentiating cells (Yan, 1995; Harada, 2001) and is predominantly expressed in terminally differentiated neurons (Lew, 1995; Dhavan, 2001; Grant, 2001). Recently, Cdk5 was established as a critical regulator of autophagy induction, once Cdk5-mediated phosphorylation of endophilin B1 is essential for autophagy induction and neuronal loss in models of PD (Wong, 2011). PP1 is phosphorylated by Cdk5 and blocking PP1 phosphorylation, by inhibiting Cdk5 activity, hinder neurite outgrowth (Li, 2007). In dividing cells, PP1 is phosphorylated by Cdk1 and Cdk2 on Thr320 (Dohadwala, 1994; Berndt, 1999; Liu, 1999). When Thr320 is phosphorylated, PP1 activity is inhibited (Dohadwala, 1994; Kwon, 1997). Cdk5 presence in LBs was revealed in several studies (Brion, 1995; Nakamura, 1997; Takahashi, 2000). Also, Cdk5 phosphorylates Parkin at Ser131 and decreases its auto-ubiquitylation (Avraham, 2007). Furthermore, the S131A mutant form of Parkin displayed a higher auto-ubiquitylation level, an increased ubiquitylation activity toward its substrates Sph1,

accumulated more into inclusions in human dopaminergic cells and increased the formation of Sph1/α-Syn inclusions; suggesting that the levels of Parkin phosphorylation and ubiquitylation may modulate the formation of inclusion bodies (Avraham, 2007). Sph1 and α-Syn interactions with Cdk5 in the databases searched (both present in STRING) were only predicted by text mining.

A proteomics study of PP1 complexes, in response to ischemia-reperfusion stress and ischemic tolerance in brain, described 14 different proteins that exhibited significant changes in their association with PP1α or PP1γ (Cid, 2007), one of them was SIAH2 interaction with PP1γ. Also, PP1γ complex with SIAH2 was abolished in a competition assay with a synthetic RVxF-containing peptide (Cid, 2007). Ubiquitin ligase SIAH (seven in absentia homolog) proteins mediate ubiquitination and subsequent proteasomal degradation of target proteins and have been implicated in the promotion of apoptotic death (Xu, 2006). Sph1 was reported to interact with SIAH1 and SIAH2 (Liani, 2004). SIAH proteins ubiquitylated Sph1, promoting its degradation by the proteasome system. Ubiquitylation is required for inclusion bodies formation, once a catalytically inactive mutant of SIAH1, which was still able to bind Sph1, failed to promote inclusions (Liani, 2004). And, like Sph1, α-Syn also associated with SIAH, but the interaction with SIAH2 was much stronger than with SIAH1. SIAH2 was shown to monoubiquitylates α-Syn. Further evidence that SIAH proteins may play a role in inclusion formation comes from the demonstration of SIAH immunoreactivity in LBs of PD patients (Liani, 2004). Sph1A was also shown to interact, *in vitro* and *in vivo*, with SIAH and to regulate its activity toward α-Syn and Sph1 (Szargel, 2009). SIAH promoted a limited ubiquitylation of Sph1A that did not lead to its degradation by the proteasome. Sph1A increased the steady-state levels of SIAH by decreasing its auto-

ubiquitylation and degradation, in addition, Sph1A decreased the monoubiquitylation of α-Syn by SIAH and the formation of α-Syn inclusions (Szargel, 2009).

SIRT2 (SIR2-like protein 2), highly expressed in brain, is a cytoplasmic deacetylase that is associated with the microtubule network and targets lysine 40 of α-tubulin for deacetylation (Afshar, 1999; North, 2004). SIRT2 has also been implicated in the control of cell cycle and proliferation (Dryden, 2003; Hiratsuka, 2003). It is a target for the mitotic kinase Cdk1, and its phosphorylation by Cdk1 is required for SIRT2 to mediate a delay in cell-cycle progression (North, 2007). Inhibition of SIRT2 rescues α-Syn toxicity, possibly by facilitating α-Syn inclusion formation or by stabilizing microtubules (Dauer, 2003; North, 2003). Treatment with microtubule destabilizing agents, vinblastin or nocodazole, reduced the number of aggregate-containing cells (Kopito, 2000). All the interactions found with SIRT2 were predicted by data mining, no experimental confirmation was made.

SH2D3C (SH2 domain-containing protein 3C) interaction with Sph1 was detected by an YTH approach that allowed the identification of 3186 interactions among 1705 proteins (Stelzl, 2005). And the interaction with PP1α was simply predicted by text mining (STRING). SH2D3C, also known as SHEP1, is a cytoplasmic protein widely expressed, including in brain, and involved in cell adhesion/migration (Vervoort, 2007). It contains an SH2 domain and an exchange factor-like domain that binds both Ras GTPases and the scaffolding protein Cas. Shep1 and Cas are both concentrated in the axons of developing olfactory sensory neurons (Wang, 2010).

From the interactions detected we can conclude that post-translational modifications, such as phosphorylation and ubiquitylation, are extremely important in inclusion bodies formation and in the regulation of Sph1/1A - α-Syn interaction and the possible complexes with PP1. Sph1/1A - α-Syn interaction, in fact, is regulated by the

phosphorylation states of both, which also interfere in the ubiquitylation and proteosome degradation of Sph1/1A and α-Syn and, as a result, in inclusion body formation.

Synphilin isoforms in testis

Sph1 was identified as a binding partner of NUB1 in an YTH screen from a human testis cDNA library (Tanji, 2006). NUB1 was also found to accumulate in inclusion bodies from brains of patients with α-synucleinopathies. NUB1 is a potent down-regulator of the ubiquitin-like protein NEDD8, because it targets the latter to the proteasome for proteolytic degradation. NUB1 physically interacts with Sph1 through its NEDD8 binding site, implying that NUB1 may also target Sph1 for proteasomal degradation (Tanji, 2006). Expression of Sph1 was also detected at the mRNA level and by IHC in several structures of the male reproductive system, including testis and epididymis (neXtProt and Human Protein Atlas platforms, Uhlen, 2010; Lane, 2012).

Spermatogenesis is the process by which large numbers of spermatozoa are produced, involving cell proliferation and differentiation, meiosis, spermiogenesis and spermiation (Yoshida, 2007; Lie, 2010). During spermiogenesis spermatides undergo morphological transformations from a rounded shape to an elongated cell, the spermatozoon.

In our study, Sph1 isoforms were found to have a parallel pattern of expression with higher levels in round spermatids and lower levels in elongating spermatids. This is similar to the expression pattern of proteins that are needed during the spermiogenesis, like the protamines (e.g. *Prmn2*) and the transition proteins (e.g. *Tpn1*) (Dastig, 2011). Protamines are nuclear proteins that replace histones in the last steps of spermatogenesis

having a role in DNA condensation and stabilization (Balhorn, 2007). Transition proteins like Tpn1 have distinct roles in histone displacement, sperm nuclear shaping, chromatin condensation, and maintenance of DNA integrity (Meistrich, 2003).

We have found that Sph1A, an isoform of synphilin-1, interacts with PP1 in human brain, by using the YTH system. The interaction was validated by overlay, using Sph1A and Sph1A mutated in its canonical PP1 binding site. The results obtained demonstrated that the interaction decreased significantly with the mutant, demonstrating that the conserved PP1 BM, RVTF, which is also present in Sph1, was crucial for the interaction. Also, Sph1A and PP1 co-localize in aggregates formed in Cos-7 cells overexpressing Sph1A-GFP, indicating that the former might act as a targeting PIP or a substrate for PP1.

Once, phosphorylation is an important regulation method involved in the formation of LBs and PD disease process, PP1-Sph1/1A complexes may modulate the phosphorylation states of key proteins in inclusion bodies thus regulating the formation of the latter as well as neuronal cell death.

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Author Disclosure Statement

No competing financial interests exist.

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Supplementary data

Table 1 | List of primers used in the qRT-PCR.

Primer	*Gene*	Sequence	Positions	Acession number
Mus musculus, Synphilin 1 - FW	*Sncaip*	ACCAACACCCAAGGCACGGAAG	1093 – 1114	NM_001199151.1
Mus musculus, Synphilin 1 - RV		TGCAGCTAGGTGTGGCAAGAGC	1200 – 1179	
Mus musculus, Synphilin 1A - FW		GACAGATCAGAATGGTCAGCTGGAG	307 – 331	
Mus musculus, Synphilin 1A - RV		TTCCTGCCCATAGCAACCTGCG	433 – 412	

Table 2 | List of in common interacting proteins of PP1, Synphilin-1 and α-Synuclein previously identified in PP1 YTH screens and present in the databases searched.

Protein	Interactions	Database
A30	SNCA	STRING
	SNCAIP	STRING
ADRBK1	SNCA	STRING HPRD Reactome
	SNCAIP	STRING
ATP13A2	SNCA	STRING
	SNCAIP	STRING
ATXN2	SNCA	STRING
	PP1	STRING
ATXN3	SNCA	STRING
	SNCAIP	STRING
BAD	SNCA	STRING HPRD BioGrid Reactome iRefIndex KEGG PATHWAY
	PP1	IntAct HPRD STRING
BAX	SNCA	STRING BioGrid Reactome iRefIndex
	PP1	InnateDB HPRD
BCL2L1	SNCA	HPRD STRING Reactome
	PP1	BioGrid IntAct STRING HPRD APID PINA
CALM1	SNCA	STRING BioGrid HPRD iRefIndex KEGG PATHWAYS ophid
	PP1	STRING Reactome
CASP3	SNCA	STRING
	SNCAIP	STRING
CCL20	SNCA	STRING
	SNCAIP	STRING
CDK4	SNCA	MINT

Protein	Interactions	Database
	PP1	APID IntAct PINA Reactome
CDK5	SNCA	STRING
	SNCAIP	STRING
	PP1	Reactome
CDK5R1	SNCA	STRING
	PP1	Y2H-PP1g2
CLTC	SNCA	STRING BioGrid
	PP1	Y2H-PP1a Y2H-PP1g1
CSNK2A1	SNCA	HPRD STRING Reactome
	SNCAIP	STRING iRefIndex STRING
CSNK2A2	SNCA	STRING HPRD iRefIndex
	SNCAIP	STRING
CSNK2B	SNCA	STRING
	SNCAIP	APID STRING
CYP2D6	SNCA	STRING
	SNCAIP	STRING
CYP2D7P1	SNCA	STRING
	SNCAIP	STRING
DBNDD2	SNCA	STRING
	PP1	Y2H-PP1g2
DRD2	SNCA	STRING
	SNCAIP	STRING
EPB49	SNCA	STRING BioGrid iRefIndex
	PP1	Y2H-PP1g2
FBXO7	SNCA	STRING
	SNCAIP	STRING
FGR	SNCA	STRING HPRD Reactome iRefIndex
	PP1	STRING
G5A	SNCA	STRING
	SNCAIP	STRING
GAK	SNCA	STRING
	SNCAIP	STRING
GBA	SNCA	STRING
	SNCAIP	STRING
GIGYF2	SNCA	STRING
	SNCAIP	STRING
GLO1	SNCA	STRING

Protein	Interactions	Database
	PP1	STRING
GPR37	SNCA	STRING
	SNCAIP	STRING
GSK3A	SNCA	STRING
	SNCAIP	STRING
	PP1	STRING
GSK3B	SNCA	MINT
	SNCAIP	STRING APID Reactome iRefIndex
	PP1	STRING BioGrid Reactome
GSTO1	SNCA	STRING
	SNCAIP	STRING
GSTO2	SNCA	STRING
	SNCAIP	STRING
HTRA2	SNCA	STRING
	SNCAIP	STRING
HTT	SNCA	STRING MINT
	SNCAIP	STRING
IAPP	SNCA	STRING
	SNCAIP	STRING
JPH3	SNCA	STRING
	PP1	Y2H-PP1a
LRRK2	SNCA	BioGrid STRING
	SNCAIP	STRING
MAP1LC3A	SNCA	STRING IntAct
	PP1	IntAct PINA
MAPK1	SNCA	STRING HPRD BioGrid Reactome KEGG PATHWAYS
	PP1	HPRD Reactome STRING
MAPK3	SNCA	STRING BioGrid KEGG PATHWAYS
	PP1	HPRD Reactome STRING
MAPT	SNCA	STRING HPRD MINT BioGrid iRefIndex ophid

Protein	Interactions	Database
	SNCAIP	STRING
NAPEPLD	SNCA	STRING
	PP1	Y2H-PP1g1
NDUFB11	SNCA	STRING
	SNCAIP	STRING
NLRP1	SNCA	STRING
	SNCAIP	STRING
PACRG	SNCA	STRING
	SNCAIP	STRING
PARK2	SNCA	Spike STRING BioGrid HPRD iRefIndex KEGG PATHWAYS ophid
	SNCAIP	Spike APID STRING BioGrid
PARK7	SNCA	STRING Reactome InnateDB iRefIndex KEGG PATHWAYS
	SNCAIP	STRING
PICK1	SNCA	STRING
	SNCAIP	STRING
PIN1	SNCA	STRING iRefIndex
	SNCAIP	STRING iRefIndex
PINK1	SNCA	STRING
	PP1	Y2H-PP1g2
PLD2	SNCA	STRING Reactome
	SNCAIP	STRING
PPP1R1B	SNCA	STRING
	PP1	HPRD Reactome STRING
PPP2R5D	SNCA	STRING Reactome
	PP1	STRING Reactome
PRKCD	SNCA	STRING Reactome
	PP1	HPRD
PRKCE	SNCA	STRING HPRD
	PP1	STRING
PSMA1	SNCA	Reactome
	SNCAIP	Reactome
PSMA2	SNCA	Reactome

