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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		Chr	Uniprot accession number
		RVxF	SILK		
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

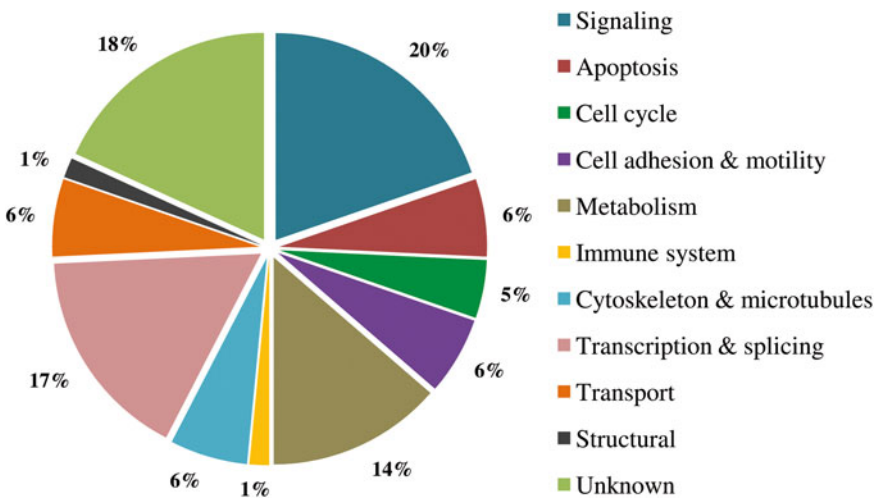

FIG. 1. Distribution of the PP1α regulators according to function. Biological functions attributed to PIPs identified; according to databases by search based on functional motifs present, interacting proteins, cellular localization, and molecular function.

(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,

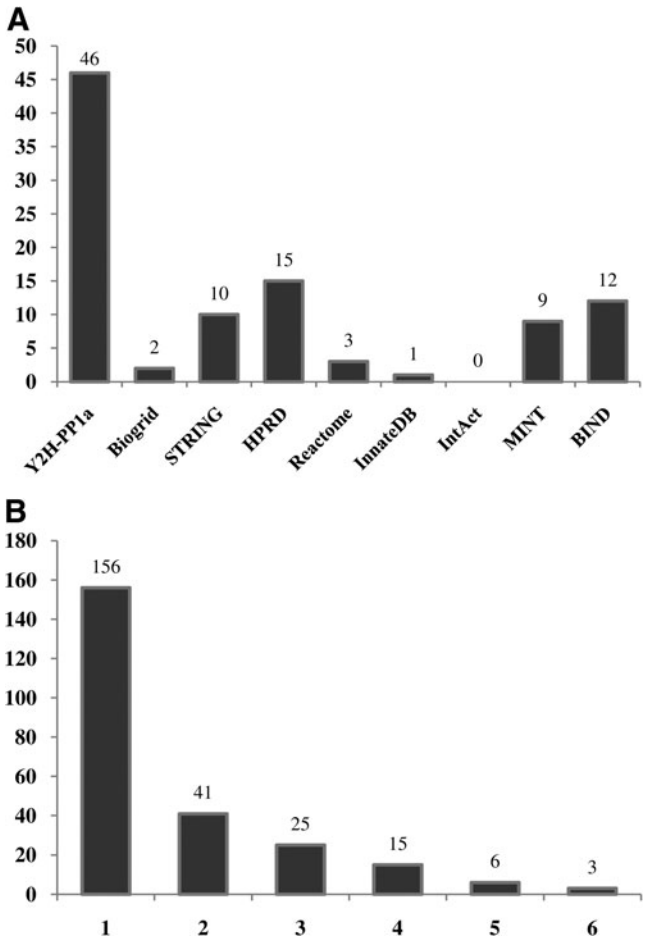

FIG. 2. Analysis of the PP1α interacting proteins in the databases searched. (**A**) Number of PIPs identified in the Yeast Two-Hybrid Screen (Y2H-PP1a) present in the different databases. (**B**) Number of PIPs identified in all the databases (including the Yeast Two-Hybrid Screen) that are present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

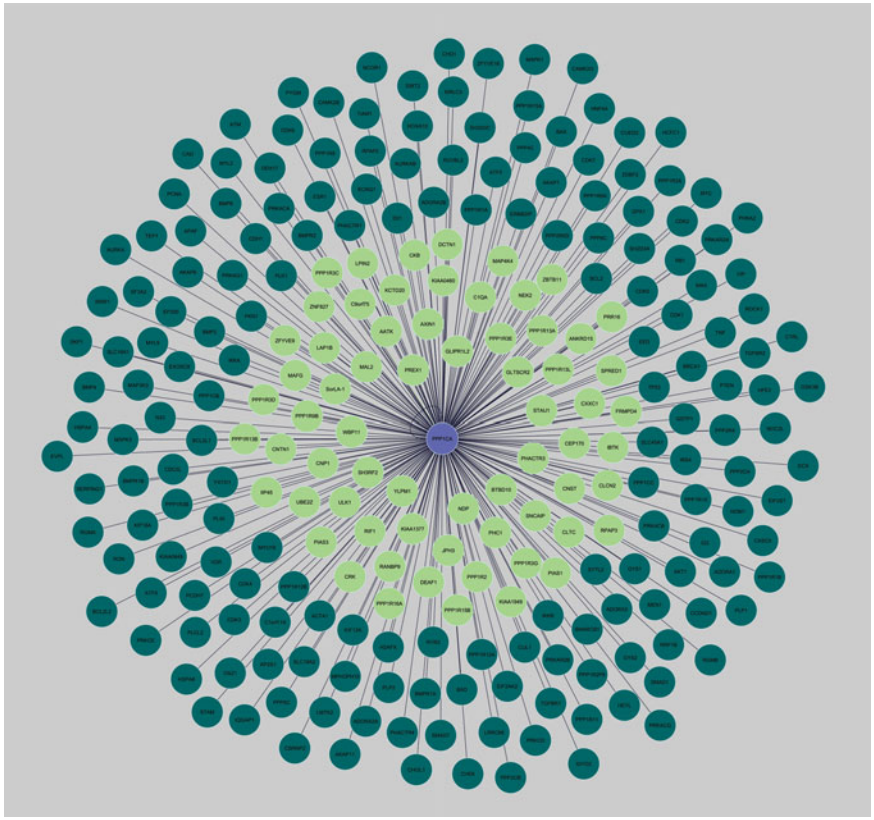

FIG. 3. PP1α interactions mapping. PP1α Interacting proteins retrieved from online databases (dark green) and identified in the Human Brain Yeast Two- Hybrid Screen using PP1α as bait (light green). The total number of interactors is 247 and the proteins obtained in the Yeast Two-Hybrid Screen is 66. 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.