Protein	Interactions	Database
	SNCAIP	Reactome
PSMA3	SNCA	Reactome
	SNCAIP	Reactome
PSMA4	SNCA	Reactome
	SNCAIP	Reactome
PSMA5	SNCA	Reactome
	SNCAIP	Reactome
PSMA6	SNCA	Reactome
	SNCAIP	Reactome
PSMA8	SNCA	Reactome
	SNCAIP	Reactome
PSMB1	SNCA	Reactome
	SNCAIP	Reactome
PSMB10	SNCA	Reactome
	SNCAIP	Reactome
PSMB3	SNCA	Reactome
	SNCAIP	Reactome
PSMB7	SNCA	Reactome
	SNCAIP	Reactome
PTEN	SNCA	STRING
	PP1	InnateDB IntAct Reactome HPRD
RAB1A	SNCA	STRING
	SNCAIP	STRING
RELN	SNCA	STRING
	SNCAIP	STRING
RNF19A	SNCA	STRING
	SNCAIP	APID STRING
RNF31	SNCA	STRING
	SNCAIP	STRING
SEPT2	SNCA	BioGrid iRefIndex STRING
	SNCAIP	STRING
SEPT4	SNCA	BioGrid STRING iRefIndex
	SNCAIP	APID STRING iRefIndex
SEPT5	SNCA	STRING
	SNCAIP	STRING
SEPT6	SNCA	STRING
	SNCAIP	STRING
SH2D3C	SNCAIP	BioGrid APID MINT Spike
	PP1	STRING
SIAH1	SNCA	STRING

Protein	Interactions	Database
	SNCAIP	APID STRING iRefIndex
SIAH2	SNCA	STRING BioGrid iRefIndex
	SNCAIP	STRING iRefIndex
	PP1	STRING
SIRT2	SNCA	STRING
	SNCAIP	STRING
	PP1	Reactome
SLC18A2	SNCA	STRING
	PP1	STRING
SLC6A3	SNCA	STRING Reactome
	SNCAIP	STRING
SNCB	SNCA	STRING HPRD BioGrid iRefIndex Reactome ophid
	SNCAIP	STRING
SPTBN1	SNCA	IntAct
	PP1	Y2H-PP1g2
SSRP1	SNCA	STRING
	SNCAIP	STRING
ST13	SNCA	STRING
	PP1	STRING
STUB1	SNCA	STRING Reactome
	SNCAIP	STRING
SUCLA2	SNCA	STRING
	SNCAIP	STRING
SUMO1	SNCA	STRING
	PP1	STRING
SUPT16H	SNCA	STRING
	SNCAIP	STRING
SYP	SNCA	STRING
	SNCAIP	STRING
SYT11	SNCA	STRING
	SNCAIP	STRING
TH	SNCA	HPRD STRING Reactome iRefIndex KEGG PATHWAYS
	SNCAIP	STRING
THY1	SNCA	STRING
	PP1	Y2H-PP1g2
UBA52	SNCA	STRING
	SNCAIP	STRING

Protein	Interactions	Database
UBB	SNCA	STRING KEGG PATHWAYS
	SNCAIP	STRING iRefIndex
UBE2L3	SNCA	STRING Reactome KEGG PATHWAYS
	SNCAIP	STRING Reactome
UBE2L6	SNCA	Reactome
	SNCAIP	Reactome
UCHL1	SNCA	STRING KEGG PATHWAYS Reactome ophid
	SNCAIP	STRING
YWHAQ	SNCA	STRING Reactome
	PP1	STRING

Table 3 | Expression levels of mRNA of Synphilin-1 isoforms. Isolated cell fractions were spermatocytes (Spc), round spermatids (rSpt) and elongated spermatids (eSpd). Relative expression values were calculated by the ΔΔ Ct-method. Increased mRNA expression is indicated by orange shading and decreased expression levels by blue shading. Light shading indicates alterations above 1.5-fold or below 0.75-fold and intense shading indicates changes in expression levels of more than 2-fold or below 0.5-fold

Gene	Isoforms	Spc	rSpd	elSpd	Hippocampus
Sncaip	Synphilin1	1,015	1,647	0,563	0,005
	Synphilin1A	1,153	1,504	0,608	0,006

Future Work

| Taperin

Taperin was shown to be a PP1α and γ binding protein, in brain and testis, and to interfere with actin dynamics in the cell, on a PP1 binding dependent way. Nonetheless, it still keeps to be establish the mechanism by which taperin regulates actin signaling. It would be important to determine if another actin binding and/or PP1 binding protein is involved in the process of actin depolimerization and the function of the complex in the nucleus. It would be interesting:

1| *To determine if both forms of taperin, resulting from cleavage, are functional and if they correspond to different pools of taperin in the cell (nuclear and cytoplasmic). A taperin construct with an N-terminal tag would be usefull.*

2 | *To determine the subcellular localization of Taperin in neuronal cell lines and cortical/hippocampal neurons and compare it to that of actin and PP1 isoforms. Once, in another study (Ferrar, 2012), taperin was shown to bind preferentially PP1α over PP1γ1. The same can be done for human sperm samples and spermatogonia cell lines. In sperm it is also necessary to analyze Taperin/PP1/actin co-localization (possibly in the acrosome). Analyzing taperin in capacitated sperm would be also important; since it may have a role in capacitation and acrosome reaction, in parallel with PP1 and actin.*

3 | *To immunoprecipitate Taperin and look for actin and PP1 as a confirmation of direct interactions with actin in the formatiom of the tri-complex.*

4 | *To determine the effect of known PP1 inhibitors like Inhibitor-1, Inhibitor-2, okadaic acid, microcystin, cantharidin, etc., and PP1 knockdown (e.g. RNAi) on PP1/Taperin/actin complex.*

5 | *To study the PP1/Taperin complex, Taperin can be isolated by immunoprecipitation, using either specific antibodies or constructs, and assay the associated PP1 activity. This method will also allow to study whether the activity of the complex changes in response to different signals and/or cellular conditions (like alterations in actin polimerization/depolimerization).*

6 | *To determine the action of Taperin in actin and cytoskeleton. For that several assays could be performed: F-actin-binding and F-actin-bundling activity; Actin dynamics and Single filament (Kits available commercially). This assays were already performed for Phostensin, a protein which shows some homology with Taperin, and that is also a PP1 binding and cytoskeleton targeting protein (Kao, 2007; Lai, 2009; Ferrar, 2012).*

| Synphilin-1A

The α-Synuclein interacting protein Synphilin-1A, a neurotoxic and aggregate prone isoform of Synphilin-1, was also found to bind PP1 in brain by the YTH technique. Both co-localize in aggregates formed in cells overexpressing Synphilin-1A. It is implicated in the process of neurodegeneration by protein aggregation and Lewy Bodies formation. α-Synuclein and Synphilin-1 interact with several kinases and are phosphorylated during the process of protein aggregation and fibrilation in α-synucleinopathies. In addition to being phosphorylated, α-synuclein is monoubiquitylated in Lewy Bodies. Phosphorylation modulates the ubiquitylation and aggregational properties of various proteins. The interaction of Synphilin-1A with different proteins involved in Parkinson`s Disease (PD) suggests that it may assemble these proteins into a multi-protein complex. In order to further reveal the importance of PP1/Synphilin-1A interaction in this process, it is essencial:

1 | *To immunoprecipitate Synphilin-1A and look for PP1 different isoforms and immunoprecipitate α-Synuclein and look for Synphilin-1A and PP1; in order to further validate the interaction. For that, specific antibodies would be necessary.*

2 | *To evaluate Synphilin-1A and PP1 co-aggregation, in neuronal dopaminergic cell lines (e.g. SH-SY5Y, PC12) and primary cortical/hippocampal neurons, and evaluate also the presence of α-Synuclein. For this end, a model of Lentivirus infection is already established, but it will be necessary to express Synphilin-1A fused with a tag, since the antibody available in the laboratory does not work properly.*

3 | *To evaluate Synphilin-1A and PP1 co-aggregation in cellular models of Lewy Body-like inclusions formation, like co-transfection of α-synuclein and Synphilin-1 and proteasome inhibition. Also, try to isolate the aggregates and analyze the presence of PP1.*

4 | *To compare the expression and localization of the PP1 and the formation of the complex PP1/Synphilin-1A in normal versus disease condition (cellular models of PD), and assay the associated PP1 activity. This method will also allow to study whether the activity of the complex changes in response to different signals and/or cellular conditions (like in disease condition).*

5 | *To determine the effect of known PP1 inhibitors like Inhibitor-1, Inhibitor-2, okadaic acid, microcystin, cantharidin, etc. and PP1 downregulation (*e.g. *RNAi) on PP1/Synphilin-1A-αSynuclein complex.*

6 | *To identify phosphorylatable residues in Synphilin-1 and Synphilin-1A, by immunoprecipitation from rat brain/cell lines (control and PD model) followed by MS analysis in order to define their phosphorylation profiles.*

7 | *To downregulate Synphilin-1/1A and PP1 (*e.g. *RNAi) and subject the extracts to a Human Parkinson's disease PCR array.*

8 | *To study PP1/Synphilin-1A complex in sperm, by immunocolocalization analysis and to immunoprecipitate Synphilin-1A from sperm/tetis and look for PP1 and vice versa.*

The proposed experiments, in addition to the preliminary results here presented, will lead to a deep knowledge of the role of PP1/Taperin and PP1/Synphilin-1A complexes.

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| Chapter IV

IV | CONCLUSION - PIPS AS THERAPEUTIC TARGETS

PPIs have emerged as important drug targets for small molecules binding to hotspots on the protein contact surfaces, being a commonly observed situation when the small molecule acts as a direct mimic of one of the protein partners (Fry, 2008). Some small molecules bind strongly to the contact surface of the target protein, and with much higher efficiencies, than do the contact atoms of the natural protein partner (Wells, 2007).

Mapping and description of PPI networks is therefore a central task in the post-genomic era. A comprehensive analysis of PPIs will be crucial for a global understanding of the cellular proteome, and is therefore a major goal in systems biology (Auerbach, 2002). Indeed, the recent exploration of entire interactomes was a consequence of high-throughput methodologies applied to the field of proteomics. These technologies are driving our understanding of protein interactomes and will be crucial to exploit their therapeutic potential (Suter, 2008). It is necessary to develop large-scale screens of a vast number of protein complexes and find putative small-molecule, peptide or antibodies binding sites overlapping with protein-protein binding sites.

PP1 is a major protein Ser/Thr phosphatase with ubiquitous distribution and broad specificity *in vitro*, but tightly regulated *in vivo*. In fact, only in this way it can be implicated in such a huge number of cellular signaling events. PP1 interactome comprises already hundreds of PPIs, consisting of specific-isoform binding partners, tissue specific interactors and specific subcellular regulators. As described in the previous chapters, the interaction of the majority of PIPs with PP1 surface is through PP1 BMs. PP1 is involved in diverse pathologies being its interacting proteins appealing targets for therapeutic interventions, once the specificity is achieved through them.

In this work a total of 263 PP1α, PPγ1 and PP1γ2 interacting proteins were identified by large scale YTH screens. A considerable number of those interactions were concluded to be novel, but also some already known PP1 interactors were detected, which confers reliability to the method used. It would be a hard and expensive task to try to characterize all the interactions obtained, so the strategy adopted was to integrate the interactions found to the ones available on PPIs databases. It became clear that PP1

interactome is vast and its cellular functions diverse. Two proteins, Taperin and Synphilin-1A, found in the YTHs were selected for a more in depth characterization of the interaction. For both the interaction with PP1 was found to be dependent on the PP1 BM RVxF, exemplifying how targeting protein contact points can be a good strategy.

Taperin was, until very recently, a completely uncharacterized protein; it was the most abundant clone in the PP1α brain YTH and was also identified in a testis PP1γ1 YTH (Fardilha, 2011). Taperin binds PP1 in the cells and translocates between cytoplasm and nucleus and in, the nucleus, seems to be implicated in DNA damage processes (Ferrar, 2012). Also, Taperin was found to be cleaved in cells into two fragments, although, the functional relevance of this cleavage is still unknown. Taperin localizes in the cell membrane with PP1 and actin and affects actin dynamics, in fact, a mutant form of taperin accumulates with actin in the nucleus and this seems to be dependent on PP1 binding.

Synphilin-1A is a protein implicated in PD through its role in LBs formation together with α-Synuclein. It was found to form protein aggregates when overexpressed in cells and to co-localize with PP1 on those structures. Both, Synphilin-1A and Taperin, were found to be expressed not only in brain but also in testis and sperm.

Future work will proceed aiming to further unravel Taperin and Synphilin-1A physiological roles and modulate their interaction with PP1 for, not only, research proposes but also therapeutic if needed.

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Manuscript 5 - The Physiological Relevance of Protein Phosphatase 1 and its Interacting Proteins to Health and Disease

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The Physiological Relevance of Protein Phosphatase 1 and its Interacting Proteins to Health and Disease

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Abstract: Protein phosphorylation is a major regulatory mechanism of signal transduction cascades in eukaryotic cells, catalysed by kinases and reversed by protein phosphatases (PPs). Sequencing of entire genomes has revealed that ~3% of all eukaryotic genes encode kinases or PPs. Surprisingly, there appear to be 2-5 times fewer PPs than kinases. Over the past two decades it has become apparent that the diversity of Ser/Thr-specific PPs (STPP) was achieved not only by the evolution of new catalytic subunits, but also by the ability of a single catalytic subunit to interact with multiple interacting proteins. PP1, a STPP, is involved in the control of important cellular mechanisms. Several isoforms of PP1 are known in mammals: PP1α, PP1β and PP1γ. The various isoforms are highly similar, except for the N- and C-termini. The current view is that since PPs possess exquisite specificities *in vivo*, the key control mechanism must reside in the nature of the PP1 Interacting Protein (PIP) to which they bind. An increasing number of PIPs have been identified that are responsible for regulating the catalytic activity of PPs. Indeed, the diversity of such PIPs explains the need for relatively few catalytic subunit types, and makes them attractive targets for pharmacological intervention. This review will summarize the PIPs identified using the Yeast Two Hybrid methodology and alternative techniques, for instance bioinformatic and proteomic approaches. Further, it compiles 129 PP1-PIP relevant physiological interactions that are well documented in the literature. Finally, the use of PIPs as therapeutic targets will be addressed.

Keywords: Protein phosphorylation, signal transduction, regulation, interactome, yeast two hybrid, drug target, cancer, Alzheimer's disease.

ABNORMAL PROTEIN PHOSPHORYLATION IN HUMAN DISEASES

Reversible protein phosphorylation is the major metabolic control mechanism of eukaryotic cells. Indeed, cellular health and vitality are dependent on the fine equilibrium of protein phosphorylation systems. Not surprisingly many diseases and dysfunctional states are associated with the abnormal phosphorylation of key proteins (e.g. cancer, diabetes, and neurodegenerative conditions).

In neurodegenerative diseases, such as Alzheimer's Disease (AD), there is evidence for abnormal regulation of protein kinases. Altered activities and protein levels of several specific kinases suggest that abnormal phosphorylation contributes to the pathogenesis of this disease. In AD, neurofibrillary degeneration results from the aggregation of abnormally phosphorylated Tau protein into paired helical filaments. Protein kinase A (PKA) and glycogen synthase kinase 3 β (GSK3β) are likely to be key kinases involved [1], and PP2A is thought to be the main Tau protein phosphatase (PP) [2]. Furthermore, activation of protein kinase C (PKC), or inactivation of protein phosphatase 1 (PP1), leads to a relative increase in the utilization of the non-amyloidogenic pathway for the Alzheimer's amyloid precursor protein (APP) [3, 4]. Increased processing *via* the non-amyloidogenic pathway at the expense of the amyloidogenic pathway results in decreased production of Abeta (beta-amyloid peptide). The latter is a product of APP cleavage events and the toxic peptide becomes deposited as senile plaques in AD patients, a characteristic hallmark of this condition [5, 6].

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Other neurodegenerative diseases with potential contributions from abnormal phosphorylation include Parkinson's Disease (PD) and Huntington's Disease (HD). Alpha-synuclein has been implicated in the pathogenesis of PD, and is a major component of Lewy bodies (a major anatomical hallmark of PD). Alpha-synuclein was demonstrated to be constitutively phosphorylated indicating that its function is regulated by phosphorylation/dephosphorylation mechanisms [7]. Motor and cognitive deficits in HD are likely caused by progressive neuronal dysfunction preceding neuronal cell death. Synapsin I is one of the major phosphoproteins regulating neurotransmitter release. In mice expressing the HD mutation, synapsin I is abnormally phosphorylated suggesting that an early impairment in its phosphorylation may alter synaptic vesicle trafficking and lead to defective neurotransmission in HD [8].

In a totally different type of disease, cancer, imbalances of protein phosphorylation systems appears to play an important role. Several families of protein kinases orchestrate the complex events that occur during cell cycle, and their activity is recurrently deregulated in cancer cells. For example, abnormalities in the functions of several tyrosine kinases, the Epithermal Growth Factor Receptors (EGFR) [9], the Vascular Endothelial Growth Factor Receptors (VEGFR) [10] and the Platelet-Derived Growth Factor Receptors (PDGFR) [11] are related to cancer development, including autonomous cell growth and invasion. In many human tumours VEGFR mRNA has been shown to be upregulated, by *in situ* hybridization [10]. PDGFR was also shown to be expressed in a number of neoplasms including glioblastomas, ovarian cancer and prostate cancer [12-14].

Altered phosphorylation has also been implicated in the etiology of heart failure [15] as well as in Diabetes [16].

Therefore, protein phosphorylation systems represent attractive targets for diagnostic and therapeutic strategies of

several neurodegenerative and non-neurodegenerative diseases.

CLASSIFICATION OF PROTEIN PHOSPHATASES

The reversible phosphorylation of structural and regulatory proteins is a major intracellular control mechanism in eukaryotes. It is involved in almost all cellular functions, from metabolism to signal transduction, cell division and memory. The phosphorylation state of a protein is a dynamic process controlled by both protein kinases and PPs. Both the protein kinases and PPs are key controlling elements, regulated by a myriad of extracellular and intracellular signals. Unlike the protein kinases that all belong to a single gene family, the PPs are divided into several distinct and unrelated protein/gene families.

STPPs, based on biochemical parameters, were initially divided into two classes: the type-1 PPs (PP1) that were inhibited by two heat-stable proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2), and preferentially dephosphorylated the β-subunit of phosphorylase kinase; and the type-2 PPs, insensitive to the heat-stable inhibitors and that preferentially dephosphorylated the α-subunit of phosphorylase kinase [17, 18]. Type-2 PPs were further subdivided into cation independent (PP2A), Ca^{2+}-dependent (PP2B) and Mg^{2+}-dependent (PP2C) classes. The use of okadaic acid, a specific PP inhibitor, further facilitated the discrimination between different classes [18]. Although widely used, this classification does not reflect the actual phylogenetic relationship between the different STPP. Molecular cloning revealed that PP2A was in fact much more related to PP1 than to PP2C [19, 20]. STPPs comprise three different gene families, the PPM, the FCP and the PPP. The PPM family comprises the Mg^{2+}-dependent PPs, such as pyruvate dehydrogenase, PP2C, and relatives [21]. The FCP family comprises the new FCP1 and SCPs 1-3 PPs that have in common specificity for the substrate RNA polymerase II [22, 23]. The PPP family includes PP1, PP2A/PP4/PP6, PP2B, PP5 and PP7 gene subfamilies that share high homology in the catalytic domains but differ in the N- and C-terminal domains [21, 24, 25]. The Tyr-specific PP family, as well as including the Tyr-specific PPs, also comprise the so-called dual specificity PPs (capable of dephosphorylating Ser, Thr and Tyr residues). Besides these intracellular PPs involved in signal transduction, there are also unrelated non-specific alkaline and acid phosphatases that are usually found either in specialized intracellular compartments or in the extracellular milieu.

The sequencing of entire genomes has revealed that approximately 3% of all eukaryotic genes encode protein kinases or PPs [26] although there appear to be fewer PPs than protein kinases. This imbalance is even more pronounced when the analysis is limited to STPP and kinases, particularly in vertebrates. The human genome, for instance, encodes approximately 20 times fewer STPP than Ser/Thr-kinases. Thus, whereas the diversity of the Ser/Thr-protein kinases has kept pace with the increasing complexity of evolving organisms, that of STPP has not. In the past two decades it has become apparent that the diversity of STPP is achieved not only by the evolution of new catalytic subunits, but also by the ability of a single catalytic subunit to interact with multiple regulatory (R) subunits [27].

THE PP1 TARGETING HYPOTHESIS

Protein phosphatase 1 (PP1), also known as phosphorylase phosphatase, has been studied since the 1940s as the enzyme responsible for the conversion of phosphorylase a to phosphorylase b [28]. The discovery that PP1 was actually a phosphatase came at the same time as the discovery of phosphorylase kinase [29, 30]. These findings marked the beginning of an era in the study of protein phosphorylation/dephosphorylation as a regulatory mechanism. Research on PP1 in the following three decades focused on defining its enzymology and role in glycogen metabolism [a review can be found in 31-35] but the progress in the isolation and characterization of PP1 activity was very slow. The study of this enzyme's enzymology is still incomplete and PP1 continues to bring surprises, as well as new questions about its cellular functions.

The key steps that led to the beginning of the understanding of the enzymology of PP1 came with the isolation of its catalytic subunits (PP1c) from liver and muscle [36, 37]. Underlying this success was the discovery that treatment of tissue extracts with denaturants (alcohol or urea) led to the release of a 35kDa catalytic subunit which could be readily isolated [38]. This 35kDa subunit was derived from a 37kDa form by proteolytic cleavage near the C-terminus [18, 35]. The denaturant treatment released the catalytic subunit from its regulatory subunits, so that the enzyme activity behaved in a monodisperse manner and was amenable to isolation by conventional chromatographic methods. Following this treatment (ammonium sulfate precipitation followed by 95% ethanol at room temperature) PP1 activity could be extracted from the pellet of denatured proteins. At the time it was clear that while phosphorylase a was the best PP1c substrate, both glycogen synthase and phosphorylase kinase were also dephosphorylated. Later studies [18] showed that this enzyme was relatively non-specific when compared, for example, to the protein kinases. PP1c isolation from rabbit skeletal muscle led to the isolation of another catalytic subunit of 35kDa, which differed in its ability to act on p-nitrophenyl phosphate [39]. This activity turned out to be the PP2A catalytic subunit [18]. Ethanol or trypsin treatment of tissue extracts led to large increases in PP1 activity, concomitant with the reduction in apparent size to the free catalytic subunit [38]. These studies resulted in the discovery of the heat-stable PP1 inhibitor proteins [40].

The existence of these inhibitors led to two important considerations in PP1 enzymology: the role of the inhibitor proteins in regulating phosphatase activity and the existence of holoenzyme forms. Subsequent studies [41] identified two such inhibitors: I-1 and I-2 [review in [18, 31, 32]. I-1 and I-2 are small proteins (165 and 204 amino acids, respectively) with estimated molecular mass of 23kDa and 19kDa. I-1 is only inhibitory when phosphorylated at a threonine residue by cAMP-dependent kinase [18]. The concept of a holoenzyme complex of the catalytic subunit with regulatory subunits was supported by the finding that PP1 activity was generally associated with high molecular weight complexes recovered by gel filtration, but that could be reduced to a low

molecular weight form by proteolysis, urea or ethanol treatment [42, 43]. A high molecular weight form of PP was first isolated as a myosin light chain PP [44]. However, studies of an inactive form of PP1 that could be activated by ATP and that had been studied by Merlevede and co-workers for many years, led to its isolation and the surprising finding that it consisted of a 1:1 complex of the 37kDa PP1c and I-2 [45, 46]. In muscle, early studies had shown the existence of a "glycogen particle" in which glycogen sedimented from muscle extracts, was shown to be associated with phosphorylase phosphatase, PP1 and phosphorylase kinase [47, 48]. The purification of the glycogen-bound form of muscle PP1 revealed it to be a heterodimer of the catalytic subunit and a glycogen binding subunit, GM [49]. GM bound both glycogen and PP1, and also modified its substrate specificity in that its activity toward glycogen synthase was enhanced. Based on these studies Cohen [18, 50] proposed that the catalytic subunit could associate with different targeting subunits, which serve to direct the enzyme to specific subcellular locations and also provide regulatory functions. This finding was followed by isolation of the myosin bound PP1 which contained two other subunits, one of which, M110, binds to myosin [51]. The targeting hypothesis is substantiated by the recent discovery of a large number of PP1 binding proteins, and the current view that PP1c exists in many heterodimeric forms explains some of the original difficulties in the isolation of PP1c.

INADEQUACY OF PROTEIN PHOSPHATASE INHIBITOR BASED THERAPY APPROACHES

One of the most significant advances in the study of Ser/Thr-PPs, and the elucidation of the cellular events they control, was the identification of several naturally occurring toxins as powerful and specific PP inhibitors. Among these are okadaic acid [52], cantharidin [53], calyculin A [54], microcystins [52, 55, 56] and tautomycin [57, 58] to name a few examples. Another class of PP inhibitors are the protein inhibitors readily available inside the cell. For example, I-1 [41, 59] and I-2 [60]. The specificity of these inhibitors for a given PP has placed them as key tools in the study of phosphorylation dependent processes.

Physiologically, the correctness of most cellular events is dependent on the fine equilibrium of protein phosphorylation systems. When key proteins suffer abnormal phosphorylation a pathological state may developed. Thus, protein phosphorylation systems represent attractive targets for diagnostics and therapeutics. However, as discussed above, unlike the myriad of known protein kinases that all belong to a single gene superfamily, the PPs belong to several unrelated families. Furthermore, relatively few PP catalytic subunits exist, exhibiting broad and overlapping substrate specificities *in vitro*. From a clinical perspective, non-selective or marginally-selective PP inhibitors have broad biological activity and are highly toxic to eukaryotic cells due to the inhibition of a number of critical cellular processes. Therefore, the development of PP inhibitors like calyculin A, microcystin or cantharidin into therapeutic agents for systemic use seems unlikely. Nonetheless, fostriecin, an antitumor antibiotic, thought to be a type II DNA topoisomerase-directed anticancer drug, like doxorubicin or etoposide [61], was later found to be a PP2A and PP4 inhibitor [62] and reached phase I clinical trial. However, due to its instability and impurity, it did not pass into more advanced stages. Nonetheless, the development of type and isoform specific inhibitors (antisense oligonucleotides) seemed very promising, although still poorly specific. Both ISIS 15534 [63] and ISIS 14435 [64] have been employed to specifically suppress the expression of human PP5 and PP1γ1, respectively in human cultured cells. These isoform specific inhibitors were never used to treat any disorder but they have already proven useful for validation of drug targets. Antisense technology is thought to have the potential to have a major impact on the clinical management of many human disorders, if difficulties associated with systemic delivery are overcome, specifically in what concerns the degradation of PIPs (see below).

More interesting, however, is the data that indicate that *in vivo*, as PPs possess exquisite specificities, both in terms of substrates and localization, the key control mechanism must reside in the nature of the proteins to which they bind. An increasing number of proteins are being identified in diverse cell types that are capable of regulating the catalytic activity of PPs. Indeed, the diversity of such PIPs explains not only the need for few catalytic subunit types, but also makes them attractive targets for pharmacological intervention. The functional diversification of PP1 is controlled *via* its interaction with regulatory proteins. A major question remaining is how the regulators exert their discrimination between virtually identical mammalian PP1 isoforms. This may be partially explained by findings from Dominguez and co-workers [65] who showed that the most divergent N- and C- terminal ends of the PP1 isoforms are flexible structures, important in confering isoform specificity when interacting with different PIPs.

ALTERNATIVE PP1 INTERACTING PROTEINS (PIPs) BASED THERAPEUTIC STRATEGIES

During the last two decades evidence has accumulated, that the substrate specificity of PP1 is achieved by its interaction with other proteins that can act as targeting subunits or activity modulators. Targeting, as a requirement for molecular juxtaposition of proteins for the generation of signaling events, is well established as a paradigm in a number of growth regulated signaling systems involving tyrosine phosphorylation [66-68], as well as in the anchoring of Ser/Thr-protein kinases by A-kinase anchoring proteins (AKAPs), one of which also binds PP2B [66, 69]. The concept of targeting as it relates to PP1, however, is rather complex given the large number of PIPs that have been reported during the past years. In effect the number of PP1 holoenzymes that may exist has exploded as has the repertoire of cellular functions involving PP1. Table **1** lists 129 PIPs that have been identified in complexes with PP1 and have been shown to have physiological relevant functions (Table **1**). Consequently, many disease states may result from complexes malfunctioning or anomalies of the PIPs, forcing us to rethink the molecular basis of many pathological conditions.

Several mammalian PIPs have been identified that can be activity regulators, targeting proteins or even substrates. Activity-modulating proteins include true inhibitors such as I-1 [70] and CPI-17 [71], that in their phosphorylated form

Table 1. Human Phosphatase Interacting Proteins (PIPs) whose interaction are Physiologically Relevant and well Documented in the Literature

Gene	Alternatives names	Interaction detection method	References supporting interaction discovery	Holoenzyme function	References supporting function	Protein
AATK	LMTK1	YTH, Co-IP	[146]	Apoptosis	[146]	NP_001073864.2
AHCYL1	IRBIT, DCAL	GST-PD	[147]	Regulation of IP3R-controlled Ca2+ signals	[147]	NP_006612.2
AKAP 1	AKAP84, AKAP121, AKAP149	Co-IP	[148]	Nuclear envelope reassembly (dephosphorylation of B-type lamins)	[149]	NP_003479.1
AKAP11	AKAP220	BM, Co-IP, IC	[150]	Coordination of PKA/PP1 signalling	[151]	NP_057332.1
AKAP9	AKAP350, AKAP450, Yotiao, CG-NAP	Co-IP, BM	[152, 153]	Synaptic transmission (NMDA receptor ion channel activity), centrosomal function	[152, 154]	NP_005742.4
AKT1	PKB, RAC	Co-IP	[127]	Apoptosis	[127, 155]	NP_005154.2
ANKRD28	PITK, KIAA0379	Co-IP, BM	[156]	Transcription	[156, 157]	NP_056014.2
ANKRD42	SARP1	YTH, BM	[81]	Putative transcription factor	[81]	NP_872409.2
	SARP2					ABL09300.1
ANP32A	LaNP, PHAP1, PHAPI, I1^{PP2A}	BM	[72]	Stimulation of PP1 and inhibition of PP2A	[72]	NP_006296.1
ANP32B	SET, PHAPII, TAF1β, APRIL, SSP29, I2^{PP2A}					NP_006392.1
APAF1	CED4	Co-IP	[111]	Apoptosis	[111]	NP_037361.1
AR	Androgen receptor	Co-IP, BM	[158]	Splicing and transcription?	[158]	NP_000035.2
ATM	AT1, ATA, TEL1	BM	[159]	Cell cycle, DNA damage responses	[159-162]	NP_000042.3
AURKA	AURORA2, STK6, STK7, STK15, ARK1	Co-IP, GST-PD, BM	[163]	Cell cycle	[163-166]	NP_003591.2
AURKB	AIK2; AIM1; ARK2; AurB; IPL1; STK5; STK12	Co-IP	[167]	Cell cycle	[168, 169]	NP_004208.2
AXIN1	Axin	Co-IP, BM	[170]	Wnt/Beta-catenin signaling	[171]	NP_003493.1
BAD	BCL2L8, BBC2	YTH, Co-IP, GST-PD	[172, 173]	Apoptosis	[108, 111, 172-175]	NP_004313.1
BAX	BCL2L4	Co-IP	[111]	Apoptosis	[111]	NP_620116.1
BCL2	Bcl-2	YTH, GST-PD, Co-IP, BM	[172]	Apoptosis	[172, 174-176]	NP_000624.2
BCL2L1	Bcl-X, Bcl-XL	Co-IP, BM	[108]	Apoptosis, RNA splicing	[108, 174, 175, 177-179]	NP_612815.1
BCL2L2	Bcl-W	Co-IP, BM	[108]	Apoptosis	[108, 174, 175]	NP_004041.1
BRCA1	IRIS, PSCP	Co-IP, GST-PD, BM	[180]	Cell cycle, DNA repair	[180-182]	NP_009225.1
C7orf16	GSBS, G-substrate	BM	[183]	Inhibition of PP1	[183-185]	NP_006649.2
CASP2	ICH1, NEDD2	GST-PD, BM	[186]	Apoptosis	[186]	NP_116764.2
CASP9	MCH6; APAF3	Co-IP, GST-PD, BM	[187]	Apoptosis	[187]	NP_001220.2

(Table 1). Contd…..

Gene	Alternatives names	Interaction detection method	References supporting interaction discovery	Holoenzyme function	References supporting function	Protein
CAV1	CAV, VIP21, CGL3, BSCL3	Co-IP, BM	[188]	Cell cycle	[189, 190]	NP_001744.2
CDC25	CDC25C	Co-IP, BM	[191, 192]	Cell cycle	[191-193]	NP_001781.2
CDCA2	Repo-Man	Co-IP	[194]	Cell viability, cell cycle	[194, 195]	NP_689775.2
CDK5R1	NCLK, CDK5P35	AC, BM	[196]	Central nervous system development	[197, 198]	NP_003876.1
DCX	Doublecortin	Co-IP	[199]	Neuronal migration	[200]	NP_000546.2
EIF2AK2	PKR	YTH, Co-IP	[201]	HSV-1 infection, Protein synthesis	[201-203]	NP_002750.1
EIF2S2	EIF2beta	Co-IP	[204]	Translation initiation	[204]	NP_003899.2
FER/fer	TYK3	Co-IP	[205]	Cell cycle	[205]	NP_005237.2
GRM1	mGluR1	GST-PD	[206]	Glutamatergic transmission?	[206, 207]	NP_000829.2
GRM5	mGluR5					NP_000833.1
GRM7	mGluR7b	YTH, BM	[208]		[207, 208]	NP_870989.1
CFC1	HCF1	Co-IP, BM	[209]	Transcription and cell cycle	[209]	NP_005325.2
HDAC10	MGC149722	AC, BM	[128]	Transcription	[128]	NP_114408.4
HSPA5	GRP78, MIF2, BIP	BM	[210]	Chaperone, modulates PP1gamma2 activity	[210]	NP_005338.1
HSPA8	HSP71	Co-IP	[211]	Platelet adhesion	[211]	NP_006588.1
I-4	pseudogene	BM	[212]	Inhibits PP1	[212]	
ITGA3	Integrin, alpha chain 3	AC, BM, GST-PD	[213]	Regulation of Integrin phosphorylation	[213]	NP_002195.1
KDM4A	JMJD2A	Co-IP	[214]	Epigenic control of gene transcription and long term memory	[214]	NP_055478.2
KIAA1949	Phostensin	YTH, Co-IP, GST-PD	[215]	Actin rearrangements	[215]	NP_001128342.1
KIF13A	Kinesin family member 13A	Co-IP, BM	[200]	Cell cycle	[200]	NP_071396.4
LMNB1	Lamin B	Co-IP	[216]	Nuclear lamina assembly	[216, 217]	NP_005564.1
LMTK2	KPI-2 CPRK, AATYK2	GST-PD	[218]	Regulation of PP1 and PPP1R2 at the membrane surface	[218]	NP_055731.2
LRRC67	TLRR	YTH, Co-IP	[219, 220]	Spermatid transformation and tail formation	[220]	NP_001013648.1
MAPK1	MAK2, ERK, ERK2, p38, ERK2	Co-IP	[221]	IGF1-induced eIF2B activation	[221]	NP_002736.3
MAPK3	ERK1					NP_002737.2
MAPK8	JNK	BM, Co-IP	[222]	Apoptosis, JNK/Bcl2/PP1 complex	[222, 223]	NP_620637.1
MAPT	TAU	AC, BM	[224]	Microtubule assembly and stability	[224]	NP_058519.2
MYEF2	MEF2, KIAA1341, FLJ11213	YTH, Co-IP, GST-PD	[225]	Neuronal cell survival, cardiac remodeling and terminal differentiation of vascular, cardiac, and skeletal muscle	[225]	NP_057216.2
MYO16	MYR8	Co-IP	[226]	Brain development	[226]	NP_055826.1
NCAM1	NCAM180	GST-PD	[227]	Development and maintenance of nervous system	[227, 228]	NP_851996.2
	NCAM140					NP_000606.3
NCL	Nucleolin	Co-IP	[229]	Cell cycle, apoptosis	[230]	NP_005372.2
NCOR1	N-Cor, TRAC1, nuclear receptor co-repressor 1	Co-IP	[231]	Regulation of differentiation of neural stem cell	[231]	NP_006302.2

(Table 1). Contd…..

Gene	Alternatives names	Interaction detection method	References supporting interaction discovery	Holoenzyme function	References supporting function	Protein
NEFL	NF-L	AC, BM	[232]	Neuronal morphology, plasticity and/or transmission?	[232]	NP_006149.2
NEK2A	NEK2A	YTH, GST-PD	[77]	Centrosomal function, chromosome segregation,	[233]	NP_002488.1
	NEK2A-T	YTH, BM	[82]	Nuclear function	[136]	AAW56418.1
NOM1	SGD1, nucleolar protein with MIF4G domain 1	Co-IP	[234]	Targets PP1 to nucleolus	[234]	NP_612409.1
OCLN	Occludin	GST-PD, Co-IP	[235]	Negative regulation of the assembly of tight junctions	[235, 236]	NP_002529.1
PARD3	Par-3, Bazooka, Baz, AZIP	Co-IP, BM	[237]	Formation of functional tight junctions?	[237]	NP_062565.2
PCDH7	Protocadherin 7-c	YTH, GST-PD	[238]	Neuronal cell-cell interaction?	[238]	NP_115833.1
PER2	FASPS, KIAA0347	Co-IP	[239]	Circadian rhythms	[239, 240]	NP_073728.1
PFKM	PFK1, PFKA, PFKX, GSD7	AC	[241]	Glycolysis?	[241]	NP_000280.1
PHACTR1	phosphatase and actin regulator 1	YTH, Co-IP	[242]	Synaptic activity and actin cytoskeleton	[242]	NP_112210.1
PHACTR3	Scapinin	YTH, BM	[243]	Associated with the nuclear nonchromatin structure and cell spreading/motility through actin cytoskeleton	[243, 244]	NP_542403.1
PHACTR4	Phosphatase and actin regulator 4	YTH, Co-IP	[245]	Regulates neural tube and optic fissure closure	[245]	NP_001041648.1
PLCL1	PRIP, p130, phospholipase C-like 1	YTH, GST-PD, BM	[246]	Regulation of synaptic inhibition by controlling the dynamics of GABA receptor phosphorylation	[247]	NP_006217.3
PPP1R1A	Inhibitor 1, I1	BM	[248]	PP1 inhibitor, glycogen metabolism, synaptic plasticity and muscle contraction, cardiac function	[249, 250]	NP_006732.3
PPP1R1B	DARPP32	BM	[251]	Neurotransmission	[252, 253]	NP_115568.2
PPP1R1C	IPP5	GST-PD, BM	[254]	Apoptosis	[255]	NP_001074014.1
PPP1R2	Inhibitor 2, I2	BM	[248]	PP1 inhibition, phosphorylated by Pro-directed kinases	[107, 256]	NP_006232.1
PPP1R3A	GM, RGL, PPP1R3, PP1G	BM	[49]	Glycogen metabolism	[90, 257, 258]	NP_002702.2
PPP1R3B	GL, PPP1R4	AC, BM	[259, 260]		[261]	NP_078883.2
PPP1R3C	PTG, PPP1R5	Co-IP, BM	[262]		[263-265]	NP_005389.1
PPP1R3D	PPP1R6	GST-PD	[266]		[258]	NP_006233.1
PPP1R3E	KIAA1443, FLJ00089	BM	[267, 268]		[268]	BAB15779.1
PPP1R3F	Hb2E	BI	[267]		[269]	NP_149992.3
PPP1R3G		BI	[267]		[267]	NP_001138587.1
PPP1R7	Sds22	Co-IP	[270]	Mitosis, regulation of sperm function and epithelial cell polarity and shape	[271-273]	NP_002703.1
PPP1R8	NIPP1, ARD-1	BM	[274]	RNA splicing	[140, 275]	NP_054829.2
	NIPP1-T	YTH	[82]	RNA splicing (testis)?	[82]	NP_612568.1

(Table 1). Contd…..

Gene	Alternatives names	Interaction detection method	References supporting interaction discovery	Holoenzyme function	References supporting function	Protein
PPP1R9A	Neurabin I	GST-PD, BM	[276]	Dendritic spine signaling targeting both glutamatergic and dopaminergic pathways, synaptic plasticity and synaptic transmission	[277-280]	NP_001159632.1
PPP1R9B	Spinophilin, Neurabin II	YTH, BM, GST-PD	[281]		[277, 279, 280, 282, 283]	NP_115984.3
PPP1R10	PNUTS, p99, CAT53	YTH, BM	[284, 285]	RNA splicing, chromosome decondensation, apoptosis, proteasomal degradation and retinal synaptic activity	[286-290]	NP_002705.2
PPP1R11	Inhibtor 3, HCG-V, TCTE5, TCTEX5, IPP3	YTH, BM	[291]	Inhibits PP1, apoptosis, sperm function	[291-293]	NP_068778.1
PPP1R12A	MYPT1, M110, MBS, M130	BM	[294]	Myosin/actin targeting	[65]	NP_002471.1
PPP1R12B	MYPT2, PP1bp55, M20 splice form	AC	[295]	Myosin/actin targeting; target subunit of myosin phosphatase in heart	[296]	NP_002472.2
PPP1R12C	p85, LENG3	Co-IP	[297]	Myosin/actin targeting	[297]	NP_060077.1
PPP1R13B	ASPP1 , p53BP2-like	YTH, Co-IP	[298]	Apoptosis	[299]	NP_056131.2
PPP1R14A	CPI-17	BM	[300]	Inhibits smooth muscle myosin phosphatase increasing muscle contraction	[300, 301]	NP_150281.1
PPP1R14B	PHI-1	BM	[302]	Modulates retraction of endothelial and epithelial cells	[301, 303]	NP_619634.1
PPP1R14C	KEPI, CPI-17like	BM	[304]	PKC-dependent PP1 inhibitor; important for drug reward/addiction	[301, 304, 305]	NP_112211.1
PPP1R14D	shorter isoform, GBPI-1, CPI17like	BM	[306]	Inhibits PP1 when phosphorylated (Brain/Stomach) - activated by PKC and inactivated by PKA	[301, 306]	NP_060196.1
	longer isoform, GBPI-2, CPI17like			Inhibits PP1 when phosphorylated (Testis) - activated by PKC and inactivated by PKA		NP_001123615.1
PPP1R15A	GADD34	YTH, BM, GST-PD	[307]	Protein synthesis, regulation of calreticulin exposure, TGFbeta signaling	[308-310]	NP_055145.3
PPP1R15B	CReP	Co-IP	[311]	Protein synthesis	[311, 312]	NP_116222.3
PPP1R16A	MYPT3	YTH, BM, GST-PD	[313]	Myosin/actin targeting, translocation of nuclear receptors	[314, 315]	NP_116291.1
PPP1R16B	TIMAP, ANKRD4	Co-IP	[316]	Regulation of pulmonary endothelial barrier	[317]	NP_056383.1
PTK2	FAK, FADK, FRNK	BM, GST-PD	[318, 319]	Focal adhesion	[318]	NP_722560.1
RAF1	CRAF	Co-IP	[320]	RAF1 activation leading to the inactivation of MEK/ERK pathway	[320, 321]	NP_002871.1
RB1	RB, p110RB	YTH, Co-IP	[322]	Inhibition of cell cycle progression to S-phase and apoptosis	[323, 324]	NP_000312.2
RIPP1		BM	[325]	Ribosomal function?	[325]	
RL1	Neurovirulen-ce protein ICP34.5, $\gamma_1$34.5	YTH, GST-PD, BM	[326]	Inhibits protein synthesis, GADD34 related	[327, 328]	NP_044661.1
RPL5	L5 ribossomal protein	YTH	[329]	Ribosomal function?	[329]	NP_000960.2
RYR1	Ryanodine receptor 1	BM, AC	[330]	Calcium ion channel activity in skeletal and cardiac muscle	[331]	NP_000531.2

(Table 1). Contd…..

Gene	Alternatives names	Interaction detection method	References supporting interaction discovery	Holoenzyme function	References supporting function	Protein
RYR2	Ryanodine receptor 2	Co-IP	[332]	Calcium ion channel activity in cardiac muscle	[331, 332]	NP_001026.2
SFPQ	PSF	YTH, BM	[333]	RNA splicing	[334-336]	NP_005057.1
SFRS13A	SRp38, TASR, NSSR, FUSIP	Co-IP, GST-PD, BM	[337]	Regulation of pre-mRNA splicing in response to cellular stress	[337]	NP_006616.1
SH3RF2	HePP1, FLJ23654	YTH, Co-IP, GST-PD	[338]	Cardiac function?	[338]	ACU24608.1
SHOC2	SUR8	GST-PD	[339]	M-Ras effector; M-Ras targets this holoenzymes to activate RAF1	[339]	NP_031399.2
SLC9A1	NHE1, FLJ42224	BM, GST-PD	[340]	Regulating the Na^+/H^+ exchanger	[340]	NP_003038.2
SLC12A2	NKCC1	Co-IP	[341]	Sodium, potassium, chloride transport	[146, 341]	NP_001037.1
SMARCB1	SNF5, INI1	Co-IP, BM	[342]	Heterotrimeric complex with PP1 and GADD34. Targeting PP1 to potential substrates associated with hSWI/SNF complex?	[342]	NP_003064.2
STAU	Staufen, FLJ25010	AC, Co-IP	[343]	mRNA translocation and translation	[343, 344]	NP_059346.2
TAT p14	TAT-1	GST-PD, BM	[345]	HIV transcription, targets PP1 to the nucleus	[345, 346]	NP_057853.1
TLX1	TCL3, HOX11	GST-PD, BM	[93]	Cell cycle checkpoint	[347]	NP_005512.1
TP53	p53, TRP53, FLJ92943	Co-IP, BM	[348]	Apoptosis	[298, 348-350]	NP_000537.3
TP53BP2	PPP1R13A, p53BP2, ASPP2	Co-IP, BM	[298]	Apoptosis	[298, 351]	NP_001026855.2
VDR	Vitamin D receptor	YTH, BM, GST-PD,	[352]	Cell cycle and differentiation	[353]	NP_000367.1
WBP11	SIPP-1, SNP70, NpwBP	YTH, GST-PD	[354]	RNA splicing	[355]	NP_057396.1
YLPM1	ZAP3	AC-MS, BM	[110, 356]	Nuclear function	[356]	NP_062535.2
YWHAZ	14-3-3-zeta, KCIP-1	Co-IP, GST-PD	[357]	Spermatogenesis	[358]	NP_003397.1
ZFYVE16	Endofin	Co-IP, BM	[359]	Enhances the recruitment of PP1 to BMP receptor 1 (ALK3 and ALK6)	[359]	NP_001098721.1
ZFYVE9	SARA	YTH, Co-IP	[360]	Enhances the recruitment of PP1 to TGFb receptor 1	[310, 360]	NP_004790.2

Legend: YTH, yeast two hybrid; Co-IP, co-immunoprecipitation; GST-PD, GST-pull down; BM, biochemical methods; IC, immunocytochemistry; AC, affinity chromatography, BI, bioinformatics.

block the activity of PP1c towards all substrates. Or they may instead act as substrate-specifiers of PP1c. For example, I-1 PP2A/PHAP-I and I-2 PP2A/PHAP-II, which are potent inhibitors of PP2A, promote the dephosphorylation of specific substrates by PP1c [72]. Targeting proteins can bind both PP1c and one of its substrates. For example, MYPT1 binds PP1c as well as specific substrates such as myosin [73-75]. Other targeting subunits do not bind the substrate directly but instead associate with a subcellular structure that contains the substrate. For example, the G subunits target PP1 to glycogen particles which also bind the substrate glycogen synthase [76]. The targeting proteins of PP1 also include scaffolding proteins that mediate the formation of protein complexes. Often, these complexes function as signaling modules that contain both protein kinases and PPs and are localized in close proximity to the substrates of these enzymes. Substrates of PP1c directly and tightly bind with it and some also function as targeting proteins. Thus, the centrosomal protein kinase Nek2 not only binds its substrate C-Nap1 but also PP1c, and both Nek2 and C-Nap1 are substrates of the associated PP1c [77]. Some PP1c-bound substrates also function as activity-modulators. For example, the retinoblastoma protein interacts with PP1c both as a substrate and as a noncompetitive inhibitor [78].

Taking into account the multiple complexes that can be formed with PP1c, the variety of functions that the holoenzymes accomplish is astonishing (Table **1**). It follows that complex malfunctioning may lead to multiple disorders. There is substantial data supporting that PP1 holoenzyme deregulation have consequences in events as diverse as cell cycle and apoptosis, glycogen metabolism, transcription, RNA processing, translation, cytoskeleton organization, sperm motility, signal transduction pathways, learning and memory, to name a few examples [79-84]. Thus, the great importance of discovering novel therapeutic drugs that target PP1 complexes and/or PIPs in specific physiological pathways/events is clearly evident and urgent.

THE RVxF MOTIF

Most PIPs have been shown to bind to PP1c *via* a short sequence that is referred to as the "RVxF motif" [85-87]. Although the sequences that correspond to the RVxF motif are degenerate (the consensus sequence is [K/R]-X0-1-[V/I]-{P}-[F/W]) [88], the evidence of its role in the binding to PP1c is convincing. First, the co-crystallization of PP1c with a synthetic fragment of the muscle-type G subunit revealed that the RVxF sequence binds tightly in an extended conformation to a hydrophobic channel of PP1c that is remote from the catalytic site [86]. Modelling suggested that the variant RVxF motif of I-2 (KLHY) can be accommodated in a similar way in this channel [89]. Additionally, it has been demonstrated for various PIPs that mutations of the hydrophobic (V/I) and/or aromatic (F/W) residue in this motif is sufficient to disrupt or weaken their interaction with PP1c [86, 89-92].

The PP1c residues that are necessary for binding to the RVxF motif (in particular residues 287-293) are invariant in all isoforms from all species [85, 86]. However, they are not conserved in the catalytic subunits of PP2A or PP2B, which explains why most regulators of PP1 do not interact with these structurally related phosphatases. Conversely, the PIPs that interact with the catalytic subunit of PP2A (HOX11, I-1 PP2A/PHAP-I and I-2 PP2A/PHAP-II) do not contain an RVxF sequence [72, 93].

Binding to the RVxF sequence does not cause significant conformational changes in the catalytic subunit [86] and usually does not have major effects on the activity of the PP [92, 94, 95]. Studies on MYPT1 and I-2 indicated that the RVxF motif can function as an anchor for PP1c enabling these PIPs to make additional contacts with the PP in an ordered and cooperative manner [89, 96]. For example, four PP-interaction sites, in addition to the RVxF motif, have been identified for MYPT1 and I-2 [74, 89, 96]. Another recurring theme is that PIPs have common or overlapping binding sites on PP1c in addition to the RVxF-binding channel. For example, the inhibition of PP1c by phosphorylated I-1 [94], DARPP-32 [95] and MYPT1 [74] have all been attributed to the binding of the phosphorylated residue at or near the catalytic site as a pseudo-substrate. The sharing of interacting sites is also in accordance with the finding that various point mutations of PP1c show altered affinity for multiple PIPs [97, 98]. As expected from the unusually high conservation of residues on the surface of PP1c, mutagenesis studies in yeast have identified many surface residues as being essential for the binding of PIPs [97, 98]. A site that lies adjacent to the RVxF–binding groove has been identified as a binding pocket for the N-terminal "IKGI" motif of I-2 [99].

The picture that emerges shows that the binding of the PIPs to PP1c is mediated by multiple, degenerate, short sequence motifs and that PIPs can share interaction sites. It should be pointed out that this combinatorial control [100] of PP1c does not rule out the possibility that some PIPs might have unique binding sites. The combinatorial control of PP1 allows the exquisite physiological regulation of PP1 holoenzymes by hormones, growth factors and metabolites at the molecular level. Work on the various holoenzymes has demonstrated that their physiological regulation involves modulation of subunit interactions, which is mediated by reversible phosphorylation or allosteric regulation of the PIPs. For three unrelated PIPs has been shown that phosphorylation of Ser residue(s) within or close to the RVxF motif disrupts the binding of this motif to PP1c [92, 101, 102]. This results in altered activity of the holoenzyme or release of the catalytic subunit. In contrast, phosphorylation of other subunits strengthens their interaction with PP1c. Examples include I-1 and DARPP-32, in which an additional binding site for PP1c is created by phosphorylation. A different type of regulation involves binding of allosteric regulators. The allosteric binding of phosphorylase a to the C-terminal tail of the liver-type G subunit (GL) abolishes the activity of the associated PP1c towards glycogen synthase [103]. An additional level of regulation of PP1 holoenzymes is provided by targeting of these enzymes to specific substrates or subcellular structures. Bollen and co-workers, by a combination of bioinformatics tools and mutagenesis studies have delineated the consensus sequence and function of three PP1 binding motifs as being [K/R]-X0-1-[V/I]-(P)-[F/W], were X denotes any residue and P any residue except proline [88]. This sequence is very similar to a consensus sequence proposed by Zhao and Lee [87], [R/K]-[K/R]-X0-2-V-[R/H]-[F/W]-X-[DE], by panning of a random peptide display library. The main differences are the presence of an N-terminal basic residue and a C-terminal acidic residue in the Zhao and Lee sequence.

The RVxF-consensus sequences function as PP1 interaction sites only when they are present in a flexible and exposed loop that can be modelled into a β-strand. Additionally, other low affinity regions on the PP1 regulators strengthen the binding. Thus, the RVxF-consensus sequence functions like an anchor and other low affinity interactions have to occur that have regulator-specific effects on PP1 activity and specificity [88]. This definition is sensitive (it covers about 90% of all known PIPs) but non-specific (it occurs in about a quarter of all proteins) [104]. A less sensitive but more specific definition was introduced by Meiselbach *et al.* [105] that proposed the consensus sequence [HKR]-[ACHKMNQRSTV]-V-[CHKNQRST]-[FW]. More recently, work from Bollen and co-workers allowed the redefinition of the RVxF motif and its flanking residues based on the sequences of 143 PIPs - [KRL]-[KRSTAMVHNQ]-[VI]-{FIMYDP}-[FW] [106].

Other PP1 binding motifs have been described: the SILK motif present in I-2 and SIPP-1 [107]; F-X-X-R-X-R, that also appears to exist in several PP1 interactors [108]; and the MyPhoNE motif (RxxQ[VIL][KR]x[YW]) present in MYPT-1 [65].

The existence of common binding sites for the PIPs explains why a relatively small protein such as PP1c can interact with numerous structural different PIPs and why the binding of most PIPs is mutually exclusive.

HIGH-THROUGHPUT IDENTIFICATION OF PIPs

Most proteins exert their biological functions through interaction with other molecules, in macromolecular complexes. Several methodologies have been developed in order to study such associations. PIPs have been identified by the yeast two hybrid (YTH) system, bioinformatic approaches based on genome scanning for proteins possessing the PP1 binding motifs and affinity purification coupled to mass spectrometry identification.

The YTH system, in combination with complementary biochemical assays, can be used to yield large numbers of interacting pairs, to unravel, for example, interaction maps of human cells [109]. Also important is the additional computational research which is also needed in order to analyse all the data obtained and to confirm its reliability and indicate the implications of the detected interactions in possible cellular processes. The majority of the PP1 regulators were identified by classical biochemical approaches and YTH screens.

A microcystin-Sepharose chromatography and mass spectrometry study, for affinity purification and identification of PIPs from rat liver nuclei, showed that many more PIPs exist in the nucleus than previously demonstrated [110]. For example, The RNA binding protein ZAP3 was established as a nuclear PIP by co-purification with PP1 on microcystin-Sepharose, by co-immunoprecipitation, by binding PP1 in an overlay assay and by the presence of a putative PP1 binding motif [110].

In order to control physiological relevant dephosphorylation reactions, that are necessary in a time and space dependent manner in the cell/tissue, PP1 diverse functions and isoforms must be independently regulated. As such, several studies and techniques were developed to identify novel PIPs in order to gain insight into the diverse physiological PP1 functions. Among these studies is a screen using an antibody array that allowed the identification of 31 novel molecular partners of PP1 [111].

More recently a displacement affinity chromatography of PP1 complexes was performed using peptides containing an RVxF PP1 binding motif, by disruption of the PP1-regulatory subunits interaction [112]. Subsequent co-immunoprecipitation experiments confirmed that each identified binding protein was either a direct PP1 interactor or was in a complex that contain PP1. It was suggested that the identification of PIPs in previous chromatography studies may have been masked when microcystin-sensitive PPs and non-specifically bound proteins were released from the matrix using other elution methods [111, 113].

By standard sequence analysis of the PIPs it was not possible to find common structural similarities given that most of the known PP1 docking motifs are short and degenerate and flanked by unconserved stretches [65, 114]. As discussed above, most of the PIPs contain a PP1 binding motif referred to as the RVxF motif [79, 86]. A recent in silico search for novel PP1 interactors through analysis of the combined Meiselbach ([RK]-X(0,1)-[VI]-{P}-[FW]) and Wakula ([ACHKMNQRSTV]-V-[CHKNQRST]-[FW]) definitions of the PP1 RVxF binding motif, as well as other additional filters [106] was carried out. This analysis was performed using 14,725 sets of orthologous human, mouse and rat protein sequences corresponding to over 14,000 genes and 115 novel putative PP1 interacting proteins were identified, 78 of which were validated by independent biochemical approaches [106]. This study demonstrated the possibility of performing bioinformatics-assisted proteome-wide screens with structurally unrelated proteins that share short motifs.

All the methods described above provide putative PIPs but the main advantages of the YTH methods are that the interactions are detected *in vivo*, there are no artificial lysing or washing steps and it has the potential to detect weak or transient interactions. It is a highly sensitive method and even low expression interacting proteins can be detected; in contrast with mass spectrometry where less abundant peptides may not be detected in the presence of more abundant ones. In addition, the YTH screens, unlike other methods, does not need the previous knowledge of the proteins to be tested and complete cellular or tissue cDNA libraries can be used. Its drawbacks are that it mostly detects binary interactions and the forced nuclear localization of the interacting proteins can create false positives or false negatives. Also, artificial fusion may inhibit certain interactions and some interactions are preferred with regard to cellular compartments.

The diverse approaches used to identify PIPs need to be complemented by independent methodologies to validate the novel PP1 interactors and to determine the physiological relevance of the same interactions. However, the techniques *per se* provide new lines of inquiry which led to new insights into the PP1 interactome and its multiple functions in the cell.

THERAPEUTIC STRATEGIES BASED ON PIP TARGETING

Since the approval of the first kinase target drug, imatinib, for chronic myelogenous leukaemia and gastrointestinal tumors, the pharmaceutical market for drugs that target protein kinases is second only to G-protein-coupled receptors [115]. Efforts are also being made by pharmaceutical companies and researchers in the discovery of therapeutical compounds that allow the manipulation of PPs. However, despite all the efforts, the only FDA-approved drugs targeting a PP are cyclosporine A and FK506, both PP2B inhibitors. These compounds are naturally occurring and are used clinically for their immunosuppressive activities [116]. Regardless of their great success in treatment of organ rejection, long-term usage can lead to nephrotoxicity and hepatotoxicity [117]. Cantharidin has long been used in China for the removal of dead tissue [118] and in US for wart treatment prior to 1960 [119], but only in the 1990's was its mechanism of action, binding and inhibition of PP1 and PP2A, described [120, 121]. The wide range of functions associated with PP1 and PP2A catalytic subunits is the reason why these enzymes are not good drug targets. Nowadays

efforts are underway to search for new semi-synthetic PP inhibitors that selectively target a specific PP. Even more ambitious would be the search for compounds that discriminate between PP isoforms.

In contrast, our perspective is that it will be better to target the proteins to which PP bind to, the PIPs, they provide a better target being much more event, tissue or subcellular compartment specific than the PP *per se*, thus limiting unnecessary toxic side effects and increasing specificity (Fig. (**1**)).

DISRUPTION OR ACTIVATION OF PP1 COMPLEXES

As already described, the vast majority of PIPs possess a conserved PP1 binding motif [106], known as the RVxF motif. This was the basis of an experimental assay developed by Cohen *et al.* [24] which assessed the total amount of PP1 associated with a particular PIP in a given cellular condition and the effect of that PIP on PP1 activity could be determined. To perform the assay, a dissociating RVxF-containing peptide was used that disrupted the immune-PP1 complex. Thus, it can be hypothesised that to interfere with different PP1 holoenzymes *in vivo* diverse peptides can be used, altering the function of PP1 complexes by disturbing them. For example, synthetic RVxF-peptides have been used to disrupt PP1 complexes and after being introduced into neuronal cells they have been shown to alter synaptic transmission [122, 123]. Moreover, synthetic RVxF-peptides have also been shown to activate PP1c [124]. A search for "RVxF mimic" compounds was undertaken and a compound resulted that activated PP1 by two-fold in *in vitro* assays [124]. Also, Garcia and co-workers [125] developed non-toxic cell permeable competing peptides that mimic the Bad PP1 interacting motif and that when delivered to cultured cells, induces cell death.

In a screen for small molecules that protect cells from endoplasmic reticulum (ER) stress, salubrinal, a selective inhibitor of cellular complexes that dephosphorylate eukaryotic eIF2alpha was identified. Salubrinal also blocks eIF2alpha dephosphorylation mediated by a Herpes simplex virus protein and inhibits viral replication [126]. Salubrinal was shown to block eIF2alpha dephosphorylation by the PP1-GADD34 complex which is activated during ER stress. Treatment of glioblastoma and prostate cancer cells with the Histone deacetylases (HDACs) inhibitor trichostatin A (TSA) disrupts PP1-HDAC6, resulting in increased dephosphorylation or acetylation of for example Akt, decreasing its activity, [127, 128]. HDACs are accepted targets for cancer therapy and several HDAC inhibitors are under clinical trials [129] but the molecular mechanism beyond their antitumor properties is not well characterized and maybe, at least for some of the inhibitors, the mechanism resides in the disruption of the PP1-HDACs complexes.

Thus, at least at the moment two complexes (PP1-GADD34 and PP1-HDAC1/6) are being disrupted by protein complex agonists (Salubrinal and TSA) with an apparent

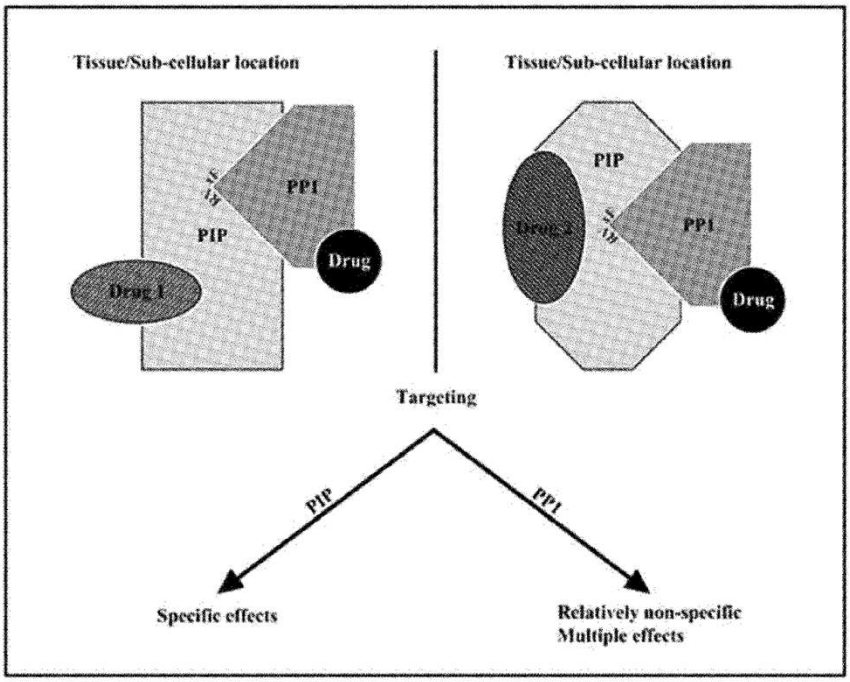

Fig. (1). Signal transduction therapeutics based on PP1 or PIPs targeting. Drugs targeting PP1 (e.g. tautomycin) would have relatively non-specific multiple effect while drugs targeting PIPs would have tissue/sub-cellular location specific effects.

therapeutic consequence on Herpes simplex virus replication inhibition and cell growth and transformation inhibition, respectively [130].

It is also worthwhile considering our recent work [131] indicating that the pathologically important peptide (Abeta) may exert some of its physiological effects *via* direct inhibition of PP1 activity. However, to date it is not known whether the effects of this peptides are mediated *via* binding of specific PP1 complexes or not. Nevertheless, it may be a feasible strategy to test modified versions of the Abeta peptide as potential modulators of specific PP1 complexes. It would be particularly pleasing if an endogenous pathologic peptide could be the basis for developing a therapeutic approach.

ANTI-SENSE OLIGONUCLEOTIDE THERAPY

Recently, there has been growing interest in alternative splicing, not only as a mechanism to increase the functional complexity of the human genome, but also as a means of providing highly specific targets for new drug development in the treatment of a variety of human diseases. Alternative splicing may represent the major factor accounting for the discrepancy between the number of genes believed to be encoded in the mammalian genome and the much larger proteome. Alternative splicing is a highly regulated process that can produce specific protein variants for different tissues, developmental stages, or even pathological states [132]. Interestingly, errors can occur during the splicing process that can lead to pathological conditions. In certain cases it is thought that some single base-pair mutations associated with human genetic diseases are related to defects in the splicing events [133]. As a result, some splice variants constitute useful diagnostic markers for certain disease conditions. Moreover, since some splice variants are tissue-specific, we propose that such alternatively spliced proteins may be useful targets for the development of highly specific new drugs that, therefore, will not affect other variants of the same protein expressed in other tissues (or conditions).

Three genes are known to encode mammalian PP1c subunits (α, β and γ) and all of them undergo specific processing to produce tissue/event-specific isoforms. For instance, PP1γ is known to undergo alternative splicing to yield an ubiquitously expressed PP1γ_1 isoform and a testis/sperm-enriched isoform, PP1γ_2 [134]. These two PP1 isoforms differ only at the extreme C-terminus. Thus, a drug targeting specifically the PP1γ2 C-terminal region should be rather specific, and should not affect the PP1γ1 isoform found in other organs besides the testis/sperm. From our own experience, we find that testis has several alternatively spliced mRNAs, including PP1γ. Indeed, an even more specific scenario would be to target a PP1γ_2 binding protein that, presumably, would be involved in a much more restricted number of cellular events than the PP catalytic subunit [135]. Our results point to a large number of putative binding PP1 proteins that are alternatively spliced, for instance Nek2C, NIPP1-T and SARP2, some of which may ultimately be used as targets to develop new drugs based on antisense oligonucleotides - ASON [81-83, 136]. By controlling alternative splicing mechanisms one of the spliced isoforms could be favored allowing for the formation of a PP1 holoenzyme with particular characteristics.

There are several examples in the literature that point to the importance of ASON as alternative RNA splicing modulators. Indeed, the BCL2L1 gene codes for two isoforms, derived by the use of two alternative 5' splice sites, with opposite functions. Bcl-xL is anti-apoptotic and Bcl-xS is pro-apoptotic, and both are required for normal cell functioning. Many cancers overexpress Bcl-xL. An ASON targeting this isoform, increased the Bcl-xS/Bcl-xL ratio, and induce apoptosis in PC3 prostate cancer cells [137, 138]. Moreover, PP1 binds Bcl family members and the latter possess the RVxF motif and a FxxRxR motif. Interestingly, Bcl-xS does not have the PP1 binding motif RVxF suggesting a weaker or absent binding to PP1. Therefore, modulating alternative splicing also interferes with PP1 holoenzyme formation. The same is true for the PIP PPP1R8 (NIPP-1) that comprises a variety of spliced isoforms α, β/δ, γ (or ARD-1) and NIPP1-T [82]. The γ isoform is the only one where the PP1 binding motif RVxF is absent, thus it does not bind PP1 and has the unique feature of having endoribonuclease activity [139]. All the others isoforms are PP1 binding proteins. PPP1R8α, and β, target PP1 to the nucleus spliceossome machinery [140, 141]. NIPP1-T is a testis-enriched isoform whose specificities are still poorly understood. Adjustment of splice variants isoforms will stimulate one or other isoform specific events.

These are just two examples of many PIPs that undergo alternative splicing, specific to a tissue, a developmental state, a certain disorder, gender, or other condition, which may in turn affect the formation of the PP1/PIP complex and ultimately the function of the complex.

Prostate-specific membrane antigen can occur as a long (PSM) or a short (PSM') alternatively spliced isoforms derived by the use of alternative 3' splice sites in exon 3. The PSM/PSM' ratio is 150 times higher in prostate cancer than in normal tissue, providing a reliable diagnostic marker for the occurence and progression of the disease [142]. PSM may be a good therapeutical target and it would be very interesting to restore the correct PSM/PSM' ratio by oligonucleotide-induced exon skipping. This illustrates how the ratio of spliced isoforms can provide diagnostic biomarkers. This approach could also be taken for PP isoforms and PIPs isoforms in a panoply of other disorders.

In what concerns the use of ASON as therapeutic tools, the approach has a great potential in the very near future since many ASON have already been approved for clinical use. The first was Vitravene for the treatment of cytomegalovirus retinitis [143]. To date, the many ASON approved are under clinical trials for the treatment of a variety of diseases, such as diabetes, cancer, infections, neurodegenerative and metabolic disorders [144].

The availability of complete genome sequences from a variety of organisms initiated the development of new approaches to deal with a huge amount of genomic information. Thus, high-throughput methods for detecting protein interactions were developed. Among them, the most used are the YTH, Affinity Purification coupled to Mass Spectrome-

try and, on a smaller scale, proteome chips [145], which have yielded vast amounts of data that can be exploited to infer protein function and regulation. These methods together with the availability of sophisticated databases are helping to decode complex interactions among proteins and to integrate interacting proteins in complex cellular pathways.

However, sequencing the genome of an organism does not mean decoding the functions of the proteins it encodes. Thus, the more challenging issue is to define the proteome of a given organism, and even more importantly, to characterize the interactions between its constituent proteins. Additionally, it is of great importance to determine when and where the proteome of an organism is expressed and to identify post-translational modifications that regulate protein function, ultimately elucidating how an organism or cell in that organism behaves. Today, people try to identify the interactions between proteins in a normal versus a disease state, being of great biomedical value in order to identify putative protein targets to cure a certain disease and identify disease markers. Thus, our ultimate goal is to develop therapeutic compounds that can disrupt or strength PP1 holoenzymes specifically, in order to interfere with a specific cellular event in a particular subcellular location or in a given organ or tissue.

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ABBREVIATIONS

AKAP = A-Kinase Anchoring Protein

AD = Alzheimer's Disease

APP = Amyloid Percursor Protein

ASON = Antisense Oligonucleotides

EGFR = Epithermal Growth Factor Receptor

GM = Glycogen binding subunit

GSK3β = Glycogen Syntase Kinase 3 β

HDAC = Histone Deacetylase

HD = Huntington Disease

I-1 = Inhibitor 1

I-2 = Inhibitor 2

PD = Parkinson's Disease

PDGFR = Platelet-Derived Growth Factor Receptor

PP1c = PP1 catalytic subunit

PIP = PP1 Interacting Protein

PSM = Prostate-specific Membrane Antigen

PKA = Protein Kinase A

PKC = Protein Kinase C

PP = Protein Phosphatase

STPP = Ser/Thr specific PP

TSA = Trichostatin A

VEGFR = Vascular Endothelial Growth Factor Receptor

YTH = Yeast Two-Hybrid

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| Appendix

APPENDIX

I. Culture Media and solutions

Bacteria 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

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).

Yeast Media:

SD synthetic medium

To 800mL of deionised H_2O add:

6.7g Yeast nitrogen base without amino acids (DIFCO)

20g Agar (for plates only)

Shake until the solutes have dissolved. Adjust the volume to 850mL 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) and 100mL of the appropriate 10X dropout solution.

10X dropout solution (DO 10X)

This solution contains all but one or more of the following components:

	10X concentration (mg/L)	SIGMA #
L-Isoleucine	300	I-7383
L-Valine	1500	V-0500
L-Adenine hemisulfate salt	200	A-9126
L-Arginine HCl	200	A-5131
L-Histidine HCl monohydrate	200	H-9511
L-Leucine	1000	L-1512
L-Lysine HCl	300	L-1262
L-Methionine	200	M-9625
L-Phenylalanine	500	P-5030
L-Threonine	2000	T-8625
L-Tryptophan	200	T-0254
L-Tyrosine	300	T-3754
L-Uracil	200	U-0750

10X dropout supplements may be autoclaved and stored for up to 1 year.

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).

2X YPDA

Prepare YPD as above. After the autoclaved medium has cooled to 55ºC add 15mL of a 0.2% adenine hemisulfate solution per liter of medium (final concentration is 0.003%).

Solutions

50X TAE Buffer

242 g Tris base

57.1 mL glacial acetic acid

100 mL 0.5M EDTA (pH 8.0)

TE Buffer (pH 7.5)

10 mM Tris-HCl (pH 7.5)

1 mM EDTA, pH 8.0

Loading Buffer (LB)

0.25% bromophenol blue

30% glycerol

STET

8% Sucrose

5% Triton X-100

50 mM Tris-HCl (pH 8,5)

50 mM EDTA

Competent Cell Solutions:

Solution I (1L)

9.9 g $MnCl_2.4H_2O$

1.5 g $CaCl_2.2H_2O$

150 g glycerol

30 mL KHAc 1M;

adjust pH to 5.8 with HAc, filter through a 0.2μm filter and store at 4ºC

Solution II (1L)

20 mL 0.5M MOPS (pH 6.8)

1.2 g RbCl

11g $CaCl_2.2H_2O$

150 g glycerol;

filter through a 0.2μm filter and store at 4ºC

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

SDS-PAGE and Immunobloting Solutions:

LGB (Lower Gel Buffer)

To 900 mL of deionised H_2O add:

181.65 g Tris

4 g SDS

Mix until the solutes have dissolved. Adjust the pH to 8.9 and adjust the volume to 1L with deionised H_2O.

UGB (Upper Gel Buffer)

To 900 mL of deionised H_2O add:

75.69 g Tris

Mix until the solute has dissolved. Adjust the pH to 6.8 and adjust the volume to 1L with deionised H_2O.

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.

Loading Gel Buffer

250 mM Tris-HCl (pH 6.8)

8% SDS

40% Glycerol

2% 2-mercaptoethanol

0.01% Bromophenol blue

1X Running Buffer

25 mM Tris-HCl (pH8.3)

250 mM Glycine

0.1% SDS

1X Transfer buffer

25 mM Tris-HCl (pH8.3)

192 mM Glycine

20% Methanol

1X TBS

10 mM Tris-HCl (pH 8.0)

150 mM NaCl

Adjust the pH to 8.0 with HCl and adjust the volume to 1L with deionized H_2O

1X TBST

10 mM Tris-HCl (pH 8.0)

150 mM NaCl

0.05% Tween

Adjust the pH to 8.0 with HCl and adjust the volume to 1L with deionized H_2O

Membrane Stripping Solution

2% SDS

62.5 mM Tris-Hcl (pH= 6.7)

100 mM β-Mercaptoethanol

Yeast Two-Hybrid Solutions:

Yeast plasmid rescue – Breaking buffer

2 % Triton X-100

1 % SDS

100 mM NaCl

10M Tris-HCl (pH 8.0)

Yeast transformation

- PEG/LiAc (polyethylene glycol 4000/lithium acetate)

	Final Conc.	To prepare 10 ml of solution
PEG 4000	40%	8 ml of 50% PEG
TE buffer	1X	1 ml of 10X TE Buffer
LiAC	1X	1 ml of 1 M LiAc (10X)

Solutions for preparation of yeast protein extracts

- Protease inhibitor solution: always prepare solution fresh just before using. Place on ice to prechill.

To prepare 688µl add in a microfuge tube:

66µl Pepstatin A (1 mg/mL stock solution in DMSO)

2µl Leupeptin (10.5 mM stock solution)

500µl Benzamidine (200 mM stock solution)

120µl Aprotinin (2.1 mg/mL stock solution)

- PMSF (phenylmethyl-sulfonyl fluoride) stock solution (100X)

Dissolve 0.1742g of PMSF in 10mL isopropanol. Wrap tube in foil and store at RT.

- Cracking buffer stock solution

To 80mL of deionised H_2O add:

48g Urea

5g SDS

4mL 1M Tris-HCl (pH6.8)

20µl 0.5M EDTA

40mg Bromophenol blue

Mix until the solutes have dissolved. Adjust the volume to 100mL with deionised H_2O.

- Cracking buffer

To prepare 1.13mL add in a microfuge tube:

1 mL Cracking buffer stock solution (recipe above)

10 µl β-mercaptoethanol

70 µl Protease inhibitor solution (recipe above)

50µl 100X PMSF stock solution

Immunoprecipitation solutions:

Lysis Buffer

50 mM Tris-HCl (pH 8)

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

Cell Culture Solutions and Immunocytochemistry:

PBS (1x)

For a final volume of 500 mL, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in deionised H_2O. Final composition:

8 mM Sodium Phosphate

2 mM Potassium Phosphate

40 mM NaCl

10 mM KCl

Sterilize by filtering through a 0.2 μm filter and store at 4 ºC

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.

Complete MEM + GLUTAMAX

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)

Antibiotics (5mL)

100 U/mL penicillin and 100 mg/mL streptomycin

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

DMEM medium

For a final volume of 1L, dissolve one pack of DMEM powder (with L-glutamine and 4500mg glucose/L, Sigma Aldrich) in deionised H_2O and add:

- $NaHCO_3$ (Sigma-Aldrich) 3.7g

Adjust to pH 7.4. Sterilize by filtering through a 0,2um filter and store at 4ºC.

Complete DMEM

For a final volume of 1L, when preparing DMEM medium adjust to pH 7.4 and before sterilizing add:

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

Antibiotics (5mL)

100 U/mL penicillin

100 mg/mL streptomycin

DMEM Components

Amino Acids:	Concentration (mg/L)
L-Arginine hydrochloride	84
L-Cystine	62,6
L-Glutamine	584
Glycine	30
L-Histidine hydrochloride	42
L-Isoleucine	105
L-Leucine	105
L-Lysine hydrochloride	146
L-Methionine	30
L-Phenylalanine	66
L-Serine	42
L-Threonine	95
L-Tryptophan	16
L-Tyrosine	103,79
L-Valine	94
Vitamins:	
Choline chloride	4
D-Calcium pantothenate	4
Folic Acid	4
Niacinamide	4
Riboflavin	0,4
Thiamine hydrochloride	4
Myo-Inositol	7,2
Inorganic Salts:	
Calcium Chloride ($CaCl_2.2H_2O$)	200
Magnesium Sulfate ($MgSO_4.7H_2O$)	97,67
Potassium Chloride	400
Sodium Chloride	6400
Sodium Phosphate monobasic ($NaH_2PO_4.2H_2O$)	109
Other components:	
D-Glucose	4500
Phenol Red	15,9

II. Bacteria and Yeast Strains

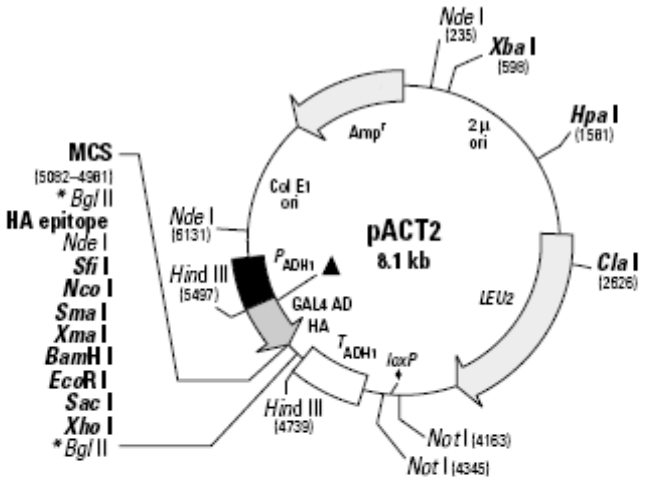
- E. coli XL1-blue: *recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacZΔM15 Tn10(Tet^r)]*

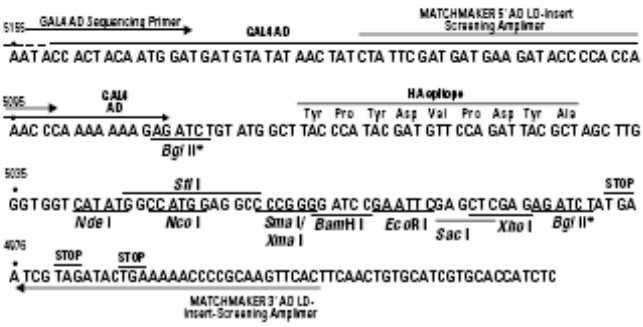
- Rosetta(DE3)pLysS: F^- ompT $hsdS_B(R_B^- m_B^-)$ gal dcm λ(DE3 (lacI lacUV5-T7 gene 1 ind1 sam7 nin5) pLysSRARE (Cam^R)

- 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

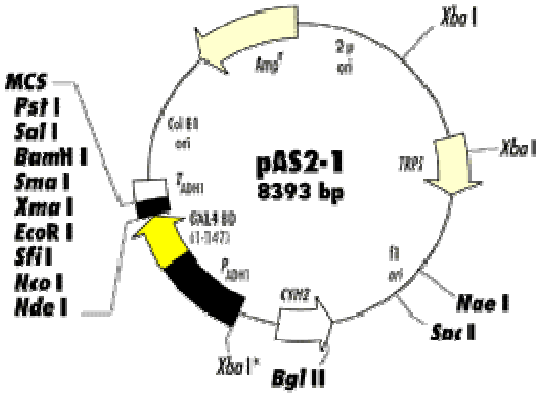
- S. cerevisiae Y187: MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met^-, gal 80Δ, URA3::$GAL1_{UAS}$-$GAL1_{TATA}$-lacZ, MEL1

III. Plasmids

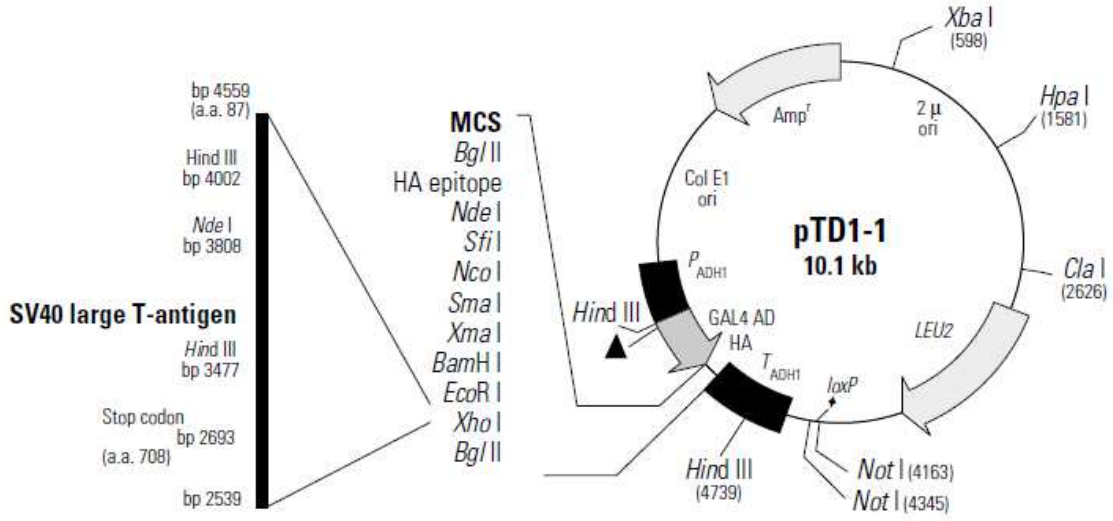




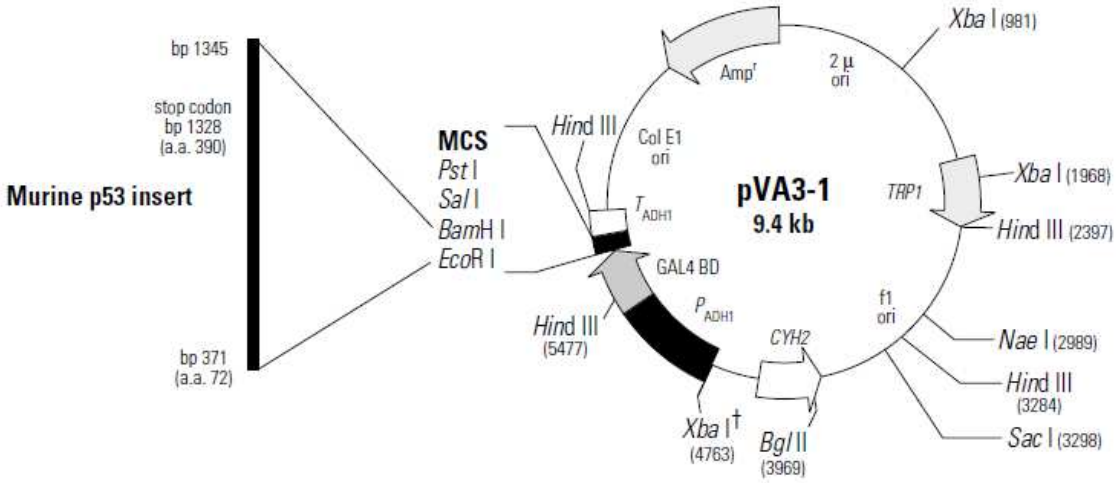
pACT2 *(Clontech) map and MCS. Unique sites are coloured blue. pACT2 is used to generate a hybrid containing the GAL4 AD, an epitope tag and a protein encoded by a cDNA in a fusion library. The hybrid protein is expressed at medium levels in yeast host cells from an enhanced, truncated ADH1 promoter 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.*



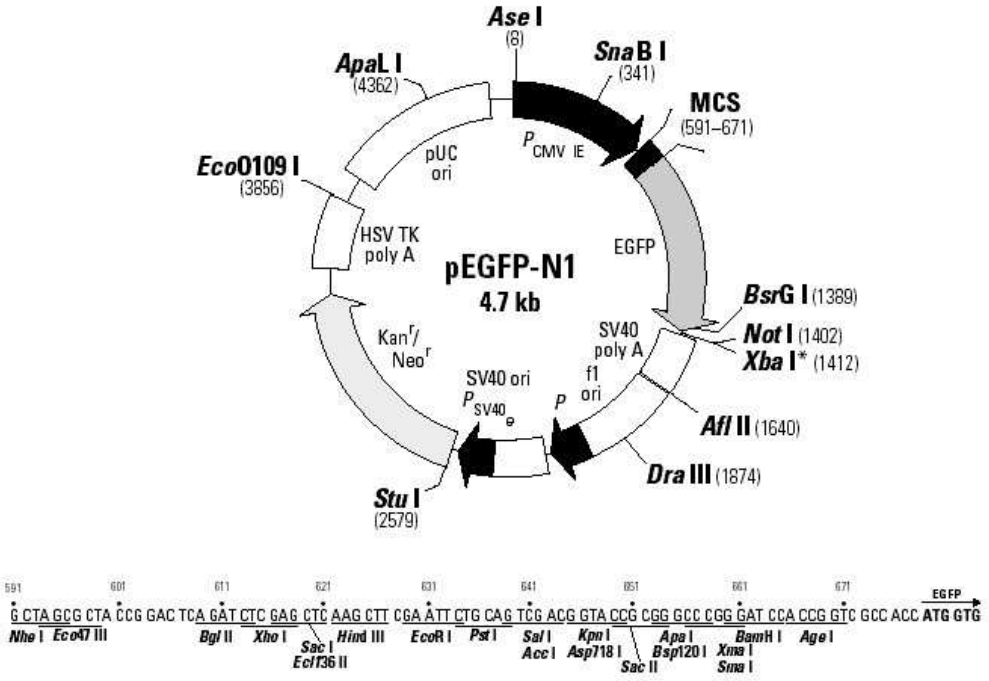
pAS2-1 (*Clontech*) *map and MCS. Unique sites are coloured blue. pAS2-1 is a cloning vector used to generate fusions of a bait protein with the GAL4 DNA-BD. The hybrid protein is expressed at high levels in yeast host cells from the full-length ADH1 promoter. The hybrid protein is target to the yeast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



pTD1-1 map. *pTD1-1 is a positive control plasmid that encodes a fusion of the SV40 large T-antigen (a.a. 87–708) and the GAL4 AD (a.a. 768–881). The SV40 large T-antigen cDNA (GenBank Locus SV4CG) was cloned into pACT2. The SV40 T-antigen insert was derived from the plasmid referenced in Li & Fields (1993); plasmid modification was performed at CLONTECH. pTD1-1 has not been sequenced and it is not known whether any of the sites are unique.*



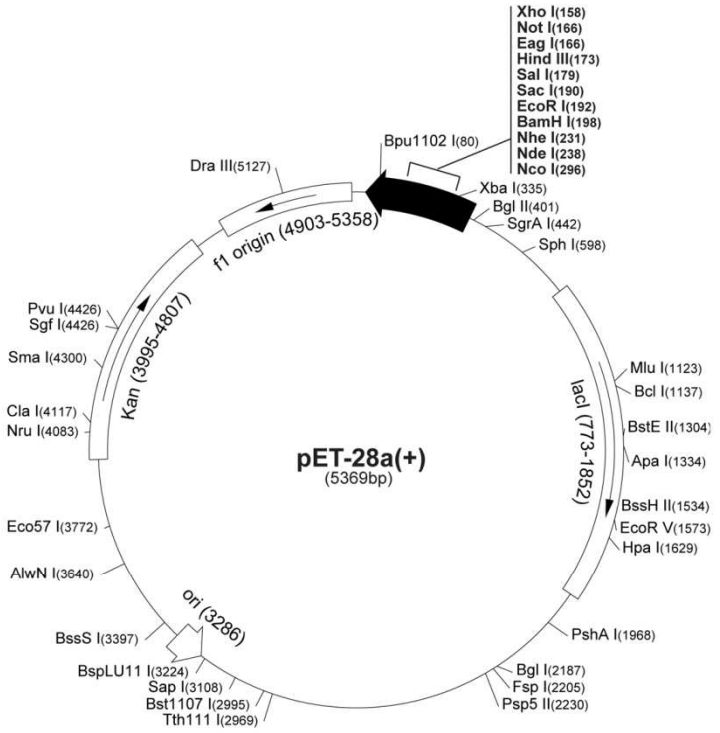
pVA3-1 map. *pVA3-1 is a positive control plasmid that encodes a fusion of the murine p53 protein (a.a. 72–390) and the GAL4 DNA-BD (a.a. 1–147). The murine p53 cDNA (GenBank Accession #K01700) was cloned into pAS1CYH2 (a precursor of pAS2-1). The p53 insert was derived from the plasmid described in Iwabuchi et al. (1993); plasmid modification was performed at CLONTECH. The Xba I site at bp 4763 (†) is methylation sensitive. pVA3-1 has not been sequenced and it is not known whether any of the sites are unique.*

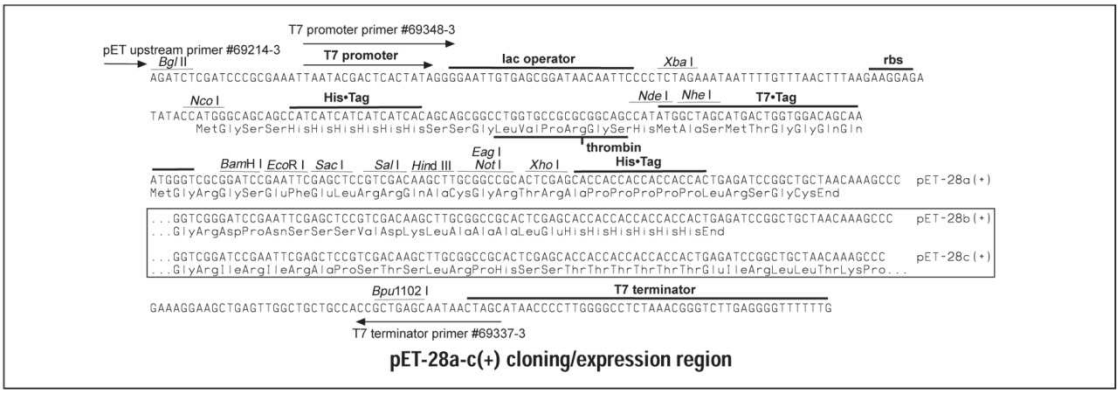


pEGFP-N1 *vector map and MCS (Clontech). This eukaryotic exppression vector was used to express GFPtag fusion protein mammalian cells.*

pET-28a(+) sequence landmarks	
T7 promoter	370-386
T7 transcription start	369
His• Tag coding sequence	270-287
T7• Tag coding sequence	207-239
Multiple cloning sites	
(*Bam*H I - *Xho* I)	158-203
His• Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.





IV. Primers

Sequencing and subcloning	
Primer	**Sequence (5'->3')**
GAL4 AD Sequencing, FW	TACCACTACAATGGATG
GAL4 BD Sequencing, FW	TCATCGGAAGAGAGTAG
Amplimer 3' Sequencing, RV	ATCGTAGATACTGAAAAACCCCGCAAGTTCAC
pEGFP-N1-FW Sequencing, FW	GTAGGCGTGTACGGTGGGAG
pEGFP-N1-RV Sequencing, RV	GCCGTCCAGCTCGACCAGG
pT7 promotor-FW Sequencing, FW	AATACGACTCACTATAG
Tprn-GFP-Fw Subcloning in pEGFP vector	CCCGGAATTCCGATGGAGACCATCCCCTTG
Tprn-GFP-RV Subcloning in pEGFP vector	ACGCGTCGACGTGAAATACAGGGCTGGCTC
Sph1A-GFP-FW Subcloning in pEGFP vector	CCCGCTCGAGATGACATATCTTATTCAG
Sph1A-GFP-RV Subcloning in pEGFP vector	ACGCGTCGACGCCAAGAAGAGTTCTTTTG
pET-Sph1A-FW Subcloning in pET-28a vector	ACGCGTCGACGCATGACATATCTTATTCAG
pET-Sph1A-RV Subcloning in pET-28a vector	GAATGCGGCCGCTTACAAGAAGAGTTCTTTT

qRT-PCR Primers

Taperin 1 (TPRN)

PCR template used: NM_175286.4

Region: 1-2825

	Sequence (5'->3')	Strand	Length	Start	Stop	Tm	GC%	Self comp.	Self 3' comp.
Forward primer	ACCGAAGGAGGTCATGCTCACAC	Plus	23	2262	2284	58.65	56.52	4.00	0.00
Reverse primer	TTGACTGGCTTCCAGGACCACC	Minus	22	2404	2383	58.43	59.09	5.00	1.00
Product length	143								
Exon junction	2277/2278 (forward primer)								

Taperin 3 (TPRN)

PCR template used: NM_175286.4 (plus the Taperin 3 corresponding region of mouse)

Region: 2260-2375

Note: forward primer was only allowed to start after 2260 and till 2290 and reverse primer was only allowed to start after 2345 and till 2375

	Sequence (5'->3')	Strand	Length	Start	Stop	Tm	GC%	Self comp.	Self 3' comp.
Forward primer	GAAGGAGGTCATGGTAAGCCAGGTG	Plus	25	2265	2289	58.81	56.00	4.00	2.00
Reverse primer	GCAGGTGTGAGCTTATCCAGGCTTC	Minus	25	2373	2349	59.61	56.00	4.00	0.00
Product length	109								
Exon junction	2273/2274 (forward primer) 2361/2362 (reverse primer)								

qRT-PCR Primers

Synphilin 1 (SNCAIP)

PCR template used: NM_001199151.1

Region (common to all isoforms): 317-1365

	Sequence (5'->3')	Strand	Length	Start	Stop	Tm	GC%	Self comp.	Self 3' comp.
Forward primer	ACCAACACCCAAGGCACGGAAG	Plus	22	1093	1114	59.54	59.09	2.00	0.00
Reverse primer	TGCAGCTAGGTGTGGCAAGAGC	Minus	22	1200	1179	59.41	59.09	6.00	2.00
Product length	108								
Exon junction	1185/1186 (reverse primer)								

Synphilin 1A

PCR template used: NM_001199151.1 (without the Synphilin 1 corresponding region of mouse)

Region: 217-417

	Sequence (5'->3')	Strand	Length	Start	Stop	Tm	GC%	Self comp.	Self 3' comp.
Forward primer	GAAGGAGGTCATGGTAAGCCAGGTG	Plus	25	2265	2289	58.81	56.00	4.00	2.00
Reverse primer	GCAGGTGTGAGCTTATCCAGGCTTC	Minus	25	2373	2349	59.61	56.00	4.00	0.00
Product length	109								
Exon junction	2273/2274 (forward primer) and 2361/2362 (reverse primer)								